Douglas L. Mayers · Jack D. Sobel
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Dror Marchaim *Editors*

Antimicrobial Drug Resistance

Clinical and Epidemiological Aspects, Volume 2

Second Edition

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Preface

Antimicrobial drug resistance is a global health problem that continues to expand as micro-organisms adapt to the antibiotics we use to treat them and as new classes of antimicrobial agents have been harder to discover and advance into the clinic. The second edition of *Antimicrobial Drug Resistance* grew out of a desire by the editors and authors to provide an updated, comprehensive resource of information on antimicrobial drug resistance that would encompass the current information available for bacteria, fungi, protozoa, and viruses. The two volumes have been extensively revised with many new authors and chapters as the field of drug resistance has evolved. We believe that this information will be of value to clinicians, epidemiologists, microbiologists, virologists, parasitologists, public health authorities, medical students, and fellows in training. We have endeavored to provide this information in a style that is accessible to the broad community of persons who are concerned with the impact of drug resistance in our clinics and across broader global communities.

Antimicrobial Drug Resistance is divided into two volumes. Volume 1 has sections covering a general overview of drug resistance and mechanisms of drug resistance, first for classes of drugs and then by individual antimicrobial agents, including those targeting bacteria, fungi, protozoa, and viruses. Volume 2 addresses clinical, epidemiologic, and public health aspects of drug resistance, along with an overview of the conduct and interpretation of specific drug resistance assays. Together, these two volumes offer a comprehensive source of information on drug resistance issues by the experts in each topic.

We are very grateful to the 197 international experts who have contributed to this textbook for their patience and support as the work came together. The editors would like to especially thank Michelle Feng He for her exceptional support and encouragement to the editors in bringing this revised textbook to print. Finally, the book would never have been completed without the patience and support of our wives and families.

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Part VII

Gram Positive Bacterial Drug Resistance: Clinical

Resistance in *Streptococcus pneumoniae*

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1 Introduction

Streptococcus pneumoniae (the pneumococcus) has been an important human pathogen for over 100 years and continues to cause a wide variety of infections, ranging from mild otitis media and sinusitis to serious lower respiratory infections, as well as life-threatening invasive infections such as meningitis or pneumonia. Worldwide, morbidity and mortality due to pneumococcal infections are highest among young children below the age of 5 years, accounting for approximately one-third of the estimated 1.3 million deaths from pneumonia in 2011 [1]. The pneumococcus is a common colonizer in the respiratory tract, especially in the nasopharynx of children where it is often exposed to antimicrobials. As well as affecting the young, *S. pneumoniae* is an important cause of morbidity and mortality in the elderly; it is the most common etiological agent of community-acquired pneumonia, often resulting in hospitalization of previously healthy individuals.

Infections caused by *S. pneumoniae* were for many years traditionally treated with penicillin or ampicillin, to which this species was exquisitely sensitive when penicillin was first introduced in the 1940s. However, exposure to antimicrobials has led to the selection of resistant strains and clones, some of which have a global distribution; resistance, which was first seen in the 1960s, has continued to increase throughout the world in more recent decades. The emergence of resistance to penicillin and other beta-lactam antibiotics in pneumococci in the 1980s and 1990s led to the increased use of macrolides, fluoroquinolones (FQs), and other non-beta-lactam antibiotics for pneumococcal infections. Pharmacodynamics predicts that high doses of parenteral β-lactams with good Gram-positive activity will currently treat most penicillin-resistant pneumococci. In contrast, oral β-lactams may fail, and high doses of amoxicillin are the best oral β-lactam drugs currently available to treat penicillin-resistant infections. Neither oral nor parenteral macrolides are able to treat macrolide-resistant pneumococcal infections. Fluoroquinolone resistance remains rare, but in patients previously exposed to these drugs, there is an increased risk of treatment failure due to selection of first-step mutants. Efforts to treat pneumococcal disease in both adults and children have been complicated by this increasing resistance to antimicrobials. The increase in antimicrobial resistance rates has been in part due to the selective pressures associated with the widespread use of antibiotics [2, 3] and the clonal expansion and spread of multiresistant pneumococcal clones [4]. More recently, the introduction of conjugate vaccine immunization of infants reduces the burden of resistance by eliminating vaccine types from invasive infections, but resistance continues to be selected in non-vaccine types. New classes of antimicrobials are needed to ensure long-term treatment options for pneumococcal infections.

This chapter will describe the emergence and incidence of antibiotic resistance in pneumococci, mechanisms, clinical implications, and impact of vaccines on resistance.

2 Epidemiology of the Pneumococcus and Risk Factors for Resistance

The incidence of pneumococcal disease remains the highest in children <2 years of age and in adults >65 years of age. Other important risk factors include underlying medical conditions such as chronic heart and lung disease, cigarette smoking, and immunodeficiency states such as asplenia, HIV, and sickle cell disease.

S. pneumoniae colonizes the upper respiratory tract and is part of the normal flora of healthy individuals. Pneumococcal colonization is a dynamic process. A particular serotype can be carried for many months before being eradicated or replaced by a different serotype. Carriage increases in the

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first few months of life, and universally, carriage rates are highest in young children (40–60 %), compared with older children (12 %), adolescents (6–10 %), and adults (3–4 %) [5, 6]. Factors associated with increased carriage include winter season, situations of overcrowding such as day-care attendance, and living in crowded conditions [7]. Prior antibiotic use does not appear to alter the rate of carriage but does promote carriage with antibiotic-resistant strains, particularly to β-lactam antibiotics [8].

Investigations of serotype prevalence from various parts of the world have shown that serotype distribution varies with geographical location and age [9]. The distribution of serotypes also varies between carriage isolates and invasive disease, and antibiotic resistance (at least in the pre-conjugate vaccine era) is most frequent in pneumococcal serotypes that are carried by children (types/groups 6, 9, 14, 19, and 23) [9]. The probable reason is the frequent use of antibiotic therapy in small children and hence exposure of strains of these serotypes to antimicrobial drugs, providing a selective advantage to resistant mutants [10].

There are multiple risk factors for acquisition of infection with antibiotic-resistant pneumococci. Most of these factors have a commonality in exposure to the drugs that select the resistance. This exposure to β-lactams can be at the level of a country [11, 12], province [13], day care [14], family [15], or individual [16]. Macrolide resistance is also a function of exposure, particularly of long-acting drugs such as azithromycin [17]. The selection of resistant strains is complicated by multiple resistances where macrolides appear to be better selectors of multiresistant strains than do β-lactam drugs [16]. Antimicrobial resistance may be seasonal, with higher rates detected during increased antibiotic use in the winter months [18]. The issue of cross-resistance extends to treatment of such diverse organisms as the malaria parasite, where treatment with Fansidar selects trimethoprim-sulfamethoxazole resistance in pneumococci [19].

Most resistance is selected as mentioned above in children, but the exception is fluoroquinolone resistance which is selected in adults [20–22] as these agents are not usually given to children. In the unusual circumstance of fluoroquinolone treatment of children, for example, as treatment of drug-resistant tuberculosis, the selection of fluoroquinolone-resistant pneumococci occurs, and these strains are associated with invasive disease [23].

Children in rural settings generally have less access to antibiotics and therefore have less resistant strains [24, 25], while in some large cities, where poorer children live in the city center with less access to care and more affluent children live in the suburbs, there may be more resistance outside the city [26].

Little is known about the impact of drug dose on the selection of resistant strains, but there is a prospective study that suggests that high dose and short duration of amoxicillin therapy may select less resistance than the same total dose given over a longer period of time [27].

Nosocomial acquisition is a major risk for resistant pneumococci [28], and the first multiply resistant strains were selected in hospital [29]. Recent hospitalization is also a risk for infection with multiply resistant pneumococci [25].

HIV infection is a risk for increased resistance in pneumococcal infections due to the frequent exposure of these patients to antibiotic prophylaxis with trimethoprim-sulfamethoxazole [30], as well as the fact that these patients, especially HIV-infected women, are at risk due to the antibiotic-resistant serotypes carried by children [31].

Children exposed to conjugate vaccine, as well as adults living in countries where these vaccines are routinely administered to children, are at lower risk for pneumococcal infections due to resistant strains as described in Sect. 10.

3 The Role of Clones in Resistance

The increase in antibiotic resistance and the introduction of conjugate vaccines have focused attention on the epidemiology of *S. pneumoniae*. Molecular typing data from numerous studies over the past few decades has added to our knowledge by showing that although there is considerable diversity among resistant strains within most serotypes, a small number of highly successful clones have emerged within countries and in some cases have achieved massive geographical spread [4]. In response to this, the Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 with the aim of standardizing nomenclature and classification of pneumococcal clones worldwide. At present, PMEN has documented 43 international clones, 26 of which are multidrug-resistant. The best characterized, and most widely spread of these international clones, is the Spain^{23F}-1 or PMEN1 originally described in Spain during the 1980s. Intercontinental spread of this clone to the USA was described in 1991 and shortly thereafter in the UK, South Africa, Hungary, and South America [32]. By the late 1990s, it was estimated that approximately 40 % of penicillin non-susceptible pneumococci circulating in the USA were members of this clone [33], and while strains belonging to this genotype continue to be isolated today in many countries all over the world, their prevalence has decreased in countries where conjugate vaccines have been introduced [34, 35]. Recent studies [32, 36, 37] looking at whole genome sequencing of pneumococci representing PMEN1 show that there is a considerable amount of genetic diversity within this lineage. This diversity, which largely results from hundreds of recombination events, indicates rapid genomic evolution and presumably allowed rapid response to selective pressures such as those imposed by vaccine and antibiotic use [36].

Clonal analyses of large surveillance collections of pneumococci have revealed the remarkable dominance of a small number of clones among the antimicrobial-resistant population. As these global clones have spread, they have been exposed to new selective pressures applied by regional variations in the use of different antibiotics. This has led to the further selection of strains belonging to these clones with varying antimicrobial resistance patterns. These resistant clones have also been exposed more recently to conjugate vaccines, and shifts in both serotype and clonal types have been documented [34, 35, 38]. For example, in the USA serotype 19A strains have been identified as the main cause of serotype replacement in both carriage and invasive disease post-PCV7 introduction; this has coincided with a significant increase in penicillin resistance and multidrug resistance among 19A clinical strains [34, 35, 39]. The majority of penicillin-resistant 19A strains belonged to emerging clonal complex 320 (CC320), which is descended from multidrug-resistant Taiwan^{19F}-14 (PMEN14). In 1999, prior to PCV7 introduction, only CC199 and three minor clones were apparent among 19A strains. In 2005 post-PCV, 11 clonal complexes were detected, including ST695 capsular variants of serotype 4 [38, 40].

4 Laboratory Detection of Resistance

Even though we can now identify pneumococci and many resistances based upon genetic features, bacterial culture and phenotypic susceptibility tests remain the gold standard approaches in clinical laboratories. Because it is a fastidious organism, however, specific methods and interpretative criteria developed by a variety of professional bodies such as the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards, NCCLS), the British Society for Antimicrobial Chemotherapy (BSAC), and the European Committee on Antimicrobial Susceptibility testing (EUCAST) must be used to ensure accurate and consistent susceptibility results [41]. Because the breakpoints are determined on the basis of microbiological, pharmacological, and clinical outcome data and since patterns of resistance to antimicrobial drugs continue to evolve, changes to breakpoints can occur during the lifetime of an antibiotic. A good example is the CLSI revised breakpoints for penicillin adopted in January 2008 to redefine the susceptibility of meningeal and non-meningeal pneumococcal isolates [42].

Culture of clinical specimens and antibiotic susceptibility testing are often slow, taking up to 48 h, and are often negative due to prior antibiotic use before sampling or autolysis of the organism. Rapid tests, based mainly on immunological or molecular techniques, have gained importance for the detection of bacteria and antibacterial resistance over the last two decades. PCR has been shown to be a useful tool for the rapid identification of *S. pneumoniae* from both clinical specimens and bacterial isolates [43, 44]. The increased use of molecular tests such as PCR for the diagnosis of bacterial infections has led in turn to an increased demand for antibiotic susceptibility testing using molecular methods. However, unlike phenotypic testing for antibiotic susceptibility, which examines all resistance mechanisms for a particular antibiotic simultaneously, molecular testing can detect only known resistance mechanisms. A variety of assays has been described to detect the presence of specific resistance genes in pneumococcal isolates and also directly from clinical specimens [44–50]. The majority of these assays are PCR based [44–47], although sequencing approaches and microarrays have also been used [49, 50].

5 Resistance to β-Lactams

With the advent of penicillin G therapy in the 1940s, the case fatality rate for pneumonia fell dramatically [51]. Pneumococcal isolates were initially extremely sensitive to the drug with MICs of ≤0.01 mg/L. Penicillin resistance was demonstrated in laboratory mutants soon after the introduction of penicillin G into clinical use but was not reported in clinical strains until 20 years later when investigators in Boston reported penicillin resistance in 2 of 200 strains [52]. Initially, the observation was not considered relevant, until a report by Hansman and Bullen [53] describing a penicillin-resistant strain (MIC 0.6 mg/L) isolated in Australia from the sputum of a patient with hypogammaglobulinemia. Subsequently, resistant strains were identified in New Guinea and Australia, and in 1974, the first clinical infection due to a penicillin non-susceptible strain was reported in the USA [54, 55]. In 1977, pneumococci resistant to penicillin began to appear in South Africa, and in 1978, the first multidrug-resistant pneumococci were documented in Johannesburg, South Africa [29, 56]. In between and after these initial reports, detection of penicillin-resistant pneumococci among clinical isolates began to be reported with increasing frequency in the clinical and microbiological setting. Today, penicillin-resistant strains are encountered in all countries in which adequate surveys are conducted. Recombination appears to be an essential mechanism in the evolution of beta-lactam resistance in nature, and resultant clonal spread of resistant strains plays an enormous role in the increase in beta-lactam resistance globally [4].

β-lactam antibiotics inhibit the growth of pneumococci by inactivation of cell wall synthesizing penicillin-binding proteins (PBPs). β-lactam resistance in pneumococci occurs by alterations in the key cell wall PBPs and the creation of *pbp* genes with decreased affinities for these antimicrobials. Six PBPs have been identified in *S. pneumoniae* (PBPs 1a, 1b,

2a, 2b, 2x, 3), of which PBP2X and PBP2B have been confirmed to be essential for cell growth [57, 58]. Resistance to β-lactams is complex and involves a multifactorial process. Depending on the selecting β-lactam, different combinations of *pbp* genes and mutations within these *pbp* genes are involved in conferring resistance. Little data exist for the role of PBPs 1b, 2a, and 3 [59, 60] as resistance determinants, and altered PBPs 2x, 2b, and 1a are the major players in the development of β-lactam resistance in most clinical isolates. The altered PBPs are encoded by genes with a mosaic structure and can undergo inter- and intraspecies recombination so that parts of the genes are replaced by allelic variants that differ by up to 20 % in DNA sequence [61]. Mosaic sequences of *pbp* genes are very difficult to classify and organize. In general, the resistance profile of particular isolates results from interactions between various combinations of altered PBPs, in conjunction with a functional *murMN* operon which encodes enzymes involved in the synthesis of branched structured muropeptides. Several other genes have been implicated in β-lactam resistance in selected clinical isolates that contribute to resistance in addition to mutations in PBP genes [61], although certain combinations of these three altered PBP genes alone appear to confer resistance.

Resistance to penicillin is associated with some degree of non-susceptibility to all β-lactam antibiotics. Mutations in PBP2x confer low-grade penicillin resistance and may be sufficient for the cell to become non-susceptible to oral cephalosporins. Alterations in PBP2b result in even higher MICs to penicillin [62], while changes in PBP1a are required for high-level penicillin resistance [60, 63] and extended-spectrum cephalosporin resistance [64, 65]. Isolates with very high levels of penicillin resistance (MICs ≥ 8 mg/L) require changes in all three PBPs (i.e., 1a, 2b, and 2x) and sometimes in additional non-PBP resistance determinants such as MurM [66].

Resistance rates reported for amoxicillin are relatively low (<5 %) as a result of the favorable pharmacodynamic properties of this agent [67, 68]. Generally, MICs to amoxicillin are equal to or two to four times less than the MIC of penicillin [69]. In the past, there have been numerous reports of strains with amoxicillin MICs (4–16 mg/L) higher than penicillin MICs (2–8 mg/L) [68, 70–72]. In particular, PBP2b appears to play a significant role in mediating the expression of this resistance phenotype [73]. In addition to typical changes in PBP1a and PBP2x, these strains have unique mutations in the 590–641 region of the PBP2b gene in close proximity to the active binding site [68, 72, 73].

Resistance to cephalosporins may develop with mutations in the pbp1a and pbp2x genes, and the close linkage of these two genes on the chromosome is conducive to the transfer of both genes in a single transformation step [64, 74]. PBP2b is not a target for cephalosporins so would remain unaltered in isolates expressing cephalosporin resistance and susceptibility to penicillin [75]. Most, but not all, extended-spectrum cephalosporin-resistant strains are also penicillin-resistant, and as with amoxicillin, the MICs of cefotaxime and ceftriaxone are usually lower than the MICs of penicillin. Newer antibiotics such as ceftaroline and ceftobiprole appear to be more active and have greater affinity for altered *pbp* genes allowing it to be active against strains with elevated MICs to other β-lactams [76, 77]. In the early 1990s in the USA, pneumococci with high-level cefotaxime and ceftriaxone (2–32 mg/L) resistance were detected [78], and this high-level resistance was due to alterations in PBPs 1A and 2X [65]. The cephalosporin MICs were in excess of the MICs of penicillin for these isolates, and specific point mutations ($Thr_{550}Ala$) in the *pbp2x* gene were associated with this phenotype [65]. These cephalosporin-resistant strains emerged within a few preexisting clones and demonstrate that point mutations as well as recombinational events are important in the development of resistance to β-lactam antibiotics in pneumococci.

6 Resistance to Macrolides

The macrolides have been used extensively to treat community-acquired respiratory tract infections worldwide, and in recent years, resistance to macrolide antibiotics (e.g., erythromycin, clarithromycin, and azithromycin) in *S. pneumoniae* has escalated dramatically. Macrolide-resistant *S. pneumoniae* are now more common than penicillin-resistant *S. pneumoniae* in many parts of the world [79]. However, both macrolide resistance rates and resistance mechanisms may vary considerably depending on location [80]. Erythromycin resistance rates range from about 15 % in Latin America to as high as 80 % recorded among isolates in Far East [81], and these differences probably reflect, in part, the variation in antibiotic prescribing behavior between different countries.

Macrolide resistance in *S. pneumoniae* is mediated primarily by two mechanisms: target modification and active efflux. The most common form of target modification is usually the result of dimethylation of the adenine residue at position 2058 on the 23S rRNA by a methylase enzyme [82]. This mechanism confers constitutive high-level resistance (MIC, >256 mg/L) to 14-, 15-, and 16-member macrolides, lincosamides, and streptogramins B, the so-called MLS_B phenotype. In *S. pneumoniae*, methylation is *erm*(B) mediated in almost all cases, although, more rarely, a methylase encoded by *erm*(A) subclass *erm*(TR) has been implicated [83]. Target modification by point mutations in domain II and V of 23S rRNA and in the genes encoding riboproteins L4 and L22 can also confer macrolide resistance and has been documented in clinical isolates from widely distributed global sites [84–86].

In certain countries, such as the USA [87], active efflux is the major mechanism for macrolide resistance. It confers low-level resistance (MIC, 1–16 mg/L) to 14- and 15-member macrolides but not to 16-member macrolides, lincosamides, and streptogramin B and is phenotypically referred to as the M phenotype. Active efflux is encoded by *mef*-class genes, which include several variants, the abundant *mef*(A) and *mef*(E), which share 90 % sequence identity, and the rare variant *mef*(I) which has only been described in two Italian clinical strains [88].

In pneumococci the three subclasses of *mef* are carried on a number of similar but distinct genetic elements. *mef*(A) is located on the defective transposon Tn*1207.1* or the closely related Tn*1207.3* [89], whereas *mef*(E) is typically carried on the mega (macrolide efflux genetic assembly) element [90]. The *mef*(I) gene exhibits 91.4 and 93.6 % homologies to the *mef*(A) gene of Tn*1207.1* and the *mef*(E) gene of the mega element, respectively [88], and is carried on a nonmobile composite structure, designated 5216IQ complex [91].

Worldwide *erm*(B) and *mef* (A or E) mechanisms account for the majority of macrolide resistance among pneumococci, and the prevalence of these genes varies considerably among countries. In recent years, the presence of both the *erm*(B) and the *mef* genes in *S. pneumoniae* clinical isolates has been increasingly recognized, particularly in Asian countries but also in Europe, S. Africa, and the USA [92, 93]. The PROTEKT study reported a 12 % global prevalence of macrolide-resistant isolates positive for both *erm*(B) and *mef*(A) in 2003–2004 [81].

The majority of dual-positive isolates exhibit multidrug resistance and are clonal lineages of Taiwan^{19F}-14, mostly multilocus sequence type 320, 271, and 236 [4, 92–94]. It appears that the global increase in macrolide-resistant strains carrying both the *erm*(B) and *mef* genes is being driven in part by the diversification and expansion of this Taiwan^{19F}-14 clone following conjugate vaccine introduction. This was especially true of the major 19A ST320 variant in the USA, which became the single most common IPD causing genetic complex in the USA prior to PCV13 implementation.

7 Resistance to Fluoroquinolones

Due to the increased rates of resistance to β-lactam and macrolide antibiotics among pneumococcal strains, fluoroquinolones (FQs) are now included among the choices for first-line therapy in clinical guidelines for the treatment of respiratory tract infections and pneumonia. A direct correlation between the use of FQs and the prevalence of resistance in *S. pneumoniae* has been described [95–97]; however, despite the increased use of FQs, the resistance of *S. pneumoniae* to the newer members of the family is uncommonly found. Reports from Europe, the USA, and Canada showed levels of resistance to levofloxacin and moxifloxacin below 2 % [95–97]. Three major events may have contributed to this low level of resistance: the replacement of the old FQ ciprofloxacin by the more active levofloxacin and moxifloxacin, the introduction of the pneumococcal conjugate vaccine, and, probably, the fact that children who are the main reservoir of pneumococci are not generally treated with FQs. This is supported by a recent study from South Africa showing a rise in FQ resistance in pneumococci isolated from children treated with FQ due to MDR tuberculosis [98]. In countries that report increasing incidence of resistance, the proportion of resistant isolates is much higher among older subjects and patients with chronic lung disease, a patient population that is frequently exposed to FQ [99].

Two mechanisms that decrease susceptibility to FQs in pneumococci have been identified: target alteration and reduced accumulation due to efflux. Resistance associated with target modification requires a combination of mutations in the quinolone resistance-determining region (QRDR) of the genes encoding the DNA gyrase and DNA topoisomerase IV subunits. First-step mutants generally result from spontaneous mutations in the preferential target for a given FQ, ParC for ciprofloxacin, and levofloxacin or GyrA for moxifloxacin, gatifloxacin, and gemifloxacin [100, 101]. Some isolates with a first-step mutation in *par*C gene have ciprofloxacin MICs that would indicate they are clinically susceptible (MIC, <4 mg/L) and these strains would not be identified using routine antibiotic susceptibility testing [102]. The population of isolates with first-step mutations is important because, compared with strains without these first-step mutations, they are more likely to develop high-level resistance during therapy with the acquisition of a second-step mutation [103, 104]. In the second-step mutants, amino acid substitutions are present in both topoisomerase IV and gyrase, most frequently affecting ParC and GyrA and less so ParE and GyrB [105].

Several mutations have been described in these enzymes, but only a few have been shown by in vitro studies to confer resistance: S81F or Y, C, or I and E85K in *gyr*A; E474K in *gyr*B; A63T, S79F, or Y or L and D83G or N in *par*C; and E474K and D435N or H in *par*E [100, 106]. Other frequently described mutations are K137N in *par*C and I460V in *par*E, which appear to not contribute to FQ resistance because they are commonly found in susceptible strains, and no evidence exists for their conferring FQ resistance in vitro [107]. A Q118K in *gyr*A together with S79F in *par*C in a FQ-resistant isolate resulted in treatment failure [108].

Another mechanism underlying non-susceptibility to FQs in some pneumococcal isolates is an increase in active efflux which affects quinolones such as ciprofloxacin [109]. In contrast to the *mef*A gene conferring macrolide resistance, the efflux mechanisms in FQ resistance are poorly characterized and have primarily been demonstrated in isolates with low-level quinolone resistance [101]. They are not encoded by

resistance genes but are thought to be overexpressed in 8–45 % of pneumococcal strains [110]. Little is known about the mechanism of the expression regulation of PmrA, but the efflux pump can be blocked by the plant alkaloid reserpine and, to a lesser degree, by verapamil [111]. Efflux may not confer complete resistance but may be able to lower intracellular FQ to sublethal concentrations, fostering the occurrence of QRDR mutations [112].

In contrast to β-lactam resistance, horizontal gene transfer and the role recombination plays in the evolution of FQ resistance are uncertain. Both intra- and interspecies transfers of FQ resistance loci have been found to occur in vivo, but the frequency of such events appears to be rare. In vitro models report a higher frequency for recombination of QRDRs between viridans group streptococci and *S. pneumoniae* compared to that of spontaneous mutations [113]; however, this level of recombination does not appear to be replicated in vivo [114]. Published studies addressing this question of recombination found evidence for horizontal gene transfer in 0–11 % of FQ-resistant isolates, and interestingly, this ratio seems to be higher in respiratory isolates than in invasive isolates [115–118].

Fluoroquinolone resistance has been reported in a number of international pneumococcal clones that have been associated with the evolution of resistance to penicillin and macrolides [119, 120]. However, the role that clonal spread plays in the increase of FQ resistance is controversial, with studies placing different significance on its importance. The increased prevalence of levofloxacin resistance that was reported from Hong Kong between 1995 and 2001 was suggested to be associated with the dissemination of strains related to the Spain^{23F}-1 clone. However, several studies have shown that clonal spread does not play a significant role in the increase of FQ resistance [120–122]. Data on levofloxacin-resistant pneumococci from 25 countries analyzed as part of the PROTEKT study (1999–2000) showed the majority were genetically unrelated, although 34 % belonged to the Spain^{23F}-1 clone [120]. These studies suggest that both clonal dissemination and the emergence of newly resistant strains contribute to the spread of FQ resistance.

8 Resistance to Newer Classes of Antibiotics

Telithromycin was the first ketolide drug approved for clinical use; however, safety issues have limited the clinical utility of this drug [123]. Both cethromycin (ABT-773) and solithromycin (CEM-101), a novel fluoroketolide, have shown improved activity against macrolide-resistant as well as telithromycin-intermediate and telithromycin-resistant organisms [124–126]. This enhanced potency shows promise for future clinical use for these compounds, subject to pharmacokinetic/pharmacodynamic, toxicity, and animal infection model studies. High-level telithromycin resistance in *S. pneumoniae* has been experimentally generated by mutations in domain II or V of 23S rRNA gene and ribosomal proteins L4 and L22 [127] and is easily created from a macrolide-resistant strain by the deletion or mutation of the region upstream of *erm*(B) [128]. In contrast, clinical telithromycin resistance in *S. pneumoniae* remains rare. Farrell reported that among a worldwide collection of 13 874 *S. pneumoniae* isolates, isolated between 1999 and 2003, only ten were resistant, with MICs ≥4 mg/L and all contained *erm*(B) gene [129]. Mutations in 23S rRNA, L4, and L22 have also been found in clinical telithromycin-resistant isolates [130, 131], and a combination of mutated genes can result in a higher telithromycin resistance than mutation of only one gene [132, 133]. Wolter and colleagues demonstrated that *erm*(B) with a deletion in the leader sequence was responsible for high-level telithromycin resistance in a strain isolated in Canada in 2007 [134].

Linezolid is the first in the class oxazolidinone that was approved for clinical use in 2000 for the treatment of nosocomial and community-acquired pneumonia. Linezolid binds to the 50S subunit of the bacterial ribosome via interactions with the central loop segment of domain V of the 23S rRNA to block the formation of protein synthesis initiation complexes. To date, linezolid non-susceptible pneumococcal strains are extremely rare [129, 135]. Recent data from the US LEADER and global ZAAPS surveillance systems show no linezolid non-susceptible isolates among 2150 *S. pneumoniae* isolates tested in 2011 [136, 137]. Reports of non-susceptibility to linezolid have been sporadic among clinical isolates of staphylococci and enterococci, and resistance has been found to be conferred by mutations in domain V of 23S rRNA [138]. In pneumococci, Wolter et al. [139] have described two clinical isolates with decreased susceptibility to linezolid (MICs 4 mg/L) which were found to contain 6-bp deletions in the gene encoding the riboprotein L4. The L4 deletions were also found to confer a novel mechanism of simultaneous resistance to macrolides, oxazolidinones, and chloramphenicol. A more recent study identified two additional linezolid non-susceptible pneumococci from the USA within the Centers for Disease Control and Prevention (CDC) Active Bacterial Core Surveillance (ABCs) program with mutations and deletions within the *rplD* gene [140]. Whole genome sequencing of linezolid-resistant laboratory-generated mutants has also revealed a role in resistance for a 23S rRNA methyltransferase (spr0333) and for the ABC proteins PatA and PatB [141]. A proteomic and transcriptomic screen suggested increased energy requirement needs associated with the burden of resistance in these laboratory-derived mutants [142]. Second-generation oxazolidinones like tedizolid, which is a protein synthesis inhibitor, are in clinical development for the treatment of

Gram-positive infections. Tedizolid has demonstrated potent in vitro activity against penicillin-resistant *S. pneumoniae*, including linezolid-resistant strains [143].

Resistance to quinupristin-dalfopristin among Gram-positive cocci has been very uncommon. Two clinical isolates among 8837 (0.02 %) *Streptococcus pneumoniae* isolates were discovered in 2001–2002 with MICs of 4 mg/L. Each had a 5-amino acid tandem duplication (RTAHI) in the L22 ribosomal protein gene (rplV) preventing synergistic ribosomal binding of the streptogramin combination [144].

9 Resistance to Other Agents

One class of antimicrobial agents previously used often in clinical practice is the tetracyclines, which are broad-spectrum bacteriostatic drugs shown to be active against pneumococci. Reflecting patterns of past usage, in some countries reported rates of non-susceptibility to tetracyclines remain the most frequently observed resistance phenotype [145]. In *S. pneumoniae* tetracycline resistance is due to the protection of the bacterial 30S ribosome subunit against antibiotic binding by the TetM or TetO [146, 147] proteins, with the *tet*(M) gene being far more common than the *tet*(O)gene in pneumococci. In streptococci, *tet*(M) is usually associated with highly mobile conjugative transposons of the T*n916*–T*n1545* type and large composite structures like T*n5253* and T*n3872*. A recent study discovered the oldest known examples of two different Tn*916*-like, *tet*(M)-containing elements identified among pneumococci dated from 1967 and 1968 [145]. These transposons often carry other resistance genes, such as *erm*(B) coding for resistance to macrolides, lincosamides, and streptogramins B which explains the persistence of tetracycline resistance (these transposons continue to be selected by macrolides). The comparison of *tet*(M) sequences in multidrug-resistant isolates reveals a high degree of allelic variation [148]. There is evidence of clonal distribution of selected alleles as well as horizontal movement of the mobile elements carrying *tet*(M) [149, 150].

The use of rifampin combined with either β-lactam antibiotics or vancomycin has been recommended for the treatment of meningitis caused by multiresistant pneumococci. Rifampin has been used in combined therapy to treat tuberculosis and resistant staphylococci, and it is extensively used in the prophylaxis of *Neisseria meningitidis* and *Haemophilus influenzae* type b exposure. The prevalence of rifampin resistance among pneumococcal isolates is low at present, and reported rates vary between 0.1 % and 1.5 % [151, 152]. Rifampin resistance has been described in several bacterial species and is caused by an alteration of the β-subunit of RNA polymerase, the target for the antibiotic. Resistance to rifampin in pneumococci has been linked to mutations in clusters N, I, II, and III of the *rpo*B gene, which encodes the β-subunit [153, 154].

Resistance to chloramphenicol in *S. pneumoniae* is due to the acetylation of the antibiotic by the production of a chloramphenicol acetyltransferase (CAT). The cat gene in pneumococcal isolates is carried on the conjugative transposon *Tn*5253, a composite transposon consisting of the tetracycline resistance transposon, *Tn*5251, and *Tn*5252 which carries the chloramphenicol resistance determinant [155]. Chloramphenicol-resistant strains have been shown to contain sequences homologous to cat_{pC194} and other flanking sequences from *S. aureus* plasmid pC194 [156].

Trimethoprim and sulfamethoxazole are used extensively in combination as the drug co-trimoxazole. Co-trimoxazole has been used in the treatment of a range of *S. pneumoniae* diseases, especially in children, because it is inexpensive and generally effective. Resistance to co-trimoxazole has increased dramatically in many regions of the world, and recent surveillance studies show rates ranging from 19 % in Europe to around 50 % associated with HIV infection in Africa and >60 % in Asia [29, 157, 158]. Resistance to co-trimoxazole is often associated with resistance to other antibiotics, especially to penicillin. Trimethoprim resistance in pneumococci has been reported to result from a single amino acid substitution (Ile-100 → Leu) in the dihydrofolate reductase (DHFR) protein [159] and often associated with mosaic alleles. Additional mutations have also been reported which seem to enhance resistance and modulate the effects of existing alterations on the affinity of DHFR for its natural substrates [160]. In many cases, resistance to sulfonamides is associated with chromosomal mutations within the gene encoding dihydropteroate synthase (DHPS). Different studies have reported the occurrence of single and/or multiple amino acid mutations in the DHPS of sulfonamide-resistant clinical isolates of *S. pneumoniae* [161–163]. The use of Fansidar therapy for malaria in Africa has been shown to increase co-trimoxazole resistance in pneumococci [19].

10 Clinical Relevance of Antibiotic Resistance

When penicillin-resistant pneumococci were first isolated from adults, there was an implicit assumption that such strains would fail intravenous penicillin therapy [164, 165]. As our appreciation of pharmacodynamics has allowed the understanding of the time-based mode of action of β-lactams, it is clear that the very high levels of penicillin achieved by intravenous therapy exceed the MICs of strains up to 8 mg/L for most of the short 4–6 h dosing interval for high-dose intravenous penicillin [166]. Such highly penicillin-resistant strains remain rare, and there is little evidence for the failure of intravenous penicillin, amoxicillin, cefotaxime, or ceftriaxone [167, 168] due to penicillin resistance. It is possible that less active intravenous agents such as cefuroxime [169]

may fail to treat penicillin-resistant infections, and β-lactams with a more Gram-negative spectrum such as ticarcillin [164] and ceftazidime [170] should not be used to treat penicillin-resistant pneumococcal infections. It is likely that oral β-lactam therapy may fail in the management of pneumococcal infections such as otitis media when the strains become intermediately (MIC ≥ 0.1 mg/L) resistant to penicillin. Poorly active cephalosporins such as cefaclor fail more often than cefuroxime [171, 172], and high-dose amoxicillin is the most active oral agent available against penicillin-resistant pneumococcal otitis media [173]. It is likely that the inferences made for otitis will be similar for sinusitis [174]. β-lactam resistance is clinically important for meningitis treatment where penicillin has been shown to fail [175, 176] even for intermediately resistant strains because of the poor penetration of penicillin through the blood-brain barrier. Extended spectrum cephalosporins fail too when there is full penicillin resistance in meningitis (MIC ≥ 2 mg/L; associated with cefotaxime or ceftriaxone MIC's ≥ 1 mg/L) [177, 178]. The empiric therapy therefore of penicillin-resistant pneumococcal meningitis is cefotaxime plus vancomycin or ceftriaxone plus vancomycin, based on the observation that these drugs in combination are able to eradicate cephalosporin-resistant pneumococci from the CSF better [178] than either drug alone [179, 180].

Macrolide resistance is associated in most instances with MICs > 2 mg/L regardless of the mechanism of macrolide resistance, and treatment of these strains with macrolides has been shown to fail [181, 182], both in the management of otitis media [171, 172] and of pneumonia [183]. These failures are in keeping with our knowledge of the pharmacodynamics of these agents [184].

Trimethoprim-sulfamethoxazole has been shown to not be able to eradicate from the middle ear, strains resistant to that agent [185].

Fluoroquinolones fail to successfully treat pneumococcal infections when preexisting resistant strains are present or even when first-step mutations in the *parC* gene are present [186]. Immunocompromised patients may be most at risk for repeated infections due to fluoroquinolones-resistant strains [187].

11 Impact of Conjugate Vaccine

The introduction of conjugate pneumococcal vaccine has not only reduced the burden of invasive disease in children [188] but has impacted on carriage and thus on the burden of disease in adults by preventing the spread of vaccine-type resistant strains to adults [189]. Direct demonstration of the impact of conjugate vaccine on antibiotic-resistant invasive disease was demonstrated in the 9-valent conjugate vaccine trial in South Africa [190], while multistate studies [191] have demonstrated a significant reduction in the proportion and absolute incidence of antibiotic-resistant pneumococci isolated from blood. Antibiotic resistance however emerged in non-vaccine-type pneumococci causing both ear infections and invasive disease following the 7-valent conjugate vaccine introduction in the USA, particularly among serotype 19A strains [192, 193]. The increase in serotype 19A post-conjugate vaccine in the USA was significantly increased among states with higher rates of community antimicrobial use in children [194]. In addition to direct protection of children from antibiotic-resistant pneumococci, and herd protection of adults to these resistant strains, through interruption of their transmission, conjugate vaccine may also contribute to reduction in selection of resistance by reducing the antimicrobial prescriptions written for vaccinated children, compared to controls [195–197].

12 Concluding Remarks

The multiply resistant pneumococcus continues to have a global distribution. Antimicrobial resistance within the pneumococcal population emerges and is maintained through a complex interplay of many factors. Attempts to reduce the burden of resistance in this pathogen are frustrated by widespread empiric therapy for respiratory infections. Both appropriate and inappropriate antibiotic uses continue to select resistance in this pathogen. Although the conjugate vaccine has reduced the burden of resistance in invasive isolates, continued antibiotic exposure is leading to the emergence of resistance in non-vaccine types.

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Antibiotic Resistance of Non-pneumococcal Streptococci and Its Clinical Impact

50

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1 Introduction

The taxonomy of streptococci has undergone major changes during the last two decades. The present classification is based on both phenotypic and genotypic data. Phylogenetic classification of streptococci is based on 16S rRNA sequences [1], and it forms the backbone of the overall classification system of streptococci. Phenotypic properties are also important, especially for clinical microbiologists. The type of hemolysis on blood agar, reaction with Lancefield grouping antisera, resistance to optochin, and bile solubility remain important for grouping of clinical *Streptococcus* isolates and therefore treatment options [2]. In the following chapter, two phenotypic classification groups, viridans group streptococci (VGS) and beta-hemolytic streptococci, will be discussed.

Antimicrobial resistance is common among VGS and beta-hemolytic streptococci isolates. Beta-lactam resistance is widespread among VGS, and resistance rates to other antimicrobials are continuously increasing. Beta-lactam resistance is uncommon in beta-hemolytic streptococci. Macrolide resistance, however, presents a clinical concern in the outpatient setting. High-level beta-lactam resistance in VGS is a real threat to the treatment of infective endocarditis and empirical treatment of sepsis in neutropenic patients. Treatment of infections, including pharyngitis, caused by macrolide-resistant beta-hemolytic streptococci may also become challenging if resistance rates continue to rise.

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Infections caused by Gram-positive organisms have increased in frequency over time and are almost as common as Gram-negative infections. This has been linked to greater use of invasive procedures and the increasing proportion of hospital-acquired infection. The regular use of broad-spectrum antibiotics in increasingly sick patients has likely resulted in increased bacterial resistance over time [3]. As a result, implementation of antimicrobial stewardship and infection control processes has become progressively more important in protecting patients, health-care providers, and communities.

This chapter summarizes the general characteristics of the streptococci groups, the current antimicrobial resistance trends, resistance mechanisms, and the clinical implication of resistance for viridans and beta-hemolytic streptococci.

2 Characteristics of Non-pneumococcal Streptococci

2.1 Viridans Group Streptococci

Viridans group streptococci form a phylogenetically heterogeneous group of species belonging to the genus *Streptococcus* [1]. However, they have some common phenotypic properties. VGS are a group of catalase-negative, Gram-positive cocci with a chaining morphology upon microscopic examination. They can be grouped as alpha- or nonhemolytic. They can be differentiated from *S. pneumoniae* by their resistance to optochin and lack of bile solubility, though the distinction between the two groups remains difficult due to similar sequence homology [2, 4]. They are leucine aminopeptidase positive, pyrrolidonylaryl amidase negative, and can be differentiated from *Enterococcus* species by their inability to grow in medium containing 6.5 % sodium chloride [2]. Nutritionally variant streptococci were once included in VGS but based on molecular data been removed to a new genus *Abiotrophia* [5]. VGS belong to the normal microbiota of the oral cavities and upper respiratory tracts of humans and animals. They can also be isolated from

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the female genital tract and all regions of the gastrointestinal tract [2, 5]. Although historically VGS are poorly classified, there are many species within the group. The six major groups include *S. mutans*, *S. salivarius*, *S. anginosus*, *S. mitis*, *S. sanguinis*, and *S. bovis* group. The *S. anginosus* group has been the source of much controversy and ambiguity regarding taxonomy and classification. This group of organisms can be alpha-, beta-, or nonhemolytic, and it is the isolates lacking beta-hemolysis that are generally considered to be a part of VGS. Due to the diverse nature of VGS, the rates and patterns of antimicrobial resistance vary greatly. Differences in species identification and patient population contribute to this variability [4].

Streptococcus mitis group organisms are resistant to more antimicrobial agents than the other VGS species [4]. The most clinically relevant species belonging to VGS are *S. mitis*, *S. sanguis*, and *S. oralis*. Lack of alpha-hemolysis does not seem to correlate with clinical outcome or severity of disease and no enzymatic or toxigenic effect has been documented as a by-product of alpha-hemolysis [4].

2.2 Beta-Hemolytic Streptococci

Beta-hemolytic streptococci can be differentiated from the heterogeneous group of streptococci by the pattern of hemolysis on blood agar plates, antigenic composition, growth characteristics, biochemical reactions, and genetic analyses. Beta-hemolytic streptococci commonly produce hemolysins, which cause complete lysis (beta-hemolysis) of red blood cells when cultivated on blood agar plates. Traditional subdividing into serological groups is based on the detection of group-specific antigenic differences in cell-wall carbohydrates. The serologic scheme of classification by Lancefield is used [6], and serogroups A, B, C, D, F, and G are those most commonly found in humans [7].

2.2.1 Group A *Streptococcus* (*Streptococcus pyogenes*)

Group A *Streptococcus* (GAS, *Streptococcus pyogenes*) is an important pathogen confined almost exclusively to human hosts [8]. *S. pyogenes* is generally associated with acute pharyngitis or localized skin infections. *S. pyogenes* is highly communicable and can cause disease in healthy people of all ages without type-specific immunity against the serotype responsible for infection [9]. Transmission can occur from those with acute infections or from asymptomatic carriers generally through hand contact or respiratory droplets. Food and waterborne outbreaks have also been documented [8]. Impetigo and pharyngitis are more likely to occur among children living in crowded homes or in suboptimal hygienic conditions. Multiple streptococcal infections may be found in the same family due to the highly contagious nature of the infection [9].

The diseases are commonly self-limiting, localized infections of the pharynx and skin. A ubiquitous organism, *S. pyogenes*, is the most common bacterial cause of acute pharyngitis, accounting for 15–30 % of cases in children and 5–10 % of cases in adults [9]. Invasion from the skin can lead to septicemia or severe deep-seated tissue infections, such as necrotizing fasciitis and myositis. Other clinical manifestations of GAS include scarlet fever, peritonsillar and retropharyngeal abscesses, otitis media, sinusitis, myositis, lymphangitis, meningitis, suppurative arthritis, endocarditis, osteomyelitis, pneumonia, erysipelas, cellulites, streptococcal toxic shock syndrome, vaginitis, and balanitis [10–13]. Primary suppurative infections may also lead to serious nonsuppurative sequelae, acute rheumatic fever, rheumatic heart disease, and acute glomerulonephritis [2, 14, 15].

Group A *Streptococcus* can be distinguished from other groups by susceptibility to bacitracin. A kirby-bauer disc contains 0.04U of bacitracin inhibits the growth of more than 95 % of group A strains, whereas 80–90 % of non-group A strains are resistant to this antibiotic [9]. Serologic typing of the M [16] and T [17] proteins has traditionally been used in epidemiologic typing of GAS [18]. More recently, molecular typing methods such as *emm* sequence typing, multilocus sequence typing, pulse field gel electrophoresis, inversion gel electrophoresis, restriction length polymorphism analysis of the *mga*-regulon (vir-typing) and random amplified polymorphic DNA analysis have provided more discriminatory power for studying the clonal relationships between GAS strains.

2.2.2 Group B *Streptococcus* (*Streptococcus agalactiae*)

Group B streptococci (GBS, *Streptococcus agalactiae*) are the most common cause of neonatal sepsis. It is one of the primary causes of bacteremia and meningitis in neonates and can cause infections in pregnant women [19, 20]. Vaginal colonization of nonpregnant and pregnant women is the principal source of GBS. However, it also can colonize the gastrointestinal tract and the upper respiratory tract of healthy humans. The portal of entry is not apparent, but possible areas include the skin, genital tract, urinary tract, and respiratory tract [21].

Neonates can acquire the organism vertically in utero or during delivery from the maternal genital tract. Although the transmission rate from mothers colonized with *S. agalactiae* to neonates delivered vaginally is approximately 50 %, with antibiotic prophylaxis, only 1–2 % of colonized neonates develop invasive group B streptococcal disease [21].

GBS may also cause invasive infections in the elderly and in nonpregnant adults with underlying or chronic diseases. The broad clinical spectrum of invasive GBS disease in adults includes skin and soft tissue infections, primary bacteremia, urosepsis, pneumonia, osteomyelitis, peritonitis, septic arthritis, meningitis, endocarditis, and intravenous catheter infection [21].

GBS has been classified into different serotypes on the basis of different chain structures of its capsular polysaccharide. Several serotypes are known—Ia, Ib, Ic, II, III, IV, V, VI, VII, and VIII. Isolation of group B streptococci from blood, cerebrospinal fluid (CSF), and/or a site of local suppuration is the only method for diagnosing invasive group B streptococcal infection [21].

2.2.3 Groups C and G Beta-Hemolytic Streptococci

Most of the Lancefield group C streptococci (GCS) produce beta-hemolysis on blood agar although nonhemolytic strains also exist [2]. Group C streptococci are mainly animal pathogens; however, beta-hemolytic strains have been isolated from normal human microbiota of the nasopharynx, skin, and genital tract [22]. The majority of group G streptococci (GGS) are beta-hemolytic [2].

More recently, group C streptococci and group G streptococci of human origin are thought to comprise a single subspecies, *Streptococcus dysgalactiae* subsp. *equisimilis*. It can be found in normal flora of the upper airways and are often asymptomatic colonizers of other areas. It may also be implicated in skin and soft tissue infections, pharyngitis, bacteremia, endocarditis, septic arthritis, osteomyelitis, puerperal infections, and meningitis [22].

3 Antimicrobial Resistance in VGS

For the purpose of this chapter, we will use data from contemporary large-scale surveillance studies to show recent resistance trends for relevant antibiotics. As both the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) breakpoints will be presented, epidemiology and resistance rates will primarily be described as defined by CLSI criteria.

3.1 Beta-Lactam Activity

Among streptococci, beta-lactam resistance is mediated by point mutations in penicillin-binding proteins (PBPs). PBPs are membrane-bound transpeptidases. They are active-site serine hydrolases, which catalyze cross-linking of the peptidoglycan subunits during bacterial cell-wall synthesis [23, 24]. Beta-lactams serve as substrates for PBPs. The active-site serine reacts with the beta-lactam ring and generates a covalently linked enzyme-beta-lactam intermediate. This acyl enzyme intermediate is not able to catalyze cross-linking of the peptidoglycan subunits [23]. In streptococci there are low and high molecular weight PBPs [25, 26]. Both of these enzymes are important for the cell-wall synthesis, but only the high molecular weight PBPs are important for the bacterial killing activity of the beta-lactam antibiotics [24]. In VGS there are two kinds of high molecular weight PBPs, PBP1 (PBP1a and PBP1b), and PBP2 (PBP2a, PBP2b, PBP2x) [25]. Homologous molecules can be found in *S. pneumoniae* and naming conventions for PBPs of VGS are adapted from *S. pneumoniae* [24–26].

VGS with wild-type PBPs are susceptible to beta-lactam antibiotics [27]. Resistance results when the high molecular weight PBPs have decreased affinity for beta-lactams. Decreased affinity can be achieved by amino acid substitutions in the transpeptidase domain of the PBPs [24, 27]. A single point mutation can result in an increase in the penicillin minimum inhibitory concentration (MIC) value. Normally more than one mutation is needed for intermediate level beta-lactam resistance. Highly resistant strains have accumulated several mutations in the PBPs, altering the PBPs significantly so the beta-lactams can no longer bind to the PBP. Accumulation of several mutations in the PBPs may also result in lethal mutations if cell-wall integrity is compromised. Based on the data obtained in *S. pneumoniae*, these highly resistant strains may also have mutations beyond those found in PBPs [24]. Streptococci have overcome this problem by horizontal transfer of functional mutated PBP coding genes or gene fragments. Transformation and subsequent homologous recombination has produced beta-lactam-resistant VGS with mosaic PBP genes. In these mosaic PBP genes, there are gene regions obtained from resistant strains dispersed through the wild-type PBP genes [28].

Penicillin resistance among VGS isolated from blood has been extensively studied. Farrell et al. at JMI laboratories performed a large-scale surveillance study to examine the susceptibility profiles of various antibiotics against 60,084 clinical isolates from 33 European region medical centers. Over 1200 viridans group streptococci isolates were collected between 2005 and 2010 and were tested for susceptibility to a range of antibiotics. The penicillin MIC_{50} and MIC_{90} was 0.06 and 1 mg/L, respectively. Per CLSI breakpoints, 77.5 % VGS were susceptible, 17 % intermediate, and 5.5 % resistant [29] (Table 50.1). The 2012 LEADER surveillance study evaluated 7429 isolates, including 526 VGS, from 60 US sites. The penicillin MIC_{50} was ≤0.06 mg/L and the MIC_{90} was 0.5 mg/L, similar to the European susceptibility pattern [30].

Overall, in VGS cephalosporins have similar susceptibility rates, MIC_{50} and MIC_{90}. The cefepime MIC_{50} and MIC_{90} were ≤0.12 mg/L and 1 mg/L, respectively, with 92.1 % of isolates susceptible. Between 3 % and 5 % of isolates showed intermediate susceptibility or were considered resistant. Ceftriaxone MIC_{50} was ≤0.25 mg/L with an identical MIC_{90} and similar percent resistance [29] (Table 50.1). US surveillance data were similar with MIC_{50} and MIC_{90} values of 0.25 mg/L and 0.5 mg/L with only 1.2 % resistance rates [30].

Table 50.1 Antimicrobial activities of ceftobiprole and comparator agents when tested against bacterial isolates from European medical centers (2005–2010)

	MIC			% of isolates susceptible/intermediate/resistant	
Organism (no. of isolates tested) and antimicrobial agent	MIC_{50}	MIC_{90}	Range	CLSI	EUCAST
B-Hemolytic streptococci (2, 981)					
Ceftobiprole	≤0.06	≤0.06	≤0.06–0.25	–/–/–	–/–/–
Penicillin	≤0.03	0.06	≤0.03–0.12	100.0/–/–	100.0/0.0/0.0
Cefepime	≤0.12	≤0.12	≤0.12–2	99.9/–/–	100.0/0.0/0.0
Ceftriaxone	≤0.25	≤0.25	≤0.25–4	99.9/–/–	100.0/0.0/0.0
Clindamycin	≤0.25	≤0.25	≤0.25 to >2	91.9/0.5/7.6	92.4/0.0/7.6
Erythromycin	≤0.25	>2	≤0.25 to >2	82.0/1.0/17.0	82.0/1.0/17.0
Daptomycin	≤0.06	0.25	≤0.06–0.5	100.0/–/–	100.0/0.0/0.0
Levofloxacin	≤0.5	1	≤0.5 to >4	99.6/0.0/0.4	95.6/4.0/0.4
Linezolid	1	1	0.25–2	100.0/–/–	100.0/0.0/0.0
Tetracycline	4	>8	≤2 to >8	49.5/2.6/47.9	49.3/0.2/50.5
Tigecycline	≤0.03	0.06	≤0.03–0.5	>99.9/–/–	>99.9/<0.1/0.0
Trimethoprim/sufamethoxazole	≤0.5	≤0.5	≤0.5 to >2	–/–/–	99.0/0.4/0.6
Vancomycin	0.25	0.5	≤0.12–1	100.0/–/–	100.0/0.0/0.0
Viridans group streptococci (1, 264)					
Ceftobiprole	≤0.06	0.25	≤0.06 to >8	–/–/–	–/–/–
Penicillin	0.06	1	≤0.03 to >4	77.5/17.0/5.5	84.3/10.2/5.5
Cefepime	≤0.12	1	≤0.12 to >16	92.1/3.4/4.5	88.1/0.0/11.9
Ceftriaxone	≤0.25	1	≤0.25 to >8	92.2/3.2/4.6	88.8/0.0/11.2
Daptomycin	0.25	0.5	≤0.06–2	99.8/–/–	–/–/–
Clindamycin	≤0.25	>2	≤0.25 to >2	88.0/0.3/11.7	88.3/0.0/11.7
Erythomycin	≤0.25	>2	≤0.25 to >2	61.6/2.2/36.2	–/–/–
Levofloxacin	1	2	≤0.5 to >4	96.8/1.1/2.1	–/–/–
Linezolid	1	1	≤0.12 to 2	100.0/–/–	–/–/–
Tetracycline	≤2	>8	≤2 to >8	62.2/2.2/35.6	–/–/–
Tigecycline	≤0.03	0.06	≤0.03–0.5	99.9/–/–	–/–/–
Vancomycin	0.5	1	≤0.12–1	100.0/–/–	100.0/0.0/0.0

Ceftobiprole medocaril is described as a fifth-generation cephalosporin with a wide spectrum of antibiotic activity. Per European surveillance data, the ceftobiprole MIC_{50} and MIC_{90} are ≤0.06 mg/L and 0.25 mg/L, respectively, for VGS [29] (Table 50.1). Ceftaroline fosamil is a broad-spectrum parenteral cephalosporin which treats certain skin infections and community-acquired bacterial pneumonia (CABP). A recent report from the SENTRY antimicrobial surveillance program tested ceftaroline against 1273 streptococci isolates between 2008 and 2011. Ceftaroline showed activity against all VGS species with the highest MIC, 1 mg/L, observed in *S. oralis*, *S. mitis*, *and S. parasanguinis* [31].

3.2 Macrolide, Lincosamide, and Ketolide Activity

Macrolides, ketolides, lincosamides, and streptogramin B antibiotics, although having different chemical structures, have similar, although not identical, antimicrobial activity against VGS since the resistance mechanisms developed by bacteria against these antimicrobials is similar. These antibiotics inhibit protein synthesis by binding to bacterial ribosomes. Macrolides can be divided into different groups according to the number of carbon atoms in their lactone ring. Fourteen- and 15-membered ring macrolides such as erythromycin and azithromycin have similar antibiotic properties. Sixteen-membered ring macrolides including spiramycin differ from 14- and 15-membered ring macrolides in their antimicrobial activity against VGS. Lincosamides such as clindamycin and streptogramins also have some differences in their activity against bacteria when compared to macrolides.

In streptococci, there are two well-characterized macrolide resistance mechanisms. These are target site modification and active drug efflux. Target site modification is mediated by methylases encoded by the *erm* (erythromycin ribosome methylation) genes or by mutations at the 23S ribosomal RNA or ribosomal proteins L4 and L22. Methylation of adenine 2058 of the peptidyl transferase

loop of 23S rRNA causes resistance to macrolides as well as to lincosamides and streptogramin B antibiotics [32]. The active efflux mechanism encoded by the *mef* (macrolide efflux) genes is more specific and causes resistance only to 14- and 15-membered ring macrolides [33]. Mutations at the macrolide binding domains of the 23S ribosomal RNA and at the ribosomal proteins L4 and L22 lower the affinity of macrolides to ribosomes [34]. Mutations can cause several different kinds of resistance phenotypes. Both *erm* and *mef* genes can be horizontally transferred between different streptococci [35].

3.2.1 Erythromycin

Erythromycin A has similar in vitro activity against VGS strains as other 14- and 15-membered ring macrolides including azithromycin [36]. Erythromycin resistance is quite common among clinical VGS isolates. In Europe, the MIC_{50} and MIC_{90} for erythromycin for VGS is ≤0.25 mg/L and >2 mg/L. Resistance levels continue to remain high with 36.2 % of isolates resistant to erythromycin [29] (Table 50.1). In the United States, macrolide MIC values and resistance rates continue to rise. Approximately 50 % of VGS isolates in the LEADER study were resistant to erythromycin with MIC_{50} and MIC_{90} values of 0.5 mg/L and 16 mg/L, respectively [30]. The most common erythromycin resistance mechanism is mediated by *mef*(A) genes azithromycin [36, 37]. Roughly 70–80 % of the erythromycin-resistant VGS strains are carrying *mef*(A) gene and about 16–20 % are carrying *erm*(B) gene [36–38]. However, the situation may vary. There is one report from France, where *erm*(B) was reported to be much more common than *mef*(A) among blood isolates of VGS [35]. The continuous surveillance of invasive VGS isolates is warranted and can guide better treatment options especially in patients with underlying diseases [39].

3.2.2 Clindamycin

Resistance to clindamycin is much less frequent among blood and normal microbiota VGS than resistance to erythromycin [40]. MIC_{50} and MIC_{90} values were similar to erythromycin (≤0.25 mg/L and >2 mg/L, respectively), with up to 12 % of VGS isolates resistant to clindamycin in both Europe and the United States [29, 30] (Table 50.1). Resistance levels are similar among both blood and the normal microbiota isolates. The reason for lower resistance levels is that the efflux mechanism mediated by *mef*(A) resistance gene does not confer resistance to clindamycin [40]. An autopsy report of a patient with a S. *mitis* strain found that the pattern of resistance in this isolate resembled an induced macrolide, lincosamide, and streptogramin B resistance (MLSB) phenotype as a result of short-term use of clindamycin. This mechanism induces resistance to both clindamycin and minocycline [41].

3.2.3 Ketolide

The binding of telithromycin to the bacterial ribosomes is much stronger than the binding of erythromycin. This is the reason why methylation of the ribosomal RNA does not increase the MIC values as much for telithromycin compared to erythromycin [42]. Mef(A) efflux pumps transport telithromycin out of the bacterial cell as well as they pump erythromycin. However, in streptococci, Mef(A) efflux does increase telithromycin MIC values when compared to the strains without *mef*(A) gene [43].

3.2.4 Streptogramin

Quinupristin-dalfopristin, a combination of streptogramin B and streptogramin A, is available for intravenous use. It has good in vitro activity against VGS. However, resistance rates vary considerably between studies. In some studies, resistant strains have not been isolated, whereas other studies show reduced susceptibility in as much as 70 % of strains and resistance in 28 % [44, 45]. VGS strains with quinupristin-dalfopristin MIC values of 16 mg/L have also been described [44]. Resistance to quinupristin-dalfopristin is linked to the streptogramin A (dalfopristin) resistance. Therefore in order to be resistant to the antibiotic combination, a strain must be resistant to streptogramin A. Streptogramin A resistance is mediated by *vga*(A), *vga*(B), *lsa*, and various *vat* genes. Thus far, these genes have been found in clinical *Staphylococcus* and *Enterococcus* strains, but the presence of the genes in VGS has not been reported [46]. Although not studied in detail [44, 47], it is possible that the resistance is mediated by ribosomal mutation as in *S. aureus* [48].

3.3 Tetracyclines and Trimethoprim-Sulfamethoxazole Activity

Tetracycline resistance in VGS is quite common. In the European surveillance study, the VGS MIC_{50} was ≤2 mg/L and MIC_{90} was >8 mg/L. Up to 36 % of VGS strains are tetracycline resistant [29] (Table 50.1). Tigecycline activity is much higher with 99.9 % of isolates susceptible and an MIC_{50} and MIC_{90} of ≤0.03 mg/L and 0.06 mg/L, respectively [29] (Table 50.1). Trimethoprim-sulfamethoxazole is not used for treatment of VGS infections but has been commonly used for prophylaxis in neutropenic patients [49]. Decreased susceptibility for trimethoprim-sulfamethoxazole is quite common among VGS strains.

3.4 Fluoroquinolone Activity

In streptococci, there are two fluoroquinolone resistance mechanisms: mutations at the quinolone resistance-determining regions (QRDRs) of the topoisomerase IV and

DNA gyrase molecules and an efflux mechanism [50–52]. In streptococci, the topoisomerase IV molecule has two subunits coded by *par*C and *par*E genes. DNA gyrase has two subunits, GyrA and GyrB, coded by corresponding genes. Topoisomerase IV is the primary target for fluoroquinolones in VGS [50]. Mutations at the topoisomerase IV genes confer low-level resistance (MIC 4 mg/L). A combination of topoisomerase IV mutations and the fluoroquinolone efflux mechanism is needed for high-level fluoroquinolone resistance (MIC ≥ 16 mg/L). Fluoroquinolone resistance determinants can be horizontally transferred between VGS and *S. pneumoniae* strains [50, 53–55]. Levofloxacin was the only fluoroquinolone evaluated in the European and US surveillance studies. Data demonstrated high susceptibility rates with a MIC_{50} of 1 mg/L and MIC_{90} of 2 mg/L. Approximately 2% of VGS isolates were determined to be resistant in Europe and 6% in the United States [29, 30] (Table 50.1).

3.5 Glycopeptide Activity

3.5.1 Vancomycin

Vancomycin, a glycopeptide antibiotic, has retained its activity against VGS. Not a single vancomycin-resistant VGS has been reported thus far [36, 56–62]. The vancomycin MIC_{50} was 0.5 mg/L and MIC_{90} was 1 mg/mL in both Europe and the United States [29, 30] (Table 50.1).

3.5.2 Oritavancin and Dalbavancin

Newer glycopeptides include oritavancin and dalbavancin. Oritavancin, a bactericidal lipoglycopeptide, was assessed in the SENTRY surveillance program in order to expand current limited in vitro data. Oritavancin has activity against many Gram-positive pathogens including streptococci with oritavancin MIC_{50} and MIC_{90} results of ≤0.008 mg/L and 0.06 mg/L, respectively, for the VGS studied [63]. The SENTRY program also evaluated in vitro activity of dalbavancin. VGS isolates were tested using CLSI reference broth microdilutions and interpretations. The MIC_{50} and MIC_{90} ranges were ≤0.03–0.25 mg/L and 0.06–0.12 mg/L, respectively [64]. Overall, all currently existing glycopeptides are potent against the VGS.

3.6 Aminoglycoside Activity

In general, the activity of the aminoglycosides against VGS is limited [65]. Aminoglycosides including gentamicin, amikacin, streptogramin, and netilmicin have been used in combination with penicillin or a cephalosporin for the treatment infective endocarditis [66] and sepsis in neutropenic patients [67]. High-level gentamicin resistance in VGS is rare. This is true with VGS isolates of blood origin [59–61] and normal microbiota [68]. MIC values are typically between 0.25 and 96 mg/L [59, 60, 69] and the MIC_{90} values are between 0.5 and 32 mg/L [59, 68]. Few high-level aminoglycoside-resistant *S. mitis* strains have been detected. In these strains gentamicin MIC values have been as high as 1000 mg/L [69].

3.7 Oxazolidinone Activity

3.7.1 Linezolid

Linezolid belongs to the oxazolidinone group of antibiotics [70]. Linezolid has been used in the treatment of vancomycin-resistant *Enterococcus faecium* infections, hospital-acquired pneumonia, and complicated skin infections [71]. The activity of linezolid against VGS strains has not been well studied. However, ongoing surveillance programs that monitor the in vitro activity of linezolid against comparator agents with Gram-positive coverage do exist. The LEADER surveillance study demonstrates MIC values of linezolid against VGS to be predominantly 1 mg/L and 100% susceptible in the United States [72]. International data through the ZAAPS program revealed similar findings [73].

3.7.2 Tedizolid

Tedizolid is the active moiety of the prodrug tedizolid phosphate. It is a novel oxazolidinone whose in vitro activity has been studied against viridans group streptococci. Fifteen VGS isolates from a phase 2 trial were obtained and tested in patients with complicated skin and skin structure infections. Susceptibility testing from phase 2 data resulted in a MIC_{50} and MIC_{90} of 0.25 mg/L [74].

3.8 Daptomycin Activity

Daptomycin is a bactericidal lipopeptide with activity against streptococci. It is used successfully to treat endocarditis caused by vancomycin-resistant enterococci and methicillin-resistant staphylococci. It is the only agent indicated for *S. aureus* bacteremia and endocarditis. Large surveillance studies have demonstrated daptomycin MIC_{50} of 0.25 mg/L and MIC_{90} of 0.5 mg/L [29] (Table 50.1). VGS has historically been considered uniformly susceptible to daptomycin; however, the development of high-level daptomycin resistance (HLDR; MIC >256 mg/L) after exposure to daptomycin has recently been reported among these isolates. In vitro studies were performed and 114 VGS strains were tested from patients diagnosed with infective endocarditis. Daptomycin susceptibilities of the baseline clinical isolates by Etest ranged between 0.03 and 1.5 mg/L for S. *mitis*, 0.023–0.12 mg/L for

S. *bovis*, 0.12–0.5 mg/L for S. *anginosus*, 0.25–0.5 mg/L for S. *mutans*, and 0.016–0.047 mg/L for S. *salivarius.* HLDR was only observed after 24 h of exposure to daptomycin in 27 % of *S. mitis* isolates, 47 % of *S. oralis* isolates, and 13 % of *S. sanguis* isolates [75]. No clinical isolates have been identified or reported to date.

4 Antimicrobial Resistance in Beta-Hemolytic Streptococci

4.1 Resistance to Macrolides

4.1.1 Incidence of Macrolide Resistance in GAS, GBS, GCS, and GGS

In 1959 Lowburry and Hurst reported the first isolate of erythromycin-resistant GAS from burns of four patients in the United Kingdom [76]. During the following years in Europe, mainly sporadic cases and small epidemics of erythromycin-resistant GAS were reported from the United Kingdom, Sweden, Italy, and Spain [76–81]. In the 1970s a large outbreak of erythromycin-resistant GAS occurred in Japan, where the proportion of resistant strains increased from 12 % in 1971 to 82 % in 1977 [82]. These strains were characterized as highly resistant (MIC values >100 mg/L) to macrolides and lincomycin and were often resistant to tetracycline and chloramphenicol as well. Strains were exclusively of the T12 serotype. From 1985 to 1987, an increase from 1 % to 17.6 % in the frequency of erythromycin-resistant GAS was seen in Australia's Fremantle area [83]. These strains represented different serotypes and exhibited overall low-level resistance to erythromycin (MIC range 2–8 mg/L) Resistance to clindamycin and tetracycline was rare. Sporadic isolates and family outbreaks with 22 % erythromycin-resistant GAS, predominantly of T4M4 serotype, was reported between 1988 and 1989 from Dundee area in the United Kingdom [84].

Resistance to erythromycin continues to be reported in GBS since 1962. The first description was from the United States [85], and in the same country macrolide resistance in GBS increased from 1.2 % among isolates collected from 1980 to 1993 to 18 % in 1997 and 1987. Increasing resistance has been reported from other countries as well. In Spain, the frequency of macrolide resistance in GBS increased from 2.5–5.6 % in 1993–1996 to 14.5–18 % in 1998–2001 [86] and in Taiwan from 19 % in 1994 to 46 % in 1997 [87]. Since the end of the 1990s, frequencies of 15–21 % have been reported in France [88–90], 13–18 % in Canada [91, 92], 40 % in Korea [93], and 22 % in Turkey [94].

Macrolide resistance among group C and G streptococci varies between different countries. Resistance is uncommon in Finland with 1 % and 3.6 % of group C streptococci found to be resistant to clindamycin and erythromycin, respectively. The most common resistance mechanism to macrolides has been via the *mef*(A) gene [95]. Similar to group C streptococci resistance rates, 3.5 % and 0.3 % of the group G streptococci have been resistant to erythromycin and clindamycin, respectively. Most of these strains have had *erm*(TR) resistance gene and one with the *erm*(B) resistance gene [95]. Higher numbers of erythromycin resistance among group C and G streptococci have been reported from Turkey. Ergen et al. reported that 1.4 % and 16.2 % of GCS and GGS, respectively, were resistant to erythromycin [28]. Erythromycin resistance among GCS and GGS in Taiwan is even more common. Resistance has been seen in 41.7 % of GCS isolates and 53.3 % of GGS isolates reported [96].

Macrolide resistance continues to rise in both European and North American countries. A total of 2981 beta-hemolytic streptococci isolates were collected from Europe, Turkey, and Israel and 950 isolates from the United States. Current cumulative surveillance data, accounting for all beta-hemolytic streptococci groups, show 7.6 % resistance to clindamycin and 17 % resistance to erythromycin in European countries. This rate is increased in the United States, with 19.4 % and 38 % resistance to clindamycin and erythromycin, respectively [29, 30] (Table 50.1).

4.1.2 Mechanisms of Macrolide Resistance in Beta-Hemolytic Streptococci

The macrolide resistance mechanism by ribosomal methylation encoded by *erm* genes was first identified in 1956 in *Staphylococcus aureus* [97]. This resistance mechanism affects macrolides, lincosamides, and streptogramin B (MLSb) antibiotics. The inducible and constitutive forms of MLSb resistance have been found in beta-hemolytic streptococci since the early 1970s [98–100]. The *erm*(B) methylase gene was the only *erm* gene class found in streptococci [101–103] until 1998, when the sequence of *erm*(TR) in *S. pyogenes* was published [104]. Its nucleotide sequence is 82.5 % identical to staphylococcal *erm*(A) and 58 % identical to *erm*(B) and, therefore, *erm*(TR) belongs to *erm*(A) methylase gene class [105]. The inducible or constitutive production of the methylase is dependent on the sequence of the regulatory region situated upstream from the structural methylase gene. Resistance is associated to structural changes in the regulatory sequence. Exposing *S. pyogenes* harboring the inducible *erm* gene to clindamycin results in highly resistant mutants of *S. pyogenes* [106].

The phenotypic expression of macrolide resistance in streptococci has been commonly studied by MIC determinations and induction tests including the double-disc test (erythromycin and clindamycin disks placed in vicinity on inoculated agar). Analysis of the Finnish GAS strains isolated in 1990 revealed a new erythromycin resistance phenotype with low- or moderate-level resistance (MIC range 1–32 mg/L) to 14- and 15-membered macrolides only

(M-phenotype). Thirty-four percent of the studied isolates represented the new M-phenotype [80]. Subsequently, the active efflux mechanism causing this phenotype and the encoding *mef*(A) and *mef*(E) (macrolide efflux) genes were characterized in *S. pyogenes* and *S. pneumoniae* [33, 107]. Isolates with this mechanism have been found among beta-hemolytic streptococci in different parts of the world. Countries where strains of GAS carrying *mef*(A) have been observed now account for the majority of macrolide-resistant isolates. These countries include Spain [108, 109], Germany [110], Greece [111], Finland [112], Taiwan [113], the United States [114], Chile [115], and Argentina [116]. Predomination of GAS strains carrying *erm*(A) have been reported from Russia, Slovakia, the Czech Republic, and Croatia [117, 118]. GBS isolates with MLS resistance caused by *erm*(B) and *erm*A predominate in most reports in Canada and other parts of the Western Hemisphere [92, 119], France [88, 89, 120], Spain [86, 121], and Taiwan [87] In both GBS and GCS, the highest proportion of isolates carrying *mef*(A) have been reported from Taiwan (37 %) and Finland (95 %) [95, 96].

In addition to familiar macrolide resistance determinants including *erm*(B), *erm*(A), and *mef*(A), a more rare mechanism has also been shown to cause resistance to macrolides. This mechanism involves mutations in the *S. pyogenes* ribosomal protein L4 and in positions 2611 and 2058 of the 23S rRNA encoding gene. Mutations in positions 2611 and 2058 of the 23S rRNA gene cause resistance to clindamycin and streptogramin B (quinupristin). Additionally, a mutation at position 2058 confers resistance to telithromycin [122–124].

The presence of a putative novel efflux system associated with *erm*(TR) in *S. pyogenes* has also been found [125]. Another gene, *mre*A, which was originally described as a macrolide efflux gene in *S. agalactiae* [107], encodes riboflavin kinase and is also found in erythromycin-susceptible GBS strains [126]. Strains with two different macrolide resistance mechanisms (*mef* and *erm*) within a single bacterial cell may coexist among GAS and more commonly among GBS [88, 94, 108, 126–129]. The phenotype of these strains is usually determined by the *erm* gene.

Resistance gene *erm*(B) has been shown to be either plasmid or chromosome associated in streptococci [105]. In earlier studies conjugative plasmids with erythromycin resistance determinants were found from group A, B, C, and G streptococci and were shown to transfer by conjugation between streptococcal species [130]. Transfer was also seen by transduction among VGS [131, 132]. However, most antibiotic resistance genes in streptococci are currently thought to be chromosomal in origin. Beta-hemolytic streptococci belonging to groups A, B, C, and G have been shown to transfer their chromosomal macrolide resistance determinants by conjugation [126, 133–135]. A composite chromosomal conjugative element, Tn3701, encoding resistance to erythromycin and tetracycline has been described in GAS [136]. Within this element the resistance genes are carried by a Tn*916*-like transposon. The presence of Tn916-Tn1545-like conjugative transposons carrying *erm*(B) and *tet*(M) has been verified, and an association of chromosomal *erm*(A) with *tet*(O) has been noted among GAS [137, 138]. An unusual chimeric genetic element containing DNA identical to *Tn*1207.1, a transposable element carrying *mef*(A) in macrolide-resistant *S. pneumoniae*, has also been found in different GAS strains. The mechanism of horizontal transfer in these strains was suggested to be transduction [139]. Furthermore, analysis of the genetic environments of the *mef*(A) and *erm*(B) genes by Southern blot experiments have indicated a remarkable heterogeneity of genetic elements carrying these genes, particularly *erm*(B). This suggests that different mobile elements can be recruited into the chromosomes of the circulating GAS population and that genetic rearrangement may also occur after a strain has acquired the resistance determinant [138]. Macrolide resistance mechanisms differ among streptococcal Lancefield groups and geographical area. New gene sequences demonstrating resistance continue to evolve.

4.1.3 Epidemiology of Macrolide-Resistant Beta-Hemolytic Streptococci

A large variety of clones of GAS are drug resistant [113, 138, 140, 141]. Increased resistance rates may be caused by clonal spread of resistant strains and by horizontal transfer of resistance determinants among the circulating microbial population. Macrolide-resistant GAS of the same clone have been found from different countries and even different continents [140]. Same clones have been found among susceptible isolates as well, but in general the heterogeneity of GAS clones seems to be lower among resistant than susceptible isolates [138, 140, 141]. Single clones of GAS with a macrolide-resistant determinant may become predominant or cause outbreaks both regionally and nationwide [128, 142–144]. For example, in 1994, 82 % of erythromycin-resistant GAS isolates collected in Finland expressed the M-phenotype. Although multiple clones were found among these isolates, increased regional resistance rates were clearly associated to T4M4 serotype with *mef*(A) [112, 134]. In the United States, isolates carrying *mef*(A) of an emm6 (M6 serotype) clone caused an epidemic among schoolchildren in 2001. In April–May of 2002, this serotype was not found in the same region when the resistance rate was high. Thirty-five percent of isolates were resistant to erythromycin, with an emm75 (M75 serotype) clone predominating [114, 143]. Cresti et al. found that a steady increase of erythromycin-resistant GAS from 9 % in 1992 to 53 % in 1997 in an area in central Italy was caused by an increase of the proportion of strains carrying inducible and constitutive *erm*(B) and *erm*(TR) determinants. These strains were of multiclonal origin. Correlation of the

erythromycin-resistant GAS clones to the heterogeneity of genetic elements carrying the *erm*(B) indicated identical genetic environments of *erm*(B) in clonally unrelated strains, but on the other hand also considerable diversity of these genetic elements both among clonally unrelated and within clonally identical strains [138]. The increase of resistance includes a complex genetic interaction within circulating streptococcal population and may be between streptococci and other species [145]. Macrolide consumption, differing immunities, and other host factors of populations may also contribute to this interplay and spread of resistance determinants and resistant clones [146–148].

4.1.4 Resistance to Clindamycin

Clindamycin resistance is almost exclusively related to MLS resistance found in beta-hemolytic streptococci. It is thus mediated by *erm* genes. In some studies, among GBS, the frequency of clindamycin resistance exceeds that of macrolide resistance suggesting another mechanism of clindamycin resistance may exists [86, 93, 149]. In one isolate of GBS from Canada, the *linB* gene encoding a lincosamide-inactivating nucleotidyltransferase was found [92]. This gene has previously been identified in *Enterococcus faecium*.

Both constitutive and inducible clindamycin resistances have increased in recent years, especially in group A and B streptococci [150]. Inducible clindamycin resistance in beta-hemolytic streptococci remains an under-recognized phenomenon of unknown clinical significance. Lewis et al. evaluated inducible clindamycin resistance through an animal model and retrospective patient chart review. In the animal model, inducible resistance impaired killing of beta-hemolytic streptococci and bacterial load by 48 h were similar to the control isolated that were constitutively clindamycin resistant. Eight of these cases resulted in both microbiological and clinical failure [151]. Thus, inducible and constitutive resistance should be detected during routine antimicrobial susceptibility testing.

European surveillance data demonstrated a clindamycin MIC_{50} and MIC_{90} of ≤0.25 mg/L with 91.9 % susceptible isolates, 0.5 % intermediate, and 7.6 % resistant [29] (Table 50.1). US data suggests a similar MIC_{50} of ≤0.25 mg/L and a MIC_{90} of >2 mg/L. Eighty percent of the 960 beta-hemolytic streptococci isolates were susceptible. Susceptibility rates in other commonly used macrolides tend to be lower [30].

4.1.5 Resistance to Erythromycin

Increased levels of erythromycin resistance in GAS have been reported in Europe. The mechanisms of erythromycin resistance in *S. pyogenes* include target site modification and active drug efflux. Target site modification is mediated by an erythromycin resistance methylase, encoded by an *erm* gene, which reduces binding of macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics to the target site in the 50S ribosomal subunit. Resistance in other beta-hemolytic streptococci groups can also be seen with recent surveillance data suggesting MIC_{50} and MIC_{90} values of ≤0.25 mg/L and >2 mg/L, respectively [29] (Table 50.1). MIC values in the US deviate from those found in other countries. Recent data report MIC_{50} values of ≤0.12 mg/L and MIC_{90} of >16 mg/L. Resistance is high with 60 % of isolates susceptible to erythromycin [30].

4.1.6 Resistance to Telithromycin

Resistance to telithromycin is currently uncommonly (<6 %) rare [152]. Few resistant strains have been isolated to date. This is due to either a constitutively expressed *erm*(B) gene or an adenine to guanine mutation at position 2058 [43, 124].

4.2 Resistance to Tetracycline

Resistance to tetracycline is common among beta-hemolytic streptococci, especially among macrolide-resistant strains. Resistance is caused by tetracycline resistance ribosomal protection proteins encoded by *tet*(M) or *tet*(O). The *tet*(M) gene is the most widely distributed and is found in GAS often in linkage with *erm*(B) on mobile elements [137]. In GBS, it is found both among macrolide-susceptible and macrolide-resistant organisms with all different macrolide resistance determinants [127]. *Tet*(O) has been found in GAS carrying chromosomal *erm*(A) or *mef*(A), and it can transfer with or without *erm*(A) and with *mef*(A) [137]. Surveillance data shows a tetracycline MIC_{50} of 4 mg/L and MIC_{90} of >8 mg/L. Of 2981 beta-hemolytic streptococci isolates tested, approximately 50 % were susceptible and 50 % resistant [29] (Table 50.1).

5 Clinical Significance of Resistance

5.1 Infections Caused by VGS

VGS are a part of the normal flora and can be found in the oropharyngeal, urogenital, and gastrointestinal microbiota. They are generally considered to have a low pathogenic potential and, however, can cause disease in immunocompromised patients as well as patients with cardiac abnormalities. As antibiotic resistance continues to rise, VGS infections are associated with significant morbidity and mortality [4]. Though other infections have been noted, this review will focus on the two predominate clinical presentations of VGS infections: infective endocarditis (IE) and neutropenic fever. It will also highlight rising challenges associated with resistance in treatment of cystic fibrosis.

5.1.1 Infective Endocarditis

Infective endocarditis most frequently presents acutely, and complete history and physical examination should be performed for source identification. The diagnosis is based off a combination of factors and may be straight forward with culture-positive endocarditis. Viridans streptococci are a common causative agent. Among 2781 patients with infective endocarditis, VGS was the underlying pathogen in 17 % of patients [153]. Several different VGS species have been reported to cause infective endocarditis, a life-threatening condition [154]. Of the VGS, *S. bovis*, *S. sanguis*, *S. mitis*, *S. oralis*, and *S. gordonii* remain some of the most common species isolated from blood or infected valves in both adults and children [66, 155, 156]. Infective endocarditis caused by S. *mitis* is a relatively common event and is empirically treated with penicillin or macrolides in immunologically stable patients. The etiology of infective endocarditis varies according to the age of the patient and the clinical nature of the disease [154, 155, 157, 158].

In adults, the epidemiology of IE caused by VGS is changing. From 1987 to 2009, the mean age of patients with native-valve endocarditis increased from 38 ± 22 years to 60 ± 16 years ($P < 0.001$). The proportion of IE cases without predisposed heart disease has progressively increased from 25 % to 67 % ($P < 0.001$) [159]. Other risk factors include dental infection as well as injection drug use, although VGS does not play a significant role in IE among intravenous drug users [155]. Although less virulent than other microorganisms, VGS continues to be the predominant cause of community-acquired IE. VGS and *Streptococcus bovis* account for 40–60 % of native-valve endocarditis in the community. In children, VGS was noted as the most common cause of IE, accountable for 32–43 % of cases [4].

Historically VGS were susceptible to many commonly administered antimicrobials including beta-lactams, macrolides, tetracycline, and aminoglycosides. As noted in the section above, there has been an increase in resistance including multiple-drug-resistant strains of *S. mitis* among patients with bacteremia. As with other pathogens, drug resistance in VGS is most clinically prevalent in patients with immunocompromised conditions. This is likely a result of exposure to hospital settings where resistant organisms are present or patients have increased exposure to multiple courses of antibiotics.

For treatment and prophylaxis, penicillin is an important antibiotic in treating VGS infections though resistance continues to present a clinical concern. A recent survey of children with Gram-positive cocci isolated in North America showed that of 182 VGS, 28.6 % were nonsusceptible to penicillin, 4.9 % of which were fully resistant [4].

Treatment recommendations depend on susceptibility patterns. The treatment recommendation per The American Heart Association (AHA) guidelines for adults with native-valve infective endocarditis caused by highly penicillin-susceptible (MIC ≤ 0.12 mg/L) VGS is intravenous penicillin G. Among the elderly, penicillin or ceftriaxone for 4 weeks is preferred. Uncomplicated episodes can also use gentamicin in combination with penicillin or ceftriaxone for 2 weeks. Patients with penicillin allergies can usually be treated with ceftriaxone; however, if patients experience immediate hypersensitivity, vancomycin for 4 weeks may be considered. Susceptibility testing of pathogens as well as repeat cultures is recommended [153].

Intermediate susceptibility is defined as MIC >0.12 mg/L and ≤0.5 mg/L. AHA guidelines recommend the same treatment as penicillin-susceptible *Streptococcus* with the addition of gentamicin in the first 2 weeks of the 4-week course. This combination has been demonstrated to be synergistic against VGS [160]; however, higher doses of penicillin and longer treatment times (4–6 weeks) are recommended [66, 160, 161]. As before, vancomycin should be considered for penicillin-allergic patients. Bacterial eradication rates greater than 98 % can be anticipated in patients who complete appropriate therapy [162]. Fully resistant strains have MICs >0.5 mg/L and recommended treatment is intravenous gentamicin for 4–6 weeks plus intravenous penicillin (4–6 weeks), ampicillin (4–6 weeks), or vancomycin (6 weeks) [153].

In recent years, beta-lactam and macrolide resistance rates among clinically isolated VGS have increased. Antimicrobial susceptibility testing for beta-lactams and macrolides suggest that mutated PBP genes in combination with the acquisition of certain macrolide resistance genes may underlie a broader resistance phenotype [41]. This is a challenge because there is limited clinical data to support alternative regimens to optimize endocarditis treatment for penicillin-resistant VGS. However, options are available and antidotal data is presented below.

The majority of VGS strains tested are susceptible to vancomycin [66, 160]. There are reports where vancomycin alone and vancomycin used in combination with ceftriaxone and gentamicin have been successfully used for treatment of endocarditis caused by resistant VGS [163, 164]. Treatment of penicillin-resistant VGS can present a more challenging clinical picture. One case showed that vancomycin treatment alone or in combination with cefotaxime and gentamicin did not completely eradicate a highly penicillin-resistant *S. mitis* strain in a human immunodeficiency virus positive man with endocarditis [165]. Vancomycin and gentamicin in combination also failed to cure endocarditis caused by highly penicillin-resistant *S. sanguis* in a 65-year-old woman with multiple medical problems. [166]. Though case reports tend to be biased toward negative outcomes, these data do demonstrate the need for new therapeutic options.

Additional antibiotics with demonstrated in vitro activity against VGS isolates include levofloxacin, moxifloxacin, quinupristin/dalfopristin, linezolid, and daptomycin. Though rare, in vitro resistance has been documented for these

antimicrobials as well. As with other antibiotics, culture and susceptibility should guide treatment. Limited clinical outcome data exists for some of the other, newer antimicrobials though typical resistance mechanisms will play a role in these antibiotics as potential treatment options. Linezolid-resistant strains are uncommon and it has been used successfully to treat endocarditis caused by vancomycin-resistant enterococci and methicillin-resistant staphylococci [71, 167]. However, oxazolinones are bacteriostatic antibiotics, and as a result their usage for treatment of infective endocarditis may be compromised [70]. Currently there is no information supporting the efficacy of linezolid in the treatment of endocarditis caused by VGS [38].

One case report of an immunocompromised patient with infective endocarditis revealed multidrug-resistant (MDR) VGS as the causative pathogen. Recurrent cycles of therapy to treat bacterial infections throughout the patients' lifecycle could have resulted in the penicillin, cephalosporin, carbapenem, macrolide, and fluoroquinolone-resistant *S. mitis*. Due to multiple complications, the patient died from pulmonary thromboembolism [41]. Another case report of a levofloxacin-resistant *S. mitis* manifested into endogenous endophthalmitis in the setting of mitral valve endocarditis as the presumed source of infection. The patient fully recovered after 6 weeks of intravenous ceftriaxone therapy based on the 2005 treatment guidelines of the AHA for patients with native-valve endocarditis caused by viridans streptococcal isolates with a penicillin MIC of 0.12–0.5 mg/L [168].

Increasing numbers of penicillin-resistant VGS strains among normal microbiota may also challenge prophylactic treatment of infective endocarditis. Amoxicillin or ampicillin is the current recommendation for endocarditis prophylaxis [66]. The prophylactic use of these antibiotics may select for penicillin-resistant VGS strains among normal microbiota, and these strains may be able to cause infective endocarditis [165]. Clindamycin is recommended for prophylaxis for patients allergic to penicillin [66]; however, it should be noted that use of macrolides can also select for clindamycin-resistant strains among streptococci in the normal flora. Telithromycin is very active against VGS strains in the normal microbiota. Despite resistance patterns, penicillin continues to serve as a widely used classical antimicrobial agent in the treatment of infective endocarditis. In patients with infective endocarditis, among other diseases, continuous surveillance of VGS isolates is warranted and can help guide appropriate treatment.

5.1.2 Neutropenic Fever

Neutropenic fever is defined as an absolute neutrophil count of less than 1500 cells/μL with a single oral temperature of >38.3 °C (101 °F) or a temperature of >38.0 °C (100.4 °F) sustained for >1 h [169]. There have been changes in the etiology of bacteremia in febrile neutropenic patients, and infections are an important cause of morbidity and mortality among this population. Gram-negative pathogens was historically the primary cause; however, up to 70 % of bacteremia cases in neutropenic patients are now associated with Gram-positive bacteria [170–173]. Bacteremia is identified in 10–27 % of febrile neutropenic patients with hematologic malignancies, and 18–29 % of the bacteremia is caused by viridans streptococci. Possible reasons for this shift are use of prophylactic antibiotics, increased use of intravenous catheters, and aggressive chemotherapies resulting in prolonged neutropenia and mucositis [171, 172, 174, 175]. VGS are an important cause of bacteremia among neutropenic patients. One study assessed 528 episodes of bloodstream infections, 15 % of which were associated with neutropenia. Thirty-five percent of the blood stream infections were caused by Gram-positive pathogens, with VGS being the most frequent causative pathogen at 22 % [176]. The proportion of VGS as a cause of bacteremia ranges between 3 % and 30 % [56, 172, 173, 177–179]. *S. mitis* followed by *S. oralis* or *S. sanguis* are the most commonly isolated species [173, 179–182]. Bacteremia caused by VGS strains often originate from the oral mucosa [183, 184]. Predisposing factors for VGS infections are severe and prolonged neutropenia, prophylactic antibiotic treatments with quinolones or trimethoprim-sulfamethoxazole, mucositis, and treatment of chemotherapy-induced gastritis with antacids or histamine type 2 antagonists [174, 182]. VGS infections can be rather asymptomatic, fever being the most common symptom [174, 181, 185–187]. Eighteen to 39 % of the patients with VGS infections develop serious complications, including septic shock, acute respiratory distress syndrome (ARDS), or both. Viridans streptococci are currently one of the most common pathogens in both adults and children, and bacteremia caused by this bacteria can result in death in up to 20 % of patients [188].

Multiple guidelines exist to combat neutropenic fever. Guidelines continue to be revised based on continued clinical evidence, experience, and advances in drug development. The Infectious Diseases Society of America's (IDSA) most recent update in recommendations in treatment of patients with fever and neutropenia discuss risk assessment. Once fever is detected, risk and severity infection should be assessed in order to help guide type, venue, and duration of empirical treatment. Updated European guidelines review the importance of appropriate initial antibiotic therapy in febrile neutropenia to minimize the collateral damage associated with antibiotic overuse and the further selection of drug-resistant pathogens. The guidelines suggest that infection control procedures and new antibiotic regimens based on local epidemiology, risk factors, escalation and de-escalation approaches, duration of empiric therapy, nonconventional therapies against MDR, and other bacterial management issues are vital to optimize antibiotic choice. For the purposes of this chapter, we will focus on IDSA-based recommendations.

Low-risk patients are defined as those having neutropenia for less than 7 days and no or few comorbid conditions. Oral empiric therapy is warranted in this population. In both low- and high-risk patients, empiric therapy should appropriately cover both Gram-positive and Gram-negative bacteria with special attention to VGS and *Pseudomonas aeruginosa* strains because infection may progress rapidly. Ciprofloxacin plus amoxicillin-clavulanate in combination is the treatment of choice, and antibiotic prophylactic treatment is not recommended in low-risk patients [189].

Per IDSA guidance, risk is affected by duration of neutropenia. High-risk is defined as neutropenia for greater than 7 days in duration with an absolute neutrophil count of ≤100 cells/mm [3] and/or significant comorbid conditions. For these patients, hospitalization and intravenous empirical treatment may be necessary. Preferred agents include an antipseudomonal beta-lactam, carbapenem, or piperacillin-tazobactam, although initiation of monotherapy with an antipseudomonal beta-lactam agent, such as cefepime, meropenem, imipenem-cilastatin, or piperacillin-tazobactam, may be used. Ceftazidime monotherapy has also been shown to be effective and continues to be used at some cancer centers. However, many experts avoid ceftazidime monotherapy because of rising resistance rates among Gram-negative bacteria and its limited activity against Gram-positive bacteria, such as streptococci, compared with newer alternatives [169]. Glycopeptides should be avoided first-line because of limited Gram-negative coverage, and empirical addition of vancomycin did not give extra benefit when compared to piperacillin-tazobactam therapy [190]. Regardless, the addition of this agent could benefit those with suspected catheter-related infection, skin or soft tissue infection, pneumonia, or hemodynamic instability [169].

For patients with methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), extended-spectrum b-lactamase (ESBL)-producing gram-negative bacteria, and carbapenemase-producing organisms, including *Klebsiella pneumoniae* carbapenemase (KPC), other agents may be added for adequate coverage. Fluoroquinolone prophylaxis should be considered for high-risk patients [191]. In multiple institutions, the use of quinolone prophylaxis in high-risk neutropenic patients is considered standard of care but the rapid development of resistance on therapy is a concern. Garnica et al. analyzed outcomes associated with quinolone prophylaxis and observed fewer episodes of febrile neutropenia and bacteremia, shorter duration of antibiotic therapy and hospitalization, as well as increased use of carbapenems and higher rates of quinolone resistance [192]. Although IDSA, among other treatment guidelines, recommend beta-lactam therapy as drug of choice, it is uncertain whether these practice guidelines can be applied to both adults and children due to potential differences in complication frequencies and antibiotic susceptibilities.

Han et al. compared clinical characteristics and antibiotic susceptibility patterns in patients with bacteremia caused by VGS in febrile neutropenic adults and children. Among the 202 episodes of viridans streptococcal bacteremia in adults and children, there were 20.8 % of cases with severe complications including 6.9 % identified deaths. Approximately 13 % of these episodes were attributable to bacteremia caused by VGS. Susceptibility testing results demonstrated that 80 % of the 199 isolates were susceptible to cefepime, and there was no association with patient age and pathogen susceptibility. This data suggests that it may not be necessary to adjust treatment guidelines between adults and children. In pediatric cancer patients, VGS strains are predominantly *S. mitis* and *S. oralis. S. mitis is* the most frequent VGS species causing bacteremia and is also most likely to be penicillin resistant [188].

Antimicrobial resistance in streptococci is rising. Studies have shown penicillin resistance is greater than 50 % and imipenem resistance is up to 25 % of *Streptococcus* from bone marrow transplant recipients [171]. As a result, some institutions may include vancomycin in the initial empiric treatment of febrile neutropenia. Studies have shown increased mortality in patients with viridans streptococcal bacteremia when vancomycin was not included in the initial empiric regimen [173]. More recently, Shelburne et al. developed a clinical prediction model for beta-lactam resistance in VGS causing bloodstream infection. The study validates use of Gram-positive spectrum antibiotics, including vancomycin, for empiric therapy of febrile neutropenia. Several assumptions were made including the definition of penicillin non-susceptibility, an MIC value ≥2 mg/L, increased risk of shock syndrome, and mortality. It was also assumed that vancomycin administered at onset of fever in neutropenic patients with VGS bacteremia will improve outcomes. Beta-lactam use in the prior 30 days, beta-lactam prophylaxis, and inpatient status at onset of febrile neutropenia correlated with a predicted MIC value ≥2 mg/L and non-susceptibility. It was determined in this one study that glycopeptides can be safely deferred until documentation of a resistant Gram-positive bacterial infection is made, despite IDSA guidelines stricter criteria [193].

5.1.3 Cystic Fibrosis

Cystic fibrosis (CF) is an inherited condition which affects the cells that produce mucous, sweat, and digestive secretions. Secretions become thick and plug passageways in the lungs and sinuses. Bacteria can adhere to this thick mucus and result in sinusitis, bronchitis, and pneumonia. Although CF has no cure, antibiotics are a staple in the treatment and prevention of lung infections. Evidence suggests S. *anginosus*, among other VGS organisms, may be important pathogens in this population. Recent studies have compared resistance patterns in CF and non-CF patient populations and have shown that both penicillin- and erythromycin-resistant VGS isolates in fibrotic

patients have reached 38.4 % and 87.9 %, respectively. Among CF isolates, resistance rates are increasing as patients are living longer and continuously face antibiotic exposure. Moreover, as the physiology of the lung is affected in these patients, so is drug penetration into their lungs. This may result in suboptimal drug concentrations at the site of infection, leading to increased selection of resistance [194].

5.2 Infections Caused by Beta-Hemolytic Streptococci

Beta-hemolytic streptococci are causative of a wide-range of diseases, both invasive and noninvasive. Some of these include streptococcal pharyngitis, neonatal sepsis, endocarditis, meningitis, and urinary tract infections. For the purpose of this review, we will focus on two clinically relevant presentations: pharyngitis and neonatal sepsis.

5.2.1 Pharyngitis

Severity of pharyngitis may vary but is traditionally defined by discomfort and pain in the throat, making it difficult to swallow. It is caused by swelling in the pharynx and may be bacterial in nature. Five to 15 % of pharyngitis cases are caused by GAS [195]. Penicillin is the drug of choice for treatment of streptococcal infections and macrolides are considered as alternative treatment for patients allergic to penicillin. Susceptibility testing should be used to confirm treatment choice and repeated cultures should be monitored for resistance development while on therapy.

Treatment eradication rates are associated with pathogen susceptibility. Specifically, studies have demonstrated the eradication rate is only 38–60 % when macrolides are used to treat macrolide-resistant strains in comparison to an eradication rate of 80–92 % when these agents are used against macrolide-susceptible organisms. [196–198]. The use of a macrolides for the treatment of macrolide-resistant GAS pharyngitis is also associated with a significantly lower clinical cure rate compared to that achieved with amoxicillin, amoxicillin-clavulanate, or cefaclor [198]. Again, emphasizing the importance of culture and susceptibility results in treatment selection. A recent study has also shown *erm* and *emm* 90 to be important resistance genes in invasive GAS [199]. Few resistant strains exist and the knowledge of resistance and resistance mechanisms is important. For example, use of clindamycin against an erythromycin-resistant isolate requires knowledge of the result of both the susceptibility testing and the determination of the macrolide resistance phenotype for a given isolate, because clindamycin should not be used to treat isolates with the MLSb-phenotype [106].

There has been debate of the remarkable stability of penicillin susceptibility in GAS and other beta-hemolytic streptococci and whether these high susceptibility rates will remain stable. Resistance to penicillin occurs in related species, such as *S. pneumoniae*, VGS, and enterococci at high rates. Reasons for the continued high susceptibility rates to penicillin in GAS include the inefficient mechanisms for genetic transfer in GAS, barriers to DNA uptake and replication, and the findings that altered PBPs expressed by penicillin-resistant laboratory mutants of GAS have defective cell-wall biosynthesis thus decreasing the viability of the penicillin-resistant organism [200, 201].

Beta-hemolytic streptococci, especially GAS and GBS, may cause serious infections and alternatives to macrolides are scarce. Limiting use of these agents should be encouraged [202, 203]. The selective pressure caused by the amount of macrolides used in the community has been shown to correlate to the level of macrolide resistance in GAS in the community, and reduction of use of these agents has been shown to lead in reduction of macrolide resistance [146–148, 202–204]. Macrolide-resistant GAS strains remain susceptible to telithromycin and therefore could be a better treatment option.

5.2.2 Neonatal Sepsis

GBS is the leading cause of neonatal infections and intrapartum antibiotic prophylaxis. Per guidelines, all pregnant women in the United States are screened and are prophylactically treated. For those at risk, intrapartum penicillin therapy is recommended, with ampicillin, clindamycin, erythromycin, and vancomycin as acceptable alternative treatments, with penicillin G being the drug of choice [205]. Previously considered a genitourinary pathogen, it has emerged as a non-nosocomial opportunistic pathogen causing serious clinical complications including bloodstream infection, endocarditis, and CNS infections. Sunkara et al. evaluated the epidemiology of GBS in nonpregnant adults. It was found that GBS is associated with younger age, higher incidence of beta-lactam allergy, and independently linked to immunosuppression. GBS are susceptible to commonly used antimicrobials including penicillins and cephalosporins and therefore are not associated with a delay in initiation of appropriate antimicrobial therapy. Resistance rates in second-line treatment options, including macrolides and clindamycin, continue to rise and should be closely monitored [206].

6 Conclusion

In this review, we discussed key global resistance data, including incidence and mechanisms of resistance. Beta-lactam resistance is primarily mediated by point mutations in penicillin-binding proteins (PBPs) and presents clinical challenges due to its role in treatment of infective endocarditis and neutropenic fever. Common macrolide resistance genes include *erm* and *mef*. Resistance with this class of antibiotics may be responsible for a variety of infections including phar-

yngitis and neonatal sepsis. Both resistance genes and mechanisms continue to evolve, and new sequences have been discovered in recent years. Antibiotic overuse, inappropriate antibiotic use, and delayed antibiotic administration are contributing factors to the rise in antibiotic resistance. Clinical studies and drug development continue to provide guidance and new treatment options; however, use of local antibiograms, implementation of infection control procedures, and antimicrobial stewardship are critical in treating patients with invasive streptococcal infections including VGS and beta-hemolytic streptococci.

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Enterococcal Infections in Adults

51

Katherine Reyes, Marcus Zervos, and Jisha John

1 Introduction

Enterococci (once called group D streptococci) were first described as human pathogens in 1899, historically thought to be endogenously acquired pathogens from human intestinal flora. *Enterococcus faecalis* is the most common pathogen, while *Enterococcus faecium* has become prevalent in hospital-acquired infections. Enterococci have resurfaced with reports of changes in epidemiology, treatment failures, and increasing complexity in antimicrobial resistance patterns. Enterococci have a variety of intrinsic antibiotic resistances and have shown the ability to acquire new resistance genes and mutations. Vancomycin resistance in enterococci is predominantly a healthcare-associated phenomenon. Urinary tract infections and bacteremia are the most common infections caused by enterococci, while endocarditis is the most serious. Enterococci are also commonly found in intra-abdominal, pelvic, and soft tissue infections, often part of a mixed flora. Less common infections are meningitis, osteomyelitis, and septic arthritis. Immunosuppression, long-term colonization, the ability to disseminate widely between patients, and the capacity to form biofilms have made enterococci major pathogens in hospitals, prompting enhanced efforts to identify optimal infection control measures and best therapeutic approaches to improve outcomes.

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2 Microbiology

Enterococci are gram-positive, facultative anaerobic cocci that are morphologically similar to streptococci, appearing on blood agar plates as gray colonies and are usually alpha-hemolytic (Fig. 51.1). Their ability to hydrolyze pyrrolidonyl-β-naphthylamide distinguishes them from certain streptococci species. For identification of newer species of enterococci, a combination of conventional biochemical tests and evaluation of DNA content is used. Twenty-three enterococcal species have been identified [1]. Sixty percent of isolates are *E. faecalis*, and 20 % are *E. faecium*. The others constitute less than 5 % of enterococcal infections, including *E. gallinarum*, *E. casseliflavus*, *E. avium*, *E. cecorum*, *E. durans*, *E. hirae*, *E. malodoratus*, *E. mundtii*, *E. pseudoavium*, and *E. raffinosus.*

Identification of enterococci to the species level is important because of resistant strains and the need to define appropriate therapy, not just for vancomycin-resistant enterococci (VRE) but also for vancomycin-susceptible enterococci (VSE). Most *E. faecalis* are susceptible to ampicillin but resistant to quinupristin-dalfopristin. Most *E. faecium* are resistant to ampicillin, susceptible to quinupristin-dalfopristin, and resistant to high levels of vancomycin. Newer tools to differentiate *E. faecalis* from other enterococcus species in blood samples include the *E. faecalis*/OE PNA FISH probe with claims of 100 % sensitivity and specificity over 90 min, and in a study, it was shown to provide the species information 2.3 days earlier than traditional phenotypic methods [2]. Pulsed-field gel electrophoresis and spa typing are used for delineation of the relatedness of strains, and polymerase chain reaction methods are used for evaluation of the possibility of gene dissemination.

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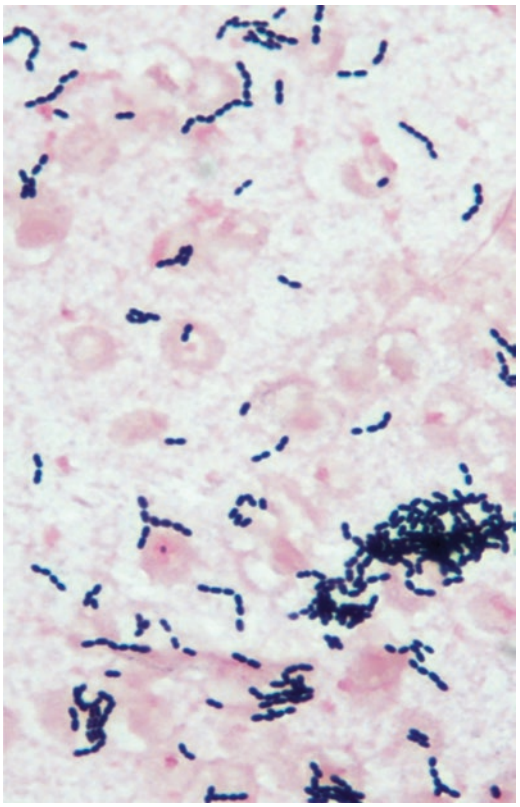

Fig. 51.1 Gram stain of *E. faecalis* in a cultured blood sample (Courtesy of Robert Tibbets, PhD.)

3 Epidemiology

Enterococci are colonizers and can survive for long periods of time in the environment. Vancomycin-resistant strains have been endemic in large hospitals with epidemics reported as well. Risk factors for colonization with VRE include antibiotic use during admission, surgery, dialysis, and discharge to another healthcare facility [3]. A systematic review showed a model-estimated median time of 26 weeks to clear colonization with VRE [4]. Asymptomatic colonization of the gastrointestinal tract is more common than is clinically recognized infection by a ratio of 10:1.

The National Healthcare Safety Network [5, 6] ranks enterococci as the second most common organism (after staphylococci) isolated from hospital infections. *E. faecalis* remains most common, but the incidence of *E. faecium* has increased. Hospitalization rates for VRE infection doubled from 2003 to 2006 [7]. The most important risk factor for VRE infection is prior antimicrobial use, with vancomycin use being both an independent risk for infection and mortality in patients with enterococcal bacteremia. Other risk factors for VRE infection include severity of illness, admission to the intensive care unit, prolonged hospital stay, exposure to other patients and contaminated surfaces with VRE, presence of invasive device, and residence in long-term care facilities. A single-institution study showed that each intensive care unit day increased the risk of acquiring VRE *faecium* by a factor of 1.03, with incidence of 21.9 newly acquired VRE cases per 1000 patient days [8]. VRE *faecalis* has been shown to occur in the community, with risk factors including non-home residence, chronic skin ulcers, previous invasive procedures, exposure to antibiotics, and presence of indwelling devices [9].

The epidemiology of spread of antimicrobial resistance is complex. It includes not only patient-related risks but also clonal transmission of strains between patients, and plasmid and transposon dissemination of resistance determinants. VRE has been shown to co-colonize or co-infect patients with methicillin-resistant *Staphylococcus aureus* [10]. Though VRE *faecalis* are relatively uncommon, isolates containing Inc18 plasmids and the vancomycin resistance transposon Tn1549 have been identified as precursors for vancomycin resistance in *S. aureus.*

4 Pathogenesis and Resistance Mechanisms

Known pathogenic determinants for enterococci include aggregation substances, cytolysis, collagen-binding proteins, *E. faecalis* endocarditis antigen, enterococcal surface protein, gelatinase, hyaluronidase, and the stress response protein G1s24 [11]. High-level gentamicin resistance (minimum inhibitory concentrations (MICs) of 600–2000 mg/ml) in enterococci is usually due to the presence of the "bifunctional" aminoglycoside-modifying enzyme [12]. Vancomycin resistance, in turn, is due to the synthesis of modified peptidoglycan precursors that have decreased affinity for vancomycin [13]. There are five major types of vancomycin resistance [14] in enterococci. VanA is the most predominant strain in the United States, Europe, and Korea. VanB retains susceptibility to teicoplanin and is predominant in Singapore and Australia [15]. VanD has low-level resistance to vancomycin and may be susceptible or intermediate resistance to teicoplanin. VanD and vanA have the same clinical and microbiological characteristics and that vanD isolates may convert to vanA phenotype after exposure to glycosides [16]. Linezolid resistance has been associated with G2576T mutations [17]. Quinupristin-dalfopristin resistance has been linked to agricultural use of streptogramin [18]. Daptomycin-non-susceptible enterococci have been associated with prior exposure to daptomycin and vancomycin resistance [19]. Infections due to enterococcal strains that demonstrate in vitro susceptibility to daptomycin but fail daptomycin therapy have been associated with vancomycin heteroresistance, cardiolipin synthase mutation, and liaF codon deletions [20–22].

5 Clinical Syndromes

5.1 Urinary Tract Infections

Urinary tract infections (UTIs) are the most common infections caused by enterococci and are usually associated with urinary catheters [23]. Among catheter-associated UTIs reported to the National Healthcare Safety Network, *E. faecalis* ranks fifth, while *E. faecium* ranks tenth among frequent pathogens [6]. It is necessary to distinguish colonization from infection, and lower tract from upper tract UTI. Diagnosis is made in the presence of signs and symptoms of infection and concurrent findings in urinalysis and quantitative urine culture. Enterococcal UTIs not accompanied by bacteremia generally require only single drug therapy, with oral agents when possible. If the organism is susceptible, amoxicillin is the drug of choice. Other oral agents include nitrofurantoin and fosfomycin which have intrinsic activity against most urinary enterococcal isolates including VRE strains [24]. Vancomycin can be used if the organism is ampicillin resistant. Linezolid or daptomycin are alternatives if the enterococcus is resistant to both ampicillin and vancomycin, and in the presence of upper UTI or bacteremia [25]. Tigecycline does not achieve good urinary concentrations and should not be used. A quinolone with a low MIC for a particular isolate may be considered as an alternative. The susceptibility of isolates to chosen agents must be determined.

5.2 Meningitis

Infections of the central nervous system, due to enterococci, are rare and are associated with older age, serious underlying diseases, presence of other sites of enterococcal infection and colonization, and indwelling central nervous system devices. Fever and changes in mental status are common presenting symptoms. The cerebrospinal fluid usually demonstrates low-grade neutrophilic pleocytosis, mild hypoglycorrhachia, elevated protein, and gram-positive stain and cultures [26]. Combination therapy is often used to achieve maximal bactericidal activity [27]. Intraventricular and intrathecal therapy are also commonly used. Antimicrobials used include penicillin or ampicillin, chloramphenicol, quinupristin-dalfopristin, daptomycin, and linezolid. Gentamicin and rifampin are used in combination regimens. Removal of an indwelling central nervous system device if present is recommended.

5.3 Intra-abdominal Infections

Enterococci when present in intra-abdominal infections are usually part of a polymicrobial infection, in which coverage for enteric bacteria and anaerobes must also be empirically administered. It used to be not necessary to routinely provide antimicrobial coverage for enterococci in the setting of intra-abdominal infection where more virulent organisms are present; however, due to increase in nosocomial infections and complexity of patients, anti-enterococcal therapy is now considered for hepatobiliary or pancreatic infections, immunosuppressed patients, and those at risk for enterococcal bacteremia. VRE enteritis after severe acute pancreatitis has been reported [28].

5.4 Skin and Soft Tissue Infections

The presence of enterococci in soft tissue cultures presents a challenge in differentiating colonization from infection. Co-colonization and/or co-infection of VRE and methicillin-resistant *S. aureus* are reported from chronic wounds, in which prior antimicrobial use is the most common risk factor. The National Healthcare Safety Network reports enterococci to cause one-third of surgical site infections after transplant surgery [6].

5.5 Bacteremia and Infective Endocarditis

Enterococcal bloodstream infections (BSI) rank second among healthcare-associated BSI [5] and are commonly associated with intravascular catheters and other devices. Also common sources of enterococcal bacteremia are hepatobiliary and genitourinary tract infections and to a lesser degree skin and soft tissue infections. The clinical presentation of enterococcal BSI varies depending on the source, and if a patient presents with shock, a workup for co-infection with more virulent organisms or a deep-seated infection source should be made. *E. faecalis* was previously a much more common BSI pathogen than *E. faecium*; however, *E. faecium* is now isolated at a rate approaching that of *E. faecalis*, with a ratio of 1 *E. faecium* for every 1.8 *E. faecalis* in blood cultures [5, 29]. Vancomycin resistance among enterococcal isolates has increased with consequent increase in the rate of VRE bacteremia from 0.06 to 0.17 infections per 1000 patient days [30]. VRE BSI when compared to VSE BSI has higher recurrence rates, mortality, and costs [31]. To distinguish endocarditis as the source of enterococcal bacteremia, a scoring system was proposed to determine the need for trans-esophageal echocardiography: heart murmur, 1 point; prior valve disease, 2 points; unknown source of bacteremia, 4 points; and continuous bacteremia, 5 points (abbreviation: NOVA, number of positive blood cultures, origin of the bacteremia, previous valve disease, auscultation of heart murmur). A NOVA score of $\geq$4 points indicates the need for trans-esophageal echocardiography [32].

Enterococcal infective endocarditis (IE) was once considered a community-acquired disease of older Caucasian men; however, now, it is more healthcare related. Enterococci are the third leading cause of IE and the second leading cause of healthcare-associated IE [5]. There is higher mortality and prolonged bacteremia with VRE *faecium* endocarditis compared with VRE *faecalis* endocarditis [2]. Risk factors for enterococcal IE include older age, male sex, immunosuppression, cancer, prosthetic valve, and presence of a central venous catheter. Older age, heart failure, and nosocomial acquisition are predictors of mortality. Enterococcal IE usually presents subacutely; the Infectious Diseases Society of America guidelines include the duration of symptoms to determine the duration of therapy [33]. *E. faecium* IE presents more frequently with stroke than *E. faecalis* [34]. The International Collaboration on Endocarditis reports that enterococcal IE is associated more commonly with heart failure than with embolic events and has an associated 1-year mortality of 28.9 % and that cardiac valve surgery is not associated with improved outcome [35]. Relapse rate from enterococcal IE is up to 3 % [15].

6 Therapy

The challenge in enterococcal antimicrobial management lies in the organisms' intrinsic resistance to numerous agents. Management of enterococcal infections includes aggressive control of the source of the infection, such as debridement of wounds and removal of invasive devices. There is no consensus whether to use monotherapy or combination therapy for enterococcal bacteremia, while it is the standard of practice to use combination therapy for endocarditis.

Cell wall-active agents inhibit enterococci. When susceptible, ampicillin is the drug of choice, and vancomycin is recommended only if the enterococcus is ampicillin-resistant and vancomycin-susceptible. *E. faecalis* can be resistant to ampicillin and penicillin when beta-lactamase production is present which usually occurs in infections with heavy burden of organisms similar to endocarditis. Nafcillin, ertapenem, aztreonam, and most cephalosporins are not active against enterococci. Ceftaroline has in vitro activity against *E. faecalis* but is inactive against *E. faecium* [36].

The Infectious Diseases Society of America recommends linezolid or daptomycin for catheter-related bacteremia caused by enterococci resistant to ampicillin and vancomycin [37]. For enterococcal endocarditis, bactericidal activity is achieved by a combination of beta-lactam antibiotics and also enhanced by combining beta-lactams and aminoglycosides [33]. For enterococci resistant to vancomycin, aminoglycosides, and penicillin, susceptibility testing for alternative antimicrobials, such as daptomycin and linezolid, is necessary.

The standard of care for enterococcal IE involves a 6-week course of combination therapy. For enterococci without high-level aminoglycoside resistance, the treatment regimen usually is ampicillin plus gentamicin; however, *E. faecalis* strains with high-level aminoglycoside resistance have increased resulting to the loss of the synergism between the aminoglycoside and the beta-lactams. An added challenge in combination therapy involving aminoglycosides is the high likelihood of nephrotoxicity [21], with prolonged therapy. One study reported that 2–3 weeks of an aminoglycoside (in combination with ampicillin) might be sufficient [38]. For *E. faecalis* endocarditis, ampicillin combined with ceftriaxone has been shown to be as effective as ampicillin combined with gentamicin, including for treatment of high-level aminoglycoside resistance strains [39, 40]. The combination of ampicillin with daptomycin has also been used for *E. faecalis* IE [41]. For patients who are at risk for gentamicin-associated nephropathy, alternative regimens to consider include the use of streptomycin instead of gentamicin, short-course gentamicin therapy (2–3 weeks), and use of a non-aminoglycoside-containing double beta-lactam regimen [33].

Treating *E. faecium* differs considerably from treating *E. faecalis* due to limited antimicrobial agents (Tables 51.1 and 51.2). Vancomycin resistance in *E. faecium* has been reported up to 80 % [5]. The Food and Drug Administration (FDA)-approved drugs for resistant enterococci include quinupristin-dalfopristin (approved in 1999) and linezolid (approved in 2000). Non-FDA-approved drugs include daptomycin and tigecycline.

The use of quinupristin-dalfopristin is limited by its adverse effects and the need for a central line. Quinupristin-dalfopristin has no activity against VRE *faecalis.*

Linezolid is a synthetic oxazolidinone agent that exhibits bacteriostatic activity against enterococci by inhibition of protein synthesis [42]. In a non-randomized program for linezolid, clinical and microbiological cure rates for enterococcal infections were 81 % and 86 %, respectively [43]. A study comparing linezolid and daptomycin for VRE bacteremia showed no significant difference in mortality [44]. Reports on linezolid for treatment of left-sided endocarditis, mostly as part of combination therapy and mostly for *E. faecalis*, show similar outcomes and good tolerability [45]. Linezolid has high oral bioavailability and may be given orally or intravenously. Toxicities associated with linezolid include development of thrombocytopenia, peripheral neuropathy, ocular involvement, and serotonin syndrome when taken with selective serotonin reuptake inhibitors. Resistance to linezolid has been reported in enterococci.

Daptomycin is a mainstay of therapy of VRE bacteremia because of its potent bactericidal activity in vitro [46]. Daptomycin is a cyclic lipopeptide that acts at the bacterial cytoplasmic membrane with multiple effects on cellular function including inhibition of lipoteichoic acid synthesis, dis-

Table 51.1 Suggested antimicrobial therapy for *Enterococcus faecalis*

E. faecalis	Antibiotic	Dose	Comments
Cystitis			
PCN-S	Amoxicillin	500 mg PO q6h	For cystitis
VRE	Nitrofurantoin	100 mg PO q6h	If isolate is susceptible
			Not for use in renal failure
	Fosfomycin	3 g PO × 1	If isolate is susceptible
Systemic infection, not endocarditis			
PCN-S	Ampicillin	3–4 g IV q6h	Adjust dose of ampicillin base on CrCl
	Penicillin G	3 million units IV q4h	
PCN-R	Vancomycin	15–20 mg/kg IV q8–12h	Monitor vancomycin serum trough levels
			Monitor CrCl
VRE	Daptomycin	8–10 mg/kg IV q24h	Monitor CPK
	Linezolid	600 mg PO/IV q12h	Monitor platelet count
Endocarditis			
PCN-S	Ampicillin plus	2 g IV q4h	PenG is also recommended 4–6 weeks duration
AMG-S	Gentamicin	3 mg/kg in 2–3 doses	To achieve serum
			Peaks of 3–4 μg/ml
			Trough <1 μg/ml
			Monitor CrCl
	Ampicillin plus	2 g IV q4h	Recommended for patients with impaired renal function
	Ceftriaxone	2 g IV q12h	Minimum of 6 weeks
PCN-allergy	Vancomycin	15–20 mg/kg IV q8-12h	Consider allergy consultation for desensitization
PCN-S or low-level resistance	Plus		Monitor vancomycin serum trough levels
AMG-S	Gentamicin	1 mg/kg q8h	Monitor gentamicin serum
			Peaks of 3–4 μg/ml
			Trough <1 μg/m
			Monitor CrCl
PCN-S	Ampicillin plus	2 g IV q4h	Adjust dose of ampicillin base on CrCl
AMG-R	Ceftriaxone	2 g IV q12h	Minimum of 6 weeks of therapy
VRE	Daptomycin +/−	8-10 mg/kg IV q24h	Monitor CPK
	Another antimicrobial		Use ampicillin or penG if susceptible

Note: PCN, penicillin; S, susceptible; AMG, aminoglycoside; R, resistant; VRE, vancomycin-resistant enterococcus; gm, grams; IV, intravenously; PO, by mouth; q, every; h, hours; UTI, urinary tract infection; IE, infective endocarditis; CrCl, creatinine clearance; CPK, creatinine phosphokinase

ruption of membrane potential, and inhibition of peptidoglycan synthesis. It displays rapid concentration-dependent killing and is bactericidal even for enterococci in the stationary phase of growth. The FDA has approved daptomycin in the United States for complicated skin and soft tissue infections at a dose of 4 mg/kg/day. Clinical trials of serious staphylococcal and enterococcal infections have used daptomycin at 6–12 mg/kg/day. Daptomycin has been reported to show synergism in vitro with aminoglycosides, rifampin, and beta-lactams against some VRE.

Issues that surround daptomycin for use in VRE infections include dosing regimens, treatment failures for isolates with high MICs to daptomycin, development of resistance on therapy, monotherapy versus combination therapy, and suitable secondary agents for combination regimens. A meta-analysis on VRE bacteremia comparing linezolid and daptomycin showed no difference in clinical and microbiological cure and adverse events [47], and another large retrospective study comparing daptomycin, linezolid, and beta-lactams did not show significant difference in mortality [48]. In a national retrospective cohort study, daptomycin was associated with better outcomes than linezolid for treatment of VRE BSI [49].

There is currently no standard recommended dosing for daptomycin for serious VRE infections. Studies using daptomycin above 6 mg/kg/day have shown clinical and microbiological success [50]. Recent evidence suggests clinical and microbiological benefit of adjunctive β-lactam therapy with daptomycin. For enterococcal blood isolates with daptomycin MIC of 3–4 mg/ml, daptomycin has shown to be less effective, suggesting concomitant beta-lactam therapy may improve clinical outcomes in this setting. The combination of ampicillin with daptomycin has shown to enhance the killing of VRE even in cases of VRE *faecium* resistant to ampicillin [51]. Daptomycin with ampicillin have been reported effective for *E. faecalis* endocarditis [41].

Table 51.2 Suggested antimicrobial therapy for VRE, usually *Enterococcus faecium*

VRE	Antibiotic	Dose	Comments
Cystitis			
	Nitrofurantoin	100 mg PO q6h	If isolate is susceptible
			Not for use in renal failure
	Fosfomycin	3 g PO × 1	If isolate is susceptible
Systemic infections, not endocarditis			
VRE	Daptomycin	10 mg/kg IV q24h	Monitor CPK
			Reports of resistance
			Developing while on
			Therapy
			+/− Another agent
	Linezolid	600 mg PO/IV q12h	Bacteriostatic Monitor platelet count
	Quinupristin-Dalfopristin	7.5 mg/kg IV q8h	Via central line
Endocarditis			
VRE	Daptomycin +/−	10–12 mg/kg per dose	Monitor CPK
	Ampicillin		Ampicillin has been shown to enhance daptomycin killing of VRE strains
	Linezolid	600 mg IV/PO q12h	Bacteriostatic
			Cardiac valve replacement may be needed for cure
	Quinupristin-dalfopristin	7.5 mg/kg IV q8h	Via central line
			Minimum of 8 weeks of therapy
			Combination therapy with ampicillin has been reported

Note: VRE, vancomycin-resistant enterococcus; AMG, aminoglycoside; S, susceptible; R, resistant; gm, grams; IV, intravenously; PO, by mouth; q, every; h, hours; UTI, urinary tract infection; IE, infective endocarditis; CrCl, creatinine clearance; CPK, creatinine phosphokinase

Ceftaroline in combination with daptomycin increases the daptomycin surface binding and also is associated with an increase in membrane fluidity and an increase in the net negative surface charge of the bacteria, resulting in increased binding and killing of daptomycin-non-susceptible VRE [52, 53]. Toxicities related to daptomycin include development of myopathy and eosinophilic pneumonia [54].

Tigecycline is a semisynthetic agent that is bacteriostatic and inhibits both *E. faecalis* and *E. faecium* at low concentrations [55]. Tigecycline is FDA approved in the United States for complicated skin soft tissue and intra-abdominal infections. The use of tigecycline for bacteremia and endocarditis is limited by inadequate serum drug concentrations. It has been used as part of combination therapy with linezolid [56] and daptomycin [57] with success, adding in vitro efficacy without demonstrating antagonism with other agents. Tigecycline is administered by intravenous infusion only and often causes nausea.

Newer agents against multidrug-resistant gram-positive bacteria include dalbavancin, telavancin, oritavancin, and teicoplanin [58]. Dalbavancin and telavancin are more potent than vancomycin against vancomycin-susceptible organisms. Dalbavancin, which is given once weekly, has shown non-inferiority to standard of care for bacteremia (it inhibits vanB type VRE at low concentrations but is not active against vanA type VRE). Telavancin, a cidal dual-action glycopeptide, is less active against VRE than against VSE, but MICs are lower than those of vancomycin against VRE [55]. Although telavancin is FDA approved, it is no longer available in the United States. Oritavancin, which has a longer half-life than dalbavancin, acts on both cell wall binding and biosynthesis and, thus, has potent in vitro activity plus synergistic activity with gentamicin for VRE but possible limitations due to protein binding [29, 59]. Teicoplanin has been shown to have activity against *E. faecalis* [60].

7 Endpoints of Monitoring Therapy

Duration of antimicrobial therapy for enterococcal infections depends on the site of infection and the clinical response. Treatment for simple UTI may require only a few days of oral or intravenous antibiotics. Bacteremia without endocarditis may require 10–14 days of antibiotics and typically depends on how quickly clinical and microbiological cures are achieved and if the source of infection is promptly removed. Echocardiography is of use in distinguishing endocarditis from bacteremia due to other foci. If the source of infection cannot be removed, such as central venous catheters that must remain in place or abscesses that cannot be drained, the duration of antimicrobial therapy may be longer. A 4-week therapy is recommended for native valve enterococcal IE, while 6 weeks is recommended for patients with

more than 3 months of symptoms or for prosthetic valve endocarditis. For endocarditis due to multidrug-resistant enterococcus, surgical valve replacement may be necessary.

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Antimicrobial Resistance in *Staphylococci aureus*: Mechanisms of Resistance and Clinical Significance

52

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Staphylococcus aureus has remained one of the most important human pathogens since the time it was discovered. The unique characteristics of the pathogen, virulence, resistance mechanisms, adaptability, and volatile nature have all been areas of interest for both basic and clinical scientists all over the world. Interestingly, despite being discovered more than two centuries ago and studied ever since, this bacterium remains a mysterious and challenging pathogen that is responsible for both substantial morbidity and major mortality. In this chapter we aim to discuss the basic epidemiology, clinical significance, resistance mechanisms, and treatment options for this pathogen and as well as the newer frontiers in infection control and prevention that are now developing.

1 Epidemiology

1.1 History

After the discovery of *Staphylococcus* in 1880 by the Scottish surgeon Alexander Ogston who was seeking the cause of suppuration [1, 2], it was further speciated by the German physician Friedrich Rosenbach who named it *Staphylococcus aureus* because of the golden pigment it produced on culture plates [3]. Over the next two centuries, this fascinating bacterium demonstrated its versatility as a pathogen both in terms of the diseases it causes and for the antibiotic resistance patterns that have emerged. Indeed, almost immediately after research began on penicillin in 1941, resistance was quickly demonstrated as early as 1942 [4]. Methicillin and vancomycin were approved for use almost simultaneously in 1958 and 1959, respectively. By 1961 the first methicillin-resistant *S. aureus* (MRSA) was reported [5]. Consequently vancomycin became the "go-to" drug for MRSA and remained so for more than half a century. Isolates with reduced susceptibility to vancomycin, called vancomycin intermediate *S. aureus* (GISA/VISA), strains were reported in 1996. The first truly vancomycin-resistant *S. aureus* (VRSA) was identified in 2002.

2 Incidence and Distribution

S. aureus is a major cause of infection throughout the healthcare spectrum, including the community as well as in the hospital setting. The true incidence of *S. aureus* infections is unknown. MRSA was initially a concern only in settings where patients were exposed to antibiotics and medical devices, becoming a widespread nosocomial pathogen by the late 1970s. However, by the early 1980s, a shift occurred, and the organisms frequently caused infections in patients who were not hospitalized and had no obvious reason for a MRSA infection. Gradually these strains became pandemic all over the world.

Although as noted, outbreaks of MRSA infections were reported from US hospitals in the late 1970s, and it was not until the 1980s that so-called healthcare-associated MRSA (HA-MRSA) became endemic in US hospitals [6, 7].

The 1990s witnessed the emergence of community-acquired MRSA (CA-MRSA) as a major pathogen in invasive infections. The most common epidemic clone reported in CA-MRSA infections in the United States is USA300 [8, 9]. The CA-MRSA has unique characteristics in regard to virulence and susceptibility when compared to HA-MRSA. From both an epidemiological and clinical point of view, the designations of healthcare-associated or community-associated have become less relevant due to mixing or combining of the genetic characteristics of the strains, making it likely a nosocomial infection could be caused by a CA-MRSA strain and a community-acquired infection could be due to HA-MRSA.

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Between 1995 and 2005, hospitalizations associated with MRSA infections in the United States more than doubled, mostly for treatment of skin and soft tissue infections and necrotizing pneumonia [10]. National Healthcare Safety Network (NHSN) data from 2003 showed that the methicillin resistance among *S. aureus* causing nosocomial infections increased to 60 % compared to the previous 5 years [11].

Emerging Infections Program/Active Bacterial Core Surveillance Activity (EIP-ABSC) at CDC started tracking invasive MRSA infections in 2005 in nine US metropolitan areas. Interestingly, although still a leading cause, recent reports show that the incidence of invasive blood stream infections from MRSA is on the decline [12]. Although the reasons are not entirely clear, an 11 % annual decline occurred in the incidence of hospital onset blood stream infections between 2005 and 2008 [13]. Overall, a 31 % decrease in the incidence of invasive MRSA infections was noted when compared to the 2005 incidence [14].

3 Colonization and Carrier State

Humans are considered the natural reservoir for *S. aureus*. Secondary analysis of data from the National Health and Nutrition Examination Survey (NHANES) showed that 31.6 % of the US population is colonized with *S. aureus*. The same data showed that the prevalence of MRSA nasal colonization in 2003–2004 was 1.5 %, up from 0.8 % in 2001–2002 [15, 16]. Additional sites of colonization are now recognized, with the throat, axilla, groin, perineum, and vagina considered important sites.

Risk factors for MRSA colonization include type 1 diabetes mellitus, hemodialysis or peritoneal dialysis, intravenous drug use, rheumatoid arthritis, and chronic sinusitis. In addition, patients receiving repeated injections for allergies, those infected with HIV and other primary immunodeficiencies, and even old age are also recognized risk factors [17, 18].

The exact risk of invasive infection among colonized patients is not clear but has been demonstrated in previous studies [19, 20].

4 Clinical Significance

S. aureus remains a major cause of community and healthcare-associated infections. The spectrum of diseases is extensive, including skin and soft tissue infections, pneumonia, blood stream infections, endocarditis, osteomyelitis, prosthetic joint, and other device-related infections. It is a major cause of prolonged hospital stay and adds to the healthcare expenditure [21, 22]. The mortality rate for MRSA exceeds that of MSSA and is in fact the highest from any infectious agent [10, 23, 24].

Recently concerns have been raised about increasing MICs to available antibiotics and the lack of appropriate validated tests and studies to guide antibiotic therapy. The Clinical Labs and Standards Institute (CLSI) decreased the breakpoint MIC for vancomycin susceptibility from 4 μg/mL to 2 μg/mL in 2006 [25], but many experts now recommend an even lower MIC cutoff for invasive infections [26]. As noted above, both vancomycin intermediate- and true vancomycin-resistant isolates have now been identified. Heteroresitance, in which a clone with vancomycin MIC between 4 and 8 μg/mL exists within apparently vancomycin-susceptible *S. aureus* (hVISA) colony, is another major concern because of lack of standardized methods for early identification [27]. It is recognized that the closer to the 2 μg/mL breakpoint, the greater the likelihood of encountering such hVISA strains [28]. The clinical significance of intermediate resistance is unclear, although studies do suggest a higher failure rate among patients treated for serious infections whose organisms possess such elevated MICs. Of even greater significance, data is increasingly accumulating that even within the susceptible range (MIC <2 μg/mL) failures of therapy are becoming common [29].

With increasing concern about the utility of vancomycin, clinicians have turned their attention to newer approaches. However, despite a plethora of recently approved antibiotics with activity against MRSA, the lack of significant advantage over vancomycin and the increasing resistance to even these newly developed antibiotics are of great concern.

Asymptomatic nasal carriage and colonization with MRSA, even among healthy individuals, could lead to persistence and spread of these organisms to vulnerable populations. Currently, there is not enough data to support universal screening and decolonization of healthy people in the community. However, screening and decolonization of healthcare workers (HCW) could have benefits although, in the absence of efficacy data, the concept is still being debated [30, 31].

As noted above, risk factors for MRSA colonization have been described; therefore, our best hope is to identify such high-risk populations and target them when planning infection control and preventive measures.

Spread of MRSA from food, the agriculture industry and even household pets is another area of major concern. Further studies are needed to understand the full clinical significance of this [32].

5 Mechanisms of Resistance

S. aureus has been described as one of the most versatile bacterium. It has been rightly called an evolving pathogen [33] in view of its adaptability and development of resistance to multiple antibiotic classes through a variety of different mechanisms. Much remains unclear about the mechanisms of resistance, but we will summarize what has been described thus far (see Table 52.1).

Table 52.1 Mechanisms of antimicrobial resistance

Antibiotic	Site of action	Mechanism of resistance	Responsible gene product	Genetic basis
Beta-lactams	Cell wall synthesis	(1) Hydrolyze the peptide bond in the beta-lactam ring and inactivate the drug	(1) Beta-lactamase (penicillinase)	(1) blaZ gene-plasmid
Penicillin		(2) Reduce affinity of binding to the PBP	(2) PBP2a	(2) mecA
Cephalosporin				
Carbapenem				
Glycopeptides	Cell wall synthesis	Vancomycin trapping in the thickened cell wall (VISA)	Thicker peptidoglycan layer with more exposed D-Ala-D-Ala residues	(1) Unclear-? agr group III polymorphism
Vancomycin		Alteration of cell wall precursor (VRSA)	Novel cell wall precursor ends in D-Ala-D-Lac	(2) Van A gene acquisition from VRE
Daptomycin	Cell membrane	Alteration of cell membrane charge	Increased L-PG (lysyl-phosphatidylglycerol) synthesis	mprF gene
Linezolid	Protein synthesis	Point mutation at 23S rRNA or 50 S L3/L4 ribosomal proteins	23S rRNA subunit target site changes	rrn gene
		Methylation of 23S rRNA subunit		cfr gene - plasmid mediated

6 Resistance to Beta-Lactams

Penicillin resistance was identified in *S. aureus* in 1942, quite immediately after the discovery of penicillin [34, 35]. It was first noted in the hospital setting followed by its appearance in the community setting. Penicillin binds to the penicillin-binding-proteins (PBPs) in the bacterial cell wall and blocks the ability of the PBPs to function, thereby inhibiting cell wall synthesis. *S. aureus* produces a penicillinase enzyme which inactivates the beta-lactam ring of penicillin by hydrolyzing the peptide bonds. This beta-lactamase is encoded by the -blaZ gene located on a plasmid that often carries additional resistance genes as well (erythromycin and gentamicin). More than 90 % of the staphylococcus isolates are now penicillin resistant.

Methicillin was introduced in 1961 as the first semisynthetic penicillinase-resistant penicillin, and reports of methicillin-resistant isolates immediately followed. The bacteria altered its cell wall content and acquired a new PBP, called penicillin-binding protein 2a (PBP2a or PBP2'), which has a lower affinity for all beta-lactam antibiotics. Accordingly, MRSA are inherently resistant to all cephalosporins and carbapenems. The PBP2a is encoded by the mecA gene that is part of a large mobile genetic element called staphylococcal cassette chromosome mec (SCCmec) [36].

It has been assumed that the mecA gene was acquired through transfer from coagulase-negative staphylococci (CONS) since no homologue of this gene exists in MSSA [37, 38]. Eleven SCCmec types have been identified to date [39].

SCCmec types 1, 4, and 5 are smaller subtypes and are only responsible for genes conferring resistance to methicillin. SCCmec subtypes 4 and 5 are smaller and are associated with community-associated CA-MRSA isolates; it has been speculated that the smaller size enhances mobility and ease of transfer between strains. SCCmec subtype 4 carries the gene responsible for production of Panton-Valentine leucocidin or PVL. A possible role for PVL in the virulence of CA-MRSA, especially in relation to serious skin-soft tissue infections and necrotizing pneumonia, has been proposed but has been challenged based on the results of recent animal studies [40].

CA-MRSA differs from HA-MRSA in regard to antibiotic susceptibility and virulence. CA-MRSA is generally considered susceptible to non-beta-lactam antibiotics (except macrolides) and appears to be more virulent. The predominant clones of CA-MRSA circulating in the United States are pulsed-field type USA300 followed by USA400. The typical USA300 clone isolate is susceptible to trimethoprim-sulfamethoxazole (TMP-SMX), clindamycin, and tetracycline and is resistant to erythromycin and gatifloxacin. Increasingly non-beta-lactam resistance has been described in USA300. Multidrug resistance in USA300 clones, conferred by large plasmids encoding resistance to mupirocin, macrolides, and clindamycin, have been described in men having sex with men in the San Francisco and Boston areas [41]. Up to 77 % of these isolates harbor chromosomally encoded resistance to ciprofloxacin as well.

SCCmec subtypes carrying additional genetic elements conferring resistance are found most notably in HA-MRSA [42].

Strains that are resistant to erythromycin occasionally possess inducible clindamycin resistance. These strains are typically identified by the phenotype of erythromycin resistance and clindamycin susceptibility. Therefore, when such isolates are found, CDC recommends further testing for inducible clindamycin resistance by the use of the D-test. SCCmec (type 2 and type 4) is most important in determining resistance, but its direct effect on virulence or invasiveness of MRSA is unknown [43].

7 Resistance to Vancomycin

Vancomycin was FDA approved in 1958 and has been the gold standard for treatment of MRSA infections ever since. In addition, due to its antimicrobial spectrum, it was increasingly used to treat a variety of other infections including *Clostridium difficile* diarrhea, MSSA infections, and enterococcal infections. Antibiotic selection pressure resulting from the widespread use of this slowly bactericidal glycopeptide resulted in emergence of resistance. Slow response to vancomycin treatment of invasive infections was reported [44] but was not considered due to reduced susceptibility. However, vancomycin intermediate-resistant *S. aureus* (VISA) infection was reported in 1996 from Japan and was followed by additional case reports [45]. Notably, this form of resistance has been associated with diminished clinical activity and treatment failures.

VISA strains were found to have increased thickness of the cell wall arising from changes in the peptidoglycan biosynthesis. These strains appear to have additional peptidoglycan in their cell wall with less cross-linking of the strands leading to exposure of more D-Ala-D-Ala residues [46, 47]. These residues bind and trap the vancomycin and prevent it from reaching the target cytoplasmic membrane. Previous exposure to vancomycin appears to be related to the development of VISA strains, and in vitro studies showed that following removal of antibiotic selection pressure the organisms may revert to a susceptible state [48]. It is interesting to note that not all VISA strains are MRSA [49].

Despite extensive study, the genetic mechanism of resistance in VISA remains unclear [50].

As noted above, reports of clinical failure of vancomycin in MRSA infections and improved understanding of pharmacodynamics led to the reevaluation of vancomycin minimal inhibitory concentration breakpoints. In 2006, CLSI redefined the breakpoints as follows: susceptible, $\leq$ 2 mg/mL; intermediate (VISA), 4–8 mg/mL; and resistant (VRSA), This should be the symbol for equal or greater than (> with a line under the >) I don't find this symbol in the options allowed. 16 mg/mL [51]. Despite this change, increasing reports of vancomycin failures even when the organisms treated were within the susceptible range caused many experts to debate whether the breakpoints should be lowered further. A phenomenon called MIC "creep" characterized as a slow increase in the median MIC to vancomycin over time was also described from many centers [52, 53]. However, there are conflicting data about this phenomenon and it appears now that "creep" may be limited to a few select centers where elevated MICs may be playing an important role in clinical practice [54, 55]. Previous antibiotic exposure and clonal replacement are considered possible reasons for the MIC creep where it exists, but its clinical significance is not established.

MRSA isolates which appear to be sensitive to vancomycin but with a subpopulation (1 in 10^5 organisms) that actually has intermediate resistance to glycopeptides called heteroVISA (hVISA) have now been reported. Although found infrequently when the MIC of the population is low, the closer the MIC approaches the 2 mg/mL breakpoint, the more likely it is to have hVISA isolates [52]. The clinical effect of hVISA infections is becoming elucidated as they are associated with persistent bacteremia, vancomycin treatment failure, metastatic complications, and high inoculum infections [56]. An association between hVISA infections and increased mortality has not yet been established [57]. A recent report describes an outbreak of infections due to hVISA organisms [58].

Vancomycin-resistant *S. aureus* (VRSA) was first reported in 2002. To date only 13 cases have been reported [59, 60]. The mechanism of resistance in VRSA strains appears to result from conjugal transfer of the vanA operon from vancomycin-resistant *Enterococcus faecalis (VRE).* The acquisition of the vanA gene allows the VRSA isolate to synthesize a cell terminal peptide D-Ala-D-Lac instead of D-Ala-D-Ala, the target site for vancomycin. This new cell wall precursor has decreased affinity to vancomycin, leading to MIC >16 mg/mL and often to >250 mg/mL [61]. Based on clinical reports, it appears these organisms are produced after prolonged exposure to vancomycin, often over months, in patients with persistent infections and co-colonization of MRSA and VRE.

8 Linezolid

Linezolid, the first oxazolidinone agent, was approved in 2000 for the treatment of MRSA infections. This protein synthesis inhibitor binds to the 50S ribosomal subunit and is 100 % bioavailable. It is available as an oral and intravenous agent and has extensive tissue penetration. Clinical uses include treatment of MRSA pneumonia, and complicated and uncomplicated SSSIs. Although linezolid has shown higher cure rates compared to vancomycin in some prospective randomized control studies [62, 63], the superiority still remains controversial secondary to conflicting studies [64, 65].

Resistance to linezolid in *S. aureus* has been observed most frequently as a result of point mutations in the binding site of the antibiotic, the 23S rRNA subunit [66]. It has also been proposed that mutations in L3/L4 proteins in the ribosomal subunit may also cause resistance alone or along with the 23S rRNA mutations [67]. In 2008, an outbreak involving 12 cases of linezolid-resistant *S. aureus* was reported from an intensive care unit in Madrid, Spain [68]. The mechanism of resistance was found to be plasmid-mediated acquisition of cfr (chloramphenicol/florfenicol resistance gene) [69]. The cfr gene mediates the methylation of the 23S rRNA subunit and the mutation can confer resistance to multiple antimicrobials including chloramphenicol, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A. This is concerning because of the potential for transmissibility and

development of resistance against other antimicrobials that act against protein synthesis.

The 9-year LEADER (Linezolid Experience and Accurate Determination of Resistance) surveillance program ranging from 2004 to 2012 detected only low-level resistance to linezolid among the *S. aureus* isolates tested (<0.03–0.15 %) [70]. Risk factors for development of resistance included previous linezolid therapy, prolonged exposure to linezolid, intensive care unit stay, and concomitant use of multiple antibiotics. This underscores the importance of antibiotic stewardship and infection control practices in preventing the development of resistance.

9 Daptomycin

Daptomycin is a lipopeptide antibiotic that is bactericidal and acts by initiating cell membrane depolarization. It was approved by FDA in 2003 for SSSIs and in 2006 for bacteremia and right-sided endocarditis. The MRSA guidelines recommend a dose of 4 mg/kg for the treatment of SSSI and 6 mg/kg for the treatment of bacteremia and endocarditis [71]. Previous vancomycin exposure, higher bacterial burden, and deep-seated infections are risk factors for daptomycin failure during treatment of MRSA infections [72, 73]. Some experts recommend using higher dose (8 or 10 mg/kg) for the treatment of bacteremia and endocarditis when vancomycin MIC > 1 or even 10 mg/kg for persistent bacteremia with resistant isolates possessing high vancomycin MICs [74–76].

Several genetic changes have been identified as reasons for daptomycin resistance, the most common being the multipeptide resistance factor (mprF) gene mutation. It involves accumulation of a variety of single nucleotide polymorphisms (SNPs) resulting in alteration of cell membrane charge leading to decreased binding of daptomycin. It has been suggested that VISA phenotypes may be associated with thicker cell wall leading to deceased daptomycin penetration to reach the cell membrane. Recently combination of daptomycin with beta-lactams has been tried in various centers for treatment of persistent bacteremia despite daptomycin treatment [77–79]. A theoretical explanation for the apparent improvement in activity is the beta-lactams' ability to reduce the positive surface charge on the organism, thus contributing to better adherence of daptomycin [80].

10 Newer Agents

Newer agents recently approved for the treatment of SSSI caused by *S. aureus* include ceftaroline, telavancin, dalbavancin, tedizolid, and oritavancin. Ceftaroline, a cephalosporin, is the first beta-lactam antibiotic that has activity agains MRSA due to its affinity for PRB2a. Telavancin was also FDA approved for the treatment of pneumonia caused by *S. aureus*. Each of these agents has simpler dosing regimens, in some cases (dalbavancin and oritavancin) due to extremely prolonged serum half-life, but no therapeutic advantage over the currently available agents. Further clinical experience would reveal clinical safety and other unique features of each of these agents.

11 Vaccine Development

Continued efforts to develop an effective vaccine against *S. aureus* have been hugely unsuccessful. Animal model studies have shown great results, but they failed to translate into clinical trials in human beings. Both passive and active immunization have been tried and failed in human studies. Fowler suggested that the limited knowledge of human protective immunity might be the reason for the inability to develop an effective vaccine against this evolving pathogen [81]. Newer studies identifying biomarkers that have a role in the virulence of *S. aureus* may ultimately influence the development of a successful vaccine [82].

12 Conclusion

The threat raised by antimicrobial resistance is real and imminent. *S. aureus*, although discovered more than two centuries ago, still remains an enigmatic organism. The study and dialogue on resistance mechanisms will help us understand this pathogen better. Much has been explained, but much remains to be discovered.

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Resistance in Aerobic Gram-Positive *Bacilli*

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1 Introduction

Aerobic Gram-positive *bacilli* comprise a variety of organisms including *Bacillus*, *Listeria*, *Erysipelothrix*, *Lactobacillus*, *Corynebacterium*, *Gardnerella*, *Actinomyces*, *Nocardia*, and *Mycobacterium*. In addition to describing the epidemiology and microbiology of *Listeria* and *Nocardia*, this chapter will focus on the *Bacillus* spp. in three different contexts: the threat of anthrax as a bioterrorist weapon, the significance of *Bacillus cereus* as an agent of foodborne illness, and the *Bacillus* spp. as occasional but important pathogens. *Bacillus* spp. are aerobic Gram-positive bacteria that are an important source of human infections. *Bacillus anthracis* is a potential bioterrorism weapon as demonstrated by the outbreak of *B. anthracis* in the United States in 2003 transmitted via intentionally contaminated letters that infected 22 persons. *Bacillus anthracis* may produce β-lactamase, and clinical failure may result when such strains are treated with penicillin. *Bacillus cereus* is a well-described cause of gastroenteritis and is usually acquired from contaminated food. *Bacillus* spp., other than *B. anthracis*, are usually resistant to penicillins and cephalosporins; they may cause serious infections including posttraumatic endophthalmitis, wound infections, bone and joint infections, and bacteremia especially in immunocompromised patients with central venous catheters. *Bacillus* spp. are generally susceptible to vancomycin, carbapenems, and quinolones. Therapy is guided by the exact infecting species, type and severity of infection, and in vitro susceptibilities.

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2 *Bacillus* spp.: Microbiology, Epidemiology, and Clinical Manifestations

2.1 Microbiology

Historically, most aerobic endospore-forming bacteria were classified as *Bacillus*. However, the use of new phylogenetic methods has led to a taxonomic transformation with the creation of a new class, *Bacilli*, within the phylum *Firmicutes* which comprises two orders, *Bacillales* and *Lactobacillales* [1]. Currently, the aerobic endospore-forming bacteria are distributed among >60 genera and seven families within the order *Bacillales* [1].

Bacillus is the type genus of the *Bacillaceae*. Members of the genus *Bacillus* are characterized as endospore forming, aerobic, catalase positive, and motile by means of peritrichous flagella. However, the genus is phenotypically diverse, and some species are asporogenous, facultatively anaerobic or strictly anaerobic, and thermophilic or psychrophilic. Although usually Gram-positive, some species show a variable reaction, especially when the stain is prepared from samples taken from the later stages of growth. When viewed under the microscope, *Bacillus* may appear as single organisms or in chains of considerable length. The size of individual rods may range from small (0.5 × 1.2 mm) to large (2.5 × 10 mm), and rod ends may appear as round or square. Formation of a single endospore in the vegetative bacterium is a dominant feature of *Bacillus*. The spore may be oval or cylindrical and may be located centrally, subterminally, or terminally. Frequently encountered species include *B. subtilis* (the type species), *B. cereus*, *B. licheniformis*, *B. megaterium*, and *B. pumilus*.

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2.2 Clinical Syndromes

Bacillus spp. are capable for causing a variety of clinical infections (Table 53.1). Clinical infections due to *Bacillus* spp. can be categorized into three broad groups: infections caused by *Bacillus anthracis* including cutaneous infections, pneumonia, and disseminated infections such as meningitis and injection related; food poisoning due to *Bacillus cereus*; and invasive infections due to non-*anthracis Bacillus* spp.

2.2.1 *Bacillus anthracis* Infection

B. anthracis is the causative agent of anthrax, which is primarily a worldwide epizootic or enzootic disease of herbivores (e.g., cattle, goats, and sheep) that acquire the disease from direct contact with contaminated soil [2–7]. However, all mammals, including humans, are susceptible. In the United States, endemic anthrax is a rare disease with only eight non-outbreak cases reported between 1989 and 2012 [8]. In 2003, the United States had 22 cases of anthrax as a result of an intentional release of *B. anthracis* [9]. Anthrax is now recognized as a potential bioterrorism agent [10, 11]. It is classified as a Category A agent by the Centers for Disease Control and Prevention: easily disseminated or transmitted person-to-person, causes high mortality, with potential for major public health impact, might cause public panic and social disruption, and requires special action for public health preparedness [12, 13].

The ultimate reservoir of *B. anthracis* is the soil (especially soil with high calcium and pH>6.1), where under proper conditions spores may persist for decades [5]. Dormant spores are highly resistant to adverse environmental conditions including heat, ultraviolet and ionizing radiation, pressure, and chemical agents and may persist in the environment for years [2, 14, 15]. In a suitable environment, spores reestablish vegetative growth. Vegetative bacteria have poor survival outside of an animal or human host; colony counts decline to an undetectable level within 24 h following inoculation into water.

Table 53.1 Classification of *Bacillus* Infections

	Blood cultures	Prognosis
A. Infections due to *B. anthracis*		
1. Cutaneous (contact)	Rarely positive	Excellent with therapy
2. Pneumonia (inhalation)	Often positive	Frequently fatal
3. Gastrointestinal (ingestion)	Sometimes positive	
4. Injection	Sometimes positive	Frequently fatal
5. Metastatic (bacteremic)	Always positive	Frequently fatal
B. *B. cereus* food poisoning	Always negative	Generally mild and self-limited disease
1. Short incubation		
2. Long incubation		
Infections due to opportunistic *Bacillus* spp.		
C. Superficial	Rarely positive	Good, occasional fasciitis or myositis
1. Wound (surgical, burn, traumatic)		
2. Skin (impetigo-like lesions)		
D. Closed space	Sometimes positive	Infection occasionally fatal and affected organ may be permanently damaged
1. Panophthalmitis/endophthalmitis		
2. Brain abscess		
3. Cholecystitis		
4. Soft tissue abscess		
5. Urogenital infection		
6. Peritonitis		
7. Necrotizing fasciitis		
8. Osteomyelitis		
9. Septic arthritis		
10. Fasciitis		
11. Myositis		
E. Severe systemic infection	Frequently positive	Frequently fatal
1. Pneumonia		
2. Empyema		
3. Meningitis/meningoencephalitis		
4. Endocarditis		

Classically, human anthrax was described as often a fatal bacterial infection that occurred when *Bacillus anthracis* endospores entered the body through abrasions in the skin, by inhalation or by ingestion [16, 17]. Recently, a fourth syndrome has emerged that is characterized by severe soft tissue infection in injection drug users [18, 19]. The source of human anthrax is direct contact with infected animal product (e.g., wool, hides, bone) or soil, ingestion of contaminated meat, or inhalation of aerosolized endospores. Rarely, direct human-to-human spread may occur [20]. The clinical manifestations of anthrax depend on the mode of acquisition being primarily coetaneous [21–25], respiratory [26–28], and gastrointestinal [29, 30]. Following initial infection *B. anthracis* may spread via the bloodstream resulting most commonly in sepsis and/or meningitis [31, 32]. The virulence of the organism is variable, determined by at least two factors: the polysaccharide capsule that prevents phagocytosis and an extracellular toxin. The anthrax toxin is comprised of three polypeptides: protective antigen (PA) binds to cellular receptors where it is cleaved by cellular furin, and oligomerizes and transports lethal factor (LF, a protease) and edema factor (EF, an adenyl cyclase) into cells [33–35]. These toxins are sufficient to produce many of the symptoms of anthrax.

Cutaneous anthrax is the most common naturally occurring form, with an estimated 2000 cases reported annually worldwide [10]. Cutaneous anthrax follows deposition of the organisms into the skin via contamination of previous cuts or abrasions. An initial pruritic macule or papule enlarges into a round ulcer by the second day. This develops into a painless, black eschar often with extensive local edema. In most cases, the eschar begins to resolve in about 10 days with complete resolution by 6 weeks. However, lymphangitis and painful lymphadenopathy may occur with associated systemic symptoms, and uncommonly cutaneous anthrax may be associated with toxemic shock [36]. The treatment of choice is medical, with ciprofloxacin or doxycycline the preferred antibiotics, but surgical biopsy may be necessary to confirm the diagnosis [37]. Without antibiotic therapy, the mortality rate has been reported to be as high as 20 % [10]. With appropriate therapy, the mortality rate is under 1 %.

Although gastrointestinal anthrax is uncommon, outbreaks continue to be reported from Africa and Asia following ingestion of insufficiently cooked meat [38–40]. The incubation period is 2–5 days. Two clinical forms of disease have been described: oral–pharyngeal and abdominal. The oral–pharyngeal form of anthrax is characterized by an oral or esophageal ulcer and development of regional lymphadenopathy, edema, and sepsis. Disease in the lower gastrointestinal tract manifests as primary intestinal lesions most commonly in the terminal ileum or cecum. Patients present with nausea, vomiting, and malaise that rapidly progresses to bloody diarrhea, development of an acute abdomen, or sepsis.

Inhalation anthrax follows deposition of spore-bearing particles into alveolar spaces. Spores are ingested by macrophages that are transported via the lymphatics to mediastinal lymph nodes, where germination occurs after a period of spore dormancy of viable and possibly extended duration [10]. Once germination occurs, clinical symptoms follow rapidly. Replicating *B. anthracis Bacilli* release toxins that lead to hemorrhage, edema, and necrosis. The mortality of inhalation anthrax, even with antibiotic therapy, remains greater than 50 %. Symptoms associated with inhalation anthrax commonly include fever and chills, sweats, fatigue, nonproductive cough, dyspnea, chest pain or pleuritic pain, and myalgias. Most patients demonstrate fever and tachycardia. Patients who present with atypical anthrax (nasal or nasopharyngeal, larynx or laryngopharyngeal, primary meningoencephalitis) present with less cough, less chest pain, or are less likely to have an abnormal lung examination than patients with typical inhalation disease [41]. Laboratory findings most commonly include a normal or slightly elevated white blood cell count often with a left shift, elevated liver transaminases, and hypoxia. The classic radiographic finding of a widened mediastinum is found in approximately 70 % of patients. Chest radiographs often demonstrate a pleural effusion and infiltrates or consolidation. The finding of mediastinal widening or pleural effusion on chest radiography is 100 % sensitive for inhalation anthrax and 71.8 % specific compared with community-acquired pneumonia and 95.6 % specific compared with influenza-like illness [42]. A chest computed tomography (CT) scan is more sensitive for detection of anthrax-associated pulmonary disease than a standard chest radiograph.

Since 2000 ~70 cases of injection of anthrax associated with intravenous drug use (principally heroin) have been reported from Europe with a case fatality rate of ~35 % [43–45]. Many of these cases have presented with severe soft tissue infection with substantial swelling or edema. Erythema and pain were not essential features at presentation, and none of the cases showed the typical eschar (i.e., a black-crusted painless lesion) of cutaneous anthrax. Laboratory-acquired anthrax has also been reported [46].

2.2.2 *Bacillus cereus* Food Poisoning

B. cereus is a well-described, but uncommon, cause of foodborne disease accounting for 3 % of outbreaks with a confirmed or suspected etiology (1 % of reported illnesses) in the United States from 1998 to 2008 [47]. Although the Centers for Disease Control and Prevention reported no deaths in these cases, death from *B. cereus*-associated food poisoning has been reported [48], including sudden death [49]. *B. cereus* strains can cause two types of food poisoning syndromes [50–57]. Type 1, "short incubation" or "emetic" syndrome, has an incubation time of 0.5–6 h; the predominant symptoms are vomiting and cramps, and less frequently,

diarrhea. The duration of illness is usually 8–10 h (range, 6–24 h). "Short incubation" strains elaborate a heat-stable peptide (toxin), "cereulide," which is capable of causing vomiting when fed to monkeys. Type 2, "long incubation" or "diarrhea" syndrome, has an incubation time of 6–24 h (occasionally >24 h); the predominant symptoms are diarrhea and abdominal cramps, and less frequently, vomiting. The duration of illness is usually 12–24 h (occasionally several days). "Long incubation" strains elaborate a heat-labile enterotoxin(s) (Hbl, Nhe, CytK implicated), which activate intestinal adenylate cyclase and results in intestinal fluid secretion. Clinical manifestations of both syndromes are usually mild and self-limited; fever is uncommon. There is no seasonality to *B. cereus* food poisoning, and secondary cases do not occur. "Early incubation" disease has most commonly been associated with contaminated fried rice or pasta, and "late incubation" disease has most commonly been associated with contaminated meats or vegetables. The usual source of contamination is raw food, rather than food-handlers or the food preparation environment. Inadequate cooking is the most important factor leading to disease outbreaks.

2.2.3 Opportunistic *Bacillus* Species Infections

Bacillus spp. have often been dismissed as contaminants in clinical specimens. However, it is now well recognized that non-*anthracis Bacillus* spp. are capable of causing serious human infections [50, 51, 58–60]. Local and systemic infections are most commonly caused by *B. cereus* and less commonly by *B. subtilis*.

Bacillus spp. have been isolated from surgical and traumatic wounds, often as part of mixed infections [61, 62]. The clinical significance of *Bacillus* in such cases is often unclear. However, *Bacillus* spp. may cause severe fasciitis and myositis resembling gas gangrene. In addition, *Bacillus* spp. may colonize or infect burn wounds. Rarely, bacteremia may accompany cutaneous or burn infection. Nosocomial wound infections have resulted from the use of contaminated plaster [63] used for preparing casts or contaminated incontinence pads [64]. Nosocomial infections have also resulted from the use of contaminated hospital linens and towels [59, 65].

Bacillus spp. may cause a variety of closed-space infections, especially ocular infections including conjunctivitis, iridocyclitis, dacryocystitis, keratitis, endophthalmitis and panophthalmitis [51, 66–71]. *B. cereus* is a well-recognized cause of panophthalmitis following penetrating ocular trauma and in intravenous drug users. Exogenous *B. cereus* panophthalmitis is characterized by rapid onset (18–24 h after injury), severe pain, chemosis, proptosis, periorbital swelling, and fever. Infection often results in evisceration and blindness. Other closed-space infections include cholecystitis, septic arthritis, osteomyelitis, intra-abdominal infection, soft tissue abscesses, and urinary tract infections.

Serious systemic infections include central nervous system infection [72–75], lower respiratory tract infections [76, 77], endocarditis [78] including prosthetic valve endocarditis [79], spontaneous bacterial peritonitis [80], and primary bacteremia with clinical sepsis [81–83]. Most patients with meningitis have predisposing factors including remote site infections, recent neurosurgery often with the use of a ventriculostomy, cancer, endocarditis, or intravenous drug use [72]. The mortality with central nervous system infection is high, approximately 50 %.

The prevalence of positive blood cultures for *Bacillus* spp. has ranged from 0.1 to 0.9 %. *Bacillus* organisms are common laboratory contaminants due to their hardy growth characteristics. Sources of *Bacillus* pseudoinfections have included contaminated broth culture, syringes, alcohol swabs used to disinfect the tops of blood culture bottles, and gloves. Approximately, 10 % of patients who have *Bacillus* isolated from a blood culture will have either recurrent *Bacillus* bacteremia or evidence of significant *Bacillus* infection. Most bacteremic patients will have underlying predisposing medical conditions, such as prematurity, intravenous drug use, indwelling central venous catheters, immunosuppressive medication, or neutropenia [84, 85]. Bacteremia has been commonly associated with clinically significant foci of infection such as meningitis and pneumonia. Endocarditis may accompany bacteremia, especially in intravenous drug users.

3 Therapy of *Bacillus* Infections

3.1 Infections due to *Bacillus anthracis*

3.1.1 In Vitro Antibiotic Susceptibility

Four caveats should be mentioned in evaluating the reports of the in vitro susceptibility of *B. anthracis* to antibiotics. First, multiple methods for determining the in vitro susceptibility have been used. The Clinical and Laboratory Standards Institute (CLSI) currently recommends that broth microdilution susceptibility testing be performed using cation-adjusted Mueller–Hinton broth (CAMHB) with incubation at 35 ± 2 °C ambient air for 16–20 h [86]. Second, CLSI provides an interpretative standard (i.e., breakpoints) only for penicillin, tetracycline and doxycycline, and ciprofloxacin [86]. Third, β-lactamase testing of clinical isolates of *B. anthracis* is unreliable and should not be performed [86]. Fourth, if MIC susceptibility testing using CLSI methods indicates that *B. anthracis* isolates are susceptible to penicillin, amoxicillin may still be considered for prophylactic use in children and pregnant women [86]. Mohammed and colleagues compared the CLSI broth microdilution method to the Etest® agar gradient diffusion method and reported no statistically significant differences between the results of these two methods for

any of the tested antibiotics; however, results for penicillin obtained by the Etest® method were 1–9 dilutions lower than those obtained by the broth microdilution method [87]. In addition, they noted that reading Etest® results through the glass of a biological safety cabinet was difficult. More recently, Luna and colleagues compared the Etest® with Sensititre® (an automated microbroth dilution method) and reported that both methods yielded "near-identical results for all antimicrobials except trimethoprim-sulfamethoxazole" [88]. A rapid susceptibility test for *B. anthracis* has been reported that yields results within 6 h [89].

Testing of clinical isolates of *B. anthracis* has revealed that strains are generally susceptible to first-generation cephalosporins, tetracyclines, quinolones, carbapenems, clindamycin, chloramphenicol, and vancomycin (Table 53.2) [88, 90–103]. *B. anthracis* also appears to be susceptible to the newer antibiotics tigecycline, linezolid, dalbavancin, and oritavancin [102–105]. Most strains are susceptible to penicillin, but clinical strains may produce a β-lactamase (see below). Most strains are resistant to second and third-generation cephalosporins, aztreonam, and trimethoprim–sulfamethoxazole.

The rapid killing of antibacterial against selected strains of *B. anthracis* has been determined using the time-kill method [106]. The most rapid bacterial killing was achieved by quinupristin–dalfopristin, rifampin, and moxifloxacin with a 4-$\log_{10}$ reduction in 0.5–4 h. The β-lactams and vancomycin demonstrated a 2–4 $\log_{10}$ reduction within 5–15 h. The macrolides, tetracyclines, and linezolid demonstrated a lower kill rate, while chloramphenicol did not kill at all. In vitro synergy of antibiotics against *B. anthracis* has also been evaluated [107]. Against two strains of *B. anthracis*, only the combination of rifampin and clindamycin were synergistic. All other combinations were either indifferent or antagonistic.

The post-antibiotic effects of a variety of antibiotics have been determined against two strains of *B. anthracis* [108]. The post-antibiotic effects observed were as follows: fluoroquinolones 2–5 h; macrolides 1–4 h; clindamycin 2 h; tetracyclines 1–3 h; β-lactams (penicillin G, amoxicillin, ceftriaxone), vancomycin, linezolid, and chloramphenicol 1–2 h; and quinupristin–dalfopristin 7–8 h.

3.1.2 Antimicrobial Resistance in *Bacillus anthracis*

Surveys of clinical and soil-derived strains have revealed resistance to penicillin G in up to 16 % of isolates tested [87, 90–92, 96, 97]. Human infection due to naturally occurring penicillin-resistant strains has been reported [109–111]. Exposure to β-lactams have been reported to induce penicillin resistance in *B. anthracis* [87, 91, 93]. The mechanism

Table 53.2 In vitro susceptibility of *B. anthracis* to antimicrobials

Highly active	Variable activity	Often resistant
First-generation cephalosporins	Penicillins	Second-generation cephalosporins
Cefazolin	Macrolides	Cefuroxime
Cefalothin	Erythromycin	Cefamandole
Carbapenems	Azithromycin	Third-generation cephalosporins
Imipenem	Clindamycin	Cefotaxime
Meropenem	Aminoglycosides	Ceftazidime
Tetracycylines	Gentamicin	Ceftriaxone
Tetracycline	Netilmicin	Fourth-generation cephalosporins
Doxycycline	Amikacin	Cefepime
Glycylcyclines		Aztreonam
Tigecycline		Trimethoprim–sulfamethoxazole
Quinolones		
Ciprofloxacin		
Levofloxacin		
Moxifloxacin		
Ofloxacin		
Macrolides		
Clarithromycin		
Rifamycins		
Rifampin		
Chloramphenicol		
Glycopeptides		
Vancomycin		
Dalbavancin		
Oritavancin		

underlying β-lactam resistance in *B. anthracis* is due to the presence of two β-lactamases, bla1 and bla2 [112]. These two β-lactamase Genes were found in the Sterne strain of *B. anthracis* and were evaluated by cloning into *E. coli* [113]. *Bla1* is a penicillinase that confers high-level resistance to ampicillin, amoxicillin, and penicillin G, while *bla2* is a cephalosporinase that confers low-level resistance to ceftriaxone, cefazolin, cefoxitin, and cefotetan [113]. More recent work has further characterized the β-lactamases of *B. anthracis* [114, 115]. Bla1 was found to preferentially hydrolyze penicillins and to be inhibited by tazobactam and clavulanic acid. *Bla1* exhibited carbapenem-, penicillin-, and cephalosporin-hydrolyzing activities.

B. anthracis has variable in vitro susceptibility to macrolides. The inducible macrolide–lincosamide–streptogramin B resistance determinant, *ermJ*, from *B. anthracis* has been cloned in *E. coli* [116].

Sequential subcultures in sub-inhibitory concentrations of antibiotics led to the development of resistance to quinolones and macrolides [117, 118]. Although the MIC of tetracycline increased, it did not reach a level that yielded clinical resistance [118]. A more recent study demonstrated that serial passages on brain heart infusion agar led to the development of resistance to quinolones, macrolides, tetracyclines, clindamycin, vancomycin, and linezolid [119]. Strains resistant to a quinolone exhibited cross-resistance to other quinolones, but not to doxycycline.

3.1.3 Recommended Antibiotic Therapy

Penicillin G has long been the standard therapy for anthrax, despite the fact that penicillin resistance was well described [17]. Prior to the intentional anthrax release in the United States in 2003, recommended therapy for anthrax included penicillin (provided the strain was penicillin susceptible), tetracycline, doxycycline, erythromycin and other macrolides, chloramphenicol, ciprofloxacin, streptomycin, first-generation cephalosporins, gentamicin, and vancomycin [10, 16]. The exact drugs, dose, and route depended on the clinical syndrome being treated (i.e., inhalation, cutaneous, or gastrointestinal).

Bacillus anthracis infections should be immediately reported to the local health department. The current therapy recommended for anthrax depends on the clinical syndrome being treated [120]. It is important to note that there are no controlled studies for the treatment of inhalation anthrax in humans. Further, there are only limited animal data using primate models of inhalation anthrax to guide therapy decisions. Ciprofloxacin, levofloxacin, and doxycycline are approved by the FDA for the treatment of inhalation anthrax in adults with ciprofloxacin and doxycycline being considered first-line therapy. If first-line agents are not available or are not tolerated, therapy could include levofloxacin or moxifloxacin, clindamycin, or amoxicillin or penicillin VK if the isolate is penicillin susceptible (the risk for development of resistance must be considered if using β-lactam drugs). Monkeys were shown to be protected from exposure to a lethal aerosol challenge (i.e., 8 LD_{50}) of *B. anthracis* by penicillin, ciprofloxacin, and doxycycline [121].

The CDC currently subdivides therapy for anthrax (systemic disease) into patients with and without possible meningitis [120]. Empiric treatment for systemic anthrax in which anthrax meningitis is suspected or cannot be ruled out should include ≥3 antimicrobial drugs with activity against *B. anthracis*, ≥1 drug should have bactericidal activity, ≥1 should be a protein synthesis inhibitor, and all should have good CNS penetration. Intravenous combination treatment for systemic anthrax with possible meningitis should be provided for ≥2 weeks or until the patient is clinically stable, whichever is longer. Preferred drugs include ciprofloxacin (alternatives: levofloxacin or moxifloxacin), meropenem (alternatives: imipenem or doripenem), and linezolid (alternatives: clindamycin, rifampin, or chloramphenicol). If the *B. anthracis* strain is susceptible to penicillin (MIC <0.125 μg/mL), penicillin G and ampicillin are acceptable alternatives to carbapenems.

With the following four exceptions, antimicrobial drug options for patients with systemic anthrax if meningitis is ruled out are similar to those for patients with suspected meningitis or when meningitis cannot be ruled out. First, treatment should include ≥2 antimicrobial drugs with activity against *B. anthracis*, ≥1 should have bactericidal activity, and ≥1 should be a protein synthesis inhibitor. Second, initial intravenous combination treatment should be given for ≥2 weeks or until the patient is clinically stable, whichever is longer. Third, if the *B. anthracis* strain is susceptible to penicillin, then penicillin G is considered equivalent to the fluoroquinolone options for primary bactericidal treatment. Fourth, treatment with antimicrobial drugs that have good CNS penetration is not a crucial factor. Thus, meropenem is recommended as an acceptable alternative option than as a first-line antimicrobial drug, and vancomycin is also an acceptable alternative. Clindamycin and linezolid are considered equivalent first-line choices for protein synthesis inhibitors. Doxycycline is added as an alternative protein synthesis inhibitor option if linezolid or clindamycin are contraindicated or unavailable.

Once patients with systemic illness who were exposed to aerosolized spores have completed initial combination treatment, they should be transitioned to single-agent oral treatment to prevent relapse from surviving *B. anthracis* spores. Antimicrobial drug options are the same as those for post-exposure prophylaxis.

The CDC provides excellent guidance on post-exposure preventive therapy for anthrax, and treatment for children, pregnant women, and immunocompromised persons [120].

In general, similar drugs are advised for children (with appropriate dose adjustment), pregnant women, and immunocompromised persons. The total duration of recommended therapy is 60 days (IV and orally combined) for bioterrorism-related cases and 7–14 days for naturally acquired cases depending on response and site of infection. The prolonged duration of bioterrorism-related cases is based on evidence such as the Sverdlovsk outbreak that following point exposure to aerosolized anthrax patients may not develop inhalation anthrax for up to 6 weeks post-exposure [122]. This is felt due to late germination of *B. anthracis* spores colonizing the upper respiratory tract. Management algorithms for the clinical assessment of patients with suspected inhalation or cutaneous anthrax are available [123].

3.1.4 Recommended Clinical Management

The CDC recommends that the initial evaluation of patients suspected of having anthrax should be similar to the standard evaluation for patients with an acute febrile illness and should have an emphasis on obtaining pretreatment blood and other appropriate cultures [120]. However, failure to fulfill systemic inflammatory response syndrome criteria should not decrease concern for sepsis because patients with systemic anthrax might not initially appear critically ill. Inhalation anthrax can have a prodromal phase followed by a fulminant phase. Patients with systemic anthrax have had debilitating symptoms, followed first by transitory improvement, and then by precipitous hemodynamic deterioration. Because of this potential for sudden decompensation, hospitalized patients should have careful hemodynamic monitoring, including continuous pulse oximetry and telemetry. Unless contraindicated, lumbar puncture should be performed to rule out meningitis.

Although there are no animal data or randomized trials to support the use of corticosteroid for human anthrax, small observational studies and the absence of apparent side effects suggest that adjunctive corticosteroids should be considered in patients who had a history of the use of corticosteroid therapy; edema, especially of the head or neck; evidence of anthrax meningitis; or vasopressor-resistant shock [124, 125]. Antitoxins used in the pre-antibiotic era have been reported to improve patient outcomes [120]. There are currently 2 antitoxins in the CDC Strategic National Stockpile: raxibacumab (GlaxoSmithKline, London, UK) and anthrax immune globulin intravenous (AIGIV) (Cangene Corporation, Winnipeg, Manitoba, Canada) [120]. Both antitoxins inhibit binding of PA to anthrax toxin receptors and translocation of the two primary toxins (LT and ET) into cells. Raxibacumab is a recombinant, fully humanized, IgG1λ monoclonal antibody. AIGIV is a human polyclonal antiserum made from plasma of persons immunized with anthrax vaccine absorbed (AVA), which might have some direct effect on LF and EF. Based on available data, an expert panel has stated that "an antitoxin should be added to combination antimicrobial drug treatment for any patient for whom there is a high level of clinical suspicion for systemic anthrax." Although there is some experience with AIGIV use in humans, "there are no major medical, operational, or logistical considerations that clearly favor the use of 1 antitoxin over another in adults with systemic anthrax" [120].

3.2 Infections Due to *Bacillus* Species Other Than *B. anthracis*

3.2.1 In Vitro Antibiotic Susceptibility

CLSI has issued guidelines for performing susceptibility testing of *Bacillus* spp. (other than *B. anthracis*) [86]. Susceptibility testing should be performed using broth microdilution using cation-adjusted Mueller–Hinton broth (CAMHB); disk diffusion testing is not recommended. Breakpoints have been provided by CLSI for selected penicillins (penicillin, ampicillin), cephalosporins (cefazolin, cefotaxime, ceftazidime, ceftriaxone), carbapenems (imipenem), glycopeptides (vancomycin), aminoglycosides (gentamicin, amikacin), macrolides (erythromycin), tetracyclines (tetracycline), quinolones (ciprofloxacin, levofloxacin), lincosamides (clindamycin), folate antagonists (trimethoprim–sulfamethoxazole), and miscellaneous agents (chloramphenicol, rifampin). Andrews and Wise reported that gradient tests (i.e., Etest®) for *Bacillus* spp. have been found to be unreliable [126]. They also demonstrated a poor correlation between penicillin resistance and detection of β-lactamase. Detection of β-lactamase production by a double-disk method was more reliable than nitrocefin or intralactam.

The in vitro antimicrobial susceptibility *of Bacillus* spp. has been evaluated in human isolates' studies as part of a comprehensive study of *Bacillus* spp. [127, 128], evaluation of specific clinical infections [70, 83, 85, 129–135], and assessments of new antimicrobials [136, 137]. *Bacillus* spp. are generally susceptible to vancomycin, imipenem, ciprofloxacin, and aminoglycosides (Table 53.3). They are generally resistant to β-lactams including third-generation cephalosporins. Preliminary studies suggest that they are susceptible to daptomycin and linezolid [127]. In vitro susceptibility testing of 10 ocular isolates of *Bacillus cereus* demonstrated that vancomycin, clindamycin, and gentamicin were all active [138]. A clindamycin–gentamicin combination demonstrated a higher rate of bactericidal synergy than a vancomycin–gentamicin combination.

3.2.2 Antimicrobial Resistance

B. cereus typically produces β-lactamases and so is resistant to β-lactam antibiotics including the third-generation cephalosporins [51, 139]. Other *Bacillus* spp. also often produce β-lactamase [126, 133]. For example, Uraz and colleagues

Table 53.3 Susceptibility of *Bacillus* to selected antibiotics

Bacillus spp.	Highly susceptible	Moderately susceptible	Rarely susceptible
B. cereus	Imipenem	Erythromycin	Penicillin
	Meropenem	Clarithromycin	Oxacillin
	Doripenem	Azithromycin	Cefazolin
	Vancomycin		Cefoxitin
	Linezolid		Cefuroxime
	Chloramphenicol		Cefotaxime
	Ciprofloxacin		Ceftazidime
	Levofloxacin		Tetracycline
	Ofloxacin		Trimethoprim–sulfamethoxazole
	Gentamicin		Clindamycin
			Amoxicillin–clavulanate
Other Bacillus spp.	Imipenem	Cefazolin	Penicillin
	Vancomycin	Cefoxitin	Ampicillin
	Erythromycin	Cefuroxime	Amoxicillin–clavulanate
	Trimethoprim–sulfamethoxazole	Cefotaxime	Oxacillin
		Chloramphenicol	
	Gentamicin	Tetracycline	
	Ciprofloxacin	Piperacillin–tazobactam	
	Levofloxacin	Clindamycin	

Highly susceptible, >95 % strains susceptible; moderately susceptible, 70–95 % strains susceptible; rarely susceptible, <70 % strains susceptible

isolated 19 *Bacillus* strains from milk of which five demonstrated β-lactamase activity [140]. Little attention has been devoted to evaluating the β-lactamases of *Bacillus* spp., and little is known except that most strains are broadly resistant to penicillins and cephalosporins including third-generation cephalosporins. Many strains are also resistant to antibiotics containing β-lactamase inhibitors (e.g., clavulanic acid) [136, 137]. However, most strains are susceptible to carbapenems.

3.2.3 Recommended Therapy

Vancomycin is generally considered the drug of choice for serious *Bacillus* infections. Alternatives include a carbapenem or a fluoroquinolone. Endophthalmitis due to *Bacillus* usually requires both intravenous and intravitreal therapy. For patients with meningitis or endocarditis, a combination of vancomycin plus gentamicin has often been used in the past. A carbapenem would be a reasonable alternative, but there is only limited clinical experience. Whether monotherapy is adequate for serious *Bacillus* infections or combination therapy is superior has not been assessed in animal models or clinical trials.

The duration of therapy for most *Bacillus* infections ranges from 7 to 14 days, depending on the site of infection, severity of illness, and underlying host defense abnormalities. Catheter removal is often required for patients with catheter-related bloodstream infections. Patients with endocarditis and osteomyelitis require prolonged therapy. For bone and soft tissue infections, oral clindamycin or ciprofloxacin may be an appropriate choice for prolonged therapy.

4 Germicide Susceptibility of *Bacillus* spp.

The Centers for Disease Control and Prevention (CDC) has estimated that healthcare-associated infections (HAIs) account for an estimated 720,000 infections and 75,000 deaths [141]. Key interventions to control healthcare-associated infections include surveillance, isolation of patients with communicable diseases or multidrug-resistant pathogens, proper skin antisepsis and hand hygiene, and appropriate disinfection and sterilization of medical devices and environmental surfaces.

Multiple nosocomial outbreaks have resulted from inadequate antisepsis or disinfection. Inadequate skin antisepsis may result from lack of intrinsic antimicrobial activity of the antiseptic, a resistant pathogen, over-dilution of the antiseptic, or use of a contaminated antiseptic. Inadequate disinfection of medical devices or environmental surfaces may result from lack of intrinsic antimicrobial activity of the disinfectant, a resistant pathogen, over-dilution of the disinfectant, inadequate duration of disinfection, lack of contact between the disinfectant and the microbes, or the use of a contaminated disinfectant.

Spore-forming *Bacilli* such as *Bacillus* spp. are intrinsically resistant to alcohols [142]. In a human challenge model, an alcohol-based hand hygiene agent did not have activity against *Bacillus atropheus* (a surrogate of *B. anthracis*) [143]. Despite the attempted decontamination with alcohol of the outside of vials containing *B. anthracis*, in one instance, these surfaces remained contaminated, resulting in cutaneous infection in a laboratory worker [144]. The use of a 70 %

ethanol solution for skin disinfection led to pseudo-outbreak of *Bacillus cereus* [145]. However, *Bacillus anthracis* has been demonstrated to be inactivated by chlorine [146–149], 4 % formaldehyde [148], 2 % glutaraldehyde [147, 149], ethylene oxide [149], and 0.025 % peracetic acid [147, 149].

5 *Listeria monocytogenes*

5.1 Microbiology and Clinical Disease

The genus *Listeria* consists of Gram-positive, non-spore-forming, facultative anaerobic, rod-shaped bacteria [150–152]. The primary habitat of *Listeria* is the environment where they exhibit a saprophytic lifestyle. *Listeria* can survive in multiple diverse habitats, including soil, water, vegetation, sewage, and food processing facilities, as well as humans and a variety of animal species. *Listeria* is an important cause of zoonoses, especially in herd animals [152]. The principal human pathogen in this genus is *L. monocytogenes*. In adults, *L. monocytogenes* causes principally sepsis, meningitis, and encephalitis [152–154]. Focal infections have been infrequently described and include endocarditis, pericarditis, arthritis, osteomyelitis, intra-abdominal abscesses, peritonitis, cholecystitis, respiratory tract infections, and brain abscesses [151, 153].

In pregnant women, *L. monocytogenes* may cause a mild, self-limited influenza-like illness or serious infection [151–154]. Infection may result in placentitis and/or amnionitis, and infection of the fetus may cause abortion, stillbirth, or, more commonly, preterm labor. It has been estimated that invasive listeriosis during pregnancy is 13-fold to more than 100-fold more frequent than in the general population [154]. Neonatal infection occurs in two forms: an early-onset sepsis syndrome often associated with prematurity and which is probably acquired in utero and late-onset meningitis that most commonly occurs ~2 weeks postpartum in full-term babies and is most likely due to acquisition of *Listeria* present in the maternal vagina at the time of parturition [152].

L. monocytogenes is a common cause of foodborne illness characterized by gastroenteritis that may be accompanied by fever, headache, arthralgias, and myalgias. The incubation period is approximately 24 h, and the duration of illness is usually around 2 days.

5.2 Antibiotic Susceptibilities and Mechanisms of Resistance

Antimicrobial testing of clinical isolates of *L. monocytogenes* should be performed using the broth microdilution method [86]. The recommended medium is cation-adjusted Mueller–Hinton broth with lysed horse blood (2.5–5 % v/v). Tubes are incubated at 35 °C, ambient air, for 20–24 h. Breakpoints are available for penicillin, ampicillin, and trimethoprim–sulfamethoxazole.

Penicillin and aminopenicillins (i.e., ampicillin or amoxicillin) are the drugs of choice for *L. monocytogenes* [151]. Although, in vitro resistance of *L. monocytogenes* to ampicillin has occasionally been reported, the methods used in these studies have been noted to be inadequate for susceptibility testing of *Listeria* [151]. In multiple studies and reviews, in vitro resistance to penicillin G and ampicillin was not detected [155–158]. *L. monocytogenes* is susceptible to trimethoprim–sulfamethoxazole [156]. Recent well-done studies of human isolates have not demonstrated emerging resistance to penicillin, ampicillin, or trimethoprim–sulfamethoxazole [158, 159]. Most *L. monocytogenes* strains isolated from the environment have been demonstrated to be fully susceptible to penicillins, gentamicin, linezolid, rifampin, and vancomycin but are intrinsically resistant to cephalosporins [157].

Rarely environment-derived strains have demonstrated resistance to erythromycin, tetracycline, and trimethoprim–sulfamethoxazole [157]. Erythromycin resistance has been due to the presence of erm(B) and erm(C). High-level trimethoprim–sulfamethoxazole resistance due to *dfrD* has been reported [160]. Resistance to tetracyclines in *Listeria* is mediated by efflux proton antiporters and ribosome protection [161].

6 *Nocardia*

6.1 Microbiology and Clinical Disease

The order *Actinomycetales* includes the family *Nocardiaceae*. At some stage all members of the order form Gram-positive rods. In direct Gram smears, organisms generally appear as very long, branching, thin, and finely beaded Gram-positive rods [162]. *Nocardia* is the most commonly isolated aerobic actinomycete from human infections. Approximately 50 species of the 85 validly names species of *Nocardia* have been reported to cause disease in humans [162].

Nocardia infections generally result from either trauma-related introduction of the organism or from inhalation with establishment of a pulmonary focus (especially in immunocompromised persons) [162]. Hematogenous spread may occur from the pulmonary focus to a variety of secondary sites, especially the central nervous system. The most common clinical syndromes are pleuropulmonary disease, central nervous system infection, and skin/soft tissue infection [163, 164]. Reviews of the clinical features and outcomes of pulmonary nocardiosis [165], central nervous system infection [166], and skin/soft tissue infection [167] have been published. The majority of patients with nocardiosis are immunocompromised [163, 164, 168]. The frequency of

nocardiosis in solid organ transplant recipients has varied between 0.7 % and 3 % and has largely been reported in heart, liver, and lung recipients [163].

6.2 Antibiotic Susceptibilities and Mechanisms of Resistance

Susceptibility testing should be performed on all isolates of *Nocardia* thought to be of clinical importance [162]. Antimicrobial testing of clinical isolates of *Nocardia* should be performed using the broth microdilution method [169]. Breakpoints are available for many antibiotics and are subdivided into primary antibiotics (amikacin, amoxicillin–clavulanic acid, ceftriaxone, ciprofloxacin {levoflocxacin}, clarithromycin {class representative for newer macrolides}, imipenem, linezolid, minocycline, moxifloxacin, trimethoprim–sulfamethoxazole, and tobramycin) and secondary antibiotics (cefepime, cefotaxime, doxycycline, and gentamicin) [169].

The antimicrobial susceptibility of both common and uncommon clinical strains of *Nocardia* have recently been published [170–173]. Susceptibility to the most commonly used therapy, trimethoprim–sulfamethoxazole, was observed in 97–98 % of isolates [172, 173]. Strains of *N. transvalensis* complex were less susceptible to trimethoprim–sulfamethoxazole (~80 %) [172, 173]. One study reported that 31 % of *N. pseudobrasiliensis* also were less likely to be susceptible (69 %) [173]. Linezolid and amikacin were also highly effective (>99 %) against all strains of *Nocardia* except *N. transvalensis* complex and *N. pseudobrasiliensis* [171–173]. For other antimicrobials, resistance was species specific. Resistance to more than one of the commonly used drugs (amikacin, ceftriaxone, trimethoprim–sulfamethoxazole, and imipenem) was highest for *N. pseudobrasiliensis* (100 %), *N. transvalensis* complex (83 %), *N. farcinica* (68 %), *N. puris* (57 %), *N. brasiliensis* (51 %), *N. aobensis* (50 %), and *N. amikacinitolerans (*43 %) [172]. Among carbapenems, doripenem and meropenem are more active than ertapenem and impenem [170]. Tigecycline has been shown to be very active in vitro [170, 173]. In conclusion, while antimicrobial resistance can often be predicted, susceptibility testing should still be considered when combination therapy is warranted, for less well-characterized species or those with variable susceptibility profiles and for patients with trimethoprim–sulfamethoxazole intolerance [172].

Trimethoprim–sulfamethoxazole is the most commonly used therapy for *Nocardia* infections, although other combination therapy and alternative antimicrobials may be used when there is a lack of response or resistance [174]. Treatment is recommended for 1–3 months in cutaneous nocardiosis, 6–12 months in pulmonary or disseminated disease, and ≥12 months for central nervous system infections [174]. Linezolid has been used to treat strains resistant to standard antibiotics but long-term use may be limited by myelosuppression, peripheral neuropathy, lactic acidosis, and retinitis [174].

As noted above, the great majority of *Nocardia* strains are susceptible to trimethoprim–sulfamethoxazole. However, there have been multiple reports of widespread resistance [175, 176]. Brown-Elliott and colleagues have re-analyzed a large number of *Nocardia* strains included in these reports and noted that 0.5 % were trimethoprim–sulfamethoxazole resistant [177]. They ascribed this difference to technical difficulties in correctly performing *Nocardia* susceptibility testing. A study of intra- and interlaboratory susceptibility testing of *Nocardia* demonstrated unsatisfactory overall reproducibility of broth microdilution testing with *N. cyriacigeorgica* and *N. wallacei* and reproducibility of tigecycline testing with *N. brasiliensis* and *N. cyriacigeorgica* and sulfonamides with *N. farcinia* and *N. wallacei* [178].

Little scientific work has been done to define the mechanisms of trimethoprim–sulfamethoxazole resistance in *Nocardia*. Valdezate and co-workers have assessed clinical strains of *Nocardia* that were resistant to trimethoprim–sulfamethoxazole by Etest® (CLSI recommends broth dilution for testing susceptibility) and reported the presence of the following resistant Genes *sul1* 93.4 %, *sul2* 78.9 %, *dfrA* (S1) 14.7 %, *bla*TEM-1 2.6 %, blaZ 2.6 %, VIM-2 1.3 %, *aph*(3')-*111a* 40.8 %, *ermA*, 2.6 %, *armB*, 77.6 %, *mefA* 14.4 %, and *msrD* 5.2 % [179].

7 Conclusions

Several genera of Gram-positive *bacilli* are capable of causing a variety human infection including *Bacillus, Listeria, Erysipelothrix, Lactobacillus, Corynebacterium, Gardnerella, Actinomyces,* and *Nocardia*. This chapter focuses mainly on *Bacillus* spp. because *B. anthracis* is considered one of the most important potential bioterrorist agents, *B. cereus* is an important cause of foodborne infections, and non-*B. anthracis* species are an unusual but important source of human infection, especially in immunocompromised patients. Reviewed also are *Listeria monocytogenes* and *Nocardia* spp. because of their importance as human pathogens.

Understanding the antibiotic spectrum of these pathogens and their common mechanisms of antibiotic resistance is crucial to the proper therapy for these pathogens.

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Part VIII

Gram Negative Bacterial Drug Resistance: Clinical

Antibiotic Resistance in *Neisseria*

54

Margaret C. Bash and Kathryn A. Matthias

1 Introduction

The genus *Neisseria* includes both pathogenic and commensal species. *N. meningitidis* and *N. gonorrhoeae* are obligate human pathogens with no reservoir outside of the human host. Commensal species *N. lactamica*, *N. sicca*, *N. subflava* (biovars subflava, flava, and perflava), *N. mucosa*, *N. flavescens*, *N. cinerea*, *N. polysaccharea*, and *N. elongata* (subspecies *elongata*, *glycolytica*, and *nitroreducens*) are likewise specific to the human niche but are rarely associated with disease. Commensal *Neisseria* species can also be found in animal respiratory tract or oral flora and include *N. canis* and *N. weaveri* in dogs, *N. denitrificans* in guinea pigs, *N. macacae* in rhesus monkeys, *N. dentiae* in cows, and *N. iguanae* in iguanid lizards.

N. meningitidis most frequently colonizes the human host without causing disease. It is only when the bacterium is able to bypass the nasopharyngeal epithelium that severe and characteristic syndromes associated with invasion, including sepsis and meningitis, develop. Likewise, acquisition of *N. gonorrhoeae* oftentimes results in asymptomatic infection (roughly 50% of women exhibit no disease symptoms) [1]. When localized disease is detected, it often presents as cervicitis in women and urethritis in men. Extension to the upper genital track can occur, leading to pelvic inflammatory disease (PID) and epididymitis, both of which are associated with long-term complications. Extragenital infections of the pharynx and rectum are also common. More rarely, disseminated infections occur, resulting in gonococcal arthritis-dermatitis syndrome, septic arthritis, and other localized forms of disseminated disease including endocarditis and meningitis [2].

Antibiotic resistance represents a substantial threat in the treatment and control of *Neisseria gonorrhoeae*. Exhibiting high transformability, resistance mechanisms have spread rapidly throughout the species, resulting in the development of clinically significant resistance to every class of antibiotics used in the treatment of gonorrheal disease. Resistance to third-generation cephalosporins, the only remaining class suitable as a single-dose single-agent therapy, has developed, and effective treatment regimens vary depending on the geographic origin of infection.

DNA analysis of *N. gonorrhoeae* and *N. meningitidis* indicates that they are closely related and very similar organisms. Diversification through genetic exchange is an important aspect of adaptation for both organisms. However, vast differences exist in the role that antibiotic resistance plays in disease treatment and prevention. While antibiotic resistance has been a major consideration in control of gonococcal disease almost since the advent of antibiotic use, it has had less dramatic effects on approaches to treating *N. meningitidis* infections.

2 *Neisseria gonorrhoeae*

N. gonorrhoeae causes one of the most common communicable diseases in humans. It is a sexually transmitted organism that usually infects mucosal surfaces. Disease presentation is highly variable, and although asymptomatic infections are common, complications of gonococcal infections are particularly burdensome in terms of both health costs and patient sequelae. Substantial evidence now suggests that gonococcal infection is also associated with increases in both the acquisition and transmission of human immunodeficiency virus (HIV), implying a direct relationship between acquisition of *N. gonorrhoeae* and other sexually transmitted pathogens [3].

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2.1 Overview of Gonococcal Disease

2.1.1 Worldwide Distribution

Gonorrhea is a disease of tremendous global public health importance. A 2008 World Health Organization (WHO) report estimated that approximately 106 million new cases occur each year, a number that adjusts for the underdiagnosis and underreporting that is characteristic of this disease [4]. Deficiencies in diagnosis and surveillance are of particular concern in settings where the disease is most prevalent, including developing nations in Africa and Asia where surveillance mechanisms are poor and treatment options are oftentimes limited. Indeed, though gonorrheal disease is distributed worldwide, both the incidence rate and the prevalence of the disease vary greatly according to geographic location. For example, the incidence rate of individuals 15–49 years of age was estimated to be approximately 13.5-fold higher in the Western Pacific Region compared to the Eastern Mediterranean in 2008 (Table 54.1) [4].

While a number of factors likely contributed to these differences, including adult population size, availability of antibiotics, and cultural attitudes regarding sexual practices, the most influential consists of the socioeconomic conditions of a given population. Even in western industrialized countries where rates of disease are considerably lower, the most marginalized communities in terms of racial and economic status exhibit the highest rates of disease. In Great Britain, for example, the overall incidence rate of gonorrhea is relatively low (63/100,000 persons), but increases substantially in urban settings, particularly in London where the incidence rate can be as high as 634/100,000 persons [5]. In the same geographic region, black ethnicity is associated with a >fourfold higher rate of diagnosis compared to white ethnicity, and rates also vary by age and gender. Similarly, in the USA in 2012, the highest rates of gonorrhea were observed among African-American men and women, with 467.7 and 456.3 cases recorded per 100,000 population, respectively (compared to an overall incidence of 107.5 cases per 100,000) [6]. The majority of cases were reported in densely populated metropolitan areas and rural, impoverished counties where public health resources are sparse or, if available, consistently overburdened.

Table 54.1 Disease burden of gonorrhea (values represent millions of individuals)

	Incidence	Prevalence
Western Pacific Region	42	13.3
Southeast Asia Region	25.4	9.3
African Region	21.1	8.2
Region of the Americas	11	3.6
European Region	3.4	1
Eastern Mediterranean Region	3.1	1

High rates of gonorrhea are currently either being maintained or increasing despite global control efforts. A resurgence of gonorrhea in homosexually active men has been noted in many parts of the developed world [7–9], and high rates of disease have been recorded in former Eastern bloc countries despite the collapse of mechanisms for case reporting [10]. Studies suggest, however, that the most significant indicators for disease acquisition include the amount of poverty and income inequality within a particular social setting [11]. These findings highlight the need for both increased surveillance mechanisms and access to cost-effective healthcare systems if the threat of gonococcal transmission is to be stemmed in the most vulnerable of communities.

2.1.2 Clinical Manifestations

Gonococcal disease is defined by the demonstration of *N. gonorrhoeae* in clinical samples. The gonococcus is an organism found only in humans and is highly adapted to its ecological niche. Most often it infects mucosal surfaces, causing sexually transmitted urethritis in men and endocervicitis in women. Anorectal and pharyngeal infections, which are more difficult to treat, may occur in both sexes, and in neonates, ophthalmic infection is acquired during passage through an infected birth canal. Endocervical, anorectal, and pharyngeal infections are commonly asymptomatic so that clinical presentation is delayed and reservoirs of infection and transmission are established.

Extension of mucosal infection may give rise to epididymo-orchitis in men or PID in women, both of which may result in infertility. In women specifically, complications may lead to an increased risk of spontaneous abortion, ectopic pregnancy, and chronic pelvic pain. Likely due to the high rate with which they experience asymptomatic infection, women are also fourfold more prone to develop disseminated gonococcal infections (DGIs), which occur in 0.5–3% of all infected patients [12]. DGIs have been associated with strains that are resistant to killing by normal human serum, and dissemination within the bloodstream may lead to infections that present as tenosynovitis, septic arthritis, or in rare cases, even endocarditis and meningitis [13]. If left untreated, gonorrhea may be transmitted to sexual partners and, in the case of pregnant women, to newborns during birth. Most often, neonatal disease presents as conjunctivitis, which may lead to corneal perforation and blindness unless antibiotics are administered.

Infection with *N. gonorrhoeae* has also been shown to significantly amplify acquisition and transmission of HIV, increasing the spread of the virus by up to fivefold [14]. Enhanced transmission is a result of the recruitment of inflammatory cells to the mucosal surface during gonococcal infection. It is these same phagocytic and CD4+ T cells that comprise the targets of HIV invasion, increasing the risk of viral acquisition. Viral load estimates of HIV in the semen of

HIV- and gonococcal-infected men are up to eightfold those of HIV-infected men without gonorrhea. This results in an increased inoculum of HIV and a greater risk of transmission. Importantly, this risk can be diminished with simple therapeutic treatment, as high viral loads revert to levels comparable to those of the uninfected cohort when effective antibiotics are administered [15].

2.1.3 Treatment and Control Strategies

It has long been recognized that a comprehensive program aimed at decreasing disease burden, transmissibility, duration of infection, and the number of sexual contacts is required for control of gonococcal disease [16, 17]. The emergence of multidrug-resistant and potentially untreatable strains of *N. gonorrhoeae* has brought a renewed focus and sense of urgency to the control and prevention of gonorrhea. Efforts have intensified in recent years to evaluate promising candidates for construction of preventative vaccines. Until such a time when a safe and efficacious vaccine becomes available, however, control of the spread of the disease must remain a primary objective.

In 2012, the World Health Organization crafted a global action plan with just such a goal in mind [17]. In addition to providing broader public awareness of existing multidrug-resistant gonococcal strains, a myriad of goals were established in an effort to successfully prevent and treat gonorrheal infections. Some of these include (1) behavioral changes, (2) improved diagnostic capabilities, (3) adequate surveillance, and (4) enhanced health-care delivery (including the provision of appropriate antibiotic treatment).

The requirement for early and effective treatment is central to this integrated approach. The treatment strategies recommended are for single-dose therapy on first presentation or diagnosis that should, at a minimum, cure 95% of all cases. The rationale behind this approach is twofold: to achieve compliance rates not possible with multidose treatments and to reduce any further disease transmission as quickly as possible (gonococci are no longer viable 12 h after effective antibiotic treatment) [16, 18, 19]. Since adequate treatment of gonorrhea is essential to the overall control of the disease, extensive efforts have been made to define, monitor, and address antimicrobial resistance in *N. gonorrhoeae*.

2.2 Antibiotic Resistance in *N. gonorrhoeae*

N. gonorrhoeae has a well-recognized potential to rapidly develop resistance to antibiotics. The organism's capacity for genetic recombination and phenotypic diversity enhance transmission and evasion of host immune systems and are essential for survival in the human host [20–22]. This propensity for genetic transformation and recombination also results in rapid spread of antibiotic resistance genes that have rendered numerous treatments ineffective in many parts of the world. This includes the penicillins, tetracyclines, quinolones, and more recently the cephalosporin group of antibiotics [23–25].

The gonococcus was originally highly susceptible to antibiotic treatment [26]. Now, in many parts of the world, only the third-generation cephalosporins, most notably ceftriaxone, remain effective and decreased susceptibility to these antibiotics has started to appear in most geographic regions. Cases of high-level cephalosporin resistance including some treatment failures have been reported [27–36]. Widespread resistance to penicillins in gonococci has necessitated demonstration of their efficacy in a given case or setting before their use is considered. The use of quinolone antibiotics is similarly restricted in many parts of the world. This means that cheap and effective oral therapy has had to be replaced by expensive and/or injectable agents. Thus, in resource-poor settings, effective antibiotics may be unavailable because the cost of the agent precludes its use [37].

In many areas where there are high rates of gonococcal disease, access to antibiotics is by means of the informal health sector. In this environment, adulterated antibiotics, off-patent preparations, and improperly stored antibiotics are all available [37–41]. The ready accessibility of these preparations means that inadequate doses may be purchased with resultant underdosing. Ironically, unrestricted drug availability leads to overuse and misuse, contributing significantly to the problem of antibiotic resistance. It is no accident that the WHO Western Pacific Region, where unregulated antibiotics are readily obtainable, has seen the sequential emergence of gonococci resistant to penicillins, tetracyclines, spectinomycin, quinolones, and now cephalosporins. Curbing these practices and ensuring proper compliance of health-care providers and their clients are of paramount importance in the WHO global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae* [17].

2.2.1 Development and Spread of Antibiotic Resistance in *N. gonorrhoeae*

Antibiotic resistance in general involves reduced access of the antibiotic to the target site or alteration of the target site itself. Access of antibiotics to the target site in gonococci may be limited by (1) reduced permeability of the cell envelope caused by changes in porin proteins, (2) active export of antibiotics from the cell by means of efflux pumps, or (3) destruction of the antibiotic before it can interact with the target [24, 25]. Alteration or deletion of the target site of the antibiotic generally results in a reduction of its affinity for the antibiotic. Genetically, these changes may be mediated by either chromosomal or extrachromosomal elements (plasmids). Multiple resistance determinants may coexist in a single organism, resulting in increased levels of resistance or, in some cases, resistance to a number of different antibiotics.

In gonococci, chromosomally mediated resistance is generally slow to emerge and disseminate. While genetic transformation (i.e., the mechanism of acquisition of these determinants) is common in *N. gonorrhoeae*, clinically relevant resistance requires multiple gene transfers [42]. Plasmid-mediated resistance in gonococci spreads more rapidly than chromosomally mediated resistance and is at present limited to penicillins and tetracyclines. Transmission requires the presence of a conjugative plasmid to mobilize the resistance plasmid. If a bacterium does not possess a conjugative plasmid, it may acquire it as a recipient strain. Once acquired, the recipient can become a donor in its own right, perpetuating dissemination not only of conjugative plasmids but also extrachromosomal resistance genes [25, 42].

Resistance to Penicillins (Penicillin, Ampicillin, Amoxicillin, Penicillin/β-Lactamase Inhibitor Combinations)

The penicillins have been widely used for the treatment of gonorrhea. Originally, *N. gonorrhoeae* was extremely sensitive and treatment with 150,000 units of penicillin was efficacious in most instances [26]. Not long after its introduction, decreased in vitro susceptibility appeared and was associated with treatment failure as early as the mid-1950s. Increasing the recommended dose of penicillin resulted in improved efficacy, but enhanced levels of resistance rapidly emerged and large numbers of treatment failures again occurred, even with high-dose regimens [19, 43]. This was a result of the additive accrual of multiple chromosomal changes to multiple loci over the span of multiple decades. The genetic basis for high-level chromosomally mediated penicillin resistance has been described and is caused by the contributions of mutations in five different genes or loci [44].

The targets of β-lactam agents are the penicillin-binding proteins (PBPs), enzymes located in the cell envelope that participate in cell wall peptidoglycan metabolism. Alterations in PBP-2 decrease their affinity for the penicillins and, thus, the susceptibility of the organism [45]. PBP-2 is encoded by the *penA* locus [46]. Changes in other loci such as *mtr* and *penB* produce additive effects. The *mtr* locus mediates resistance to a wide range of antibiotics, detergents, and dyes through an active efflux system [47, 48]. Mutations in the *penB* locus, in turn, affect the major outer membrane porin protein, resulting in reduced permeability of the cell envelope to hydrophilic antibiotics and other compounds [42, 49–51]. An additional contribution to resistance by *ponA1* encoding a mutation in PBP-1 has been shown, but only in the presence of a mutation in *penC* (*pilQ2*), which interferes with the formation of the high molecular weight PilQ secretin complex [44, 52]. The combined effect of *penA, penB, mtr, penC,* and *ponA1* is to increase the minimum inhibitory concentration (MIC) of penicillin by 120-fold. Gonococci exhibiting these changes are termed chromosomally resistant *N. gonorrhoeae* (CMRNG) [53]. The CMRNG phenotype has been associated with strains expressing the P1. B allele of the porin that is the target for serologic typing antibodies and is found in strains designated serotype IB (or WII/WIII) [54, 55].

Resistance to penicillin is also mediated by a plasmid-borne, inducible TEM-1 type β-lactamase which is believed to have been initially acquired by the gonococcus from *Haemophilus* species [56–60]. This enzyme hydrolyzes the β-lactam ring of penicillins, thus inactivating them. In contrast to the slow evolution and incremental increase in resistance associated with chromosomal changes, acquisition of the plasmid confers resistance in a single step. In 1976, penicillinase-producing *N. gonorrhoeae* (PPNG) were detected at the same time in both the UK [61] and the USA [62]. The first isolates were imported, respectively, from Africa and the Far East. Although the same TEM type of β-lactamase was present in both instances, the gene was carried on plasmids of different sizes which became known as the "African" and "Asian" plasmids, respectively. Transmission of resistance by conjugation required the presence of a mobilizing plasmid that was present in the Asian PPNG upon initial isolation, but was not found in the African strains until 1981 [60]. Thus, resistance due to the Asian plasmid disseminated more quickly and widely compared to the African plasmid. A number of related PPNG carrying plasmids of different sizes have since been described [42].

Lactamase production (PPNG) and chromosomal changes (CMRNG) can coexist in the same isolate. Attempts have been made to negate the effects of penicillinase production by combining a β-lactamase inhibitor with a penicillin, e.g., amoxicillin with clavulanic acid. Treatment of gonorrhea with such a combination has met with only limited success [63, 64]. Although lactamase inhibitors may neutralize the effect of the hydrolyzing enzyme and leave the penicillin to act on the organism unhindered, if underlying chromosomally mediated mechanisms of resistance are also present, the organism will still be intrinsically resistant [65].

Tetracyclines

Tetracycline antibiotics are generally not recommended for treatment of gonorrhea because they must be administered in multiple doses over several days, often resulting in decreased compliance or an inadequate regimen. Despite these problems, tetracyclines remain widely used, particularly in the informal health sector where poverty makes them especially appealing due to their inexpensive cost.

Both chromosomal and plasmid-borne tetracycline resistance mechanisms are found in gonococci, the latter being responsible for high-level resistance. As with the penicillins, chromosomal resistance is linked to alterations in the *mtr* and *penB* loci [42]. In addition, a third locus, tet-2, has been identified as a single point mutation in the *rpsJ* gene

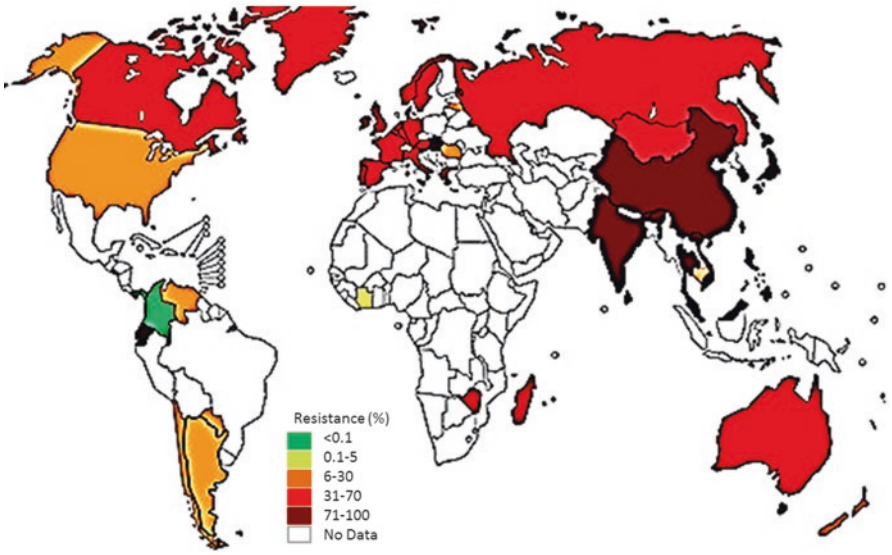

Fig. 54.1 Monitoring gonococcal antimicrobial susceptibility. Proportion of *N. gonorrhoeae* strains resistant to ciprofloxacin and/or other quinolones reported in countries, 2010 [154].

encoding the ribosomal protein S10 [46, 66]. The combination of these and other chromosomal mutations result in clinically significant resistance [67].

High-level tetracycline resistance in gonococci (TRNG) results from the acquisition of the *tetM* determinant and was first reported in 1986 [68]. *tetM* exists as two slightly different "Dutch" and "American" types, located on a self-mobilizing plasmid [69] that is widely dispersed in the normal genital tract flora. A study of the molecular epidemiology of the *tetM* genes by PCR suggests that the Dutch type may have originated in the Far East and the American type on the African continent [70]. The mobility of the plasmid and the selective pressure created by the use of tetracyclines to treat other sexually transmitted infections (STIs) has contributed greatly to the widespread distribution of the TRNG phenotype [53, 71–75].

Sulfonamide-Trimethoprim Combinations

Sulfamethoxazole and trimethoprim (co-trimoxazole) have been combined in an oral formulation that is used as a multidose treatment for gonorrhea. As discussed above for the tetracyclines, the need for multiple doses has implications for the development of resistance due to poor compliance. Trimethoprim is not particularly active against gonococci and is in fact used for bacterial growth in primary culture plates, a result of the reduced affinity of gonococcal dihydrofolate reductase for the microbial agent. Increased production of dihydrofolate reductase or decreased cell permeability may also contribute to resistance [76]. Resistance to the sulfonamides can develop separately [42, 77].

Quinolone Antibiotics

Oral, single-dose fluoroquinolone therapy, such as ciprofloxacin and ofloxacin, was recommended for the treatment of genital *N. gonorrhoeae* infections starting in the early 1990s [78]. Over the past two decades, quinolone-resistant gonococci (QRNG) have been isolated with increasing frequency worldwide (Fig. 54.1), diminishing the usefulness of quinolone antimicrobials [24, 25, 79, 80]. In 2007, the Centers for Disease Control and Prevention (CDC) called for the discontinuation of their use for treatment of gonorrhea in the USA [81].

Fluoroquinolone resistance has been attributed to point mutations in bacterial genes *gyrA* and *parC*, which code for the target enzymes DNA gyrase and topoisomerase IV, respectively [82]. Sequence analysis suggests that multiple mutations in *gyrA* or the combination of *gyrA* and *parC* mutations are generally associated with ciprofloxacin resistance (ciprofloxacin MIC $\geq$ 1 μg/mL) and are clinically expressed as treatment failure [82–88]. Additionally, porin changes and efflux mechanisms may contribute to resistance [89, 90]. Newer quinolones with enhanced ParC activity have been released. However, this target site is less important in gonococcal resistance than GyrA, so these agents are unlikely to be effective in areas where high-level resistance to quinolones is already well established [91].

Cephalosporin Antibiotics

Increased resistance of *N. gonorrhoeae* to fluoroquinolones and the subsequent discontinuation of their use have left the cephalosporins as the sole remaining single-dose single-agent antibiotic class to which widespread gonococcal resistance has not developed. As β-lactam agents, they function like the penicillins to bind PBPs present in the cell membrane and inhibit the synthesis and cross-linking of the bacterial cell wall. Cephalosporins, however, exhibit less susceptibility to the action of β-lactamases than penicillin antibiotics, and cephalosporinases, which are constitutively expressed by many Gram-negative genera, have not been detected in *Neisseria sp.* [23]. Thus, altered susceptibility to cephalosporins in gonococci is chromosomally mediated and

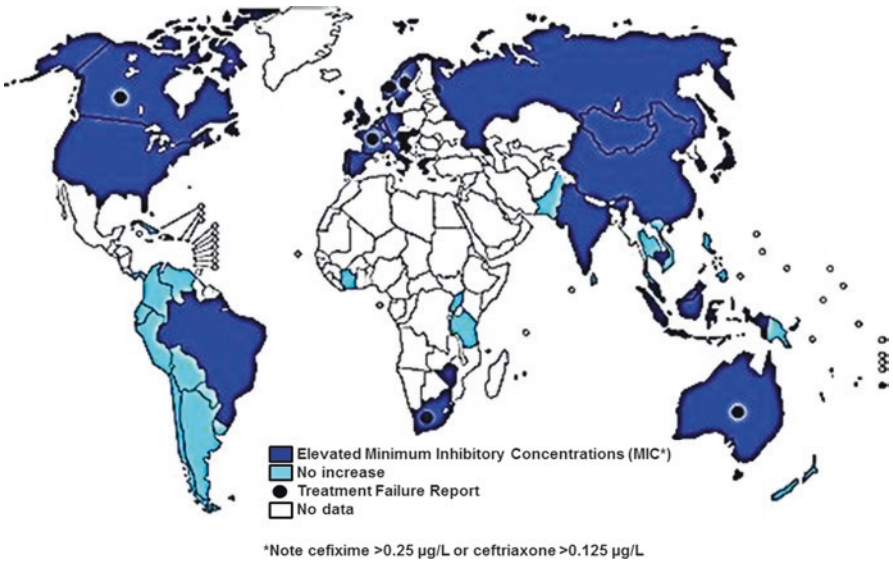

Fig. 54.2 Monitoring gonococcal antimicrobial susceptibility. Countries with documented elevated minimum inhibitory concentrations to cefixime and/or ceftriaxone, 2010 [154].

is due to similar changes that account for decreased penicillin susceptibility in CMRNG [42, 92].

Data suggest that, like penicillins, mutations in multiple genes including *penA*, *ponA*, *mtrR*, and *penB* (*porB*) [93–95], contribute to increased cephalosporin resistance, though *pilQ* is unlikely to affect susceptibility [96]. Indeed, cross-resistance between penicillins and early generation cephalosporins (such as cefuroxime) has been demonstrated [42, 92, 97], though this is not the case for third-generation cephalosporins like ceftriaxone and cefixime [98]. Resistance to these extended spectrum cephalosporins (ESCs) is most often associated with multiple mutations in the *penA* gene that likely induce a conformational change in the β-lactam binding site of PBP-2 [99]. This mosaicism alone is sufficient to significantly increase gonococcal resistance to ESCs, but the effect is enhanced in the presence of *ponA*, *mtrR*, and *penB* mutations.

penA mosaicism in *N. gonorrhoeae* is believed to have arisen from recombination events with the *penA* genes of closely related commensal species, including *N. flavescens*, *N. perflava*, *N. subflava*, *N. cinerea*, and *N. meningitidis* [100]. Regions of homology were identified in the *penA* transpeptidase domain when the sequence of ESC-resistant isolates was compared to that of the commensals [95, 101]. Since the niche of these commensals is oropharyngeal, it is likely that genetic transfer takes place at that site, a hypothesis bolstered by the steady increase in the recovery of ESC-resistant isolates from sex workers and men-who-have-sex-with-men (MSM), in addition to multiple reported treatment failures of pharyngeal infection with ceftriaxone [28, 42, 92, 102].

The first verified treatment failure of a patient administered oral cefixime was recorded in Japan in 2002 [36]. Since that time, further treatment failures have been reported in countries including South Africa, Canada, Austria, France, Spain, the UK, and Norway [27, 29, 30, 34, 35]. Concurrent with treatment failures, the percentage of isolates exhibiting elevated MICs to cefixime (≥0.25 µg/mL) continued to rise (Fig. 54.2) until a decade later when, in 2012, the CDC ceased recommendation of the antibiotic for use in gonococcal treatment [103]. Currently, the CDC recommends dual therapy treatment, utilizing injectable ceftriaxone in combination with either oral azithromycin or doxycycline. Yet, treatment failures of ceftriaxone-based therapies [33, 102, 104] and the emergence of extensively drug resistant (XDR) gonococcal strains [28, 32, 33] have recently been reported, highlighting the anticipated loss of these treatment options in the future.

Spectinomycin and Aminoglycosides

In *N. gonorrhoeae*, high-level resistance to spectinomycin or aminoglycosides occurs via linked single-step chromosomal mutations in ribosomal genes, allowing bacterial translation to continue unimpeded [42, 105, 106]. Spectinomycin-resistant gonococci appeared in Korea in the 1980s following widespread use. When spectinomycin treatment was discontinued, however, antibiotic-resistant strains disappeared. Spectinomycin-resistant strains of *N. gonorrhoeae* are uncommonly encountered and specifically have not been seen in Korea for many years following discontinuation of their use [75], suggesting the possibility that they may constitute effective agents for therapy in the future. Yet, apparent treatment failure has been reported where in vitro sensitivity to the antibiotic was demonstrated, likely due to inadequate dispersal from the site of injection [77]. Additionally, though spectinomycin is efficient in treating urogenital and anorectal gonorrhea, it is less effective in the treatment of pharyngeal infections [107], demonstrating the importance of the site of infection in selecting a proper antibiotic regimen.

Aminoglycoside antibiotics, primarily kanamycin and gentamicin, are low-cost injectable agents, sometimes used as first-line treatments. Susceptibility testing data indicates, however, the emergence of low-level gentamicin resistance, and a 2012 study of documented urogenital gonorrhea cases demonstrated that while single-doses of gentamicin were effective at treating the majority of infections, the percentage of subjects that cleared *N. gonorrhoeae* without requiring further treatment fell below the current CDC criteria for recommended therapy (≥95% efficacy) [108]. As the MIC for defining gentamicin resistance has still not been well defined [109, 110], future studies examining the correlation of in vitro susceptibility to clinical treatment failures are clearly needed.

Newer Macrolides

A number of newer macrolides have been made available for treatment of gonococcal infection concomitant with *Chlamydia trachomatis*, most notably azithromycin. As with erythromycin [47], chromosomal resistance to azithromycin is dependent on expression of the *mtr* phenotype [111]. Mutations encoding resistance to erythromycin have been localized to the ribosome [112, 113] and promoter region of *macAB*, which promote unimpeded translation and increased transcription of the genes encoding the MacA-MacB efflux pump [114]. Treatment failures have been reported with low-dose (1 g) azithromycin regimens [115–117]. However, a recent dual therapy study utilizing oral azithromycin has proven much more promising, as combination treatment with injectable gentamicin or oral gemifloxacin resulted in 100% and 99.5% efficacy, respectively, in clearing urogenital gonorrhea [118]. One hundred percent efficacy was also demonstrated against pharyngeal and anorectal infections for both combinations, highlighting the importance of dual therapy treatments in the elimination of bacterial reservoirs that may contribute to antibiotic resistance.

Chloramphenicol/Thiamphenicol

Data on in vitro susceptibility is often lacking, but that which does exist suggests gonococcal resistance to these agents [71, 119].

2.2.2 Laboratory Determination of Resistance

In principle, laboratory methods for susceptibility testing of gonococci are similar to those for other bacteria. However, *N. gonorrhoeae* has specialized growth requirements that have led to the development of tests with numerous variations in methodology.

Agar Dilution (Agar Incorporation) Methods

The agar dilution MIC is the definitive susceptibility test. It is a labor-intensive method and is only performed in specialized laboratories, but is relatively inexpensive when large numbers of strains are tested in batches. The methods currently in use are not uniform and different MIC values expressed in mg/l may be obtained in different laboratories [120]. MICs are generally accepted to be accurate to a value of plus or minus one doubling dilution.

Disk Diffusion Methods

Disk diffusion susceptibility tests are widely used and are practical because of low cost and technical simplicity. Their utility and accuracy in assessing gonococcal susceptibility is debated since the method was initially standardized for rapidly growing organisms and the slow rate of bacterial growth and increased time of incubation for *N. gonorrhoeae* greatly affect inhibition zone diameters. Attempts have been made to correlate inhibitory zones with MICs in order to develop interpretive criteria. Although not standardized, comparable data can be generated in different laboratories [16].

E-Test

This is a quantitative susceptibility test that uses a strip impregnated with a predefined antibiotic gradient. When performed under reference laboratory conditions, the E-test has compared favorably with the conventional agar dilution MIC. However, the methods were less comparable in a field study in Malawi [109]. MICs obtained with this method in reference laboratories tend to be slightly lower than those obtained by conventional agar dilution methods.

Comparability of MIC Data

Many problems with comparability of MIC data exist. Recently, however, it has been suggested that resistance rates obtained by different methods can be compared if certain test parameters are defined and controls that are internationally agreed upon are used [77, 90]. For example, the MIC value for chromosomal resistance to penicillin is defined as ≥2 μg/mL in the USA and Canada and ≥1 μg/mL in the UK and Australia. However, qualitative classifications of the strains (i.e. as sensitive or resistant) are the same when the relevant interpretive criteria are applied. The validity of this approach has been demonstrated in the quality assurance aspects of the continuing program of surveillance in the WHO Western Pacific Region [121, 122].

Detection of β-Lactamase (Identification of PPNG)

PPNG express an inducible TEM-type β-lactamase that is encoded on plasmids and can be detected by a number of methods, including a commercially available chromogenic test. The clinical utility of these tests may be limited since resistance to the penicillins is widespread, and CMRNG are not detected by this means. Resistance due to chromosomal mutations can only be detected in PPNG after strains are cured of plasmids.

Special Test Requirements for Some Antibiotics

Although not generally recommended, co-trimoxazole has been used extensively in some regions to treat gonorrhea because of its availability and low price. Testing susceptibility to this drug requires that the growth medium be free of substances that interfere with its activity. Azithromycin susceptibility testing is pH dependent. Since carbon dioxide (needed for the growth of *N. gonorrhoeae*) can alter the pH of the medium, robust controls must be used when assessing the activity of azithromycin.

DNA Probe and Hybridization Techniques for Susceptibility Determination

Chromosomally mediated resistance in *N. gonorrhoeae* is the result of multiple genetic changes, for which there is no simple probe. Probes have been tested that identify known mutations in *gyrA, parC*, and *gyrB* genes associated with QRNG [123–125] and those of *penA* and *ponA* that are associated with penicillin resistance [126]. Because new mutations that affect levels of resistance are continually being discovered, an alternative approach that uses probes to identify the absence of mutations (wild-type sequence) in resistance determining regions may be a useful screening strategy for detecting antibiotic susceptibility [127]. Mutations that may partially reverse resistance (e.g. suppressed expression of the *mtr* phenotype by the *env* mutation) [42] illustrate the complicated nature of identifying phenotypic resistance by molecular-based methods. Yet, identification of resistance markers by DNA testing will add significantly to conventional susceptibility tests if the ability to reliably and rapidly test non-cultured direct clinical specimens can be developed.

2.3 Clinical Significance of Resistance in *N. gonorrhoeae*

2.3.1 Epidemiology

The epidemiology of *N. gonorrhoeae* is complex. Although gonococci are generally considered to be non-clonal, outbreaks of antibiotic-resistant gonorrhea have been caused by strains that are phenotypically and/or genotypically related [67, 128, 129]. Studies suggest that the establishment of antibiotic-resistant gonorrhea in a community progresses through several characteristic stages. Initially, resistant isolates are primarily imported and are sporadic with little or no secondary spread. At this stage, resistant isolates are diverse. Sustained local transmission of a resistant strain may develop, establishing endemic transmission. This transition is usually associated with infection of core transmitters, such as sex workers [130]. During this stage, one strain or a few closely related strains account for a large proportion of resistant isolates, suggesting a unique window of opportunity for public health intervention before resistance genes become widely distributed in the population. In regions where high rates of resistance have been established for several years, multiple genetically diverse resistant strains can be found.

The spread of QRNG is an interesting example. The emergence of QRNG has been remarkable, particularly in Asia, and since 1999 has accounted for over 50% of Southeast Asian isolates [122, 131–133]. Analysis of variability in mutation patterns and typing characteristics demonstrate worldwide isolate diversity [84, 91, 134, 135], and the initial introduction of QRNG to communities has been characterized by low numbers of diverse strains imported from endemic regions via travelers [134]. Rapid increases in the prevalence of QRNG in a community signal the beginning of endemic transmission, which is typically clonal. The pattern of clonal spread of a highly resistant strain establishing high rates of endemic QRNG has been shown in studies conducted in the UK, Australia, Japan, the USA, Israel, and Sweden [84, 133, 134, 136–139].

2.3.2 Surveillance

Since antibiotic-resistant gonococci arise and spread rapidly, crossing national and regional boundaries with ease, data on in vitro susceptibility of prevalent gonococci are needed to establish and maintain effective treatment guidelines [140–145]. While in vitro susceptibility data reliably predicts clinical outcome, these examinations are generally not performed on an individual basis and treatment must routinely be provided before results of this testing become available. To circumvent these problems, an epidemiological approach is utilized to determine susceptibilities of prevalent gonococci and trends in gonococcal resistance patterns. The spread of antimicrobial resistance has been exacerbated by international travel, as documented by the abundance of data on PPNG and QRNG [146, 147]. In the absence of reliable in vitro testing, therefore, it is of critical importance for successful disease treatment to understand both the origin of an infection and the local and global patterns of gonococcal-specific antimicrobial resistance.

Unfortunately, severe limitations in resources impact the surveillance process. While national schemes have been in existence in developed countries for several decades [6, 148–152], there are few examples of adequate data arising in less developed countries [4]. Some regional activity has been implemented, however, and data are progressively being generated, assessed, and validated by international groups [121, 153]. Widespread use of nucleic acid-based amplification assays for the diagnosis of STIs, while a powerful tool, has diminished the use of culture-based techniques that permit the performance of susceptibility testing. Protocols that require bacterial isolation and antibiotic susceptibility testing will need to be adopted in the future if gonococcal surveillance programs are to remain successful.

Regional Surveillance Data

Due to the substantial regional differences in rates of gonococcal antibiotic resistance, a number of surveillance programs for *N. gonorrhoeae* currently exist [4, 121]. The Gonococcal Antimicrobial Surveillance Program (GASP) has continuously monitored the emergence of antibiotic resistance in the WHO Western Pacific Region since 1992 [75, 121]. Similar, less developed programs exist in Latin America and Southeast Asia, and a West African GASP has been established, as well. Attempts are underway to establish a global program of gonococcal susceptibility surveillance [154, 155].

Country-Based Data

In addition to regional programs, there are also a number of national programs that conduct surveillance for the progression of antibiotic resistance in *N. gonorrhoeae*. The Australian Gonococcal Surveillance Program (AGSP), for example, was established in 1979 [151] with the US Gonococcal Isolate Surveillance Program (GISP) [148] following in 1986. Additional country-based programs exist in Canada, the UK (Gonococcal Resistance to Antimicrobials Program, GRASP) [152], Sweden, Denmark, Singapore, China, Hong Kong, Bangladesh, and France, among others. The current data from the AGSP, GISP, and GRASP is sufficient alone to reveal the loss of utility of penicillins, quinolones, and increasingly cephalosporin antibiotics in gonococcal treatment [6, 149, 150]. Surveillance from other countries has likewise demonstrated clinically significant trends in diminished antibiotic susceptibility. In Scandinavian surveys, for instance, multi-resistant gonococcal infections acquired overseas are prominent, while data from China, Hong Kong, and Bangladesh all point to major problems with multiply resistant strains. In data available from Africa and Latin America, quinolone resistance is not as pronounced as in Asia, but penicillin and tetracycline resistance rates are high [77]. Clearly, improved surveillance results in a greater ability to monitor global trends in antibiotic resistance and to use that knowledge to treat gonorrhea at a local level.

2.4 Treatment

2.4.1 Management of *N. gonorrhoeae* Infections

In developed countries, the usual practice is to establish an etiological diagnosis in individuals presenting with symptoms of STIs. Bacterial culture was the typical diagnostic approach, but DNA-based diagnostic techniques are increasingly becoming the standard diagnostic method. Nucleic acid amplification tests (NAAT) have improved case finding, particularly in reference to asymptomatic infection common among women. In contrast, in many parts of the world where diagnostic facilities are nonexistent or rudimentary, treatment algorithms based on syndromic approaches have been developed [156]. Treatment is aimed at those infecting agents most likely to be involved in a particular clinical situation. The syndromic approach presumes that clinical symptoms are not only present but also that they are of sufficient magnitude to induce the patient to seek treatment. However, in the case of asymptomatic women or those that only experience minimal discomfort, patients who fail to present often place themselves at risk of complications and can serve as a reservoir of infection for others [156, 157].

Regardless of the level of diagnostic capability, initial treatment is empiric and the choice of antibiotic used is predetermined by the patterns of antibiotic resistance demonstrated in recently isolated gonococci. Disaggregated local information, as opposed to pooled country-based information, is relevant to tailoring treatment schedules to specific geographic regions. For example, while penicillins remained suitable for use in some remote settings in rural Australia, treatment regimens in Sydney were adjusted to utilize ceftriaxone to account for high rates of penicillin resistance as well as significant increases in QRNG [158]. In practice, once resistance to an individual antibiotic in a gonococcal population reaches 5% or more, it is recommended to be removed from treatment schedules [145].

Concomitant infection of *N. gonorrhoeae* with other treatable STIs is common. In particular, infection with *Chlamydia trachomatis* frequently accompanies gonococcal infection and can be asymptomatic or produce symptoms similar to those seen with gonorrhea. As some population studies have demonstrated coinfection in 20–40% of those with gonorrhea, it is common to include anti-chlamydial treatment with initial anti-gonococcal therapy, unless the presence of chlamydia has been specifically excluded [144, 159–161].

Follow-up evaluation of treated patients is standard practice in developed countries. This does not uniformly include a repeat laboratory examination, but cultures should be obtained if symptoms persist or recur to ensure that the individual patient is cured and, in the case of treatment failure, to determine antibiotic susceptibility [162]. It is important to identify treatment failures due to new or spreading forms of resistance so that control measures may be implemented in a local setting. Oftentimes though, it is difficult to differentiate between failure of antibiotic treatment and gonococcal reinfection. Comparisons of pre- and posttreatment cultures can assist in this distinction. Controlled monitoring can also alert practitioners to the existence of novel forms of resistance in the gonococcus. The timing of repeat evaluation needs to be carefully considered. Even if the organism is resistant to an antibiotic or antibiotic combination, symptoms and signs may be temporarily relieved and recur after cessation of therapy. In this context, anti-chlamydial therapy administered at the same time may have a suppressive, but not curative, effect [116, 163]. In less developed settings, access to treatment and clinics is often limited and follow-up assessments are infrequent.

While the focus here is on antibiotic resistance and treatment strategies, individual case management should include a comprehensive approach to the patient's needs for reproductive health. Counseling, contact tracing, and identification of other possible STDs are all essential to the management of gonorrhea.

2.4.2 Current Antibiotic Recommendations

Optimal practice requires cure of a minimum of 95% of cases by single-dose antibiotic therapy on initial presentation. Ideally, the selected regimen should be cost-efficient and orally administered under direct supervision, followed by an appropriate clinical review. Due to the propensity for *N. gonorrhoeae* to develop resistance rapidly, any treatment regimen should also be designed and administered so as to prevent emergence of new forms of resistance. Standard protocols that fulfill the above criteria have been developed either for universal application or else for use in individual countries [141, 142, 144, 145, 164]. Recommendations incorporate different requirements for treatment of genital and extragenital disease (pharyngeal, rectal, and ophthalmic infection), for complicated gonococcal disease, and for the special cases of infections in neonates, children, and during pregnancy. These recommendations are regularly revised to take account of changing susceptibility of gonococci to existing antibiotics, the introduction of new agents or new experience with older agents, the availability of currently used treatments, and the different prevalence of disease in different settings. Updated treatment recommendations for the USA are provided by the CDC (http://www.cdc.gov/std/tg2015/). Published protocols also include procedures for the prophylaxis of neonatal ophthalmia neonatorum. Treatment protocols generally utilize the antimicrobial agents described below.

Cephalosporins

Of the antibiotics currently recommended for the treatment of gonorrhea, the third-generation cephalosporins are the most efficacious. Prior to 2012, regimens including oral cephalosporins to treat gonorrhea were recommended, but the rapid development of resistant strains has in recent years diminished their use. The CDC now recommends administration of a single intramuscular injection of ceftriaxone (250 mg) in conjunction with a single dose of oral azithromycin (1 g) or a 7-day course of doxycycline (100 mg, twice a day) [144]. If oral cefixime is instead administered, a follow-up appointment is recommended one week later to confirm the absence of treatment failure. Ceftriaxone is suitable for use in all forms of gonococcal disease, including pharyngeal gonorrhea, which is often difficult to treat. Intravenous inpatient treatment is usual for DGI, including meningitis and endocarditis. Ophthalmic infections of neonates, older infants, and adults also respond to ceftriaxone. This class of antibiotics can be administered during pregnancy.

Quinolones

Fluoroquinolones, primarily ciprofloxacin or ofloxacin, are widely recommended as standard treatments for gonorrhea in geographic regions where resistance remains low. Recommended doses include 500 mg of ciprofloxacin or 400 mg of ofloxacin administered as a single oral dose. Initially, low-dose regimens (e.g., 250 mg doses of ciprofloxacin) were used, but the higher dose recommendation was implemented following reports of treatment failures due to low antibiotic levels. Fluoroquinolone agents given orally are effective in the treatment of anogenital and pharyngeal infection, but should not be administered during pregnancy/lactation or given to prepubertal children. Parenteral ciprofloxacin (500 mg) or ofloxacin (400 mg) may be administered every 12 h to treat DGI and systemic gonococcal infection. However, ceftriaxone is used preferentially for this complication. Earlier generation non-fluorinated quinolones, which are less efficacious, are not recommended.

Spectinomycin

Spectinomycin is an aminocyclitol compound given by intramuscular injection as a 2 g dose. It is relatively expensive but effective in the treatment of anogenital infection. Use in the treatment of pharyngeal gonorrhea is not recommended. Side effects are few, and as such, spectinomycin is usually regarded as a "reserve" agent in the treatment of gonorrhea (e.g. for those intolerant of cephalosporins or quinolones). It can also be administered to pregnant women in standard doses and for DGI if given twice daily for up to seven days.

Suboptimal or Obsolete Treatments

Some previously efficacious regimens have become ineffective due to developing resistance and should only be used if the infecting organism has clearly been demonstrated to be susceptible. However, in some regions, antibiotic susceptibility screening is not possible and inefficient, readily available treatments continue to be used because more effective therapy is simply unaffordable. Penicillins, including ampicillin and amoxicillin (with or without clavulanate and/or probenecid), were once standard treatments for gonorrhea but are now rarely used as a consequence of resistance. The most effective form of administration is a single 3 g oral dose of amoxicillin. Probenecid (1 g), if given at the same time, will delay renal excretion of the penicillins.

Azithromycin is widely used as a treatment for chlamydia as a single 1 g dose. It is now routinely recommended as a component of dual therapy for gonorrhea based on the theoretical benefit of using two antibiotics with different mechanisms of action to improve effectiveness and delay the development of resistance to the cephalosporins [144]. As a single agent, this antibiotic exhibits some anti-gonococcal activity but treatment failures are unacceptably high, and resistance has appeared during therapy [165]. The additive

anti-gonococcal effect of azithromycin combined with a specific gonococcal therapy is unquantified and should not be relied upon to affect a cure for gonorrhea [116, 117, 166]. Oral tetracyclines, like azithromycin, are also used as anti-chlamydial agents in conjunction with anti-gonococcal treatments and have been shown to exhibit some anti-gonococcal activity. Yet tetracyclines, when used for gonorrhea treatment, require multidose regimens and thus are not generally recommended due to insufficient compliance.

Despite this, the ready availability of tetracyclines and their low-cost result in their continued use in some settings. Some guidelines also mention kanamycin (2 g, intramuscular injection) as an alternative treatment where in vitro resistance rates are low. However, data on efficacy and in vitro criteria of resistance are poorly documented [109, 110]. Co-trimoxazole (trimethoprim/sulfamethoxazole combination), like the tetracyclines, is a multidose oral treatment and as such is not recommended. Its use should be guided by in vitro data demonstrating susceptibility to the agent, though considerable technical requirements must be fulfilled before reliable in vitro data can be obtained. Chloramphenicol/thiamphenicol antibiotics are still widely used for the treatment of many diseases in resource-poor settings, even though they are not recommended.

2.4.3 Infection Control Measures

Availability of proper treatment in a community contributes significantly to reductions in both disease and complication rates, including those of enhanced HIV transmission. It has been estimated that effective treatment of 100 women with gonorrhea, 25 of whom were pregnant, would prevent 25 cases of PID, one ectopic pregnancy, six instances of infertility, and seven cases of neonatal ophthalmia [16]. These estimates have been supported by results of longitudinal studies in Sweden where a decrease in the incidence of gonorrhea coincided with a reduction in the incidence of PID [167–169]. It has also been estimated that proper treatment of gonococcal disease in a cohort of 100 high-frequency gonorrhea transmitters would cumulatively prevent 425 new cases of HIV over a period of 10 years [170]; the decreased incidence of new HIV infection observed in a study of improved STD treatment in Mwanza, Tanzania, lends support these projections [171].

The multidisciplinary approach needed for control of antimicrobial resistance in *N. gonorrhoeae* includes (1) rapid and accurate diagnostic testing, (2) ready access to effective antibiotics administered in a setting with an established regulatory framework that oversees drug evaluation and approval, (3) enforcement of prescription-only drug access, (4) reliable drug delivery systems, (5) and an informed prescriber base and laboratory systems with good and evaluable diagnostic standards [172]. Prevention measures are also essential, including those aimed at effecting behavioral changes. While control was seemingly achieved in some countries when a concerted effort combining these elements was in place, the reduction in rates has been reversed as "safe sex" practices have been abandoned [7, 173].

Some progress has been made in efforts to control gonococcal antimicrobial resistance. For example, the WHO is developing systems of inexpensive diagnostics to underpin syndromic management algorithms for STIs. Additionally, a series of easily accessible color-density maps showing distribution of gonococcal-specific antibiotic resistance by region and country has been developed. The data for these maps have been provided by effective gonococcal surveillance systems, which have been implemented in settings with few resources at relatively little cost [4, 121]. Utilization of these control measures and others aimed directly at treatment on a local level has been demonstrated in practice to be effective at reversing gonococcal resistance trends. In Hawaii, for example, a reduction in the rate of fluoroquinolone-resistant gonococci was observed in the 2001–2002 period (from19.6% to10.1%) after initiation of control measures including universal antimicrobial resistance testing, followed by partner identification, and treatment of all cases of fluoroquinolone-resistant gonorrhea [174]. Reversal of resistance trends is rare though, and effective measures frequently only succeed in slowing the rate of emerging resistance.

3 *Neisseria meningitidis*

3.1 Overview of Meningococcal Disease

3.1.1 Epidemiology and Clinical Manifestations

Neisseria meningitidis causes both endemic and epidemic disease worldwide but, as with gonococcal infections, there are significant differences between developed and less developed settings. The clinically significant serogroups, as determined by their capsular polysaccharides, are groups A, B, C, W, Y, and recently serogroup X. Group A is particularly associated with recurrent epidemics and hyperendemic meningococcal disease in sub-Saharan Africa often called the "meningitis belt" and parts of Asia. It is estimated that in the 10 years from 1995 to 2004, outbreaks of meningococcal disease in Africa caused about 700,000 cases and 60,000 deaths [175]. Serogroup X is also primarily localized to the meningitis belt, but was considered to be only a source of sporadic disease until it was determined to be responsible for an outbreak in Niger, Uganda, Kenya, Togo, and Burkina Faso during the 2006–2010 period [176]. In western industrialized countries, infections with serogroup B or C meningococci predominate. In the USA, serogroup Y accounts for over one third of meningococcal disease [177] and has been increasing recently in the UK [178]. For endemic disease, the peak incidence is in the very young (less than four years)

with a predominance of serogroup B infections; a secondary peak occurs in adolescents and young adults, and serogroup C clusters and outbreaks are common in this age group.

Asymptomatic carriage of the organism in the nasopharynx is common. In a study of healthy individuals observed over 32 months, 18% were found to be carriers at some point [179]. Invasive meningococcal disease occurs when the organism penetrates the epithelial surface of the nasopharynx. Sepsis and meningitis are the most typical presentations of invasive disease, but pneumonia, arthritis, and recurrent bacteremia can occur. Mortality rates vary and are influenced by many factors, including type of care available, clinical presentation, age of patient, and serogroup. Case-fatality rates in industrialized countries in non-epidemic situations are around 8% [180, 181] but are often much higher during epidemics in less developed countries. Morbidity is substantial, even in the setting of optimal medical treatment, and can include hearing loss, neurologic sequelae, and limb loss from auto-amputation.

3.1.2 Treatment and Control

The first successful report of antibiotic treatment for meningococcal disease was on the use of sulfonamides in 1937 [182]. Penicillin became an effective therapy in the 1940s, followed soon after by chloramphenicol, and most recently the third-generation cephalosporins. Current recommendations are for early empiric treatment with ESCs (cefotaxime or ceftriaxone), followed by 5–7 days of therapy with high-dose penicillin, ampicillin, or cephalosporin once a microbiologic diagnosis is made. For severe penicillin allergy, chloramphenicol is the recommended alternative when available [183]. Chemoprophylaxis with rifampin, ceftriaxone, ciprofloxacin, or azithromycin is indicated for close contacts of patients. Effective and cheap antibiotic treatment is difficult to deliver in the meningitis belt and currently heavy reliance for treatment of individual cases is placed on the use of long-acting chloramphenicol given by intramuscular injection [175]. A trial conducted in Niger has suggested that ceftriaxone, administered as a single 100 mg/kg intramuscular injection, is a suitable alternative to current treatments in terms of efficacy, ease of use, and cost [184].

Control of meningococcal disease has been through the use of vaccines, treatment of identified cases, and prophylaxis of case contacts. Licensed bivalent (A/C) or quadrivalent (ACYW) polysaccharide vaccines have been used in selected "at-risk" populations or in outbreak control. For example, vaccination with a polyvalent ACYW vaccine became mandatory for all pilgrims undergoing the Hajj following a series of outbreaks between the years 1987 and 2001 [185]. Since the induction of compulsory vaccination, the rates of incidence and invasive meningococcal disease decreased in both pilgrims and residents of Mecca and Medina [186]. Until recently, control of epidemic meningococcal disease in sub-Saharan Africa relied on "reactive vaccination" programs utilizing polysaccharide vaccines to interrupt outbreaks, in addition to administration of antibiotic treatment for established cases. Since polysaccharide vaccines fail to induce immunological memory and are less immunogenic in infants and young children, routine preventive vaccination was not utilized, and the overall success was limited by the ability to rapidly identify an outbreak and mount a vaccination response [175].

Unlike polysaccharide vaccines, conjugate vaccines induce immunological memory in children by stimulating T-cell responses that may be boosted upon repeat vaccination and are generally highly immunogenic in infants. The first licensed meningococcal conjugate vaccines were monovalent serogroup C preparations used in universal vaccination campaigns in the UK. Monovalent meningococcal C conjugate vaccines are part of the national immunization programs of several countries in Europe, as well as in Australia and Canada [187–189]. Multivalent conjugate vaccines effective against serogroups A, C, Y, and W have subsequently been licensed, and in the USA routine immunization of adolescents is recommended [183]. The recent development and large-scale administration of a serogroup A conjugate vaccine, MenAfriVac®, in sub-Saharan Africa is bringing about the elimination of epidemic meningitis in that region [190, 191]. Developed in response to an epidemic in the meningitis belt between 1996 and 1997 that resulted in 250,000 cases of disease and 25,000 deaths, MenAfriVac® has since been delivered to more than 217 million people in 15 countries [192, 193]. To date, there have been no cases of serogroup A meningococcal disease in vaccinated individuals. Vaccination campaigns with MenAfriVac® in Chad were undertaken by region. The impact of this vaccine was demonstrated by a 94% reduction in the incidence of meningitis (all cause) in vaccinated regions compared to the unvaccinated regions [194]. This is likely connected to a dramatic reduction in carriage rates which have been determined to remain at low levels years after vaccination [195, 196].

Although the development and use of meningococcal conjugate vaccines has resulted in a marked reduction in disease where universal vaccination programs have been introduced, control of serogroup B outbreaks has been challenging. This is due to the fact that the group B polysaccharide is poorly immunogenic and is chemically identical to material found in the human central nervous system. Thus, formulation of serogroup B vaccines has necessarily relied on the study of non-capsular antigens as vaccine candidates.

Serogroup B meningococci are often of diverse subtypes, but individual subtypes may give rise to epidemic or hyperendemic disease [197]. Preparations of outer membrane vesicles (OMVs) derived from the specific serogroup B strain associated with an outbreak or hyperendemic meningococcal disease have exhibited efficacy in Norway, Cuba, Brazil, Chile, and New Zealand [198–205]. Vaccines for prevention of endemic serogroup B disease caused by antigenically

diverse strains have been the focus of new meningococcal vaccine development, and protein-based serogroup B vaccines have been licensed in parts of Europe, Canada, Australia, and recently the USA. Because disease rates are low, clinical end-point efficacy studies of these vaccines were not possible. Antibodies generated in response to these vaccines have been shown to be bactericidal against selected strains, but additional studies and epidemiologic surveillance following widespread use will provide additional information about their effectiveness [206–210].

3.2 Antimicrobial Resistance in *N. meningitidis*

Fortunately, unlike with the gonococcus, antibiotic resistance has not yet had a major impact on clinical disease management of *N. meningitidis* infections. One explanation for this, at least for the penicillins, has been provided by Antignac et al. who noted differences in the gene coding for PBP-2, *penA*, suggestive of variation arising after an event separating the two species [211]. That is, meningococci and gonococci have evolved differently in regard to their capacity to develop resistance. Nevertheless, meningococcal susceptibility patterns have been changing over the past two decades [212].

3.2.1 *N. meningitidis* Resistance to Agents Used for Treatment

Penicillins

Resistance to penicillin is due in part to development of altered forms of PBP-2, which have decreased affinity for penicillin [211, 213, 214]. Mutations in *penA* have been correlated with decreased susceptibility to β-lactam antibiotics [215], though β-lactamase production is not an important resistance mechanism in meningococci. Decreased membrane permeability [216] and efflux [217] may also contribute to resistance.

Isolates with a decreased susceptibility to penicillin were first identified in Spain in 1985 [218]. Since then, they have been found across much of Europe, North America, Australia, and parts of Africa, though with widely varying rates. While numerous reports show the prevalence of moderately penicillin susceptible strains (MIC 0.125–1 μg/mL) are increasing, the degree of resistance appears stable [212, 219]. Indeed, a 2007 genomic study analyzing the *penA* sequence from 1670 meningococcal isolates collected from 22 countries and spanning 60 years demonstrated the frequent emergence of altered PBP-2 alleles that occurred independent of clonal expansion [220]. A mechanism that explains the deficiency of increased resistance levels has been recently described, as alterations in peptidoglycan modifications arising from *penA* mutation lead to decreased Nod1-dependent inflammatory responses and meningococcal colonization [221]. Thus, *penA* acquisition comes at a fitness cost to the organism.

Chloramphenicol

Chloramphenicol is no longer commonly used in developed nations and has not been standard therapy for meningococcal meningitis in Vietnam since the 1980s. It is used frequently in topical, especially ophthalmologic, preparations, and it remains standard parenteral treatment in many developing countries, particularly in Africa. Despite its widespread use there, a 2001 study of 33 serogroup A isolates from nine countries collected between 1963 and 1998 demonstrated chloramphenicol susceptibility in all cases [222]. Only one of the isolates demonstrated the possible presence of the chloramphenicol acetyltransferase (*catP*) gene, possibly acquired from *Clostridium perfringens*, which mediates chloramphenicol resistance. As sequencing was not conducted and results relied solely upon the performance of the polymerase chain reaction, the authors concluded the presence of *catP* in this isolate to be a likely false positive.

While meningococcal susceptibility to chloramphenicol is common, there have been two reports of isolates verified to exhibit chloramphenicol resistance [223]. In the first, two serogroup B isolates were recovered from patients in Australia in 1994 and 1997. In the second, strains were isolated from the CSF of 12 patients between 1987 and 1996. Eleven of these were epidemiologically unrelated strains from Vietnam, and one was from France from a patient with no history of travel to Southeast Asia. While all 12 strains were serogroup B, they were also genetically diverse. MICs for these strains were 64 mg/l, and disk-agar dilution tests showed them to also be resistant to sulfonamides and streptomycin, but susceptible to penicillins, cephalosporins, tetracyclines, macrolides, rifampin, and quinolones. In the case of both reports, the presence of *catP* gene was confirmed.

3.2.2 *N. meningitidis* Resistance to Agents Used for Prophylaxis

Resistance of meningococci to sulfonamides was identified in the USA as early as 1963 and is now widespread. Although sulfonamides were no longer used for treatment of clinical disease, they were widely prescribed for prophylaxis prior to the identification of resistance [224]. Resistance is due to mutations in the gene for dihydropteroate synthase [225].

Resistance to rifampin has also been identified [226, 227], a result of mutations in the *rpoB* gene and changes in membrane permeability [180, 223, 228]. A 1997 study from the CDC found 3 of 97 isolates to be resistant to rifampin [229]. A larger study from Australia involving 1434 isolates obtained over six years in the 1990s found only eight isolates resistant to rifampin (MIC 1 mg/l) and one with reduced susceptibility to ciprofloxacin (MIC 0.25 mg/l) [228]. A 2003 study in France found only one out of 2167 isolates to be resistant to rifampin and one additional isolate with reduced

susceptibility [180], though three cases of patients infected with a serogroup C rifampin-resistant strain were recently described [230, 231]. Other studies in Europe and Latin America found similar patterns [213, 232, 233]. A 1996 study in the USA showed the development of rifampin resistance during prophylaxis treatment. Oropharyngeal cultures were obtained before and three weeks after prophylaxis treatment was instituted in a middle school in Seattle. No secondary cases occurred, but resistance developed in 12% of the isolates, all of which were group B [234].

There have been several reports of reduced susceptibility to ciprofloxacin, including, among others, a serogroup C strain from a patient with invasive disease in Australia, a group B strain from a carrier in France, and a group B strain isolated from the CSF of a patient in Spain [235, 236]. The first documented cases of ciprofloxacin-resistant meningococci in North America were reported at the northern border between Minnesota and North Dakota between 2007 and 2008 [237]. More recently, a study of 374 isolates in China collected between 1965 and 2013 highlighted the changing patterns of fluoroquinolone resistance in Asia, as the number of isolates that were nonsusceptible to ciprofloxacin increased from 0% in 1965–1985 to 84% in 2005–2013 [102]. This rapidly evolving resistance is a result of the ability of *N. meningitidis* to develop mutations in the *gyrA* and *parC* genes that are similar to the mutations in QRNG [238].

3.3 Clinical Significance of Resistance in *N. meningitidis*

A study from the UK specifically examined the question of a link between reduced susceptibility to penicillin and fatal outcome from meningococcal infection [181]. The authors retrospectively analyzed over 11,000 cases reported between 1993 and 2000 in England and Wales. During this time period, the frequency of penicillin intermediate strains increased from less than 6% to greater than 18% (12.6% overall), with a higher frequency among serogroups C and W135. The overall case-fatality rate was around 8%, and while there was an association between fatal outcome and specific serogroups and serotypes, there was no link with reduced susceptibility to penicillin. In a Spanish study of isolates from 1988 to 1992, 34% of strains (72 of 213) showed decreased susceptibility to penicillin. Higher morbidity/mortality was associated with these strains, even though penicillin was not used for therapy in all cases [239].

Isolated case reports raise the possibility of treatment failure associated with decreased susceptibility to penicillin. A report from the UK describes an 18-year-old with meningococcal meningitis who was treated with IV benzylpenicillin and, after an initial clinical response, remained ill several days later. The CSF culture was positive when repeated. After treatment was changed to chloramphenicol, this patient rapidly improved. The *N. meningitidis* isolated from CSF initially and upon repeat culture had a penicillin MIC of 0.64 (reduced susceptibility). Of note, the dose of penicillin was lower than some use [240]. Another report from Argentina also suggests possible treatment failure of penicillin [241].

Prophylaxis failures associated with rifampin resistance have been reported [226, 242]. In Israel, three small clusters of disease that occurred in the military were examined. In one, the initial case was rifampin sensitive, but two secondary cases occurred among the contacts who had taken rifampin, and the strains were identified to be rifampin resistant. All three strains were group C:NT:P1.2 [227]. In the 1997 CDC study, nine contacts of individuals with rifampin resistant *N. meningitidis* received prophylaxis with rifampin and none developed disease [229].

3.4 Treatment and Infection Control Recommendations

Penicillin G remains the recommended treatment for invasive meningococcal disease in the USA and elsewhere. The dose is 250,000 U/kg per day (up to a maximum of 12 million U/day), divided every 4–6 h. Cefotaxime, ceftriaxone, and ampicillin are acceptable alternatives. Recent data suggest that shorter courses of treatment than the usually accepted 7–10 days of therapy are adequate for management of meningococcal disease [184, 243]. Chloramphenicol is recommended for patients with penicillin allergy characterized by anaphylaxis. For disease that may have been acquired in regions of the world where decreased susceptibility to penicillin is common or resistance has been reported, cefotaxime, ceftriaxone, or chloramphenicol is recommended. Chemoprophylaxis is recommended for persons who have had close contact with infected individuals, for whom the risk of developing invasive disease is increased. This includes household contacts, childcare or nursery school contacts during the seven days prior to disease onset in the index patient, and individuals who have been exposed to the index patient's secretions, such as by kissing or sharing eating utensils during the seven days prior to the onset of illness. In addition, health-care workers who administered mouth-to-mouth resuscitation or were unprotected during endotracheal intubation should receive chemoprophylaxis. The patient should also receive prophylaxis to eliminate carriage unless the infection was treated with ceftriaxone or cefotaxime. Recommended regimens include rifampin, 600 mg (or 10 mg/kg for children over one month of age and 5 mg/kg for infants under one month of age), every 12 h for two days; ceftriaxone, 250 mg intramuscularly as a single dose (125 mg for children less than 15 years of age); or ciprofloxacin, 500 mg as a single oral dose [183].

Secondary cases of meningococcal disease can occur several weeks after onset of disease in an index patient. Therefore, vaccination can be used as an adjunct to chemoprophylaxis.

4 Commensal *Neisseria* Species

N. lactamica, *N. sicca*, *N. subflava* (biovars subflava, flava and perflava), *N. mucosa*, *N. flavescens*, *N. cinerea*, *N. polysaccharea*, and *N. elongata* subspecies *elongata*, *glycolytica*, and *nitroreducens* are human commensal organisms that are rarely associated with disease in a normal host. *N. elongata* subspecies *elongata, N. subflava*, and *N. sicca/perflava* have been occasionally associated with infective endocarditis that occurs on damaged or normal heart valves and in congenital heart disease [244, 245]; *N. sicca* and *N. perflava* have been described in pulmonary and disseminated infections in patients with AIDS [246].

Antibodies to *N. lactamica* developed during carriage are thought to provide immunity to meningococcal disease. Interest in *N. lactamica* has thus been focused mainly on identifying the "immunizing" characteristics and dynamics of carriage of this organism. There are, however, few large studies on antimicrobial resistance in the commensal *Neisseria* sp. derived from these carriage studies.

The commensal *Neisseria* are by their nature longer-term inhabitants of the nasopharynx than the more transient, invasive subtypes of *N. meningitidis.* For this reason, they are exposed more often and for a greater duration to resistance pressures arising from use of antimicrobials, prescribed for any reason. It is therefore reasonable to suggest that the commensal *Neisseria* may harbor a reservoir of resistance genes available for acquisition by the invasive species. Penicillin-binding proteins, encoded by chromosomal *penA* genes, are the target site for the penicillins. It has been shown that mosaic *penA* genes present in the highly transformable commensal *Neisseria* confer intermediate resistance to penicillin. These resistance genes are then transferred to other commensal *Neisseria* and also to *N. meningitidis* by recombination [247, 248].

One report of antibiotic resistance in 286 *N. lactamica* isolates collected in Spain in 1996 and 1998 during studies on meningococcal carriage demonstrated raised penicillin MICs (0.12–1 mg/l) in all isolates tested [249]. Additionally, about 2% of isolates in this study showed decreased quinolone susceptibility many years before its appearance in pathogenic *N. meningitidis* in that country. In another carriage study conducted following the outbreak of fluoroquinolone-resistant meningococci in the USA, *N. lactamica* strains were determined to exhibit the same mutation in *gyrA* that was observed in isolates recovered from infected patients, suggesting occurrence of a horizontal gene transfer event [250]. Likewise, examination of *N. gonorrhoeae* isolates in Japan that had decreased susceptibility to cefixime identified mosaic PBP-2 contained fragments that were identical to the PBP-2 of *N. cinerea* and *N. perflava* [251].

Isolates of *N. elongata* subspecies *elongata, N. subflava*, and *N. sicca/perflava* found in occasional instances of systemic infection often display resistance or decreased susceptibility to penicillins and other agents. Thus, misuse of antibiotics may have profound consequences on the commensal members of this genus in terms of both the emergence and spread of resistant genes. Because the pathogenic *Neisseria* species also occupy the same niche as the commensals, during which time the opportunity for genetic transfer arises, "downstream" effects on both meningococci and gonococci may also be observed.

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Mechanisms of Resistance in *Haemophilus influenzae* and *Moraxella catarrhalis*

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Michael R. Jacobs

1 Introduction

Haemophilus influenzae and *Moraxella catarrhalis* are found as both respiratory tract commensals and respiratory and invasive pathogens. While it is ideal to tailor chemotherapy to a known pathogen with a known drug susceptibility profile, it is often difficult or impractical to isolate the causative agent, and many infections are treated empirically [1]. It is therefore important to know the activity of antimicrobial agents against the pathogens associated with diseases being treated empirically and the effect of resistance mechanisms on in vivo activity. Antimicrobial agents should be used rationally, avoiding overuse, tailoring treatment to identified pathogens as much as possible, and basing empiric treatment on the disease being treated and the susceptibility of the predominant pathogens at breakpoints based on pharmacokinetic (PK) and pharmacodynamic (PD) parameters [2]. The current status of resistance mechanisms found in *Haemophilus influenzae* and *Moraxella catarrhalis* against the antimicrobial agents recommended for empiric and directed treatment of the diseases caused by these pathogens forms the basis of this review.

2 Carriage of *Haemophilus influenzae* and *Moraxella catarrhalis*

Many infections, particularly those of the respiratory tract, are superinfections of inflammatory processes, such as viral infections, by bacteria colonizing the nasopharynx and oropharynx. Bacteria normally residing in the mouth and respiratory tract include streptococcal species, especially *Streptococcus pneumoniae*, *H. influenzae*, *M. catarrhalis*, *Neisseria* species, various anaerobes, and staphylococcal species. Carriage of *S. pneumoniae*, with over 90 serotypes, *H. influenzae*, both encapsulated and nonencapsulated strains, and *M. catarrhalis* changes over time as immunity develops to each strain and different strains are acquired from other persons [3, 4]. Carriage of these species is also influenced by use of protein-conjugated capsular polysaccharide vaccines, *H. influenzae* type b (Hib) and the 7-, 10-, and 13-valent pneumococcal vaccines [5].

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3 Major Diseases Caused by *Haemophilus influenzae* and *Moraxella catarrhalis*

The major diseases caused by these pathogens are childhood meningitis and bacteremia, community-acquired pneumonia in adults and children, acute otitis media, acute sinusitis, and acute exacerbations of chronic bronchitis. Empiric and directed antimicrobial therapy of these diseases will be briefly reviewed to establish the range of antimicrobial agents of clinical importance, and therefore where resistance needs to be considered.

3.1 Meningitis

While Hib vaccination has greatly reduced the incidence of Hib meningitis in countries where it is used, meningitis remains a serious problem in children under 7 years of age in areas where the vaccine is not used [6, 7]. Current WHO estimates are that, while Hib vaccine was used in 189 countries by the end of 2013, overall worldwide coverage with three doses of Hib was estimated at 52 % in 2013, being particularly low in the Western Pacific (18 %) and in Southeast Asia (27 %) regions, with 199,000 vaccine-preventable deaths per year [8]. The empiric antimicrobial treatment of meningitis recommended by the Infectious

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Diseases Society of America for this age group is vancomycin plus a third-generation cephalosporin such as cefotaxime or ceftriaxone [9]. If a Gram stain of cerebrospinal fluid shows Gram-negative bacilli presumptively identified as *H. influenzae*, a third-generation cephalosporin alone is recommended. Alternative therapies for *H. influenzae* include chloramphenicol, cefepime, and meropenem. Once the pathogen has been isolated and identified, and susceptibilities are known, the antibiotic choices can be narrowed or changed if necessary. For β-lactamase-negative *H. influenzae*, ampicillin is recommended as standard therapy, with a third-generation cephalosporin, cefepime, or chloramphenicol as alternate regimens. β-Lactamase-positive *H. influenzae* should be treated with a third-generation cephalosporin, with cefepime or chloramphenicol as alternatives. Meningitis caused by *H. influenzae*, usually untypeable strains, can also occur in patients who have suffered basilar skull fractures. These patients should be treated with the same agents discussed above, with the addition of moxifloxacin to the list of alternative agents recommended for adult patients only.

3.2 Childhood Pneumonia and Bacteremia

In regions where protein-conjugated Hib and pneumococcal capsular polysaccharide vaccines are not used, the most common bacterial causes of childhood pneumonia between 6 months and 5 years of age are *S. pneumoniae*, *H. influenzae* type b, and *M. catarrhalis* [10, 11]. *Mycoplasma pneumoniae* and *Chlamydia* (*Chlamydophilia*) *pneumoniae* become more common at school age, with *M. pneumoniae* more common in the 5–10-year-old cohort and *C. pneumoniae* more common after age 10 [12, 13]. Bacteremia with *S. pneumoniae* and *H. influenzae* type b occurs with or without the presence of pneumonia.

High-dose amoxicillin (90 mg/kg/day), either alone or with the addition of clavulanic acid, is the first-line drug of choice for empiric treatment of outpatients with childhood-presumed bacterial pneumonia [14]. If oral antibiotics are not tolerated, daily intramuscular (IM) ceftriaxone has good coverage for the three major bacterial pathogens. In older children with a higher probability of *C. pneumoniae* or *M. pneumoniae*, addition of a macrolide is recommended [13–17]. Oral cephalosporins should be avoided due to a lack of coverage for penicillin-resistant pneumococci. Recommended empiric therapy for inpatients includes ceftriaxone or cefotaxime to provide coverage for penicillin-non-susceptible *S. pneumoniae* and β-lactamase-positive *H. influenzae*. The addition of azithromycin or erythromycin is recommended to provide coverage for atypical pathogens in older children. Vancomycin or clindamycin should be added for life-threatening pulmonary infections in which *Staphylococcus aureus* is a suspected pathogen as virulent, community-acquired, methicillin-resistant strains are increasingly being encountered. Directed parenteral therapy for pneumonia due to *H. influenzae* includes ampicillin for β-lactamase-negative strains and ceftriaxone, cefotaxime, or cefuroxime for β-lactamase-positive strains.

3.3 Community-Acquired Pneumonia (CAP) in Adults

The most common causes of CAP are *S. pneumoniae* (26–60 %), *M. pneumoniae* (10–37 %), untypeable *H. influenzae* (2–12 %), *Legionella pneumophila* (2–6 %), *C. pneumoniae* (5–15 %), and *M. catarrhalis* (2–3 %) [1]. Treatment guidelines for management of CAP in immunocompetent adults have been established by the American Thoracic Society and the Infectious Diseases Society of America [18]. Recommendations for outpatients with no comorbidities include azithromycin, clarithromycin, and doxycycline if no antibiotic therapy had been administered in the past 3 months; if antibiotic therapy had been administered in the past 3 months, recommendations are levofloxacin, gemifloxacin, or moxifloxacin as single agents or combination macrolide-β-lactam therapy [azithromycin or clarithromycin with amoxicillin (3 g/day) or amoxicillin-clavulanate (4 g/250 mg/day)]. Recommendations for outpatients with comorbidities include azithromycin, clarithromycin, levofloxacin, gemifloxacin, or moxifloxacin if no antibiotic therapy had been administered in the past 3 months; if antibiotic therapy had been administered in the past 3 months, recommendations are levofloxacin, gemifloxacin, or moxifloxacin as single agents or combination macrolide-β-lactam therapy [azithromycin or clarithromycin with amoxicillin-clavulanate (4 g/250 mg/day)]. Amoxicillin-clavulanate or clindamycin is recommended for suspected aspiration pneumonia. High-dose amoxicillin, high-dose amoxicillin-clavulanate, cefpodoxime, cefprozil, cefuroxime axetil, levofloxacin, gemifloxacin, or moxifloxacin is recommended for influenza with bacterial superinfection. Recommendations for inpatients in medical wards include levofloxacin, gemifloxacin, or moxifloxacin alone or azithromycin or clarithromycin plus cefotaxime, ceftriaxone, ampicillin-sulbactam, or ertapenem. Recommendations for patients requiring intensive care are the same plus inclusion of an antipseudomonal agent if infection with *Pseudomonas aeruginosa* is a concern. The guidelines emphasize that the infectious etiology be determined whenever possible and that pathogen-directed therapy be used once the organism has been identified. Virulent, community-acquired, methicillin-resistant *S. aureus* (MRSA) are increasingly being encountered, and the addition of vancomycin or other anti-MRSA agents should also be considered [19].

Ceftaroline and tigecycline have recently been approved for the treatment of CAP in adults [20]. Ceftaroline is highly potent against *H. influenzae*, while MICs of tigecycline are close to its susceptibility breakpoint (Figs. 55.8 and 55.10). The efficacy and safety profile of ceftaroline was comparable

to ceftriaxone in patients hospitalized with CAP. Tigecycline is approved for the treatment of CAP but has a safety warning regarding an increased mortality risk compared to other agents used to treat pneumonia.

3.4 Acute Otitis Media (AOM)

AOM is one of the most common pediatric infections, second only to the common cold in prevalence, occurring most often between 6 months and 3 years of age, especially in children with frequent viral upper respiratory infections [29]. The principal bacterial causes of AOM are *S. pneumoniae* (25–50 %), untypeable *H. influenzae* (23–67 %), and *M. catarrhalis* (12–15 %) [30, 31]. In the USA introduction of the conjugate pneumococcal vaccine in children has resulted in untypeable, β-lactamase-producing *H. influenzae*, and ampicillin-resistant serotype 19A *Streptococcus pneumoniae* is becoming more prevalent in patients failing first-line amoxicillin therapy [32–34]. Recent guidelines for empiric treatment of AOM include the following [35]. Amoxicillin (80–90 mg/kg/day) or amoxicillin-clavulanate (90/6.4 mg/kg/day) is recommended as initial therapy, with cefdinir, cefuroxime axetil, cefpodoxime, or ceftriaxone (50 mg/kg/day IM or IV for 1–3 day) as alternatives for patients with penicillin allergy. Amoxicillin-clavulanate (90/6.4 mg/kg/day) or ceftriaxone (50 mg/kg/day IM or IV for 3 days) is recommended for patients not responding to treatment after use of amoxicillin for 48–72 h. Addition of clindamycin and diagnostic tympanocentesis are offered as additional options.

3.5 Acute Sinusitis

Although most cases of acute sinusitis are viral, *S. pneumoniae*, untypeable *H. influenzae*, and *M. catarrhalis* are the predominant pathogens when bacterial superinfection occurs, with *M. catarrhalis* being more common in children [22]. Recommended first-line therapy for adults is amoxicillin-clavulanate (1.5 g/375 mg/day–1.75 g/250 mg/day), while recommended second-line therapy is amoxicillin-clavulanate (4 g/250 mg/day) or doxycycline [36]. Doxycycline, levofloxacin, or moxifloxacin is recommended for β-lactam allergic patients. Ampicillin-sulbactam, levofloxacin, moxifloxacin, ceftriaxone, or cefotaxime is recommended for hospitalized patients. Recommended first-line therapy for children is amoxicillin-clavulanate (45/6.4 mg/kg/day), while recommended second-line therapy is amoxicillin-clavulanate (90/6.4 mg/day). Levofloxacin, clindamycin plus cefixime, or cefpodoxime is recommended for β-lactam allergic children. Ampicillin-sulbactam, levofloxacin, ceftriaxone, or cefotaxime is recommended for hospitalized children.

3.6 Acute Exacerbations of Chronic Bronchitis (AECB)

Acute bacterial exacerbations of chronic bronchitis are predominantly caused by the typical upper respiratory bacteria, untypeable *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis*, which make up 85–95 % of cases, with *H. influenzae* usually the most frequent pathogen [37]. In addition, *H. parainfluenzae*, *P. aeruginosa*, *S. aureus*, *M. pneumoniae*, *Legionella pneumophila*, and opportunistic Gram-negative organisms are occasionally implicated, with the latter found principally in severe disease. The presence of a new strain of *H. influenzae*, *S. pneumoniae*, or *M. catarrhalis* from the sputum of a patient with chronic bronchitis increases the relative risk of an exacerbation twofold [4].

Recommendations for treatment of AECB are stratified by the presence of baseline patient factors (pulmonary function, comorbid illnesses, recurrent exacerbations, chronic steroid use, home oxygen use, and hypercapnia) and severity of the exacerbation. Severity of the exacerbation is based on the presence of increased dyspnea, increased sputum volume, and increased sputum purulence. A "mild" exacerbation is one featuring only one of these three symptoms and does not require antibiotic treatment. "Moderate" or "severe" exacerbations require the presence of any two of the three symptoms, and treatment is determined by the severity of baseline patient factors. Recommendations for patients without the baseline risk factors listed above include azithromycin, clarithromycin, doxycycline, cefuroxime axetil, cefpodoxime, and cefdinir. Recommendations for patients with any of the baseline risk factors listed above include amoxicillin/clavulanic acid, levofloxacin, gemifloxacin, and moxifloxacin; ciprofloxacin should be considered if *Pseudomonas aeruginosa* is suspected. Patients with worsening clinical status or inadequate response in 72 h should be reevaluated and have sputum cultures performed [38, 39]. First-line, narrow spectrum antibiotics (amoxicillin, ampicillin, trimethoprim/sulfamethoxazole, and doxycycline) are recommended for elderly patients who are likely to have a low probability of resistant organisms, while second-line, broader spectrum agents (amoxicillin/clavulanic acid, second- or third-generation cephalosporins, and respiratory fluoroquinolones) are suggested for patients with significant risk factors for resistant organisms or who failed initial antibiotic treatment [40].

4 Baseline Susceptibility and Development of Resistance

Every bacterial species typically has a baseline, wild-type population with a defined, usually narrow, range of intrinsic susceptibility to antimicrobial agents at the time of introduction of a new antimicrobial drug class [41]. This defines the

initial spectrum of activity of each antimicrobial agent, and this in turn depends on the dosing regimen and the site of infection. Species can then be studied based on baseline susceptibilities and susceptibilities of strains with decreased susceptibility, should they be present initially or should they develop. Susceptibility breakpoints between susceptibility of baseline, wild-type populations and those of populations with acquired resistance can be used and are referred to as "microbiological breakpoints" [41]. Such breakpoints are very useful but do not necessarily correlate with clinically relevant breakpoints. Unfortunately, many breakpoints in common use for *H. influenzae* are microbiological breakpoints that are of little clinical use, and the current CLSI (formerly NCCLS) interpretation guideline for *H. influenzae* states that results of susceptibility testing using breakpoints provided for the oral β-lactam, macrolide and ketolide agents "are often not useful for the management of individual patients" but "may be appropriate for surveillance or epidemiologic studies" [42].

Clinically relevant susceptibility breakpoints are also typically developed for each agent, enabling isolates to be classified as susceptible, intermediate, or resistant. Such breakpoints should be based on pharmacokinetic and pharmacodynamic (PK/PD) parameters and appropriate clinical studies and should be the same for all species associated with each clinical syndrome, e.g., pneumonia, meningitis, cystitis, otitis, etc. Many breakpoints were developed before these principles were introduced, and some breakpoints in clinical use have been shown not to be appropriate, particularly for oral agents. This is especially the case for *H. influenzae*, as noted earlier, while CLSI does not have breakpoints for *M. catarrhalis*, although other groups such as EUCAST do have these [23]. To overcome this problem, breakpoints based on PK/PD parameters where available, and adequate clinical studies have been developed and will be used in this review to enable meaningful use of the terms clinical susceptibility and clinical resistance [26, 43].

5 Mechanisms of Resistance of *H. influenzae* and *M. catarrhalis*

5.1 β-Lactams

β-Lactams exert an antimicrobial effect by interfering with the formation and maintenance of the peptidoglycan layer of the bacterial cell wall [44, 45]. The cross-linking of stem peptides is facilitated by peptidases, which are located on the extracellular surface of the cell membrane [46]. β-Lactams exert their antimicrobial effect by irreversibly binding to these peptidases, which are frequently referred to as penicillin-binding proteins (PBPs) [47]. Resistance is achieved when genetic alterations result in a PBP that has a reduced affinity for β-lactam antibiotics or when β-lactamases are produced [25, 48, 49]. β-Lactamases are structurally related to PBPs and have a high affinity for β-lactam antibiotics; the interaction between β-lactams and β-lactamases causes a permanent opening of the β-lactam ring, thereby inactivating the antibiotic (Fig. 55.1) [45, 50]. Unlike the interaction between the antibiotic and PBPs, the interaction between β-lactams and β-lactamases does not result in a covalent bond, and the enzyme is free to inactivate other β-lactam molecules.

The predominant mechanism of β-lactam resistance in *H. influenzae* is β-lactamase production, and the genes encoding for β-lactamases in *H. influenzae* are found primarily on plasmids; however, in some cases, these genes are incorporated into the bacterial chromosome [51]. Two distinct β-lactamases are produced by strains of *H. influenzae*: TEM-1 and ROB-1, of which, the TEM-1 β-lactamase is more common [52]. Three β-lactamases are produced by *M. catarrhalis*: BRO-1, BRO-2, and BRO-3, which are structurally similar to each other, but are distinct from the TEM-1 or ROB-1 β-lactamases [53].

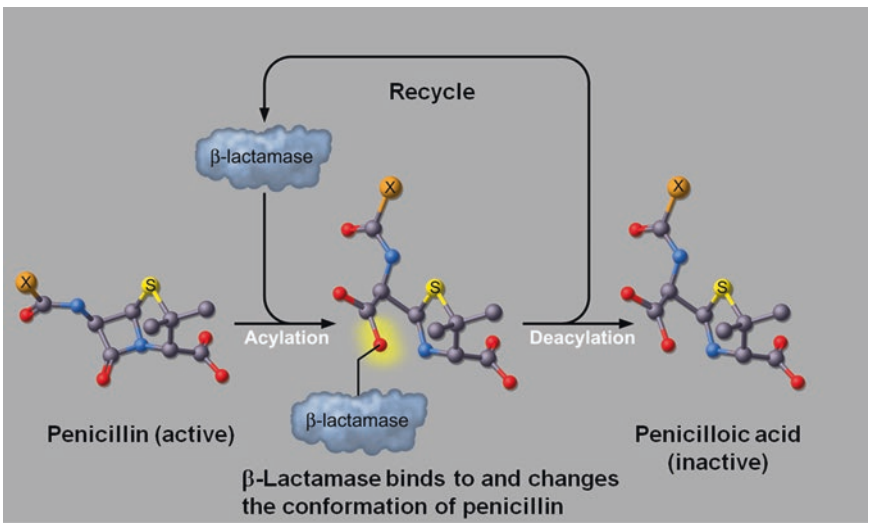

Fig. 55.1 Antibiotic inactivation of penicillins by β-lactamases of *H. influenzae* and *M. catarrhalis*. Copyright Michael R. Jacobs, used with permission

Resistance via β-lactamase production cannot be overcome by increasing the dose of the β-lactam antibiotic (i.e., the concentration at the site of infection) because the β-lactamase enzyme is regenerated following each interaction with—and subsequent inactivation of—an antibiotic. However, this mechanism of resistance can be overcome by using a combination of a β-lactam antibiotic with a β-lactamase inhibitor (e.g., amoxicillin-clavulanate) or by using β-lactam antibiotics that are stable to the actions of β-lactamases (e.g., ceftriaxone, cefuroxime, cefpodoxime, cefixime, provided the pharmacokinetic properties of the agent are adequate). β-Lactamase inhibitors act as "suicide substrates," forming a covalent bond between the enzyme and the β-lactamase inhibitor, inactivating the enzyme, and preventing it from destroying more β-lactam molecules (Fig. 55.2) [54]. β-Lactamase-stable agents evade the action of β-lactamases due to stereochemical blocking of the attachment site of β-lactamases by the side chains of these agents. Extended-spectrum β-lactamase (ESBL) variants of TEM-1 with increased antibiotic resistance to broad-spectrum β-lactam antibiotics and, in some cases, β-lactamase inhibitors (e.g., clavulanic acid) have appeared in *Enterobacteriaceae*, but have not been detected in clinical isolates of *H. influenzae*, although they have been expressed in cloned strains [55]. ESBLs have been reported in two South African isolates of *H. parainfluenzae* that produced a TEM-15 enzyme and had cefotaxime MICs of >16 μg/mL [56].

Non-β-lactamase-mediated resistance to β-lactams due to PBP alterations have occurred in *H. influenzae*, both type b and untypeable strains, mediated via changes in PBP3, which is encoded by the *ftsI* gene [25, 57]. This PBP is made up of an N-terminal hydrophobic region, a central penicillin-binding domain, and a C-terminal domain, and the active site of transpeptidase activity is formed by three conserved amino acid motifs, SXXK, SSN, and KTG (Fig. 55.3). These motifs occur at amino acid positions 326–330, 379–381, 512–514 in PBP3 of *H. influenzae* [28]. Strains with specific

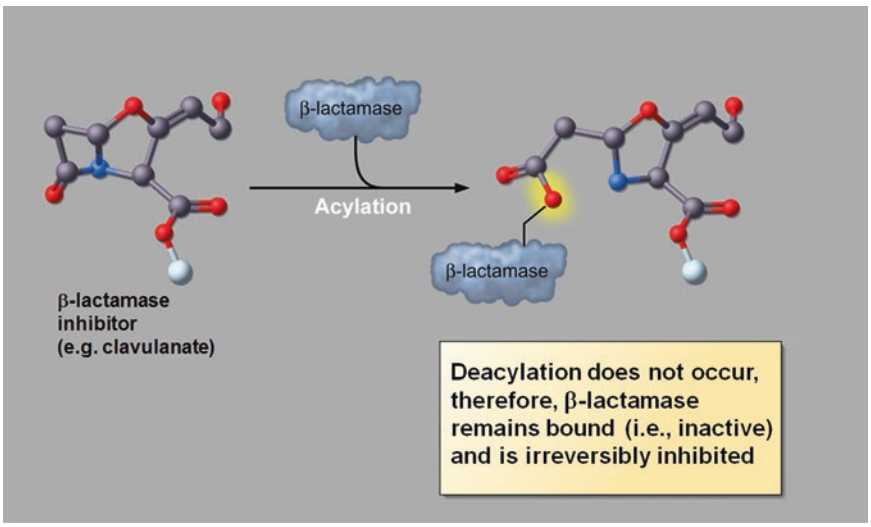

Fig. 55.2 Irreversible binding of a β-lactamase inhibitor to β-lactamase. Copyright Michael R. Jacobs, used with permission

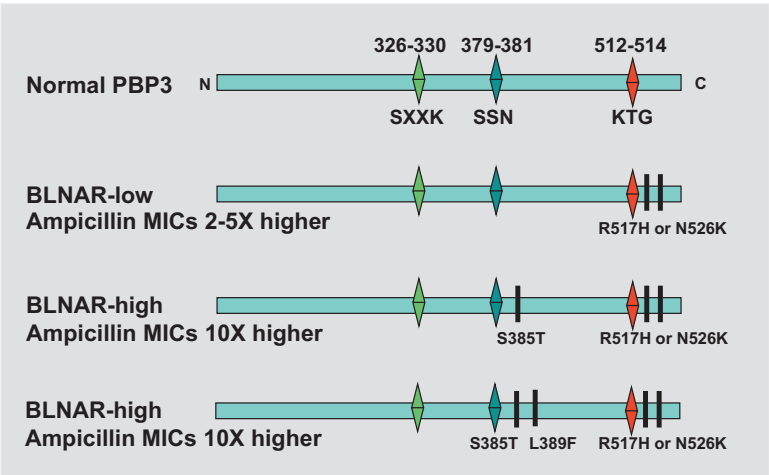

Fig. 55.3 Primary structures and positions of motifs making up the active transpeptidase sites of PBP3 of *Haemophilus influenzae* and mutations associated with low- and high-level BLNAR strains. Adapted from Ubukata et al. [28], Dabernat et al. [58], and Hasegawa et al. [25]. Copyright Michael R. Jacobs, used with permission

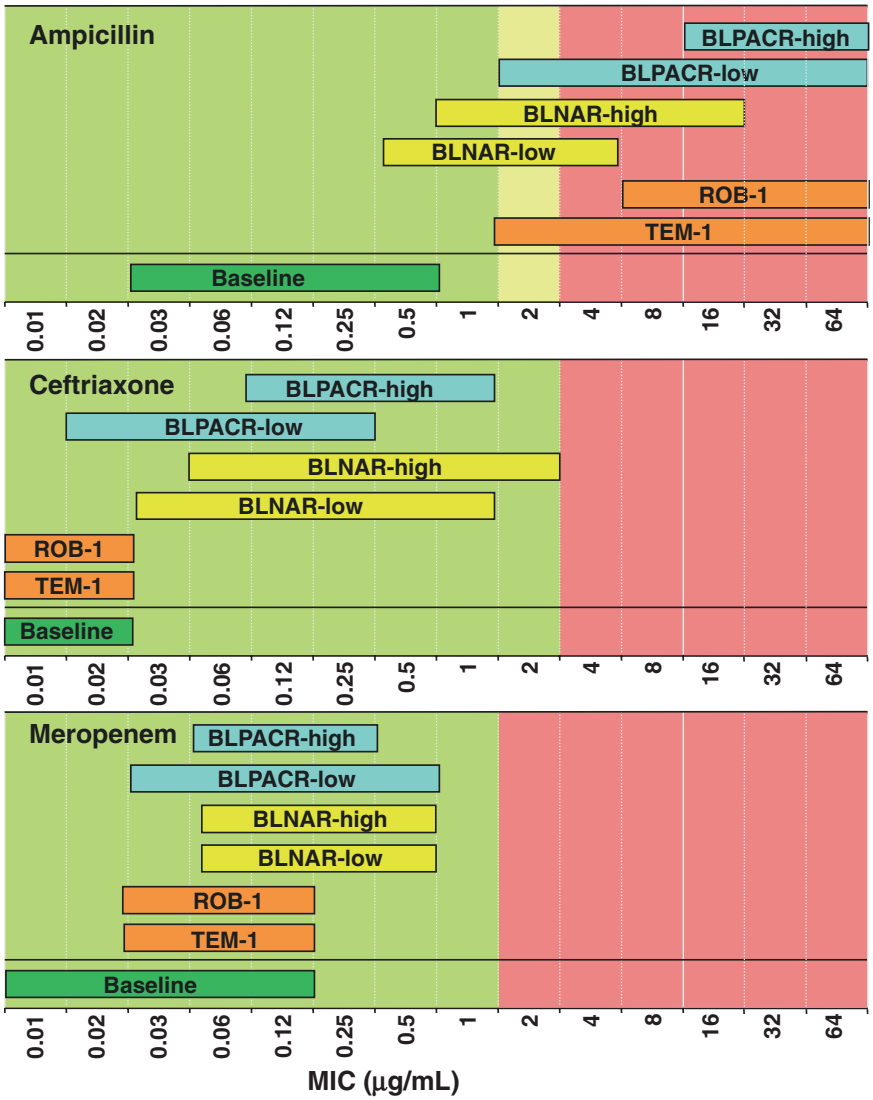

Fig. 55.4 Correlation between β-lactam resistance mechanisms and susceptibility of *H. influenzae* to ampicillin, ceftriaxone, and meropenem. Background color indicates susceptibility based on PK/PD parameters: green, susceptible; yellow, intermediate; red, resistant. Adapted from Hasegawa et al. [6] and Sanbongi et al. [59]. Copyright Michael R. Jacobs, used with permission

mutations in or around these motifs are referred to as β-lactamase negative, ampicillin resistant (BLNAR) or β-lactamase positive, amoxicillin-clavulanate resistant (BLPACR) if they are also β-lactamase positive [57]. Strains are further divided into low- and high-level resistant: low-level BLNAR strains have ampicillin MICs of 0.5–4 µg/mL (compared to a modal value 0.12 µg/mL for wild-type strains), and high-level BLNAR strains have ampicillin MIC of 1–16 µg/mL (Fig. 55.4). Low-level BLNAR and BLPACR strains have N526K or R517H substitutions close to the KTG motif in the *ftsI* gene, while high-level BLNAR and BLPACR strains additionally have S385T or S385T and L389F substitutions close to the SSN motif (Fig. 55.3) [28, 58, 59]. Horizontal transfer of the *ftsI* gene in *H. influenzae* has been demonstrated within and between *H. influenzae* and *H. haemolyticus* [60]. MICs of all β-lactams are higher against strains with *ftsI* mutations than against wild-type strains, and the clinical significance varies based on the PK/PD breakpoint for each agent (Figs. 55.4 and 55.5).

Low-level BLNAR and BLPACR strains are fairly common in many countries, accounting for up to 10 % of isolates, while high-level BLNAR and BLPACR strains to date are rare in most areas, accounting for fewer than 1 % of isolates [61–63]. However, in Japan low-level BLNAR and BLPACR strains have been reported from 26 % of nonmeningeal and 40 % of meningeal isolates, while high-level BLNAR and BLPACR strains account for 13 % of nonmeningeal and 24 % of meningeal isolates [6, 25]. Similar findings in nasopharyngeal isolates from Japanese children with AOM have also been reported [48]. High-level BLNAR and BLPACR strains have also been reported from Korea and Spain [64–66].

5.2 Protein Synthesis Inhibitors

Several classes of agents inhibit protein synthesis [67]. Although these agents are chemically and structurally distinct, they all exert an antimicrobial effect by binding to the

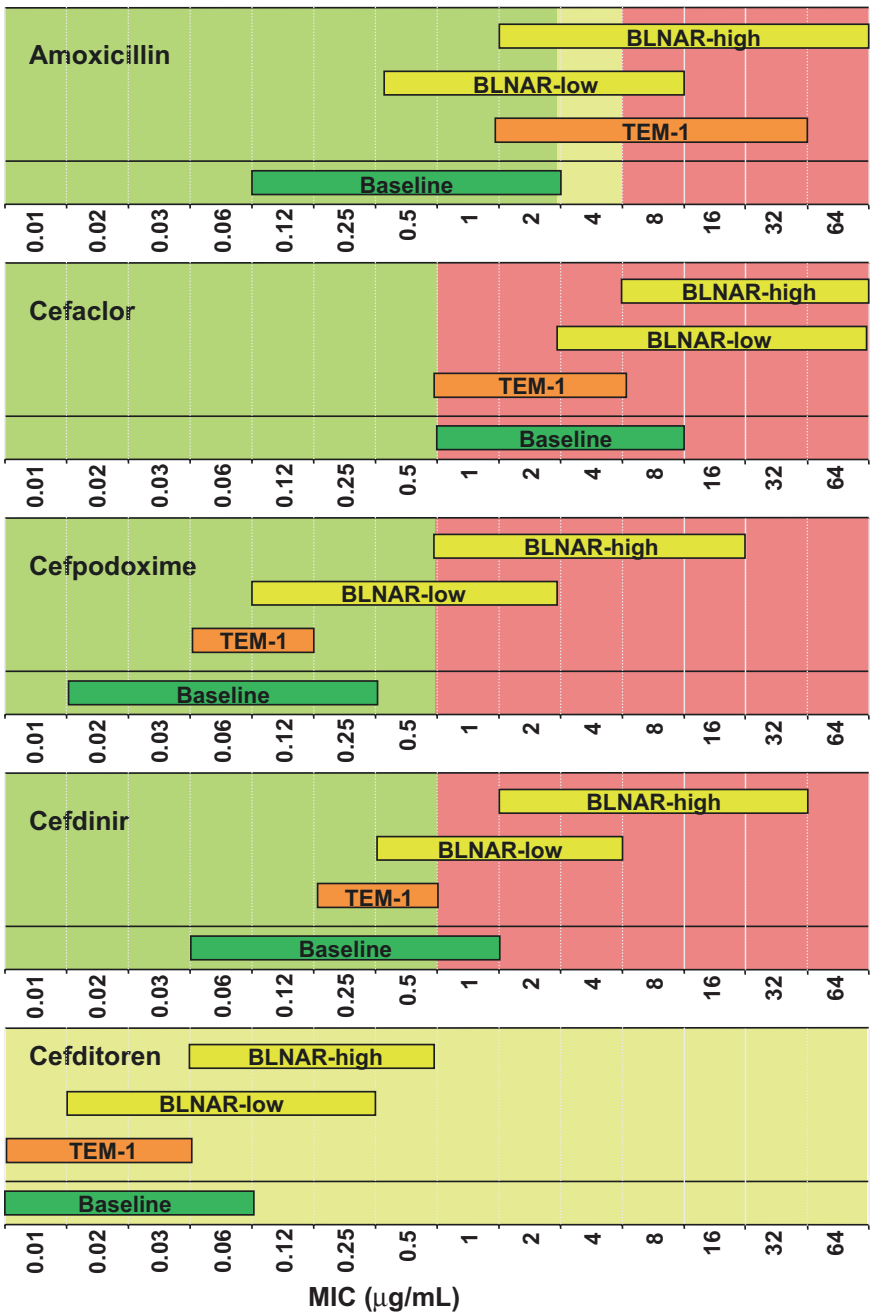

Fig. 55.5 Correlation between β-lactam resistance mechanisms and susceptibility of *H. influenzae* to amoxicillin, cefaclor, cefpodoxime, cefdinir, and cefditoren. Background color indicates susceptibility based on PK/PD parameters: green, susceptible; yellow, intermediate; red, resistant. Adapted from Hasegawa et al. [6] and Sanbongi et al. [59]. Copyright Michael R. Jacobs, used with permission

23S component of the 50S subunit of bacterial ribosomes and disrupting protein synthesis [68]. The number of 70S ribosomes in a typical bacterium ranges from 20,000 to 70,000, each of which consists of two subunits: 50S and 30S. The 50S subunit is comprised of 34 ribosomal proteins and two strands of ribosomal RNA (rRNA; 23S RNA and 5S RNA). The rRNA provides structure to the 50S subunit and determines the position of the ribosomal proteins. Tetracyclines prevent the binding of charged tRNA to the A site of the ribosome; chloramphenicol inhibits the peptidyl transferase reaction of the large subunit of the ribosome; MLS antibiotics, which include macrolides (e.g., erythromycin, clarithromycin), azalides (e.g., azithromycin), lincosamides (e.g., clindamycin), ketolides (e.g., telithromycin), and streptogramins, block the ribosome exit tunnel, thereby preventing movement and release of the nascent peptide.

5.3 MLS Agents and Ketolides

Macrolide resistance mechanisms include efflux pumps, either intrinsic or acquired, ribosomal methylase, and alterations in ribosomal proteins and RNA [69, 70]. *H. influenzae* is intrinsically resistant to MLS agents and ketolides. This is

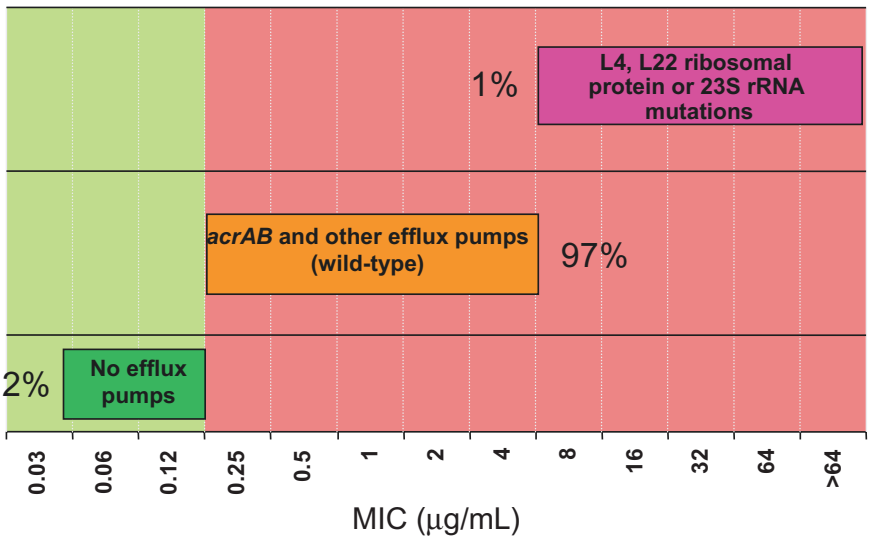

Fig. 55.6 Correlation between azithromycin MICs and resistance mechanisms of *H. influenzae*. Adapted from Peric et al. [74] Copyright Michael R. Jacobs, used with permission

associated with the presence of an *acr*AB efflux pump homologous to this mechanism in *E. coli*, explaining the limited activity of these agents against most wild-type strains of this pathogen [71–73]. Occasional strains of *H. influenzae* lack this efflux pump and have lower MICs than typical wild-type strains, while a few strains have higher MICs associated with mutations in L4 or L22 ribosomal proteins or 23S rRNA (Fig. 55.6).

5.4 Tetracyclines

Tetracyclines exert an antimicrobial effect by binding to the 30S subunit of bacterial ribosomes and preventing tRNA from binding to the A or P sites [75]. Tetracycline resistance in *H. influenzae* is produced by a cell membrane-associated efflux mechanism encoded by the *tet*(B) gene, which is usually located on conjugative plasmids [76, 77]. The efflux protein encoded by the *tet*(B) gene confers resistance to both tetracycline and minocycline, but not glycylcyclines [76]. Tetracycline resistance is often transmitted on conjugative plasmids carrying ampicillin-chloramphenicol-tetracycline-kanamycin resistance genes, which have been described in *H. influenzae* type b isolates in Belgium, Spain, and Cuba [78, 79].

5.5 Quinolones

The quinolones have a broad spectrum of activity and exert an antimicrobial effect by interfering with DNA replication, and subsequently, bacterial reproduction. Two enzymes that are important in the replication process are DNA gyrase and topoisomerase IV, and resistance to quinolones among strains of *H. influenzae* occurs via alterations in the quinolone resistance-determining region (QRDR) of these genes [80, 81]. These alterations can occur via spontaneous mutations or via the acquisition of DNA from other bacteria. The newer quinolones are potent against *H. influenzae*, and the prevalence of resistance among clinical strains is low [49, 63, 82]. However, spontaneous quinolone-resistant mutants are readily selected in vitro by exposure to quinolones, and this has resulted in development of considerable resistance to this drug class in other species [83, 84]. Quinolone-resistant isolates of *H. influenzae* have been shown to have high mutation frequencies [85].

5.6 Chloramphenicol

Chloramphenicol resistance in *H. influenzae* is usually associated with plasmid-mediated production of chloramphenicol acetyltransferase (CAT) encoded by the *cat* gene, with occasional strains having a penetration barrier [86, 87]. The *cat* gene is carried on conjugative plasmids ranging in molecular weight from 34×10^6 to 46×10^6, and these plasmids often carry genes encoding for resistance to tetracycline and ampicillin as well. These conjugative plasmids can also be incorporated into the chromosome [88]. The CAT enzyme produced resembles the type-II CATs produced by enterobacteria. Resistance associated with a permeability barrier is due to the loss of an outer membrane protein [86].

5.7 Folic Acid Metabolism Inhibitors

Trimethoprim and sulfamethoxazole (used alone or in combination) exert an antimicrobial effect by interfering with cellular metabolism and replication by sequentially blocking the production of tetrahydrofolate. During normal cellular metabolism, dihydrofolate is reduced to tetrahydrofolate by

the enzyme dihydrofolate reductase (DHFR) [89]. Tetrahydrofolate is an important cofactor in many cellular reactions, supplying single-carbon moieties for the production of thymidylate, purine nucleotides, methionine, serine, glycine, and other compounds [90]. Inhibiting the production of tetrahydrofolate causes the bacterial cells to die because the lack of thymine prevents DNA replication [91]. Trimethoprim is a substrate analog of dihydrofolate and blocks the reduction of dihydrofolate to tetrahydrofolate by DHFR, whereas sulfamethoxazole is a substrate analog of para-aminobenzoic acid, which is involved in the production of dihydropteroate, a precursor compound of dihydrofolate, blocking the enzyme dihydropteroate synthetase (DHPS) (Fig. 55.7) [89]. Thus, the use of these compounds in combination limits the production of dihydrofolate and prevents the conversion from dihydrofolate to tetrahydrofolate. Both compounds, trimethoprim and sulfamethoxazole, selectively inhibit bacterial metabolism with little toxicity to humans because humans do not synthesize folic acid; rather, the necessary levels of folic acid are obtained from dietary sources.

Resistance to trimethoprim occurs via alteration in the affinity between trimethoprim and DHFR. The decreased affinity is the result of altered genes that encode for DHFR, which often are carried on plasmids or transposons and probably originated from closely related bacteria. Studies have shown that substitutions in the amino acid sequence of DHFR result in resistance to trimethoprim without affecting the affinity of the natural substrates [92–94]. Resistance to trimethoprim-sulfamethoxazole among strains of *H. influenzae* is common and is caused by an increase in the production of DHFR with altered affinity for trimethoprim [95]. Resistance to trimethoprim-sulfamethoxazole also has been noted among strains of *M. catarrhalis*, which is intrinsically resistant to trimethoprim [96–99].

Resistance of *H. influenzae* to sulfonamides is associated with two mechanisms [100]. The first is mediated via the *sul2* gene, a common mediator of acquired sulfonamide resistance in enteric bacteria, which encodes for drug-resistant forms of DHPS. The second is mediated via the mutations in the chromosomal gene encoding DHPS, *folP*, associated with insertion of a 15 bp segment together with other missense mutations.

6 History of Geographical Spread

Bacterial antibiotic resistance results from antibiotic pressure and natural selection and can be spread either through clonal expansion or horizontal transfer, usually through plasmids, phage vectors, or natural transformation systems. The key antimicrobial class to which resistance in *H. influenzae* and *M. catarrhalis* has developed has been the β-lactams, predominantly due to β-lactamase production.

6.1 *Haemophilus influenzae*

Cases of ampicillin treatment failure in *H. influenzae* meningitis were first reported in 1973 [101] and confirmed in 1974 [102, 103] at which time β-lactamase production was identified as the mediating cause [104]. These cases were dispersed throughout the USA, England, and New Zealand. By the late 1970s, ampicillin resistance in *H. influenzae* in the UK was already reported to be at 6.2 %, 92 % of which was β-lactamase mediated [105]. In the early 1980s, BLNAR strains of *H. influenzae* began to be isolated in the USA, the UK, New Zealand, and Japan [105–107]. BLNAR and BLPACR strains are now common in Japan, Korea, and

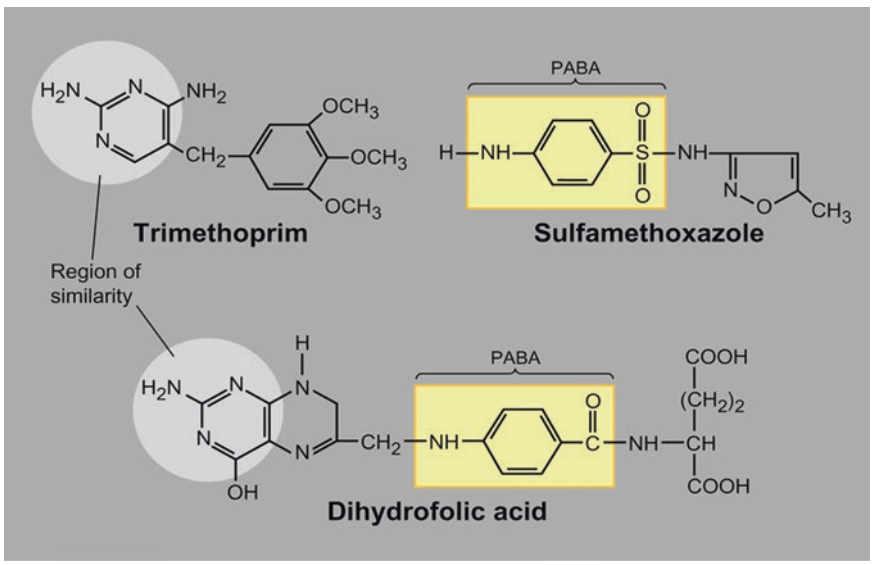

Fig. 55.7 Mechanism of action of trimethoprim and sulfonamides is by mimicry of dihydrofolic acid components, blocking the enzymes involved in conversion of PABA to dihydrofolic acid and dihydrofolic acid to tetrahydropholic acid, the active form of the enzyme. Regions of similarity of trimethoprim and sulfamethoxazole with dihydrofolic acid are highlighted. Copyright Michael R. Jacobs, used with permission

Spain [6, 59, 65, 66]. β-Lactamase production among strains of *H. influenzae* has generally increased from the 1980s, but has been stable for the past two decades [63, 108]. During the early 1980s, the proportion of strains that produced β-lactamases in the USA was approximately 10–15 %, whereas more recent surveillance studies have demonstrated an overall global prevalence of 20 % [63]. Prevalence of β-lactamase production in various countries varied from 4.2 % in Russia to 29.6 % in the USA (Fig. 55.8) [21]. Clonally related, multidrug-resistant *Haemophilus influenzae* PBP3-mediated resistance to broad-spectrum cephalosporins has recently emerged in Norway [109].

The activity of macrolides against *H. influenzae* has remained essentially unchanged throughout the past 30 years, although a few hyper-resistant strains have developed [26, 74]. Resistance to tetracyclines and chloramphenicol has developed, associated with plasmids carrying ampicillin-chloramphenicol-tetracycline-kanamycin resistance genes as noted earlier, predominantly in type b isolates [110]. Resistance to quinolones among clinical isolates of *H. influenzae* is also rare; however, surveillance studies have identified a few clinical strains with increased quinolone MICs, and an outbreak of a highly resistant clone was detected in a long-term care facility [21, 26, 111]. Twelve of 457 isolates

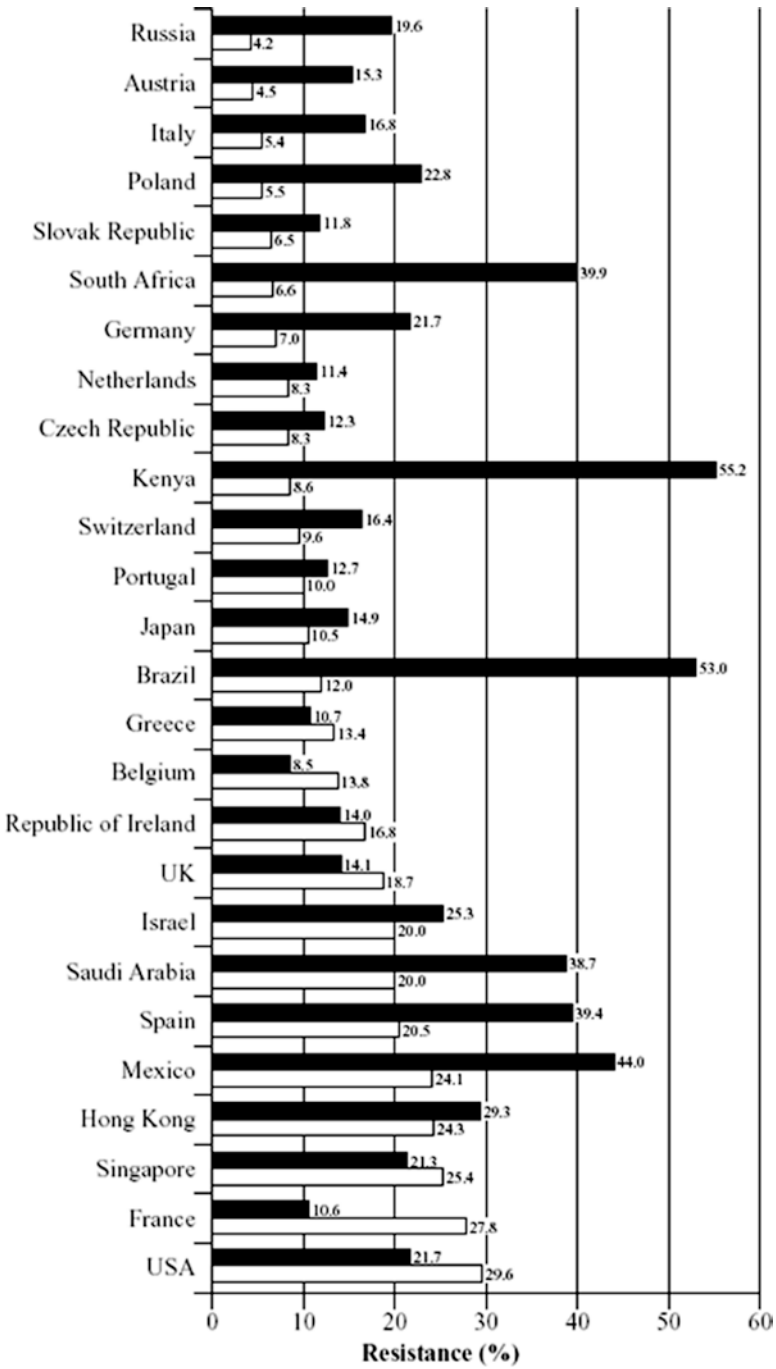

Fig. 55.8 Prevalence of trimethoprim-sulfamethoxazole resistance (MIC ≥ 1 μg/mL) (black bars) and β-lactamase production (white bars) in *H. influenzae*, Alexander Project 1998–2000. Reproduced with permission from Jacobs et al. [21]

(2.6 %) of *H. influenzae* isolated in Hokkaido prefecture, Japan, during 2002–2004, were quinolone resistant, with resistant isolates found only in patients over 58 years of age [82]. In contrast, resistance to trimethoprim-sulfamethoxazole has increased over the past two decades, with resistance varying from a low of 8.5 % in Belgium to a high of 55.2 % in Kenya (Fig. 55.8) [21].

6.2 *Moraxella catarrhalis*

β-Lactamase production among strains of *M. catarrhalis* also is prevalent. β-Lactamase-mediated resistance first appeared in the late 1970s and is now present in at least 90 % of worldwide isolates. Walker and Levy, working from a 10-year veterans administration hospital collection of *M. catarrhalis* isolates, examined the genetic changes that accompanied the transition from less than 30 % to greater than 95 % of isolates being β-lactamase positive in that comparatively brief period [112]. A surveillance study noted that nearly 100 % of strains of *M. catarrhalis* produced β-lactamases [21]. Amoxicillin-clavulanate is active against *M. catarrhalis*, with MICs of 0.12–0.25 μg/mL. β-Lactamase-stable cephalosporins, macrolides, and fluoroquinolones all are active against the majority of strains of *M. catarrhalis*.

7 Clinical Significance

Significant advances have recently been made in understanding the relationships between in vitro susceptibility and in vivo response to infection based on PK/PD correlations. In the absence of human studies or to complement limited human data, susceptibility breakpoints can be established based on animal models and pharmacokinetic parameters. Clinically relevant susceptibility breakpoints can then be derived based on applying these PK/PD parameters to standard dosing regimens. For nonmeningeal infections breakpoints can be derived from nonprotein-bound plasma drug levels present for 25–50 % of the dosing interval for time-dependent agents such as β-lactams and from AUC/MIC ratios exceeding 30 for concentration-dependent agents such as most non-β-lactam agents. These principles have repeatedly been validated in animal models and in bacteriologic outcome in human studies of AOM, AECB, and sinusitis [2, 43, 113–116]. Breakpoints for agents recommended for use against *H. influenzae* and *M. catarrhalis* based on PK/PD parameters, as well as current CLSI and EUCAST breakpoints, are shown in Table 55.1. While PK/PD and EUCAST breakpoints are very similar, many CLSI breakpoints for *H. influenzae* are considerably higher and generally represent microbiological rather than clinical breakpoints as discussed earlier. Susceptibility of worldwide isolates of *H. influenzae* and *M. catarrhalis* to agents recommended for treatment of diseases due to these pathogens is shown in Table 55.2, with regional differences in susceptibility of *H. influenzae* in Table 55.3.

The relationships between MIC distributions and susceptibility breakpoints are important as they determine the clinical activity of agents. MICs of clinically useful agents should be below PK/PD breakpoints, and the greater the difference between MICs and breakpoints, the greater the likelihood that the agent will be successful in clinical use. It is therefore important to examine MIC distributions in relation to breakpoints, and several patterns are found with *H. influenzae* (Figs. 55.9, 55.10, and 55.11) [21, 22, 24–28, 57].

- A unimodal MIC distribution with modal MIC value fourfold (i.e., two doubling dilutions) or more below the breakpoint. This is the case with cefuroxime (parenteral), amoxicillin-clavulanate, cefixime, cefpodoxime, and the quinolones. These agents are therefore highly active against *H. influenzae* and are most suitable for empiric use.
- A unimodal MIC distribution with breakpoint within the MIC distribution, as seen with cefuroxime (oral), cefdinir, cefprozil, and doxycycline. These are agents with limited clinical activity, and their use should be limited to circumstances where other more suitable agents cannot be used.
- A unimodal MIC distribution with breakpoint below the MIC distribution, as seen with cefaclor, erythromycin, azithromycin, clarithromycin, and telithromycin. These agents have intrinsic resistance due to pharmacokinetic limitations and have essentially no clinically useful activity against *H. influenzae*.
- A bimodal MIC distribution with a clearly defined susceptible population below the breakpoint and a clearly defined resistant population, typically with defined resistance mechanisms. This is the case with the ampicillin- and amoxicillin-resistant populations associated with β-lactamase production, tetracycline-resistant population associated with *tetB* gene, trimethoprim-sulfamethoxazole-resistant population associated with mutations in DHFR and DHPS, and chloramphenicol-resistant population associated with *cat* gene. These agents are suitable for directed use against *H. influenzae* and for empiric use where resistance is low, or the consequences of treatment failure are minor, although drug toxicity also needs to be considered.

Comparison of these PK/PD-based susceptibility interpretations with current recommendations for treatment of diseases associated with *H. influenzae* reveal the following:

- **Meningitis.** Current empiric therapy recommendations, vancomycin plus a third-generation cephalosporin such as cefotaxime or ceftriaxone or a third-generation cephalosporin alone if Gram stain enables presumptive pathogen

Table 55.1 Breakpoints (μg/mL) used to determine susceptible (*S*), intermediate (*I*), and resistant (*R*) categories, based on PK/PD, EUCAST, and CLSI interpretative breakpoints [21, 23, 42, 108, 115, 117]. PK/PD breakpoints are applicable to both species

			EUCAST breakpoints						CLSI breakpoints					
	PK/PD breakpoints		*H. influenzae*			*M. catarrhalis*			*H. influenzae*			*M. catarrhalis*		
Antimicrobial	*S*	*R*	*S*	*I*	*R*	*S*	*I*	*R*	*S*	*I*	*R*	*S*	*I*	*R*
Parenteral agents														
Ampicillin	≤2	≥4	≤1	–	≥2	≤1	–	≥2	≤1	2	≥4[a]	–	–	–
Ampicillin-sulbactam	≤2	≥4	≤1	–	≥2	–	–	–	≤2	–	≥2	–	–	–
Piperacillin-tazobactam	≤8	≥16	–	–	–	–	–	–	≤1	–	≥2	–	–	–
Cefuroxime sodium	≤4	≥4	≤1	2	≥4	≤4	8	≥16	≤4	8	≥16	–	–	–
Cefotaxime	≤2	≥4	≤0.125	–	≥0.25	≤1	2	≥4	≤2	–	–	≤2	–	–
Ceftriaxone	≤2	≥4	≤0.125	–	≥0.25	≤1	2	≥4	≤2	–	–	≤2	–	–
Cefepime	≤4	≥8	≤0.5	–	≥1	≤4	–	≥8	≤2	–	–	–	–	–
Ceftazidime	≤8	≥16	–	–	–	–	–	–	≤2	–	–	≤2	–	–
Ceftaroline			≤0.03	–	≥0.06							–	–	–
Meropenem	≤4	≥8	≤2 (0.25)[e]	– (0.5–1)[e]	≥4 (≥2)[e]	≤1	–	≥2	≤0.5	–	–	–	–	–
Imipenem	≤4	≥8	≤2	–	≥4	≤0.5	–	≥1	≤4	–	–	–	–	–
Doripenem	≤4	≥8	≤1	–	≥2	≤2	–	≥4				–	–	–
Ertapenem	≤1	≥2	≤0.5	–	≥1	≤2	–	≥4	≤0.5	–	–	–	–	–
Parenteral and oral agents														
Erythromycin	≤0.25	≥0.5	≤0.5	1–16	≥32	≤0.25	0.5	≥1	–	–	–	≤2	–	–
Clarithromycin	≤0.25	≥0.5	≤1	2–16	≥32	≤0.25	0.5	≥1	≤8	16	≥32	≤1	–	–
Azithromycin	≤0.12	≥0.25	≤0.12	0.5–4	≥8	≤0.25	0.5	≥1	≤4	–	–	≤0.25	–	–
Doxycycline	≤0.25	≥0.5	–	–	–	–	–	–	–	–	–	–	–	–
Trimethoprim-sulfamethoxazole[d]	≤0.5	≥1	≤0.5	1	≥2	≤0.5	1	≥2	≤0.5	1–2	≥4	≤0.5	1–2	≥4
Ciprofloxacin	≤1	≥2	≤0.5	–	≥1	≤0.5	–	≥1	≤1	–	–	≤1	–	–
Ofloxacin	≤2	≥4	≤0.5	–	≥1	≤0.5	–	≥1	≤2	–	–	–	–	–
Gemifloxacin	≤0.25	≥0.5	≤0.25	–	≥0.5	≤0.25	–	≥0.5	–	–	–	–	–	–
Levofloxacin	≤2	≥4	≤1	–	≥2	≤1	–	≥2	≤2	–	–	≤2	–	–
Moxifloxacin	≤1	≥2	≤0.5	–	≥1	≤0.5	–	≥1	≤1	–	–	–	–	–
Rifampin	ND	ND	≤1	–	≥2–	–	–	–	≤1	2	≥4	–	–	–
Chloramphenicol	≤2	≥4	≤2	–	≥4	≤2	–	≥4	≤2	4	≥8	–	–	–
Oral agents														
Amoxicillin (1.5 g/day; 45 mg/kg/day)	≤2	≥4	≤2	–	≥4	–	–	–	–	–	–	–	–	–
Amoxicillin (3–4 g/day; 90 mg/kg/day)	≤4[b]	≥8[b]	–	–	–	–	–	–	–	–	–	–	–	–
Amoxicillin-clavulanate (1.5 g/250 mg/day; 45/6.4 mg/kg/day)	≤2[b]	≥4[b]	≤1	–	≥2	≤1	–	≥2	≤4	–	≥8[c]	≤4	–	≥8
Amoxicillin-clavulanate (4 g/6.4 mg/day; 45 mg/kg/day)	≤4[b]	≥8[b]	–	–	–	–	–	–	–	–	–	–	–	–
Cefaclor	≤0.5	≥1	–	–	–	–	–	–	≤8	16	≥32	≤8	16	≥32
Cefuroxime axetil	≤1	≥2	≤0.25	0.5–1	≥2	≤0.12	0.25–4	≥8	≤4	8	≥16	≤4	8	≥16
Cefixime	≤1	≥2	–	–	–	≤0.5	1	≥2	–	≥1	–	–	–	–
Cefprozil	≤1	≥2	–	–	–	–	–	–	≤8	16	≥32	–	–	–
Cefdinir	≤0.5	≥1	–	–	–	–	–	–	≤1	–	–	–	–	–
Cefpodoxime	≤0.5	≥1	≤0.25	0.5	≥1	–	–	–	≤2	–	–	–	–	–
Ceftibuten			≤1	–	≥2							–	–	–
Telithromycin	≤0.5	≥1	≤0.12	0.25–8	≥16	≤0.25	0.5	≥1	≤4	8	≥16	–	–	–
Tetracycline	≤2	≥4	≤1	2	≥4	≤1	2	≥4	≤2	4	≥8	≤2	4	≥8
Doxycycline	ND	ND	≤1	2	≥4	≤1	2	≥4				–	–	–

ND, not defined

–, no breakpoint available

[a]CLSI breakpoint used to define BLNAR isolates and to regard BLNAR strains of *H. influenzae* as resistant to amoxicillin-clavulanate, ampicillin-sulbactam, cefaclor, cefamandole, cefetamet, cefonicid, cefprozil, cefuroxime, loracarbef, and piperacillin-tazobactam despite apparent in vitro susceptibility of some BLNAR strains to these agents [42]

[b]Breakpoints are expressed as amoxicillin component in a 2:1 ratio of amoxicillin/clavulanic acid

[c]Breakpoint used to defined BLPACR isolates

[d]Breakpoints are expressed as trimethoprim component in a 1:19 ratio of trimethoprim/sulfamethoxazole

[e]Meningitis breakpoints in parentheses

Table 55.2 Susceptibility of worldwide isolates of *H. influenzae* (*N* = 8523) and *M. catarrhalis* (*N* = 874) to 23 antimicrobials and MIC_{50}s and MIC_{90}s. Alexander Project 1998–2000. Adapted from Jacobs et al. [21]

	H. influenzae					*M. catarrhalis*		
	MIC_{50}	MIC_{90}	PK/PD	CLSI		MIC_{50}	MIC_{90}	PK/PD
Antimicrobial	(μg/mL)	(μg/mL)	*S* (%)	*S* (%)	*R* (%)	(μg/mL)	(μg/mL)	*S* (%)
Ampicillin	0.25	>16	NA	81.9	17.0	8	16	NA
Amoxicillin	0.5	>16	81.6	83.2	16.8	8	16	22.7
Amoxicillin-clavulanate, lower dose	0.5	1	98.1	99.6	0.4	≤0.12	0.25	100
Amoxicillin-clavulanate, higher dose	0.5	1	99.6	NA	NA	≤0.12	0.25	100
Cefaclor	4	16	1.4	89.7	3.6	2	4	10.9
Cefuroxime axetil	1	2	83.6	98.1	0.7	1	2	61.9
Cefixime	0.03	0.06	99.8	99.8	NA	0.12	0.5	100
Ceftriaxone	≤0.004	0.008	100	100	NA	0.12	1	97.4
Cefprozil	2	8	22.3	92.5	2.6	4	8	16.0
Cefdinir	0.25	0.5	92.0	97.6	NA	0.25	0.5	100
Erythromycin	4	8	<0.5	NA	NA	≤0.5	≤0.5	99.7[a]
Clarithromycin	8	16	<0.3	79.6	0.9	≤0.5	≤0.5	99.9[a]
Azithromycin	1	2	<1.2	99.5	NA	0.06	0.12	99.3
Chloramphenicol	0.5	1	98.1	97.9	1.9	0.5	0.5	100
Doxycycline	0.5	1	28.9	NA	NA	0.12	0.25	95.8
Trimethoprim-sulfamethoxazole	0.12	>4	78.3	78.3	17.0	0.25	1	72.0
Ciprofloxacin	0.015	0.03	99.9	99.9	NA	0.03	0.06	99.9
Ofloxacin	0.03	0.06	99.9	99.9	NA	0.12	0.12	99.8
Gemifloxacin	0.004	0.015	99.9	NA	NA	0.008	0.015	99.8
Levofloxacin	0.015	0.015	99.9	99.9	NA	0.03	0.06	>99.5
Moxifloxacin	0.015	0.03	99.8	99.8	NA	0.06	0.06	100

NA, not available

[a]For *M. catarrhalis*, the percentage susceptibility to erythromycin and clarithromycin was based on the lowest concentration tested (0.5 mg/L) instead of at the breakpoints of 0.25 (μg/mL)

Table 55.3 Regional differences in susceptibility (%) of *H. influenzae* to antimicrobials based on PK/PD breakpoints (refer to Table 55.1 for breakpoints). Alexander Project 1998–2000. Adapted from Jacobs et al. [21]

Region/country	*N*	Ampicillin	Ampicillin, β-lactamase negative	Amoxicillin	Amoxicillin, β-lactamase negative	Amoxicillin-clavulanate, lower dose	Amoxicillin-clavulanate, higher dose	Cefaclor	Cefuroxime axetil	Cefixime	Ceftriaxone	Cefprozil	Cefdinir	Chloramphenicol	Doxycycline	Trimethoprim/sulfamethoxazole	Ofloxacin
Africa	361	91.4	98.2	90.6	97.3	97.5	100	0.8	80.1	99.4	100	19.7	93.5	96.1	26.6	57.6	100
E. Europe	1393	93.6	99.8	93.6	99.8	99.7	100	0.5	88.0	99.9	99.9	26.4	95.4	99.3	26.5	82.1	100
W. Europe	3064	85.5	99.1	85.7	99.3	99.1	99.9	0.2	87.4	100	100	22.4	93.5	98.7	35.1	83.5	100
Hong Kong	379	74.9	99.0	72.3	95.5	96.0	99.5	0.5	73.4	100	100	8.4	87.3	90.8	16.4	70.7	99.7
Japan	457	81.0	90.5	80.1	89.5	87.1	94.5	0.4	53.8	96.7	100	9.2	66.2	95.4	18.4	85.1	99.8
Saudi Arabia	225	79.1	98.9	78.2	97.8	98.2	100	0.0	80.0	100	100	9.8	88.8	92.0	16.9	61.3	100
Brazil	183	88.5	100	89.1	100	100	100	9.3	95.6	100	100	60.7	100	94.5	49.7	47.0	100
Mexico	191	75.4	99.3	75.4	99.3	99.5	100	5.2	88.0	99.5	100	30.4	94.0	99.5	45.5	56.0	100
USA	2073	69.7	98.8	69.2	98.2	98.5	99.8	3.2	83.5	100	100	23.4	93.9	99.7	23.6	78.3	99.8
All isolates	8523	81.9	98.6	81.6	98.2	98.1	99.6	1.4	83.6	99.8	100	22.3	92.0	98.1	28.9	78.3	99.9

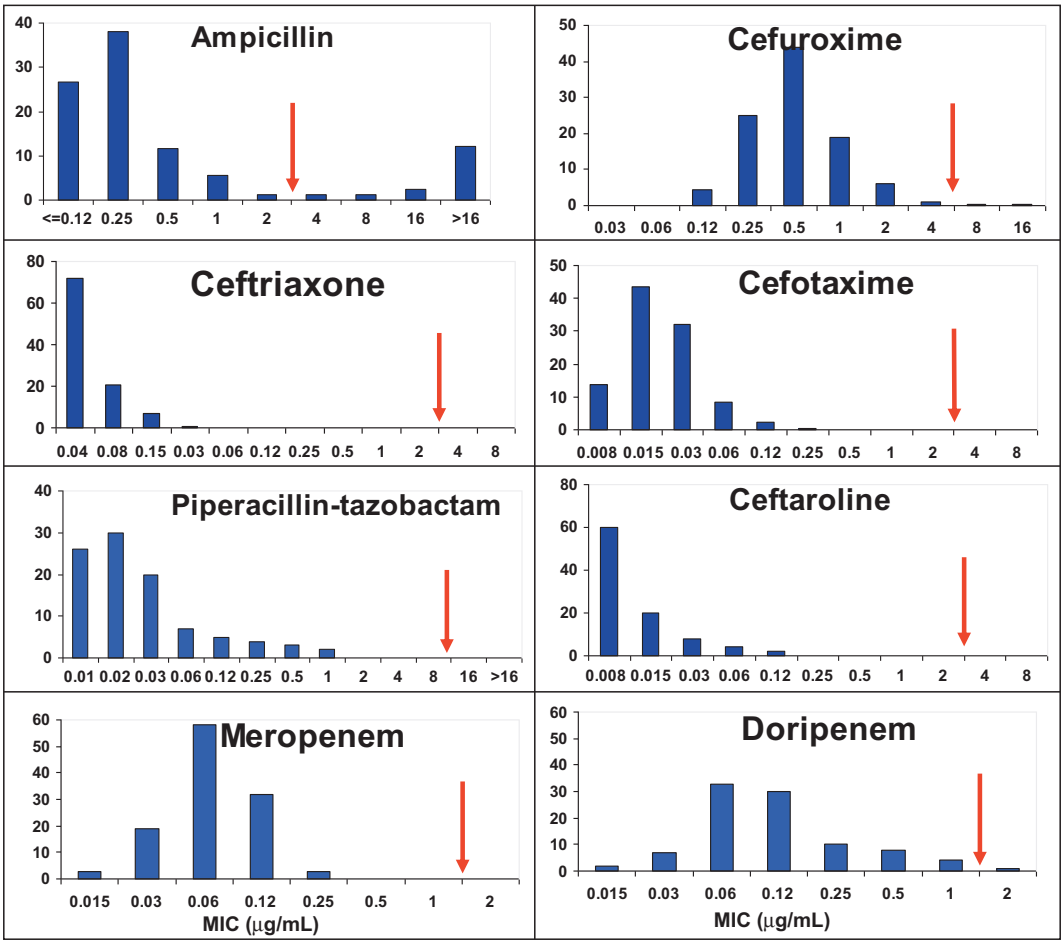

Fig. 55.9 MIC distributions of selected parenteral β-lactam antimicrobial agents for *H. influenzae*. Arrows indicate PK/PD breakpoints. Data adapted from literature or the databases used to generate these publications [21–28]. Copyright Michael R. Jacobs, used with permission

identification as *H. influenzae*, are still valid except in areas where high-level BLNAR and BLPACR strains are prevalent and Hib is not in use [6]. Suggested therapy for areas where high-level BLNAR and BLPACR strains occur is cefotaxime or ceftriaxone plus meropenem based on no additional loss of affinity of meropenem for PBP3 between low and high BLNAR strains (Table 55.4 and Fig. 55.4) [6, 59]. Alternative therapies recommended for *H. influenzae*, chloramphenicol, cefepime and meropenem appear to be valid except in areas where chloramphenicol-resistant strains or high-level BLNAR and BLPACR strains are prevalent. Moxifloxacin is recommended as an alternative agent for adults, which is a valid option as virtually all *H. influenzae* are currently susceptible. The use of quinolones in children should also be considered if other options are contraindicated. Development of susceptibility breakpoints for *H. influenzae* applicable to meningitis would be very worthwhile.

- **Childhood pneumonia and bacteremia.** Current empiric and directed treatment recommendations are valid except for areas where BLNAR and BLPACR strains are prevalent, where the efficacy of oral amoxicillin and amoxicillin-clavulanate against high-level BLNAR strains may be compromised. The efficacy of parenteral cephalosporins and meropenem may also be compromised, but MICs of high-level BLNAR strains are currently below PK/PD breakpoints (Tables 55.4 and 55.5 and Fig. 55.4) [6, 59].
- **CAP in adults.** Recommendation of azithromycin, clarithromycin, or doxycycline for outpatients with no comorbidities and of azithromycin or clarithromycin for outpatients with comorbidities if no antibiotic therapy had been administered in the past 3 months is problematic as these agents have little if any clinical activity against *H. influenzae*, and the activity of these agents against macrolide-resistant pneumococci remains a concern [118]. The remaining recommendations for CAP are valid except for areas where BLNAR and PLPACR *H. influenzae* are found as discussed earlier, in which case respiratory quinolones and meropenem are suitable agents.

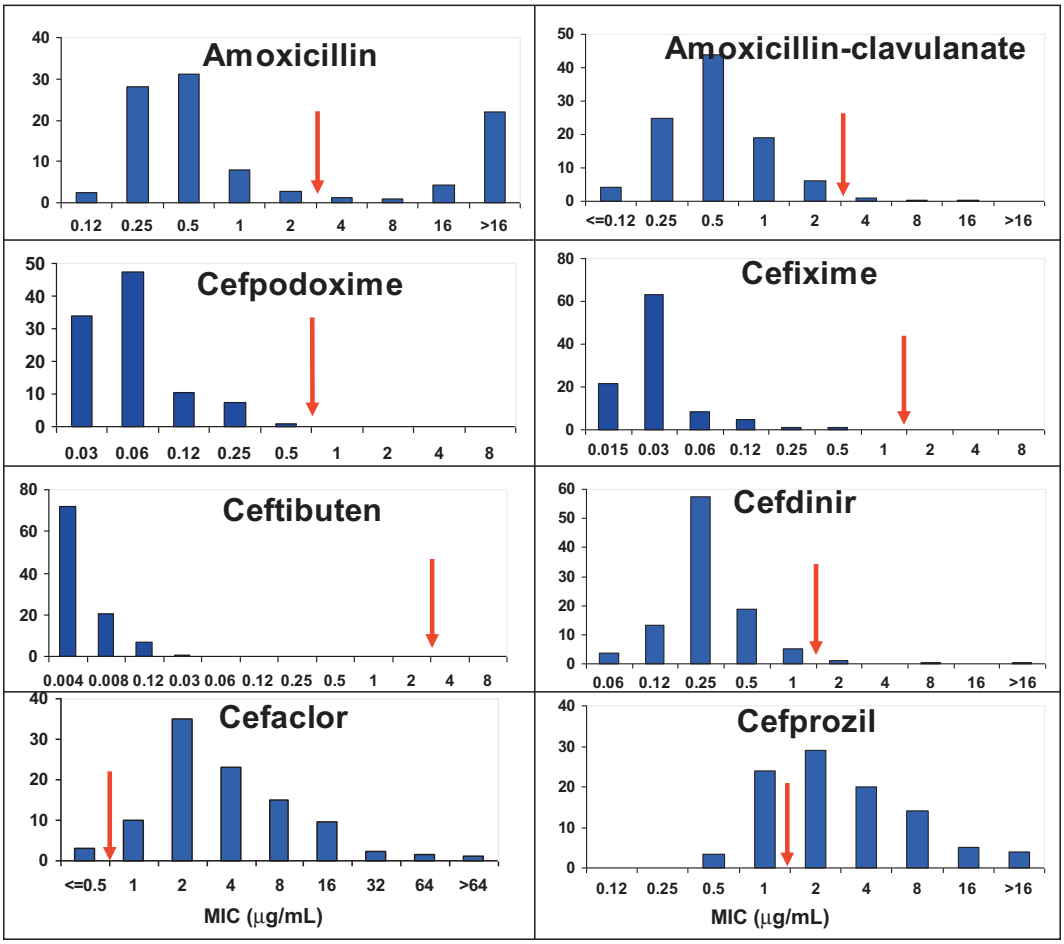

Fig. 55.10 MIC distributions of selected oral β-lactam antimicrobial agents for *H. influenzae*. Arrows indicate PK/PD breakpoints. Data adapted from literature or the databases used to generate these publications [21–28]. Copyright Michael R. Jacobs, used with permission

- **AOM.** Current recommendations are valid with the exception again of areas where BLNAR and PLPACR *H. influenzae* are found, where MIC_{90}s of amoxicillin, cefaclor, cefpodoxime, and cefdinir against high-level BLNAR and PLPACR strains are above PK/PD breakpoints (Table 55.5). The MIC_{90} of cefditoren against high-level BLNAR strains is 0.25 µg/mL; however, the PK/PD breakpoint for this agent has not been established but is likely to be lower than the MIC_{90} value [119, 120]. Cefixime may have clinically useful activity against high-level BLNAR strains, but additional information is needed, and the use of quinolones should be considered [34, 120, 121].
- **Acute sinusitis.** As is the case for AOM, current recommendations for sinusitis are valid with the exception of areas where BLNAR and PLPACR *H. influenzae* are found.
- **AECB.** Recommendations for patients with baseline risk factors, amoxicillin-clavulanate, and respiratory quinolones are valid as *H. influenzae* is the predominant pathogen, and these agents are active based on PK/PD breakpoints. However, of the agents recommended for patients without baseline risk factors, azithromycin, clarithromycin, telithromycin, doxycycline, cefuroxime axetil, cefpodoxime, and cefdinir, only cefpodoxime is active against *H. influenzae* based on PK/PD breakpoints. The rationale for these latter recommendations of agents that will not be effective for the patient group with a high probability of spontaneous resolution is unclear.

8 Laboratory Determination of Susceptibility

8.1 MIC Determination

Susceptibility testing of *H. influenzae* by MIC determination has been well standardized, with CLSI, EUCAST, and other methods generally providing comparable results [42,122]. The main requirements for testing are to ensure that concentrations

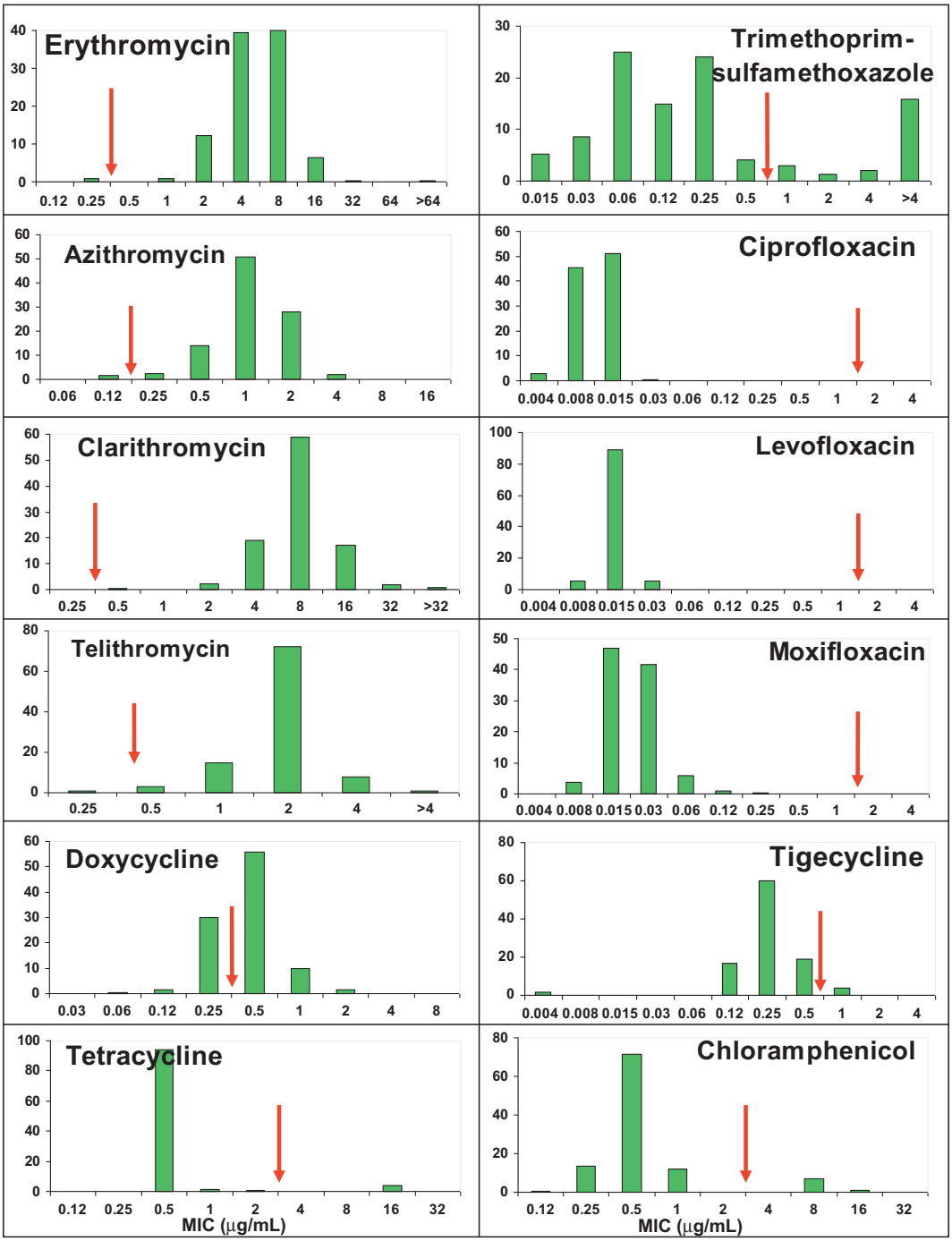

Fig. 55.11 MIC distributions of selected non-β-lactam antimicrobial agents for *H. influenzae*. Arrows indicate PK/PD breakpoints. Data adapted from literature or the databases used to generate these publications [21–28]. Copyright Michael R. Jacobs, used with permission

of hematin, hemoglobin, blood or other iron source, and NAD adequately support growth, that the inoculum size is correct, and that appropriate quality control strains are included in each test batch. The medium specified by CLSI is Haemophilus Test Medium (HTM), which consists of cation-supplemented Mueller-Hinton broth supplemented with 5 mg of yeast extract per mL, 15 µg of NAD per mL, and 15 µg of hematin per mL [42, 123]. The medium specified by BSAC is Iso-sensitest agar or broth supplemented with 5 % horse blood (lysed for the broth formulation) and 20 µg of NAD per mL [124]. Results obtained with these two methods and other variations are comparable [125, 126]. The media containing hematin should be fresh as hematin tends to precipitate out of solution on storage [125]. CLSI specifies susceptibility testing of *M.*

Table 55.4 MIC_{50}/MIC_{90} values of 621 meningeal isolates of *H. influenzae* type b, Japan 2000–2004, based on β-lactam resistance mechanisms. Adapted from Hasegawa et al. [6]

	MIC_{50}/MIC_{90} values (μg/mL) for isolates based on resistance mechanism					
	None (*N* = 155, 25 %)	TEM-1[a] (*N* = 68, 11 %)	Low-level BLNAR[b] (*N* = 189, 30 %)	High-level BLNAR[c] (*N* = 138, 22 %)	Low-level BLPACR[d] (*N* = 59, 10 %)	High-level BLPACR[e] (*N* = 12, 2 %)
Ampicillin	0.25/0.5	8/16	1/2	2/4	16/32	32/64
Cefotaxime	0.016/0.03	0.016/0.03	0.06/0.125	0.5/1	0.06/0.125	0.5/1
Ceftriaxone	0.004/0.008	0.004/0.008	0.016/0.03	0.125/0.25	0.016/0.03	0.125/0.25
Meropenem	0.03/0.06	0.06/0.06	0.125/0.25	0.125/0.25	0.125/0.25	0.125/0.25

[a]TEM-1, TEM-1 β-lactamase gene present
[b]N256K or R517H substitution in *ftsI* gene
[c]S385T substitution with either N256K or R517H substitution in *ftsI* gene
[d]TEM-1 β-lactamase gene and N256K or R517H substitution in *ftsI* gene
[e]TEM-1 β-lactamase gene and S385T substitution with either N256K or R517H substitution in *ftsI* gene

Table 55.5 MIC_{50}/MIC_{90} values of 296 Japanese and 100 US respiratory isolates of untypeable respiratory isolates of *H. influenzae*, 1999, based on β-lactam resistance mechanisms. Adapted from Hasegawa et al. [25]

	MIC_{50}/MIC_{90} values (μg/mL) for isolates based on resistance mechanism									
	None		TEM-1[a]		ROB-1[b]		Low-level BLNAR[c]		High-level BLNAR[d]	
Country	Japan	USA	Japan	USA	Japan	USA	Japan	USA	Japan	USA
N (%)	163 (55 %)	45 (46 %)	9 (3 %)	26 (26 %)	–[e]	10 (10 %)	78 (26 %)	13 (13 %)	39 (13 %)	–
Ampicillin	0.25/0.5	0.25/.025	4/32	8/32	–	16/64	1/2	1/1	2/8	–
Amoxicillin	0.5/0.5	0.5/0.5	4/32	8/32	–	16/64	2/4	2/8	4/16	–
Piperacillin	0.016/0.06	0.016/0.03	1/32	4/32	–	16/64	0.03/0.06	0.03/0.125	0.06/0.25	–
Cefotaxime	0.016/0.03	0.016/0.03	0.016/0.03	0.016/0.016	–	0.008/0.016	0.06/0.25	0.06/0.06	0.5/1	–
Ceftriaxone	0.004/0.008	0.004/0.008	0.004/0.008	0.004/0.004	–	0.004/0.008	0.016/0.03	0.016/0.03	0.125/0.25	–
Cefaclor	2/8	2/8	2/4	2/4	–	16/64	16/64	16/64	32/64	–
Cefpodoxime	0.06/0.125	0.06/0.125	0.06/0.125	0.06/0.06	–	0.06/0.06	0.25/1	0.25/0.5	2/8	–
Cefdinir	0.5/0.5	0.25/0.5	0.25/0.5	0.25/0.5	–	0.25/0.25	1/4	0.5/1	8/16	–
Cefditoren	0.016/0.03	0.016/0.03	0.016/0.06	0.008/0.016	–	0.016/0.016	0.03/0.125	0.03/0.03	0.25/0.25	–
Meropenem	0.06/0.125	0.06/0.06	0.06/0.125	0.06/0.06	–	0.06/0.06	0.125/0.5	0.12/0.25	0.25/0.5	–

[a]TEM-1, TEM-1 β-lactamase gene present
[b]ROB-1, ROB-1 β-lactamase gene present
[c]N256K or R517H substitution in *ftsI* gene
[d]S385T substitution with either N256K or R517H substitution in *ftsI* gene
[e]–, not applicable

catarrhalis with Mueller-Hinton broth or agar [117]. BSAC recommends Iso-sensitest agar supplemented with 5 % horse blood [124]. EUCAST recommends MH-F medium, consisting of Mueller-Hinton broth supplemented with 5 % lysed horse blood and 20 mg/L β-NAD, for testing *Haemophilus* spp. and *Moraxella catarrhalis* [127]. Agar versions of these media are used for disk and gradient diffusion methods.

8.2 Disk Diffusion Testing

CLSI and EUCAST both show interpretative disk diffusion criteria for a number of agents against *Haemophilus* species [42, 128]. However, most MIC distributions are unimodal, and testing of these agents is best performed by MIC determination rather than disk diffusion. Agents showing bimodal MIC distributions, such as ampicillin, amoxicillin, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole, are the most suitable for testing by disk diffusion. EUCAST and CLSI also have some disk diffusion interpretations for *M. catarrhalis* [23, 117]. A major limitation of disk diffusion testing is that interpretative criteria for many agents are based on microbiological rather than PK/PD breakpoints, so their clinical relevance is limited.

8.3 Gradient Diffusion (E-Test)

This method has been widely used and is generally comparable to standard MIC methods for testing *H. influenzae* [129]. MICs of macrolides, ketolides, and quinolones are generally twofold higher by E-test with incubation in a 5–10 % CO_2

atmosphere, so this needs to be considered when interpreting and comparing results [130, 131]. The accuracy of E-test for differentiation of BLNAR and BLPACR strains from baseline strains has not been adequately established [57].

8.4 β-Lactamase Detection

This is best determined by the chromogenic cephalosporin method using nitrocefin, which is converted from a yellow to a pink compound when hydrolized by β-lactamases, or other comparable agents [132].

9 Infection Control Measures

Prior to the introduction of the Hib vaccine, *H. influenzae* type b was the most common cause of bacterial meningitis in children between the ages of 2 months and 5 years. Prevention, through widespread use of the Hib vaccine, has been highly effective. In areas where the vaccine is unavailable or in unvaccinated children, *H. influenzae* type b meningitis remains a childhood threat [8, 133]. The disease remains communicable as long as the organism is present in the nasopharynx and until 24–48 h after beginning effective antibiotic treatment. Contacts, particularly those under 6 years of age, should receive prophylactic treatment with rifampin (20 mg/kg in children and 600 mg in adults, once daily, by mouth for 4 days).

Prevention of AOM due to untypeable *H. influenzae* using a novel vaccine containing polysaccharides from 11 *S. pneumoniae* serotypes each conjugated to *H. influenzae*-derived protein D has been demonstrated [134]. In addition to protection against pneumococcal AOM, efficacy of this vaccine was also shown by a 35.5 % reduction in episodes of AOM caused by untypeable *H. influenzae*.

10 Conclusions

H. influenzae and *M. catarrhalis* are major pathogens associated with common respiratory tract infections, and *H. influenzae* type b is an invasive pathogen of unimmunized children. Treatment of these infections is limited by both intrinsic and acquired resistance, and mechanisms of resistance continue to evolve in these pathogens. Application of PK/PD principles to these pathogens is essential to understand the clinical relationship between in vitro susceptibility and in vivo response. Judicious use of antimicrobial agents is the key to preserving the activity of these agents and to prevent further development of resistance.

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Enterobacteriaceae

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David L. Paterson and Yohei Doi

1 Introduction

Gram-negative bacteria of the Enterobacteriaceae family are important causes of urinary tract infections (UTIs), bloodstream infections, hospital- and healthcare-associated pneumonias, and various intra-abdominal infections. Within this family, *Escherichia coli* is a frequent cause of UTIs, species of *Klebsiella* and *Enterobacter* are important causes of pneumonia, and all of the Enterobacteriaceae have been implicated in bloodstream infections and in peritonitis, cholangitis, and other intra-abdominal infections. Additionally, organisms such as *Salmonella* produce gastroenteritis and, subsequently, in some patients, invasive infection. Emerging resistance in Enterobacteriaceae is a significant problem. Resistance related to production of extended-spectrum beta-lactamases (ESBLs) and carbapenemases is a major problem in the management of infections with the Enterobacteriaceae. The emergence of carbapenemases in Enterobacteriaceae is of particular concern since these organisms often present with extensive drug resistance (XDR) and sometimes even pan-drug resistance (PDR).

2 Overview of Resistance Trends

Approximately 50–60 % of *E. coli* isolates are resistant to ampicillin [1, 2]. This resistance is mediated by broad-spectrum (but not extended-spectrum) beta-lactamases such as TEM-1 [3]. The addition of beta-lactamase inhibitors, such as clavulanic acid, can protect penicillins from hydrolysis by TEM-1. Thus, rates of resistance of *E. coli* to amoxicillin-clavulanate are only approximately 5 % [3, 4]. When all Enterobacteriaceae are considered, almost one quarter of isolates are resistant to amoxicillin-clavulanate [4]. This is likely because of production of beta-lactamases, such as AmpC beta-lactamases, by organisms such as *Enterobacter cloacae,* that are not inhibited by clavulanate.

The third-generation cephalosporins were developed, in part, because of the advent of broad-spectrum beta-lactamases such as TEM-1. The most frequent acquired mechanism of third-generation cephalosporin resistance in Enterobacteriaceae is production of extended-spectrum beta-lactamases (ESBLs). In the United States, the Centers for Disease Control and Prevention (CDC) has ranked ESBL-producing Enterobacteriaceae as a serious threat with an estimated 26,000 cases occurring annually with 1,700 subsequent deaths. In the most recent report by the CDC-coordinated National Healthcare Safety Network (NHSN) System (comprising 2009–2010 data), 28.8 % of *Klebsiella* spp. isolates implicated in central line-associated bloodstream infections (CLABSI) in the United States were resistant to third-generation cephalosporins [5]. This number remained stable compared with the prior period (2007–2008). Resistance to third-generation cephalosporins was observed in 37.4 % of *Enterobacter* species, which also remained stable. Notably, however, the third-generation cephalosporin resistance rate in *E. coli* increased substantially from 12.3 to 19.0 % between these periods. In some countries in Europe, even higher rates of invasive *K. pneumoniae* isolates resistant to third-generation cephalosporins (up to 74.8 % in Bulgaria) have been reported for the year of 2012 (http://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2012.pdf).

Rates of resistance to fluoroquinolones are on the rise as well, especially in *E. coli*, increasing from 37.7 % in 2007–2008 to 41.8 % in 2009–2010 in the aforementioned NHSN survey [5]. In Europe, the rates of fluoroquinolone resistance range from 9.7 % in Iceland to 42.0 % in Cyprus

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and Italy in 2012. Non-susceptibility rates to fluoroquinolones in species other than Enterobacteriaceae are generally lower at 9.7 % in *K. pneumoniae*, 10.1 % in *Enterobacter cloacae*, 17.0 % in *Citrobacter freundii*, and 4.3 % in *Serratia marcescens*, among inpatient urinary tract infection isolates in the United States between 2009 and 2011.

Rates of ciprofloxacin resistance in *Salmonella* spp. are increasing as well, going from just 0.4 % in 1996 to 2.4 % in 2009 according to the CDC's National Antimicrobial Resistance Monitoring System (NARMS) data [6].

Aminoglycosides have maintained good activity against Enterobacteriaceae. Rates of amikacin non-susceptibility were 1.3 % in *E. coli*, 5.5 % in *K. pneumoniae*, and 1.0 % in *E. cloacae* among inpatient UTI isolates in the United States between 2009 and 2011 [7]. Gentamicin non-susceptibility was 16.8 % in *E. coli*, 22.4 % in *K. pneumoniae*, and 7.0 % in *Enterobacter* spp. among pneumonia isolates from the United States and Europe in 2012 [8]. Methylation of 16S ribosomal RNA is emerging as a mechanism of high-level resistance to aminoglycosides across the board (amikacin, gentamicin, and tobramycin) in Enterobacteriaceae [9]. However, this mechanism likely accounts for just a small percentage of aminoglycoside-resistant isolates at the present time.

Carbapenem resistance in Enterobacteriaceae used to be extremely rare, but this has changed dramatically in the last decade. In the most recent report by the NHSN comprising 2009–2010 data, 12.8 % of *Klebsiella* spp. isolates implicated in central line-associated bloodstream infections in the United States were resistant to carbapenems [5]. Unfortunately more recent national surveillance data from the United States is not available. However, in the recent report on antibiotic resistance threats, carbapenem-resistant Enterobacteriaceae (CRE) were ranked one of the three "urgent" threats. It was estimated that 9,300 cases occur annually with 610 deaths. High rates of carbapenem-resistant *K. pneumoniae* have been reported in some countries in Europe, reaching 60.5 % in Greece and 28.8 % in Italy (http://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2012.pdf). Carbapenem resistance in species other than *Klebsiella* spp. remains less common, with 1.9 % for *E. coli* asnd 4.0 % for *Enterobacter* spp. showing resistance to this class among CLABSI isolates in the United States [5].

Acquired resistance to tigecycline and polymyxins is uncommon but has been reported in association with the use of these agents [10–13]. Of note, tigecycline lacks activity against *Proteus* spp., *Providencia* spp., and *Morganella morganii*, and polymyxins are not active against *Proteus* spp., *Providencia* spp., and *Serratia marcescens* [14, 15].

3 ESBL-Producing Enterobacteriaceae

3.1 General Issues and Nomenclature

Infections caused by ESBL-producing Enterobacteriaceae are serious concerns in the current environment. Some ESBLs represent enzymes that have evolved from broad-spectrum (but non-ESBL) beta-lactamases such as TEM-1, TEM-2, and SHV-1. The CTX-M-type ESBLs appear to be derived from chromosomally encoded beta-lactamases produced by *Kluyvera* spp. [3]. ESBLs can hydrolyze most cephalosporins and penicillins. However, ESBLs are typically not active against cephamycins (e.g., cefotetan, cefoxitin, or cefmetazole) or carbapenems (doripenem, imipenem, ertapenem, and meropenem) and can generally be inhibited by beta-lactamase inhibitors, such as clavulanate, sulbactam, or tazobactam. Unlike most ESBLs that have been found in *E. coli*, *K. pneumoniae*, and other Enterobacteriaceae, OXA-type ESBLs have been found mainly in *Pseudomonas aeruginosa* and only rarely in Enterobacteriaceae [16].

ESBLs should be distinguished from other beta-lactamases capable of hydrolyzing extended-spectrum cephalosporins and penicillins. Examples include AmpC-type beta-lactamases and carbapenemases. Carbapenemases may be further grouped as either metallo-beta-lactamases (class B) or serine carbapenemases (classes A and D). Like ESBLs, AmpC beta-lactamases hydrolyze third-generation or expanded-spectrum cephalosporins, but unlike ESBLs, they are also active against cephamycins and are resistant to inhibition by clavulanate or other beta-lactamase inhibitors [17, 18]. In addition, AmpC beta-lactamases do not efficiently hydrolyze fourth-generation cephalosporins such as cefepime and cefpirome. Carbapenemases generally have broader-range activity, inactivating carbapenems as well as expanded-spectrum cephalosporins [17, 19].

3.2 In Vitro Susceptibility Profiles and Clinical Outcomes

Rates of resistance of ESBL-producing Enterobacteriaceae to the cephalosporins should be reviewed with caution. In general, a much greater proportion of Enterobacteriaceae used to be genotypically defined as ESBL producers than would be suggested by examining resistance rates to third-generation cephalosporins according to the Clinical and Laboratory Standards Institute (CLSI) criteria prior to 2010 [20]. This had clinical relevance. In a study of patients with ESBL-producing *K. pneumoniae* bacteremia, 54 % of patients receiving treatment with a susceptible cephalosporin, as determined by old CLSI criteria, experienced clinical failure [20]. These results

were consistent with those from a variety of observational studies, which show rates of clinical failure of >90 %, approximately 67 %, and <30 % with cephalosporin MICs of 8, 4, and ≤2 μg/mL, respectively, when third-generation cephalosporins were used to treat ESBL producers [21, 22]. To address this problem, the CLSI has lowered the susceptibility break points for key cephalosporins in clinical use. Under these revised break points, detection of ESBL is considered optional. This is based on the consideration that the use of cephalosporins will be avoided for most ESBL-producing Enterobacteriaceae by the treating providers since they would report as resistant to them. Still, nearly 40 % of ESBL-producing Enterobacteriaceae are reported as susceptible to ceftazidime due to the low catalytic efficiency of CTX-M-type ESBLs toward this agent [23]. The clinical significance of this phenomenon has not been established.

3.3 Treatment of ESBL Producers

The presence of ESBL-producing Enterobacteriaceae complicates therapy, especially since these organisms are often multidrug resistant. When isolates from a patient indicate an ESBL-producing organism, the first thing to consider is whether the patient has a true infection versus colonization. Patients with positive isolates from urine or perhaps the respiratory tract may be only colonized, and, clearly, there is no indication for treatment in those situations. Assuming the patient has a serious infection due to ESBL-producing Enterobacteriaceae, the choice of empirical therapy is made difficult by the likelihood of multidrug resistance and the fact that there are no data from large, randomized, controlled trials (RCTs) designed to compare one antibiotic therapy with another for infections caused by ESBL-producing organisms. One such RCT is underway (the "MERINO" trial; NCT02176122), but results are not expected until 2017. The major controversy is whether piperacillin/tazobactam can be used for serious infections due to ESBL producers and whether outcomes with this beta-lactam/beta-lactamase inhibitor combination (BLBLIs) are as good as those observed with carbapenem therapy.

Proponents of meropenem superiority point to a number of lines of evidence. Firstly, carbapenems are not typically hydrolyzed by ESBLs nor do they undergo a significant inoculum effect. Several clinical studies have shown a clinical benefit of carbapenem therapy. A prospective, observational, international study of patients with *K. pneumonia* bacteremia reported an all-cause 14-day mortality rate of 3.7 % (1 out of 27) with the use of a carbapenem alone, compared with rates of 36.3 and 44 % with fluoroquinolone and non-carbapenem beta-lactam monotherapy, respectively [22]. For patients infected with ESBL-producing *K. pneumoniae*, the corresponding 14-day mortality rates were 4.8 % (2 out of 42) among patients receiving carbapenem monotherapy or combination therapy and 27.6 % (8 out of 29) among those receiving treatment with a non-carbapenem antibiotic. A number of other studies showed similar results, and a meta-analysis of these published in 2012 found a superiority of carbapenems over non-carbapenem regimens, including BLBLIs [24]. Two subsequent observational studies have also shown superiority of carbapenems over piperacillin/tazobactam [25, 26].

On the contrary side, by definition, ESBLs are inhibited by beta-lactamase inhibitors such as tazobactam [3]. However, *E. coli* or *Klebsiella* may produce multiple beta-lactamase types some of which are resistant to inhibition by tazobactam. Additionally, in some cases outer membrane protein loss may contribute to resistance to tazobactam [27]. Despite these limitations, observational studies suggest that piperacillin/tazobactam may have a role in the treatment of ESBL-producing organisms. The largest observational studies with an analysis by treatment outcome were published in February 2012 by Rodriguez-Bano and colleagues [28] and in October 2012 by Peralta et al. [29]. Rodriguez-Bano performed a post hoc analysis of six published cohorts of patients with bacteremia due to ESBL-producing *E. coli*. In all cohorts, analysis was restricted only to those infections with an organism susceptible to the antibiotic analyzed. In this study, carbapenems (such as meropenem) were not superior to beta-lactam/beta-lactamase inhibitor combinations (such as piperacillin/tazobactam). Specifically, in the definitive therapy cohort, mortality rates at 30 days were not significantly different: 9.3 % for those who received a beta-lactam/beta-lactamase combination (such as piperacillin/tazobactam) and 16.7 % for those who received a carbapenem ($p > 0.20$) [28]. Peralta et al. reviewed the outcome of 387 patients with ESBL-producing *E. coli* or *Klebsiella* infections. Overall inpatient mortality was 20.9–18.2 % for those receiving piperacillin/tazobactam and 25.7 % for those receiving a carbapenem [29]. Again, no superiority was seen when the outcome of those treated with a carbapenem was compared to piperacillin/tazobactam. A recently completed retrospective international cohort study (the "INCREMENT" study) comparing patients receiving definitive therapy with a BLBLI versus carbapenems should add further to the debate on the use of non-carbapenems for ESBL producers.

While ESBLs do not effectively hydrolyze cephamycins (such as cefoxitin or cefotetan), Enterobacteriaceae may exhibit resistance to those agents due to plasmid-mediated expression [30] or overexpression [31] of AmpC beta-lactamases. The development of porin-deficient mutants may also contribute to resistance to cephamycins [32]. Such occurrences have argued against the use of cephamycins in patients with serious infections due to ESBL-producing Enterobacteriaceae. ESBL-producing organisms may be susceptible to cefepime. Subgroup analysis from a randomized,

evaluator-blind trial comparing cefepime with imipenem in patients with nosocomial pneumonia showed that 100 % of patients (10 out of 10) receiving imipenem for pneumonia caused by an ESBL producer experienced a positive clinical response compared with only 69 % of patients (9 out of 13) treated with cefepime [33]. In a retrospective study of 145 patients with bloodstream infections due to ESBL-producing organisms, multivariate analysis showed that empirical therapy with cefepime for BSI due to an ESBL-producing pathogen was associated with a trend toward an increased mortality risk and empirical carbapenem therapy was associated with a trend toward decreased mortality risk [34].

Similarly, fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazole (TMP-SMX) are generally not appropriate initial therapeutic choices for serious infections caused by ESBL-producing Enterobacteriaceae because ESBL producers are often resistant to these drugs as well [35–37]. With fluoroquinolones, even in the presence of apparent susceptibility, there may be a substantial failure rate. In the international study discussed earlier, 36.4 % of patients who received treatment with a fluoroquinolone for bacteria caused by ESBL-producing *K. pneumoniae* died within 14 days [22], and a recent meta-analysis of non-randomized studies suggested that non-carbapenem, non-beta-lactamase beta-lactamase inhibitor agents (such as fluoroquinolones) were associated with higher mortality compared with carbapenems for definitive treatment of ESBL-producing Enterobacteriaceae infections [24].

3.4 Community-Acquired ESBLs

ESBL-producing Enterobacteriaceae are prevalent in the hospital setting, and there is now evidence that they, in particular ESBL-producing *E. coli*, are emerging and spreading in the community as well [38]. Most cases of ESBL-producing organisms in the community have been reported internationally although reports from the United States are also emerging [39, 40]. Most commonly, the cases of community-acquired ESBL producers involve urinary tract infections (UTIs), although gastrointestinal infections in the community may also be important. A population-based laboratory surveillance study of ESBL-producing *E. coli* bacteremia in the Calgary Health Region of Canada reported that 76 % of patients had community-onset disease [41]. The study did not address whether the ESBL-producing *E. coli* were necessarily acquired in the community, but the data do speak to the high prevalence of infections associated with ESBL-producing species in the community [38]. The most common ESBL type in *E. coli* isolated from patients with community-onset infections is of the CTX-M type, in particular CTX-M-14 and CTX-M-15, many of which are produced by a single clonal lineage of *E. coli* belonging to sequence type (ST) 131 and the sublineage *H*30 within ST131 [42–44].

The typical clinical picture for community-associated infection involving ESBLs is UTI (sometimes associated with bacteremia) due to CTX-M-producing *E. coli*, with elderly women being most commonly affected. Isolates are resistant to typical first-line agents for UTI, such as ciprofloxacin, TMP-SMX, gentamicin, and ceftriaxone. So there is now the very real risk that treatment of community-acquired infections with *E. coli* may be compromised because of multidrug resistance. In a study conducted at hospitals in Michigan between 2010 and 2011, 16 and 18 % of ESBL-producing *E. coli* cases were community associated [45]. The healthcare community needs to be aware of this emerging problem of community-acquired ESBL producers, especially now that detection of ESBL is not always performed by microbiology laboratories.

ESBL-producing pathogens may also be involved in gastrointestinal infections acquired in the community. Bacterial species that have been reported to produce ESBLs leading to drug-resistant gastroenteritis include *Salmonella* species, *Shigella*, and Shiga toxin-producing *E. coli* [46–50]. The possible emergence and spread of *Salmonella* strains resistant to antibiotics commonly used as treatment are concerns, because those infections can be invasive. TEM-, SHV-, and CTX-M-type ESBLs, as well as AmpC beta-lactamases, have been identified in infection-causing *Salmonella* [46, 47, 51]. Within the United States, the mechanism of *Salmonella* resistance to third-generation cephalosporins has been linked to production of AmpC beta-lactamases [52–54]. In particular, resistance has been associated with the plasmid-mediated AmpC beta-lactamase known as CMY-2. More recently, *Salmonella*-producing ESBL has been reported among both human and animal isolates in the United States as well [55, 56]. Resistant to third-generation cephalosporins is of concern because (1) ceftriaxone and, secondarily, fluoroquinolones are the drugs of choice for invasive *Salmonella* disease and (2) fluoroquinolones are not indicated for use in children. Fortunately, ceftriaxone-resistant *Salmonella* are currently rare in the United States, but they represent an area that bears further watching.

4 Antibiotic Resistance in *Enterobacter* Species

Enterobacter species are significant causes of nosocomial infection and are intrinsically resistant to aminopenicillins, cefazolin, and cefoxitin due to production of constitutive chromosomal AmpC beta-lactamases [57]. Moreover, beta-lactam exposure is capable of inducing expression of AmpC beta-lactamases in *Enterobacter* species—with consequent resistance to third-generation cephalosporins. Furthermore, mutations can result in permanent hyperproduction and persistent resistance. Treatment of *Enterobacter* infections with third-generation cephalosporins may select for mutant strains

associated with hyperproduction of AmpC beta-lactamase. The prevalence of *Enterobacter* species resistant to third-generation cephalosporins has increased since the introduction and common use of these antibiotics. For example, in one study, resistance to third-generation cephalosporins emerged in approximately 20 % of patients during treatment for *Enterobacter* bacteremia [58]. Multidrug-resistant *Enterobacter* species in initial positive blood cultures were significantly more prevalent ($P < 0.001$) among patients who had previously received third-generation cephalosporins than among patients who had previously received other antibiotic treatments, and they were associated with higher mortality rates [58].

Third-generation cephalosporins should be avoided as treatment for serious infection with *Enterobacter* species because their use in such situations results in selection of derepressed mutants which hyperproduce AmpC. In contrast, cefepime is comparatively stable to AmpC beta-lactamases and therefore has been regarded as a suitable option for treatment of *Enterobacter* infections [57]. However, ESBL-producing *Enterobacter* species, particularly *E. cloacae*, have been identified in the United States [59–62], Europe [63], and Asia [64–66]. Those producing CTX-M- and SHV-type ESBLs may have elevated cefepime MICs which compromise the activity of the antibiotic [67].

5 Fluoroquinolone Resistance

Fluoroquinolones have been used widely for the treatment of serious *E. coli* UTIs and may also be used to treat other infections caused by other members of the Enterobacteriaceae family [68, 69]. Hence, fluoroquinolone resistance in Enterobacteriaceae may lead to treatment failures and is a significant concern, as is the emergence of plasmid-mediated resistance to fluoroquinolones. In the early 2000s, means of 7.3 and 8.2 % of *E. coli* isolates were fluoroquinolone resistant from patients in ICUs and non-ICU areas of US hospitals, respectively [70]. By 2010, 36.5 and 47.1 % of *E. coli* CLABSI isolates from patients in ICUs and non-ICU areas were resistant to fluoroquinolones, respectively [5]. Prior receipt of a fluoroquinolone has been shown to be an independent risk factor for fluoroquinolone resistance [71].

Quinolone resistance in Enterobacteriaceae is usually due to alterations in target enzymes (DNA gyrase and/or topoisomerase IV) or to impaired access to the target enzymes, occurring either because of changes in porin expression or because of efflux mechanisms [72]. Both of these principal means of resistance are caused by chromosomal mutations. The recent deterioration of fluoroquinolone susceptibility in *E. coli* has coincided with worldwide dissemination of *E. coli* ST131 *H*30, which is resistant to fluoroquinolones due to DNA gyrase and topoisomerase IV alterations. Plasmid-mediated fluoroquinolone resistance has also emerged in *K. pneumoniae* and *E. coli*. The first case of plasmid-mediated resistance to fluoroquinolones in *K. pneumoniae* was reported in the United States in 1998 and was from a strain isolated at the University of Alabama in 1994 [71]. The plasmid, pMG252, confers multidrug resistance and was shown to greatly increase fluoroquinolone resistance when transferred to strains of *K. pneumoniae* deficient in outer membrane porins. The gene associated with that resistance has been designated *qnr*. Fluoroquinolone resistance associated with *qnr*-containing plasmids has now emerged in *E. coli* and *K. pneumoniae* strains [73–75]. A study in the United States reported that 11.1 % of *K. pneumoniae* strains from six states exhibited plasmid-mediated fluoroquinolone resistance associated with the *qnr* gene, although none of the *E. coli* strains examined contained *qnr* [76]. Some of the strains contained the original pMG252 plasmid, but *qnr* was carried on different plasmids for others. The mechanism of fluoroquinolone resistance associated with *qnr*-containing plasmids involves inhibition of fluoroquinolone binding with DNA gyrase [77]. Other mechanisms of plasmid-mediated fluoroquinolone resistance in Enterobacteriaceae include acetylation by AAC(6')-lb-cr, a variant of the gene encoding the aminoglycoside acetyltransferase AAC(6')-lb [78], and efflux pump QepA1 [79, 80].

The emergence of this new plasmid-mediated mechanism of fluoroquinolone resistance is particularly worrisome because it provides a mechanism for the rapid development and spread of fluoroquinolone and multidrug resistance to important members of the Enterobacteriaceae family.

5.1 Carbapenem Resistance

The emergence and spread of carbapenem-resistant Enterobacteriaceae (CRE) is one of the most recent and worrisome developments in antimicrobial resistance. The problem is most acute with *K. pneumoniae*. While resistance to carbapenems may involve several combined mechanisms including modifications to outer membrane permeability and upregulation of efflux systems, the recent surge in CRE is mostly mediated by production of carbapenemases. Among various carbapenemases, the most frequently encountered one in the United States and Europe is KPC. In the United States, carbapenem resistance has been observed in strains of *K. pneumoniae*-producing class A carbapenemases, mostly KPC-2 or KPC-3 [81–88]. These enzymes are apparently obtained via plasmid conjugation and are capable of hydrolyzing and inactivating all carbapenems. KPC-producing strains have generally been shown to exhibit multidrug resistance that includes piperacillin-tazobactam, third- and fourth-generation cephalosporins, fluoroquinolones, and aminoglycosides, as well as carbapenems [88]. Loss of outer membrane proteins appears to be a required cofactor for high-level resistance in KPC-2- and KPC-3-producing

strains [83, 84, 89]. The spread of KPC-producing *K. pneumoniae* is understood as a highly clonal process, where most isolates found in the United States as well as worldwide belong to a single lineage ST258 or related STs [90]. As with metallo-beta-lactamase-producing Enterobacteriaceae, susceptibility testing may falsely indicate the clinical susceptibility of KPC-producing *K. pneumoniae* due to an inoculum effect [81, 86, 87].

On a global scale, other emerging carbapenemases of concern include NDM-1 and OXA-48. NDM-1 was initially described in 2009 as a novel metallo-beta-lactamase produced by *K. pneumoniae* and *E. coli* isolates in a patient who returned from India to Sweden [91]. Subsequently, NDM-1-producing Enterobacteriaceae has been found to be highly prevalent in the Indian subcontinent [92, 93] and has spread worldwide in a matter of several years [94]. OXA-48 is a class D serine carbapenemase that was initially reported in *K. pneumoniae* in Turkey [95]. Detection of OXA-48-producing Enterobacteriaceae poses a challenge since, unlike other carbapenemases, OXA-48 hydrolyzes penicillins and carbapenems but not cephalosporins. This means that some OXA-48 producers are susceptible to cephalosporins and may be easily missed unless the carbapenem MICs are high enough for them to be classified as resistant to carbapenems. Nonetheless, it is becoming clear that OXA-48-producing Enterobacteriaceae have a broad geographic distribution, covering North Africa, the Middle East, Turkey, and India [96].

5.1.1 Treatment of Carbapenemase Producers

Options for treating patients infected with carbapenem-resistant *K. pneumoniae* are limited as they are typically resistant to all beta-lactam agents including carbapenems, but some strains remain susceptible to gentamicin, and most remain susceptible to tigecycline and colistin. Agents consistently shown to have in vitro activity against KPC-producing *K. pneumoniae* include tigecycline (65–100 % susceptible), colistin (73–93 %), aminoglycosides (gentamicin 30–63 %, amikacin 6–77 %), and tetracyclines (32–67 %) [81, 88, 97–99].

Colistin is a cationic cyclic polypeptide linked to a fatty acid chain and is often the only agent against carbapenemase-producing Enterobacteriaceae that achieves adequate serum levels exceeding the MICs. However, it has to be administered as a microbiologically inactive prodrug, and due to this unique pharmacokinetic property as well as potential for nephrotoxicity limiting the dosing range, it is often given in combination with another agent as part of combination therapy.

Among 889 patients included in a systematic review of treatment outcome of carbapenemase-producing *K. pneumoniae* infections, 441 received combination therapy and 346 received monotherapy [100]. The mortality rates were 27.4 % for combination therapy and 38.7 % for monotherapy ($p < 0.001$). The mortality rates within monotherapy were 40.1 % for carbapenem, 41.1 % for tigecycline, and 42.8 % for colistin. The mortality rates for combination therapy were 30.7 % for carbapenem-sparing combinations and 18.8 % for carbapenem-containing combinations, suggesting that adding a carbapenem in the combination may provide survival benefit. A large proportion of patients in this analysis were derived from four studies conducted in the United States, Greece, and Italy addressing the clinical outcome of carbapenemase-producing *K. pneumoniae* bacteremia, most of which were due to KPC-producing isolates. These data support the use of combination therapy that includes colistin and/or tigecycline along with carbapenem in the therapy of invasive infections due to carbapenem-resistant *K. pneumoniae*. On the other hand, patients may fare well regardless of therapy for noninvasive infections such as uncomplicated urinary tract infection. In a retrospective study examining the clinical outcome of 21 patients with urinary tract infection due to carbapenem-resistant *K. pneumoniae*, 90 % of them had clinical success regardless of therapy given, and the overall 30-day mortality was low at 6 % [101].

There are several new agents with activity against CRE which have been recently approved or are in late clinical development that merit mention. Avibactam is a non-beta-lactam beta-lactamase inhibitor that is active against known Ambler class A and C beta-lactamases with activity against some Ambler class D enzymes as well [102]. It is not active against MBLs (e.g., NDM, VIM, IMP) due to the absence of the active site serine residue in these enzymes [103, 104]. Of note, avibactam has a potent inhibitory activity against KPC that is substantially greater than that of clavulanate and tazobactam [105]. Ceftazidime-avibactam is FDA approved for the treatment of complicated intra-abdominal infections and complicated urinary tract infections. It may play a role in the empirical monotherapy of invasive infections suspected to be caused by resistant Enterobacteriaceae pathogens and also potentially definitive therapy of KPC-producing Enterobacteriaceae infection.

Plazomicin is a novel aminoglycoside that is designed to resist most clinically relevant aminoglycoside-modifying enzymes and holds promise for the treatment of infections caused by CRE, including KPC-producing *K. pneumoniae* [106]. It is currently undergoing a phase 3 superiority trial targeting CRE bacteremia and pneumonia, where plazomicin-based regimens will be compared with colistin-based regimens. It is however not active against most NDM-producing isolates due to the coproduction of 16S ribosomal RNA methyltransferase.

6 Conclusions

Enterobacteriaceae are significant causes of serious infections, and many of the most important members of this family are becoming increasingly resistant to currently available antibiotics. It is a troubling trend and one that requires vigilance

and intensified measures to control the further spread of resistance by these important Gram-negative pathogens. It should be emphasized that improvements in infection control and antibiotic stewardship are necessary if the steady rise in ESBL-producing and carbapenemase-producing Enterobacteriaceae and in other forms of resistance in these species is to be slowed or stopped.

Infection control is a key aspect in restriction of the emergence and spread of resistant Enterobacteriaceae. With regard to ESBL and KPC producers, there is ample evidence of person-to-person spread. Combining reductions in third-generation cephalosporin use with traditional infection control measures—such as the use of gloves, gowns, and hand hygiene in the care of colonized or infected patients—has been reported to control the hospital spread of multidrug-resistant *K. pneumoniae*. As the list of antibiotics with potential activity against those strains continues to shrink, measures that prevent and slow the spread of multidrug-resistant Enterobacteriaceae strains throughout the world need to be put in action.

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Pseudomonas

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1 Introduction

Pseudomonas species are non-fermentative Gram-negative bacteria that are ubiquitous in diverse environments. *Pseudomonas aeruginosa* is an opportunistic pathogen for humans, and is a major cause of infections among hospitalized patients, especially those with impaired immune function. It is a common cause of hospital-acquired pneumonia, bloodstream, and urinary tract infections. Owing to its low outer membrane permeability, and the expression of several multidrug efflux pumps and chromosomal β-lactamase, *P. aeruginosa* is intrinsically resistant to many antimicrobial agents. Moreover, it has a remarkable capability to acquire additional drug resistance through several pathways, such as the horizontal transfer of resistance determinants and the acquisition of resistance mutations that alter the expression and/or function of chromosomally encoded resistance mechanisms. Multidrug-resistant *P. aeruginosa* in intensive care units have severely limited our therapeutic options. Thus, the increasing emergence of multidrug-resistant *P. aeruginosa* isolates in hospital settings should be regarded as a serious health hazard. A concerted effort is urgently needed to curtail the spread of resistance. The objective of this chapter is to review the current knowledge on *P. aeruginosa* with an emphasis on its antibiotic resistance mechanisms, including intrinsic, acquired, and adaptive mechanisms. Key strategies for prevention and management of *P. aeruginosa* resistance are also discussed.

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2 Overview: Pseudomonas spp.

Pseudomonas bacteria are Gram-negative aerobes that belong to the family Pseudomonadaceae [1]. These bacteria are abundant in the environment. The number of organisms belonging to pseudomonas genus has increased steadily over the years to more than 200 species [2].

Several strains of pseudomonas genus have been identified as human pathogens. For example *P. oryzihabitans* (previously known as *Flavimonas oryzihabitans*) is an emerging pathogen that could cause infections in humans such as bacteremia, urinary tract, and catheter-associated infections [3–6]. *P. fluorescens* is a widespread bacterium that is found in a variety of environments such as refrigerated food and water [7]. It has also been suggested to play a role in Crohn's disease [8, 9] and associated with several outbreaks of bacteremia among hospitalized patients [10, 11]. *P. putida* has been infrequently associated with infections in humans such as bacteremia, urinary tract infections, and pneumonia [12, 13]. Antibiotic resistance in some of these pseudomonas species has been reported [6, 14, 15]. However, these species are generally considered of low virulence and of little clinical significance compared with *P. aeruginosa*, the most important human pathogen belonging to the pseudomonas genus.

P. aeruginosa was first isolated in 1882 by Gessard from green pus [16]. The majority of *P. aeruginosa* clinical isolates produce the green-blue pigment pyocyanin, which is responsible for their characteristic green color [17]. *P. aeruginosa* is very versatile biochemically and could inhabit different environments such as soil and water. It can also adapt efficiently to hospital environment; *P. aeruginosa* was found in cultures of samples taken from hospital sinks, drains, bathtubs, ventilators, and, occasionally, the hands of clinical staff [18]. *P. aeruginosa* also has the capability to tolerate hostile environmental conditions that are deemed inhospitable to the majority of other microorganisms, which makes this bacterium very difficult to eradicate [19].

P. aeruginosa is an opportunistic pathogen that is commonly associated with nosocomial infections, especially

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among immunocompromised patients [20]. Chronic lung infection due to *P. aeruginosa* is very common among cystic fibrosis patients and is considered to be the leading cause of mortality in these patients [21]. *P. aeruginosa* is also an important cause of urinary tract infections, community- and hospital-acquired pneumonia, bacteremia, and soft-tissue infections [22]. A wide array of virulence factors mediate the pathogenicity of this bacterium such as production of proteases, endotoxin A, lipases, phospholipases, and pyocyanin [23]. Additionally, a major virulence determinant is the expression of the type III secretion system (T3SS). By means of T3SS needlelike appendages, effector proteins are translocated from the bacterium into the host cells. Four effectors have been identified in *P. aeruginosa*: ExoY, ExoS, ExoT, and ExoU. These effectors are believed to promote cell injury and antagonize wound healing. Thus, infections due to T3SS-expressing phenotypes are usually invasive and associated with increased mortality [23].

P. aeruginosa infections are generally more challenging to treat; thus, they are associated with high morbidity and mortality [24, 25]. This is attributed to *P. aeruginosa* remarkable capability to resist antimicrobial chemotherapy. In fact, the majority of the known mechanisms of antibiotic resistance, including enzymatic and mutational mechanisms, could be seen in this bacterium [26]. *P. aeruginosa* is intrinsically resistant to a variety of structurally unrelated antimicrobial agents. The complete genome sequencing of the wild-type strain PAO1, achieved in 2000, has provided valuable information on the molecular aspects of this inherent property [19]. Intrinsic resistance in *P. aeruginosa* is attributed primarily to its outer membrane impermeability and the activity of several multidrug efflux pumps [27]. Moreover, *P. aeruginosa* could readily develop additional resistance during treatment through horizontal transfer of resistance determinants and/or resistance mutations. Acquired resistance has been reported to all classes of antipseudomonal drugs: penicillins, cephalosporins, carbapenems, monobactams, fluoroquinolones, aminoglycosides, and polymyxins. Mechanisms of acquired resistance in *P. aeruginosa* include the production of drug-inactivating enzymes, overexpression of drug efflux pumps, alterations in target site, or further reduction in outer membrane permeability. These mechanisms could also present simultaneously resulting in multidrug resistance, which could significantly compromise treatment and adversely affect clinical outcomes [28].

3 Epidemiology of *P. aeruginosa* Infections

3.1 Respiratory Tract Infections

P. aeruginosa is responsible for several pneumonia syndromes. Community-acquired pneumonia (CAP) due to *P. aeruginosa* is rare. However, reported cases of CAP due to *P. aeruginosa* are usually very rapidly progressive and fatal even among previously healthy subjects [29, 30]. Risk factors include lung diseases, especially chronic obstructive pulmonary disease, smoking, HIV infection, previous hospitalization, and intubation [31]. On the other hand, hospital-acquired pneumonia (HAP) due to *P. aeruginosa* is very common, especially in intensive care units (ICUs) and among immunocompromised patients. In fact, *P. aeruginosa* is the leading cause of nosocomial pneumonia and the most frequently isolated bacterium from respiratory tract in the ICUs [32]. *P. aeruginosa* is an established causative pathogen of bronchoscope-associated pneumonia (BAP) through the use of contaminated bronchoscopes [33] and healthcare-associated pneumonia (HCAP) [34]. According to a recent surveillance study, *P. aeruginosa* was the most common cause of ventilator-associated pneumonia (VAP) in different geographic regions, accounting for approximately 26 % of all VAP cases [35]. Duration of hospital stay was identified as a possible risk factor for VAP due to *P. aeruginosa* [36]. Even with appropriate antibiotic therapy, mortality due to *P. aeruginosa* VAP could exceed 40 % [31, 37].

In addition, *P. aeruginosa* is commonly associated with chronic lung infections in cystic fibrosis patients. Chronic lung infection due to *P. aeruginosa* is seen in approximately 80 % of adult cystic fibrosis patients and accounts for the majority of the attributed mortality in these patients [38]. Chronic *P. aeruginosa* lung infections are also common among patients with chronic bronchiectasis and chronic obstructive pulmonary disease [23].

3.2 Urinary Tract Infections (UTIs)

UTIs are the most frequent infections acquired by hospitalized adult patients with an estimated prevalence of 30–40 % of all nosocomial infections. The incidence of nosocomial UTI in critically ill patients ranges between 7 and 31 %. Gram-negative bacteria account for approximately 71 % of UTIs [39]. The organisms most frequently implicated in nosocomial UTIs are *Escherichia coli*, *Proteus mirabilis*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *Enterococcus faecalis* [40]. *P. aeruginosa* is the third most common cause of urinary tract infection [38]. UTIs due to *P. aeruginosa* are more common among ICUs and patients with indwelling urinary catheters [22].

3.3 Skin and Soft-Tissue Infections (SSTIs)

SSTIs include necrotizing infection, infections associated with bites and animal contact, diabetic foot infections, surgical site infections, and burn infections [41, 42]. Risk factors for SSTIs include diabetes, renal failure, cirrhosis, and conditions that impair the immune function such as glucocorticoid use, chronic immunosuppressive therapy, and HIV infection [43].

Infections due to Gram-negative aerobes such as *P. aeruginosa* are frequently reported. *P. aeruginosa* is often identified as the most frequent bacterial pathogen in burn units. *P. aeruginosa* is also the fourth most common cause of surgical site infection following gastrointestinal surgery [38].

3.4 Bacteremia

Bacteremia is a serious and potentially life-threatening medical condition. Although the majority of bacterial bloodstream infections are caused by Gram-positive strains [44], *P. aeruginosa* is also an important causative agent of bacteremia. *P. aeruginosa* bacteremia is associated with high mortality. Reported crude mortality rates due to *P. aeruginosa* bacteremia in ICUs are higher than 50 % among immunosuppressed patients [45]. Immunocompromised patients and patients with malignancy or neutropenia are also at high risk of *P. aeruginosa* bacteremia. Risk factors for mortality in patients with *P. aeruginosa* bacteremia include septic shock, pneumonia, having a severe underlying disease, neutropenia, inappropriate empirical therapy, delay in starting effective antimicrobial therapy, and multidrug resistance [45–48].

4 Intrinsic Resistance Mechanisms

A major challenge in the treatment of *P. aeruginosa* infections is that this bacterium is intrinsically resistant to many structurally unrelated antimicrobial agents [27]. This could be attributed in part to its low outer membrane permeability, owing to its naturally incompetent porin systems, which selectively restricts antibiotic uptake [49]. Porins are trans-outer-membrane proteins that form water-filled channels through which hydrophilic molecules diffuse into the bacterium. Several important families of porins have been identified in *P. aeruginosa* [19]. OprF porins are the most abundantly expressed on the outer membrane of *P. aeruginosa*. Unlike other porin proteins, OprF porins are nonspecific, allowing the diffusion of large as well as small molecules, with an exclusion limit estimated at 3000 Da [50]. Therefore, they are regarded as general-diffusion porins. OprF porins are formed mostly of small channels and a few large channels, which promote the uptake of larger substrates. However, owing to the limited number of large channels, the uptake capacity of large substrates, including antimicrobial drugs, is somewhat restricted. Therefore, the significance of their contribution to antibiotic permeability through the outer membrane of *P. aeruginosa* is debatable [51, 52]. In addition to the nonspecific OprF family, several specific porins have been identified. OprB porins are carbohydrate-selective porins that are primarily responsible for glucose and other sugars uptake such as fructose, mannitol, and xylose [53, 54]. Some evidence suggests that they might also play a role in the uptake of tobramycin [55]. OprD porins promote the uptake of basic amino acids and small peptides [56]. They also play a role in the uptake of carbapenems, in particular imipenem [57–59]. OprD porins are moderately expressed on the outer membrane of *P. aeruginosa*, so mutations that result in deletion of these porins contribute considerably to the reduced susceptibility of this bacterium to carbapenems [60]. Other porin families have not been shown to play a significant role in susceptibility of *P. aeruginosa* to antibiotics [61].

Although poor outer membrane permeability plays a significant role in the reduced susceptibility of *P. aeruginosa*, it does not solely account for its high level of intrinsic antibiotic resistance. Several studies that involved the use of inhibitors or *P. aeruginosa* knockout mutants suggested that the expulsion of antibiotics by energy-dependent multidrug efflux systems constitutes the most important mechanism of intrinsic resistance [62]. The efflux pumps that are associated with clinically significant intrinsic resistance are those belonging to the resistance-nodulation-cell division (RND) family. Whole-genome sequence information suggested the presence of more than ten RND family multidrug efflux pumps in *P. aeruginosa* [19]. Members of RND family that are expressed at basal levels by *P. aeruginosa* wild-type strains and have been shown to confer intrinsic multidrug resistance are MexAB–OprM and MexXY–OprM. The former was the first system to be identified. It is a tripartite system, consisting of an inner membrane drug-proton antiporter (MexB), an outer membrane channel-forming component (OprM), and a periplasmic membrane linker protein (MexA) [63, 64]. It plays a role in the intrinsic resistance to a wide range of antibiotics including fluoroquinolones, some β-lactams such as carbenicillin, tetracycline, macrolides, chloramphenicol, novobiocin, trimethoprim, and some sulfonamides [65–69]. MexAB-OprM expression is regulated by the repressor protein MexR, which is responsible for the negative downregulation of expression [67, 70]. The other efflux system, MexXY-OprM, has the ability to efflux aminoglycosides, tetracycline, and erythromycin [71–73]. However, it appears that their activity is contingent upon the induction of MexXY expression by wild-type strains in the presence of certain antimicrobial agents [72]. Similar to MexAB-OprM, the MexXY-OprM system also utilizes OprM as its outer membrane component [71, 72, 74]. MexZ protein appears to repress the expression of mexXY [73].

In addition to the previously mentioned mechanisms, some strains of *P. aeruginosa* constitutively produce chromosomal AmpC β-lactamase, which hydrolyzes β-lactams by cleaving the amide bond of β-lactam ring [75, 76]. Wild-type strains of *P. aeruginosa* produce insignificant levels of AmpC β-lactamase and are, therefore, susceptible to penicillins, cephalosporins, and carbapenems. Induction of β-lactamase

expression occurs upon exposure to some β-lactams, resulting in reduced susceptibility to β-lactams including the inducing agent [77].

It is worth mentioning that these resistance mechanisms do not appear to function independently; collaboration takes place between different mechanisms resulting in very low susceptibility to antimicrobials. For instance, interplay takes place between poor outer membrane permeability of *P. aeruginosa* and AmpC β-lactamase overproduction, resulting in significant elevation in β-lactams MIC [61, 78]. Another study also suggested that interplay could also take place between efflux and AmpC β-lactamase activity [75]. Therefore, it seems that each mechanism acts to facilitate the function of another mechanism, resulting in the substantial intrinsic resistance observed for this bacterium. Moreover, the intrinsic resistance of *P. aeruginosa* could be further potentiated via target-site mutations.

5 Acquired Resistance Mechanisms

Although the inherent intrinsic resistance is always a concern, the biggest problem with *P. aeruginosa* is its extraordinary ability to acquire additional resistance via several pathways. Segments of DNA, such as plasmids, integrons, or phages that carry antibiotic resistance genes, can rapidly spread resistance among bacterial strains. This mechanism is known as "horizontal transfer of resistance determinants." This type of resistance was demonstrated against several classes of antipseudomonal drugs including aminoglycosides and β-lactams [79–81]. For example, genes encoding aminoglycoside-modifying enzymes, extended-spectrum β-lactamase (ESBLs), metallo-β-lactamases (MBLs), and 16S rRNA methylases could be transferred among *P. aeruginosa* strains through this mechanism [81–86]. Horizontal transfer is particularly critical because it is associated with rapid and broad dissemination of resistant determinants among bacterial populations.

Another important mechanism is acquisition of resistance mutations that have the capability to alter the expression and/or function of chromosomally encoded mechanisms. For example, mutations that compromise the expression of efflux repressor genes *mexR* or *mexZ* could result in overproduction of MexAB-OprM or MexXY-OprM efflux systems, respectively, resulting in reduced susceptibility to their antibiotic substrates [67, 87–90]. Other mutations could result in the induction of MexCD-OprJ and MexEF-OprN [91–94]. These are multidrug efflux systems that are not naturally produced by wild-type *P. aeruginosa* stains. Their expression in response to mutations in *nfxB* and *nfxC* genes results in considerable resistance to a variety of antimicrobial agents [65, 69, 93, 95–97].

Several other resistance mutations such as those resulting in reduced outer membrane permeability via the alteration of porins or mutations in antibiotic-binding sites have been reported in the literature [98, 99]. Such mutational resistance pathways are commonly associated with considerable clinical resistance. Furthermore, it was also suggested that synergistic interplay between different resistance mutations in *P. aeruginosa* could result in high-level resistance [100, 101].

In addition to these pathways, several low-level resistance mutations have been recently described for *P.* aeruginosa [102–105]. Although these mutations were not individually associated with a significant increase in the level of resistance, accumulation of several of these mutations could result in stepwise evolution of high-level resistance. This phenomenon was termed "creeping baselines" [27, 106] and was precisely demonstrated by El'Garch et al. Using a set of generated double-, triple-, and quadruple-PAO1 mutants in their study, they were able to demonstrate the cumulative effects of different nonenzymatic mutations on aminoglycoside resistance, with MICs increasing from 2-fold for the double mutants to 64-fold in the quadruple mutant compared with the wild-type strain PAO1 [107]. This phenomenon has also been shown to affect the susceptibility of *P. aeruginosa* to other classes of antibiotics [106]. Therefore, the gradual accumulation of several low-impact mutations in *P. aeruginosa* genome, as seen with isolates obtained from patients with cystic fibrosis, could ultimately give rise to clinically significant resistance.

Certain types of mutational resistance have been suggested to elicit dramatic modifications in *P. aeruginosa* phenotype. Mutations that inactivate the DNA mismatch repair system (MMR), which is responsible for the maintenance of the genetic material by repairing DNA replication errors, result in rapid increase in the bacterial mutation frequency [108]. These strains are known as hypermutable strains or "mutators," and they are highly prevalent among cystic fibrosis patients who are chronically colonized with *P. aeruginosa* [109]. Mutations in *mutS*, *mutL*, or *mutU* (also known as *uvrD*) are primarily responsible for hypermutation [110]. Mutators have the capability to acquire resistance and multidrug resistance to β-lactams, aminoglycosides, and fluoroquinolones [111–115]. Furthermore, some evidence suggests that mutators acquire additional resistance mechanisms much more rapidly than non-mutator strains [112, 116].

Specific mechanisms of acquired resistance to different antibiotic classes are further discussed later in this chapter.

6 Adaptive Resistance Mechanisms

Adaptive resistance is a set of resistance mechanisms to one or more antimicrobial agents that are induced in response to drastic environmental conditions or the exposure to a certain triggering agent [117]. In contrast to acquired resistance, this class of resistance is characterized by being non-mutational, not

inheritable, and of transient nature (i.e., upon removal of the triggering factor, the MICs gradually revert to near-baseline level). Adaptive resistance was first discovered in the 1960s, but owing to its transient nature, this class of resistance has been overlooked for decades. It is now being increasingly recognized as an important contributor to the poor clinical outcome of *P. aeruginosa* infections along with intrinsic and acquired resistance mechanisms.

Triggering factors for adaptive resistance include subinhibitory concentrations of antibiotics as well as some environmental signals such as pH, anaerobiosis, cation levels, and formation of biofilms [106]. These triggering signals appear to cause the dysregulation of one or more resistance genes, resulting in alteration in function of efflux systems, the outer membrane permeability, and/or the enzymatic activity. Several mechanisms of adaptive resistance have been identified in *P. aeruginosa*. For example, induction of *ampC* gene-encoded β-lactamases in response to exposure to some β-lactams has been previously described [118]. Overexpression of gene encoding the transporter MexY of the MexXY-OprM efflux system has also been observed following exposure to aminoglycosides [119]. However, since adaptive resistance has gained attention only recently, the majority of the molecular mechanisms involved in this type of resistance are still not completely understood.

7 Mechanisms of Resistance to Important Antipseudomonal Drugs

7.1 Resistance to β-Lactams/Carbapenems

β-lactams act by inhibiting the synthesis of bacterial cell wall via blocking the action of transpeptidases, also known as penicillin-binding proteins (PBPs) [120]. This class includes penicillins, cephalosporins, carbapenems, monobactams, as well as β-lactam/β-lactamase-inhibitor combinations [121]. Wild-type strains of *P. aeruginosa* are intrinsically resistant to penicillin G, aminopenicillins, as well as first- and second-generation cephalosporins. They are otherwise susceptible to the majority of the remaining β-lactams such as carboxypenicillins, ureidopenicillins, aztreonam, some third- and fourth-generation cephalosporins, and group 2 carbapenems [122]. *P. aeruginosa* could additionally acquire resistance to β-lactams through the production of β-lactamases, overexpression of several efflux systems, alteration of outer membrane permeability, and/or alteration of PBP (target site) [26].

7.1.1 Production of β-Lactamases

β-Lactamases are enzymes that disrupt the amide bond of β-lactam ring and thus inactivate them. Production of β-lactamases is the most important mechanism of resistance to β-lactams in *P. aeruginosa* [123]. Hundreds of β-lactamases have been reported; they are commonly categorized based on substrate specificity or proteomic homology. Four major classes of β-lactamases belonging to Ambler's molecular classification system have been identified in *P. aeruginosa*: A–D [124, 125]. Class A, C, and D β-lactamases inactivate the β-lactam ring via a catalytically active serine residue [126]. Class B metallo-β-lactamases (MBLs) operate through a different mechanism. They are characterized by having divalent cations, usually zinc, as metal cofactors in their active centers [127].

Carbenicillin-Hydrolyzing β-Lactamases (CARBs)

These enzymes belong to class A of β-lactamases. Four types have been identified in *P. aeruginosa*: CARB-1 (PSE-4), CARB-2 (PSE-1), CARB-3, and CARB-4 [128]. These enzymes can hydrolyze carboxypenicillins, ureidopenicillins, and cefsulodine but not caftazidime or carbapenems. Their activity is inhibited by commercially available β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam [129].

AmpC β-Lactamase (Cephalosporinase)

AmpC β-lactamase belongs to molecular class C of β-lactamases. This enzyme is encoded by the gene *ampC*, which is expressed by wild-type *P. aeruginosa* in low quantities [130, 131]. However, induction of *ampC* expression could occur in the presence of some β-lactams, such as imipenem, resulting in increased enzymatic activity (up to thousandfold). Increased production of AmpC β-lactamase in *P. aeruginosa* confers resistance to third-generation cephalosporins and is a common finding in clinical isolates [132–135]. Although imipenem is an excellent inducer of *ampC*, its resistance to hydrolysis preserves its efficacy against *P. aeruginosa* strains that overexpress AmpC [136, 137]. However, *ampC* overexpression, alone or in combination with OprD inactivation, is correlated with reduced susceptibility to doripenem and meropenem, resulting in two- to four-fold increase in MICs [137]. Additionally, extended-spectrum AmpC, which has the ability to inactivate imipenem as well as oxyiminocephalosporins, has been reported in clinical *P. aeruginosa* isolates [138]. The activity of AmpC β-lactamase is not inhibited by commercially available β-lactamase inhibitors except avibactam [127, 139]. Plasmid-mediated transfer of AmpC β-lactamase has not been detected in *P. aeruginosa*.

Oxacillinases

They are also known as OXA-type enzymes. They belong to class D of β-lactamases. Classical oxacillinases (not belonging to extended-spectrum β-lactamases) include OXA-1, OXA-2, and OXA-10 types and they hydrolyze carboxypenicillins and ureidopenicillins [26, 140, 141]. OXA-50 type, which is chromosomally encoded in *P. aeruginosa*, has been shown to exhibit weak hydrolytic activity against imipenem and, to a much lesser extent, meropenem.

The activity of OXA-50 is weakly inhibited by tazobactam and clavulanic acid [142].

Extended-Spectrum β-Lactamases (ESBLs)

These are a group of β-lactamases that can hydrolyze a wide range of β-lactams including penicillins, narrow- and extended-spectrum cephalosporins, and aztreonam. However, they do not hydrolyze cephamycins and carbapenems [127, 143]. Transfer of ESBL-encoding genes in *P. aeruginosa* is plasmid or integron mediated [141,143]. Several ESBLs have been detected in *P. aeruginosa*, which belong to molecular classes A and D [144].

Class A ESBLs: Seven types have been detected in *P. aeruginosa*: TEM, SHV, PER, VEB, GES/IBC, BEL, and PME [145–152]. Class A ESBLs confer resistance to carboxypenicillins, ureidopenicillins, aztreonam, and extended-spectrum cephalosporins such as ceftazidime, cefepime, and cefpirome. However, they do not confer resistance to carbapenems [144]. Their activity can be inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam [152, 153].

Class D ESBLs: They are also known as OXA-type ESBLs. OXA-11 was the first OXA-type ESBL to be discovered in *P. aeruginosa* [154]. These enzymes confer resistance to cefotaxime, ceftazidime, cefipime, cefpirome, moxalactam, meropenem, and aztreonam [142, 155]. They are not inhibited by β-lactamase inhibitors such as clavulanic acid or tazobactam except for OXA-18 and OXA-45 [125, 127, 143].

Class B Metallo-β-Lactamases (MBLs)

MBLs are considered to be ESBLs as they have the capability to hydrolyze all β-lactams including the carbapenems (imipenem, meropenem, and doripenem) except for aztreonam. Owing to their carbapenem-hydrolyzing capability, they are often referred to as carbapenamases [156]. MBLs are not inhibited by commercially available β-lactamase inhibitors; however, they are susceptible to inhibition by metal ion chelators such as EDTA [157]. Transfer of class B MBL-encoding genes in *P. aeruginosa* is plasmid or integron mediated [85]. The types of MBLs that have been identified in *P. aeruginosa* are IMP, VIM, SPM, GIM, NDM, AIM, and FIM [158–164].

7.1.2 Efflux

MexAB-OprM efflux system contributes to the intrinsic resistance of wild-type *P. aeruginosa* strains to the majority of β-lactams [165]. Moreover, overexpression of MexAB-OprM, which occurs primarily through mutations in *mexR*, *nalC*, *nalB*, or *nalD* genes, is associated with further reduction in susceptibility to β-lactams [67, 70, 166, 167]. Overproduction of MexAB-OprM reduces *P. aeruginosa* susceptibility to penicillins, cephalosporins, monobactams, and meropenem but not to other carbapenems (imipenem or panipenem) [58]. Overexpression of MexCD-OprJ system, due to mutations in the *nfxB* gene, confers resistance to cefepime and cefpirome [168, 169]. MexXY-OprM expression is associated with resistance to cefepime [170]. Mutations in the *nfxC* gene resulting in overexpression of MexEF-OprN pumps confer resistance to carbapenems, especially imipenem. However, this resistance appears to be attributed to the decreased expression of OprD porin observed in *nfxC* mutants [171].

7.1.3 Alteration in Outer Membrane Permeability

Clinical *P. aeruginosa* strains that lack OprD porins due to mutations in *oprD* gene show increased resistance to imipenem but not to other β-lactams [58, 171, 172]. Inactivation of *oprD* is also associated with reduced susceptibility to meropenem and doripenem. However, clinical resistance to these carbapenems appears to be contingent upon the presence of additional mechanisms such as the overproduction of AmpC or MexAB-OprM in the *oprD* mutants [137].

7.1.4 Alteration of Target Site

Production of modified PBP with low affinity to β-lactams by *P. aeruginosa* or reduced transcription of *pbp* is associated with increased resistance to β-lactams [173–175]. Alteration of PBP1a/b pattern has been linked to β-lactam resistance in *P. aeruginosa* clinical isolates [176]. Downregulation of *pbp3* has also been shown to play a role in carbapenem resistance [177]. However, the presence of other underlying resistance mechanisms in the clinical isolates examined makes it difficult to draw a definite conclusion on the relative contribution of such mechanism to the observed resistance.

7.2 Resistance to Aminoglycosides

Aminoglycosides are polycationic molecules that exhibit bactericidal activity against *P. aeruginosa.* These agents act primarily by binding to the bacterial 30S ribosomal subunit, and thus impairing bacterial protein synthesis [178]. The most important mechanism of *P. aeruginosa* resistance to aminoglycosides is through the synthesis of aminoglycoside-modifying enzymes (AMEs). Other important resistance mechanisms include active efflux as well as modification of target site [179].

7.2.1 Aminoglycoside-Modifying Enzymes (AMEs)

AMEs inactivate aminoglycosides by attaching a functional group to the antibiotic molecules, and thus compromising

drug binding to the target site (the bacterial 30S ribosomal subunit) [178, 180]. AME-encoding genes are horizontally transferred through mobile genetic elements such as plasmids and integrons [179]. Three classes of AME have been identified in *P. aeruginosa*:

Aminoglycoside Acetyltransferases (AACs)
They are responsible for acetylation of aminoglycosides. They confer resistance to gentamicin, tobramycin, netilmicin, amikacin, and arbekacin [179, 181–183].

Aminoglycoside Nucleotidyltransferases (ANTs)
They are also known as aminoglycoside adenyltransferases (AADs). They act by adenylation of aminoglycosides such as gentamicin, tobramycin, isepamicin, amikacin, and streptomycin [179, 184].

Aminoglycoside Phosphotransferases (APHs)
They are responsible for phosphorylation of aminoglycosides. They confer resistance to gentamicin, kanamycin, neomycin, streptomycin, isepamicin, tobramycin, and amikacin [179, 185, 186].

7.2.2 Efflux and Alteration in Outer Membrane Permeability

Several studies published in the 1970s and 1980s suggested that reduction in *P. aeruginosa* outer membrane permeability could result in reduced intracellular concentration of aminoglycosides, and thus reduced susceptibility to all aminoglycosides. Impermeability was believed to be the most common resistance mechanism among isolates from cystic fibrosis patients [187–190]. However, it is now known that reduced intracellular accumulation is attributed to active aminoglycoside efflux rather than reduced uptake [73, 191, 192]. Exposure to aminoglycosides has been shown to induce the expression of MexXY-OprM efflux system resulting in reduced susceptibility to aminoglycoside [192]. This induction is attributed to overexpression of the gene encoding the transporter MexY [73, 119, 191], mutations in the repressor *mexZ* gene [193, 194], or other mutations [195]. It has also been suggested that the novel outer membrane protein OpmG and its closely related paralog OpmI are involved in aminoglycoside efflux [196].

7.2.3 Alteration of Target Site

Mutations resulting in alterations in bacterial ribosomes have been associated with high-level resistance to aminoglycosides. Acquisition of the *rmtA* gene, which encodes 16S rRNA methylase, via mobile genetic elements is associated with high-level pan-aminoglycoside resistance [86]. Similarly, acquisition of *rmtD* or *armA* gene, which encodes novel 16S rRNA methylases, confers pan-aminoglycoside resistance [197–199].

7.3 Resistance to Quinolones/ Fluoroquinolones

Quinolones and fluoroquinolones are bactericidal agents that interact with two enzymes that are essential for bacterial DNA replication, transcription, recombination, and repair: DNA gyrase (topoisomerase II) and topoisomerase IV [200]. This interaction results in inhibition of bacterial DNA synthesis as well as RNA synthesis at higher drug concentrations [201]. The two major mechanisms of *P. aeruginosa* resistance to quinolones are the alteration of target enzyme and active efflux.

7.3.1 Alteration of Target Site

Fluoroquinolone resistance mutations occur most commonly in the quinolone resistance determining regions (QRDRs) of the genes encoding DNA gyrase and/or topoisomerase IV. Mutations in *gyrA/gyrB* genes, which encode DNA gyrase, result in production of modified enzyme with reduced affinity to quinolones. This mechanism is associated with a dramatic reduction in the susceptibility of *P. aeruginosa* to ciprofloxacin as well as other quinolones [98, 202, 203]. Mutations in *parC/parE* genes, which encode topoisomerase IV, are also associated with reduced susceptibility to quinolones [92]. Multiple mutations in *gyrA* and/or *parC* gene are associated with high-level resistance [203–205].

7.3.2 Efflux

Quinolones are substrates for all four major efflux pumps identified in *P. aeruginosa*: MexAB-OprM, MexXY-OprM, MexEF-OprN, and MexCD-OprJ. Therefore, mutations resulting in overexpression of any of these pumps generally result in an increase in quinolone MICs, including ciprofloxacin. Active efflux appears to be the most prevalent mechanism of fluoroquinolone resistance among cystic fibrosis isolates [92, 169, 206–209]. A study has also provided evidence that MexVW-OprM system (a newly identified member of the RND family) could be involved in resistance to fluoroquinolones [210].

Although alteration of target enzyme and active efflux are commonly regarded as the only two mechanisms contributing to resistance, a recent study suggested that additional unidentified mechanism(s) may contribute to the high-level fluoroquinolone resistance observed in clinical isolates [211]. In this study, mutations in the QRDRs were introduced into the susceptible *P. aeruginosa* reference strain PA14 and the efflux regulator-encoding genes were inactivated generating mutants that overexpress the MexCD-OprJ,

MexAB-OprM, MexXY, and MexEFOprN efflux pumps. The results indicated that these two mechanisms might not be sufficient to explain the level of fluoroquinolone resistance observed in clinical *P. aeruginosa* isolates. Further studies are needed to corroborate these findings.

7.4 Resistance to Polymyxins

The primary mode of action of polymyxins is through the interaction with lipid A component of the bacterial outer membrane lipopolysaccharide, resulting in alteration in permeability and disruption of cell homeostasis [212]. *P. aeruginosa* can develop resistance to polymyxin as well as other cationic antimicrobial peptides via the modification of lipid A with 4-amino-L-arabinose, which interferes with polymyxin interaction with the outer membrane. Genes in the *arnBCADTEF-pmrE* operon (also known as *pmrHFIJKLME* [PA3552-59]) encode enzymes responsible for the synthesis and the addition of 4-amino-L-arabinose to lipid A [213, 214]. Transcription of *arnBCADTEF-pmrE* operon is stimulated in response to antimicrobial peptide exposure through three two-component regularity systems, PmrAB, PhoPQ, and ParRS [215–217]. Mutations in *pmrB*, *phoQ*, or *parR* genes that result in the activation of this mechanism have been associated with low- to moderate-level polymyxin resistance in clinical *P. aeruginosa* strains [218–220]. Recently, two additional two-component regulatory systems, CprRS and ColRS, have been found to play a role in polymyxins and other cationic antimicrobial peptide resistance in *P. aeruginosa* [221, 222].

8 Biofilm Resistance and Nonreplicating Persisters

Biofilms are populations of one or more types of bacteria that are attached to surfaces and enclosed in exopolysaccharide matrices [223]. Biofilms can grow on medical implants, central venous catheters, urinary catheters, endotracheal tubes, and prosthetic heart valves leading to serious nosocomial infections [224, 225]. Several bacterial species have been shown to grow in biofilms including *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *and Staphylococcus aureus* [226]. Chronic lung infections in cystic fibrosis patients are commonly associated with *P. aeruginosa* biofilms [227].

Formation of biofilms is regarded as a form of adaptive resistance [106]. It has been established that when bacteria are enclosed in biofilms, they become between 10 and 1000 times more resistant to antimicrobials compared with planktonic (free-living) bacteria [228]. This is particularly problematic for cystic fibrosis patients and could explain, at least in part, why in vitro susceptibility testing fails to accurately predict the in vivo efficacy of therapy for infections due to *P. aeruginosa* [229].

Several mechanisms are responsible for *P. aeruginosa* biofilm resistance. It has been suggested that the exopolysaccharide matrix acts as a physical and chemical barrier to the diffusion of antimicrobial agents through the biofilm. Using different in vitro models, it has been shown that the biofilm matrix could limit the interaction between the bacteria and the antimicrobial agent [230, 231]. The growth rate of bacterial cells in biofilm could also contribute to resistance. *P. aeruginosa* cells are metabolically less active and grow at a slower rate in biofilms compared with planktonic cells. This is generally accompanied by increased resistance to antimicrobial agents that target metabolically active cells [232, 233]. The slow growth of bacteria in biofilms could be attributed to slow diffusion of nutrients through the matrix resulting in nutrient deficiency as well as waste product accumulation.

It was also suggested that the high bacterial cell density within biofilm could trigger a general stress response [234]. Two alternative sigma factors, RpoS and AlgT, are known to protect the cell against environmental stresses. These two factors are highly expressed in *P. aeruginosa* cells within biofilm. These two factors have also been shown to increase the resistance of *P. aeruginosa* cells to oxidative biocides and thus they contribute to the observed biofilm resistance [235]. Furthermore, some studies have shown that a population of bacteria within a biofilm does not react uniformly to the action of antimicrobials. Depending on the availability of nutrients, cells that are closer to the exposed side of the biofilm grow at a faster rate compared with cells that are deeply embedded within the matrix. Thus, the location of the bacterial cells within the biofilms seems to play a role in their physiological response to antimicrobials [236, 237]. It has also been suggested that *P. aeruginosa* biofilm cells display particular phenotypes that are profoundly different from planktonic cells and have the ability to combat the damaging effects of antimicrobial agents [238, 239]. For example, a novel ABC-family efflux system that is preferentially expressed in biofilm cells has been shown to confer resistance to aminoglycosides [240].

In addition to the previously mentioned resistance mechanisms, nonreplicating persister cells represent another challenge in the eradication of *P. aeruginosa* biofilms [241]. Persister cells are a group of multidrug-tolerant bacterial cells that constitute a small subpopulation within the bacterial community. These cells can withstand therapeutic concentrations of antibiotics and are able to replicate after the antibiotic pressure is withdrawn, resulting in recurrence and relapse [241]. Persisters are present in substantial numbers in *P. aeruginosa* biofilms and they are increasingly recognized as an important factor in biofilm resistance [242, 243]. The

key difference between persistent cells and drug-resistant cells is that upon regrowth of persisters, the proliferated population will exhibit similar sensitivity to the original bacterial population. On the other hand, acquired resistance mechanisms alter the bacterial genome permanently and are inheritable upon regrowth [241].

Recalcitrant chronic infections with *P. aeruginosa* in cystic fibrosis patients are strongly linked to persisters. Using a longitudinal study design, Mulcahy et al. compared persistence between isolates collected from 15 cystic fibrosis patients at the onset of the chronic infection with *P. aeruginosa* and after 96 months. They were able to demonstrate that formation of persisters increased dramatically for the late isolates without significant increase in drug resistance, which suggested that persisters played a significant role in recalcitrant cystic fibrosis infection [227].

9 Multidrug Resistance

Multidrug resistance among *P. aeruginosa* isolates is an emerging threat that severely limits our therapeutic options, especially in the ICUs [244]. The prevalence of these multidrug-resistant isolates is increasing worldwide at an alarming rate. Clinicians are obligated to adopt more aggressive treatment strategies, such as prolonged and continuous infusion of β-lactam antibiotics, or the use of older antibiotics such as polymyxins, despite their toxicity [245, 246].

A major problem appears when attempting to assess the problem of multidrug resistance in *P. aeruginosa*; a consensus of the definition of multidrug resistance is lacking, which hinders the direct comparison of the findings from different studies. Multidrug resistance in *P. aeruginosa* has been arbitrarily defined in the literature as resistance to at least two, three, four, or eight antipseudomonal drugs [247]. Similarly, many definitions of extensive-drug resistance and pan-drug resistance are being used in the medical literature. Recently, a joint initiative by the European Centre for Disease Prevention and Control (ECDC) and the Centers for Disease Control and Prevention (CDC) led to the creation of a standardized international terminology to describe acquired resistance profiles in all the bacteria that are prone to multidrug resistance: Multidrug resistance was defined as acquired non-susceptibility to at least one agent in three or more antimicrobial classes, while extensive-drug resistance was defined as non-susceptibility to at least one agent in all but one or two antimicrobial classes. Pan-drug resistance should be reserved for isolates that are resistant to all available therapeutic options [248].

Several surveillance studies have attempted to elucidate the specific mechanism(s) of multidrug resistance in *P. aeruginosa*. Multidrug-resistant phenotypes were generally attributed to multiple sequential resistance mutations or acquisition of resistance genes via horizontal transfer, each conferring resistance to one class of antibiotics. It could also be mediated via a single mechanism such as overexpression of multidrug efflux pumps(s). Overexpression of the efflux pump(s) of the RND family was a common finding among multidrug-resistant clinical isolates. In particular, overexpression of MexB of the MexAB-OprM system, which confers resistance to a broad spectrum of antimicrobial agents, was frequently detected in clinical isolates [133, 135, 249, 250]. Overexpression of MexXY-OprM was also highly prevalent (up to 72 % in some series) [135, 166, 249–252]. Loss of outer membrane porin OprD, which confers resistance to carbapenems, was frequently detected in up to 100 % of the isolates [135, 249, 250, 252]. Mutations in QRDR of *gyrA* and *parC* were common in multidrug-resistant isolates [133, 249]. Finally, overexpression of chromosomal AmpC β-lactamase [135, 249, 253] as well as acquisition of MBL (e.g., VIM-2, and VIM-4) [133, 249, 254], ESBLs [250, 253], and OXA-type β-lactamases [249, 250] were also reported.

Several risk factors for the isolation of multidrug-resistant *P. aeruginosa* in clinical settings have been identified recently. Prior exposure to antibiotics, especially quinolones, was an important risk factor for nosocomial infections with multidrug-resistant isolates in several studies [255–260]. Prior exposure to β-lactam antibiotics or aminoglycosides was also associated with increased risk for isolation of multidrug-resistant phenotypes [25, 244, 256, 260, 261]. Other risk factors included ICU stay [244, 262], being bedridden, the use of invasive devices [244], a history of *P. aeruginosa* infection during the preceding year, a history of chronic obstructive pulmonary disease [261], corticosteroid therapy [260], and mechanical ventilation [25].

10 Effect of Antibiotic Resistance on Fitness and Virulence

Whether different resistance mutations are associated with a cost in *P. aeruginosa* fitness and virulence is debatable. Several studies have suggested that resistance mutations come at a physiological cost to the bacterium, which compromises its ability to develop stress adaptation mechanisms. This cost is manifested by the reduced bacterial survival under suboptimal growth conditions such as in an animal host or in a nutrient-limited environment [263–267]. For example, resistant mutants that overexpress one or more efflux system are usually less fit compared with their wild-type counterparts [263, 267]. Thus, in the absence of an antibiotic selective pressure, resistant phenotypes were less capable of competing with their susceptible counterparts coexisting in the environment. However, bacterial adaptation through the acquisition of compensatory mutations could act to restore the bacterial fitness without a significant reduction

in bacterial resistance [268, 269]. Metabolic compensation of fitness costs without acquiring compensatory mutations was also recently reported [270]. Thus, the notion that resistant isolates are usually less fit compared with their wild-type counterparts remains controversial.

Although infections resulting from resistant isolates are generally associated with worse clinical outcomes [244, 271, 272], several reports suggested that some multidrug-resistant *P. aeruginosa* isolates were less pathogenic than nonresistant isolates, as reflected by reduced production of virulence determinants and higher bacterial clearance rates [62, 266, 273, 274]. Thus the adverse outcomes in patients infected with resistant isolates could be attributed, at least in part, to factors other than virulence such as decreased effectiveness of second-line antibiotics or a delay in the initiation of appropriate antimicrobial therapy. However, some reports observed that certain multidrug-resistant *P. aeruginosa* genotypes displayed a highly virulent phenotype; isolates harboring the *exoU* gene of the T3SS were rapidly cytotoxic and had the greatest impact on disease severity compared with the exoS genotype [275, 276]. Additionally, some resistance mutations have been shown to enhance virulence. For example, *P. aeruginosa* strains that lacked OprD porin were more pathogenic compared with strains expressing this porin [277]. Therefore, it seems that a detailed phenotypic analysis is essential in order to accurately predict the fitness and virulence of the clinical isolates.

11 Prevalence of Resistance and Multidrug Resistance

Resistant *P. aeruginosa* isolates are highly prevalent worldwide. Resistance is particularly a problem in ICUs and among cystic fibrosis patients; aminoglycoside resistance is common among isolates from cystic fibrosis patients, while resistance to β-lactams and fluoroquinolones is highly prevalent among ICU isolates.

The trends in the incidence of resistance among clinical *P. aeruginosa* isolates are somewhat controversial. A national surveillance of the resistance rates among *P. aeruginosa* isolates obtained from ICU patients in the USA between 1993 and 2002 revealed a significant decline in susceptibility to all antipseudomonal drugs over the years [278]. The most significant increase in resistance rate was observed for ciprofloxacin (15–32 %), imipenem (15–23 %), and tobramycin (9–16 %). The rate of multidrug resistance, defined as resistance to ≥3 of the following drugs, ceftazidime, ciprofloxacin, tobramycin, and imipenem, also increased steadily over the 10-year period (4–14 %). The resistance rates to different antipseudomonal agents for isolates from 2002 are shown in Table 57.1. On the other hand, another national surveillance for *P. aeruginosa* isolates from US ICUs between 1993 and 2004 failed to demonstrate an increasing trend in emergence of resistance for the majority of the tested antipseudomonal drugs, except for ciprofloxacin (11.2–28.9 %), imipenem (10.6–14.5 %), and tobramycin (7.8–13.7 %) [279]. Nevertheless, a significant increase in the rate of multidrug resistance, defined as resistance to at least one extended-spectrum cephalosporin, one aminoglycoside, and ciprofloxacin, was observed over the 12-year period (1.7–9.3 %). The resistance rates among isolates collected between 2002 and 2004 from the latter study are reported in Table 57.1. Likewise, a summary of antimicrobial resistance patterns for healthcare-associated infections reported to the National Healthcare Safety Network (NHSN) at the CDC in 2009 and 2010 revealed that the proportion of resistant *P. aeruginosa* isolates did not change significantly from that in the previous 2 years [310]. It also showed that the resistance rates did not significantly differ by critical care location status. Interestingly, the reported resistance rates to different antipseudomonal agents were comparable to those observed in 2002 by Obritch et al. as shown in Table 57.1. This suggests that the resistance rates in the USA were mostly consistent over the past decade.

In Europe, the reported trends in resistance rates were also not consistent. Similar to the inference drawn by Obritch et al., the annual report of the European antimicrobial resistance surveillance network (EARS-Net) revealed a steady increase in the rate of *P. aeruginosa* resistance to all antipseudomonal drug classes between 2008 and 2011 in several countries [280]. For example, a significantly increasing trend in resistance to piperacillin, ceftazidime, fluoroquinolones, aminoglycosides, and carbapenems was observed in France. A significantly increasing trend in multidrug resistance, defined as resistance to ≥3 antibiotic classes, was also observed. However, the observations from another study that assessed the trends in *P. aeruginosa* resistance using isolates collected from six French hospitals between 2001 and 2011 contradicted those reported by the EARS-Net [282]. Slekovec et al. observed a significantly increasing trend in resistance to carbapenems only. Moreover, they observed a significantly decreasing trend in resistance to fluoroquinolones, aminoglycosides, and aztreonam. The discrepancy in the observed resistance trends between the two studies could be attributed, at least in part, to the difference in the resistance definitions; Slekovec et al. used a constant definition of resistance in accordance with the EUCAST 2013 breakpoints while the susceptibility testing results in EARS-Net report were based on the clinical breakpoint criteria used by the local laboratories of the reporting countries. Therefore, the lack of consensus in resistance definitions makes the direct comparison of the findings from different studies difficult [248].

The first national prospective surveillance study to assess antimicrobial resistance in Canada was performed in 2008 and included 373 *P. aeruginosa* isolates from patients in 10

Table 57.1 Reported rates of antimicrobial resistance among *P. aeruginosa* isolates

	% of *P. aeruginosa* isolates exhibiting resistance				
	National ICU surveillance[a], USA, 2002 (n = 951) [278]	National ICU surveillance[a], USA, 2002–2004 (n = 3550) [279]	National Healthcare Safety Network[b], USA, 2009–2010 (n = 6111) [310]	EARS-Net annual report, Europe, 2008–2011 (n > 9300) [280]	National hospital surveillance[a], Canada, 2008 (n = 373) [281]
β-Lactams					
Cefepime	25.0	12.5	23.3[c]		7.2
Ceftazidime	19.0	4.5		14.2	
Ceftriaxone		48.0			32.7
Meropenem			22.5[d]	18.6[d]	5.6
Imipenem	23.0	14.5			
Piperacillin	15.0	16.0	15.5[e]	16.5[e]	
Piperacillin/tazobactam	10.0	13.2			8.0
Ticarcillin/clavulanate	17.0				
Aztreonam	32.0	17.8			
Aminoglycosides			10.0	17.7	
Amikacin	10.0	3.5			3.5
Gentamicin					12.3
Tobramycin	16.0	13.7			
Fluoroquinolones			29.6	22.5	
Ciprofloxacin	32.0	28.9			19.0
Levofloxacin	34.0				24.1
Polymyxins					
Colistin					0.8
Multidrug resistance	14.0 [f]	9.3 (in 2004) [g]	13.5 [h]	15.3 [i]	5.9 [j]

[a]In accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines
[b]Overall resistance rates calculated from values reported for central line-associated bloodstream infections, catheter-associated urinary tract infections, ventilator-associated pneumonia, and surgical site infections
[c]Combined rate for cefepime and ceftazidime reported
[d]Combined rate for meropenem and imipenem reported
[e]Combined rate for piperacillin and piperacillin/tazobactam reported
[f]Multidrug resistance defined as resistance to ≥3 of the following: ceftazidime, ciprofloxacin, tobramycin, and imipenem
[g]Multidrug resistance defined as resistance to at least one extended-spectrum cephalosporin, one aminoglycoside, and ciprofloxacin
[h]Multidrug resistance defined as resistance or intermediate susceptibility to at least one drug in three of the following classes: extended-spectrum cephalosporins, fluoroquinolones, aminoglycosides, carbapenems, and piperacillin or piperacillin/tazobactam
[i]Multidrug resistance defined as resistance to ≥3 antibiotic classes
[j]Multidrug resistance defined as resistance to ≥3 of the following: cefepime, piperacillin/tazobactam, meropenem, amikacin or gentamicin, and ciprofloxacin

different hospitals [281]. The reported resistance rates were generally lower than those reported in the studies from the USA and Europe (Table 57.1). Based on hospital ward location, the resistance rates were the highest among isolates from cystic fibrosis clinics and ICUs. The rate of multidrug resistance, defined as resistance to ≥3 of the following, cefepime, piperacillin/tazobactam, meropenem, amikacin or gentamicin, and ciprofloxacin, was 5.9 %. The lower rate of multidrug resistance compared with that reported in the studies from the USA and Europe could be attributed in part to the slightly more restrictive definition of multidrug resistance used in this study. Thus, in the absence of a standard definition for the multidrug-resistant phenotype, the prevalence of multidrug-resistant *P. aeruginosa* might not be easily compared across different studies that used different definitions.

The increasing prevalence of multidrug-resistant *P. aeruginosa* is particularly concerning because infections due to these isolates are generally associated with less favorable clinical outcomes [28]. A study to examine the impact of multidrug-resistant *P. aeruginosa* bacteremia on patient outcomes revealed that multidrug resistance was independently associated with 30-day mortality [odds ratio (OR): 6.8]. Furthermore, the time to mortality was significantly shorter among patients with multidrug-resistant *P. aeruginosa* bacteremia (p = 0.011) [45]. Another study that evaluated the clinical outcomes among patients with *P. aeruginosa* bloodstream infections in two Italian university hospitals revealed

that multidrug resistance and inadequate initial antimicrobial therapy were independently associated with 21-day mortality (OR: 3.31 and 2.73, respectively) [260]. In addition to increased mortality, infections due to multidrug-resistant *P. aeruginosa* were also associated with increased morbidity. Isolation of multidrug-resistant *P. aeruginosa* was associated with a higher incidence of surgery (i.e., surgical removal of infection source), increased number of invasive procedures such as bronchoscopy or catheter implantation, increased length of hospital stay, and increased frequency of patient discharge to chronic care facility [244]. However, it is worth mentioning that the adverse outcomes in patients infected with resistant isolates are not always attributed to enhanced virulence of these strains compared with their susceptible counterparts (as discussed earlier under "Effect of antibiotic resistance on fitness and virulence"). Other factors, such as decreased antibiotic effectiveness or a delay in the initiation of appropriate antimicrobial therapy, could also contribute to the clinical outcome of these infections.

A few studies have addressed the economical burden of multidrug-resistant *P. aeruginosa* infections. These studies generally suggested that these infections were associated with increased hospital charges as well as length of stay [28]. For example, a case series that included 22 patients hospitalized between August 1994 and December 1997 aimed to examine the economic outcome of infections due to multidrug-resistant *P. aeruginosa.* They found that multidrug resistance was associated with significantly higher mean hospitalization charges compared with susceptible *P. aeruginosa* infections. This was attributed in part to the need for surgery to remove infection source among patients with multidrug-resistant infections, which increased both hospitalization costs and length of stay [283]. A more recent retrospective study of all hospital admissions between January 1, 2005, and December 31, 2006, was carried out in a tertiary-care teaching hospital in Spain to assess the hospital costs of nosocomial multidrug-resistant *P. aeruginosa* acquisition. The study included 402 *P. aeruginosa*-positive cultures. Compared with nonresistant isolates, resistant and multidrug-resistant isolates were independently associated with an increased hospital total cost (more than 70 % increase per admission) [284]. Thus, finding appropriate strategies to curb the emergence and spread of multidrug-resistant infections are essential to not only improve the clinical outcomes but also limit the heavy economic burden associated with the management of these patients.

12 Strategies for Prevention and Management of Resistance

Effective prevention and management of antibacterial resistance require coordination of different strategies for prompt detection, infection control, and effective treatment.

12.1 Development of Reliable Clinical Prediction Tools

Identifying patients at risk for adverse outcomes resulting from *P. aeruginosa* infection, such as development of drug resistance or death, is very important. Development of validated tools predicting these adverse outcomes can improve medical decision making. For example, clinical prediction tools could be derived to quantify the risk of resistant *P. aeruginosa* for a given subject with *P. aeruginosa* infection. Using factors predictive of multidrug resistance among patients with *P. aeruginosa* respiratory tract infections, Lodise et al. developed an institution-specific tool to estimate the probability of multidrug resistance among this patient group [285]. This model could assist clinicians in their empirical decision-making process and thus improve the therapeutic outcome. Similarly, several models have been developed to predict mortality among patients with *P. aeruginosa* bacteremia [286, 287]. Such models could be valuable to clinicians in the identification of patients at high risk of mortality and, thus, implementation of patient-targeted monitoring/interventions to decrease mortality.

12.2 Early Detection and Isolation

Patients at increased risk for acquiring nosocomial *P. aeruginosa* infections, such as immunocompromised patients or those requiring mechanical ventilator, should be recognized and monitored closely for any signs of infection. The delay in the detection of *P. aeruginosa* infections could potentially result in the loss of an opportunity to initiate early appropriate antibiotic therapy, which could result in poor prognosis. Novel techniques for the rapid detection of *P. aeruginosa* include quantitative PCR (qPCR), peptide nucleic acid fluorescence in situ hybridization (PNA FISH), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Several studies have suggested that qPCR and MALDI-TOF MS were highly sensitive and specific tools for early detection of *P. aeruginosa* compared with specimen culture [288, 289]. Similarly, a recent study that assessed the utility of PNA FISH for rapid detection of *P. aeruginosa* showed that this technique offered a sensitivity and specificity of 100 % and 95 %, respectively, for *P. aeruginosa* clinical isolates [290]. However, these methods cannot reliably predict the antibiotic susceptibility when used alone. Clinical *P. aeruginosa* isolates exhibit diverse resistance mechanisms and antimicrobial susceptibilities. Thus, in vitro susceptibility testing remains important to guide the selection of appropriate chemotherapy. Specimens for culture and susceptibility testing should still be collected promptly, preferably before administration of any antibiotics.

12.3 Infection Control and Transmission Reduction

Since *P. aeruginosa* is primarily associated with nosocomial infections, strict compliance with infection control practices is critical to avoid hospital outbreaks. This may include policies for contact isolation of patients colonized or infected with multidrug-resistant isolates, to minimize the risk of patient-to-patient transmission [291]. Other measures include frequent hand washing, sterile insertion techniques for intravascular catheters, and cleaning and disinfecting devices such as the stethoscope's diaphragm.

12.4 Implementation of Antibiotic Stewardship Programs (ASPs)

ASPs are sets of coordinated hospital-based strategies dedicated to improving antibiotic use by enhancing the patients' outcomes, minimizing resistance development, and avoiding unnecessary treatment costs. The core element of a successful hospital ASP is to implement interventions that could improve antibiotic use such as antibiotic "time-outs," dose adjustment and optimization, tracking and reporting antibiotic use, and evaluation of patients' outcomes. Other important ASP elements include leadership support and continuous staff education by providing regular updates on the most recent local antibiotic resistance trends, antibiotic prescribing, and strategies for infectious disease management that address both national and local problems.

There is substantial amount of evidence that demonstrated the success of ASPs in improving the quality of patient care, reducing treatment failures, and minimizing antibiotic resistance. Regal et al. compared *P. aeruginosa* susceptibility patterns before and after reduction in β-lactam use as part of an ASP. They reported an increase in *P. aeruginosa* susceptibility to ceftazidime, piperacillin, imipenem, and aztreonam [292]. A significant increase in *P. aeruginosa* susceptibility to imipenem was also observed by Goldstein et al. after starting ertapenem use in a community teaching hospital [293]. In view of the benefits of ASPs and the pressing need to improve antibiotic use in hospitals, in 2014 the CDC recommended that all acute-care hospitals implement these programs [294].

12.5 Appropriate Empiric Therapy

Once an infection due to *P. aeruginosa* is diagnosed, prompt administration of appropriate empiric therapy is critical to clinical outcome. For uncomplicated lower urinary tract infections, antibiotic monotherapy is recommended except in case of neutropenic patients or when bacteremia is suspected. Combination therapy with two or more antipseudomonal agents is commonly used for severe infections such as bacteremia and hospital-acquired pneumonia [295]. The rationales for administration of combination therapy are to increase the probability of appropriate empirical coverage, minimize selection of resistant mutants, and achieve synergetic antibacterial effect. A combination of one aminoglycoside and one β-lactam is commonly used for treatment of bacteremia due to *P. aeruginosa*. Several studies reported an advantage for appropriate empirical combination therapy over appropriate empirical monotherapy [296–298]. However, some recent studies provided additional insights into this claim. Bowers et al. compared the outcomes of patients receiving appropriate empirical combination versus monotherapy for *P. aeruginosa* bacteremia. After adjusting for baseline APACHE II scores and lengths of hospital stay prior to the onset of bacteremia, they found no statistical differences in 30-day mortality, hospital mortality, or time to mortality following appropriate empirical combination versus monotherapy [24]. Pena et al. also reported similar results; they found that treatment with combination antimicrobial therapy did not reduce the mortality risk compared with single-drug therapy for *P. aeruginosa* bloodstream infections after adjustment for the potential confounders [299]. A comprehensive review that analyzed data from 18 studies including 426 nonneutropenic patients with *P. aeruginosa* sepsis compared all-cause mortality following the use of β-lactam/aminoglycoside combinations versus β-lactam monotherapy. They observed no significant differences between the study groups. Additionally, they observed that the adverse events, particularly the nephrotoxicity, occurred significantly more frequently in the combination study group [300]. Thus, as long as the empiric therapy was appropriate, no advantage to antipseudomonal combination therapy over monotherapy was observed in terms of clinical outcomes. Furthermore, several studies showed that the β-lactam/aminoglycoside combination therapy was not superior in suppressing resistance emergence compared with β-lactam monotherapy [301]. Large, prospective, randomized, controlled trials are needed to further evaluate the validity of the empirical combination therapy for treatment of *P. aeruginosa* infections in different patient populations.

12.6 Alternative Routes of Drug Administration

In case of difficult-to-treat *P. aeruginosa* infections, alternative routes of antibiotic administration might be considered as adjunct therapy. For example, inhaled aminoglycosides such as tobramycin are used to eradicate *P. aeruginosa* in cystic fibrosis patients as well as lung infections in non-cystic fibrosis patients with bronchiectasis [302]. Inhaled

aminoglycosides or colistin could also be beneficial in the treatment of ventilator-associated pneumonia due to multidrug-resistant *P. aeruginosa* [303]. Topical polymyxin B is also used in conjugation with systemic antibiotics for the treatment of clinically infected wounds [304]. Piperacillin/tazobactam combination is used for treatment of endophthalmitis due to multidrug-resistant *P. aeruginosa* via intravitreal instillation [305]. Intrathecal colistin has been successfully used to treat central nervous system infections post-neurosurgery due to multidrug-resistant *P. aeruginosa* such as meningitis and ventriculoperitoneal shunt infections [306–308]. In addition to the direct antibiotic delivery to the infection site, these routes of administration reduce the drug systemic exposure and consequently minimize the adverse effects.

13 Conclusions

P. aeruginosa is a major cause of nosocomial infections and a challenging pathogen to combat in healthcare settings. Resistance to all classes of antipseudomonal drugs is increasingly reported. Unfortunately, only very few new antibiotics for the treatment of drug-resistant *P. aeruginosa* strains are expected to become commercially available within the next decade [309]. Thus, it appears that *P. aeruginosa* infections will continue to be a problem for many years to come. High standards of infection control and judicious antimicrobial use are crucial to prevent the situation from deteriorating.

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Acinetobacter baumannii and *Acinetobacter* spp.

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1 Introduction: Challenges in Microbiology and Classification

Acinetobacter baumannii is a bacterial pathogen increasingly identified in the clinical microbiology laboratory as a cause of infection in humans. Upon microscopic examination, *A. baumannii* appears as a Gram-negative coccobacillus, and it produces clear colonies when grown on MacConkey agar, indicating its inability to ferment lactose. The taxonomy of *Acinetobacter* genus, part of the γ subclass of the *Proteobacteria* phylum, is complex and currently comprises 43 species (http://www.bacterio.net/acinetobacter.html), defined mostly by genomic DNA–DNA hybridization.

The original description of *A. baumannii* dates back to 1986 (Bouvet and Grimont). Although *A. baumannii* is the most frequently identified nosocomial pathogen in the genus *Acinetobacter*, several other species are becoming increasingly important in the clinic as occasional causes of infections and outbreaks in humans. These include *A. nosocomialis* and *A. pittii*, which are phenotypically similar to *A. baumannii* and therefore very difficult to distinguish in the clinical microbiology laboratory by traditional biochemical methods. This limitation can be partially overcome when "state-of-the art" technologies such as matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry or PCR/electrospray ionization mass spectrometry (PCR/ESI-MS) and nucleic acid sequencing are employed. With considerable less frequency, *A. ursingii*, *A. haemolyticus*, *A. lwoffii*, *A. parvus*, and *A. junnii*, among others, are also found as a cause of infection in humans. The most recently identified species of the genus is *A. seifertii*, found among clinical isolates from as early as the 1990s. The taxonomic effort that led to the recognition of the new species *A. seifertii* required a multifaceted analysis, which included similarities in the sequence of housekeeping genes, as well as the determination of a sufficiently low (<95 %) average nucleotide identity (AIN) relative to the whole genome sequence of other *Acinetobacter* species. Also, in order to differentiate *A. seifertii*, proteomic analysis with whole cell MALDI-TOF mass spectroscopy has been employed, as well as metabolic and physiologic testing and sequencing [1–8]

2 Insights from Genomic Analysis of *Acinetobacter baumannii*

With advances in genomic tools, several analyses have focused on *A. baumannii* and other representatives of the genus and revealed insights into its genetic diversity and evolutionary dynamics. A more comprehensive analysis of various genomes of *Acinetobacter* spp. has also been carried out, with the goal of covering the entire range of diversity of the species. In the case of *A. baumannii*, various genotypes defined by multilocus sequence typing (MLST) were also included. The analysis revealed that the average genome had a size of 3.87 Mb (ranging from 2.7 to 4.9 Mb). For *A. baumannii*, the core genome had 1590 orthologous protein families, which correspond to 44 % of the size of the smallest *A. baumannii* and with a gene repertoire

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relatedness of only 78 %, therefore indicating the great genetic variability within the species and suggesting frequent horizontal gene transfer. Also, genomic analysis reveals that the genus of *Acinetobacter* is very ancient, approximately 5 million years old, based on the differences in the sequence between *A. baumannii* and the most distant species of the genus [8–15].

There are various mechanisms that make it possible for *A. baumannii* to accumulate such genetic diversity. Among these, one of the most important are integrons of the *intI1* class. Such integrons demonstrate tremendous abundance in *A. baumannii*, as revealed by genomic analysis. Insertion sequences (IS) have also been implicated in genetic diversity and especially in the regulation of antibiotic resistance. Diverse IS are frequently found in the genome of *A. baumannii*. In this regard, the role of IS*Aba1* serving as a promoter determining the expression of carbapenemase genes located downstream has achieved special recognition [16]. Although not established as widely, the process of natural transformation among *Acinetobacter* spp. is also known to occur [9, 17, 18].

Horizontal transfer of genetic material in *Acinetobacter*, including antibiotic resistance determinants, may also be facilitated by transduction with phages. Comparative genomic analysis reveals the frequent presence of phage sequences in *A. baumannii.* Similarly, there is evidence from the genomic analysis of the predominance of mobilizable small elements over large conjugative elements. Similarly, the conservation of the genetic machinery required for transformation in the majority of *Acinetobacter* genomes suggests that most members of the genus are naturally transformable given certain conditions (see above). In order to preserve their genome from "infection" with transmissible genetic elements such as plasmid and viruses, bacteria possess clustered regularly interspaced short palindromic repeats (CRISPR), which, in conjunction with other sequences (such as the *cas* genes and Cas proteins), form the CRISPR-Cas, an adaptive immune system. Within *A. baumannii*, most CRISPR-Cas systems are of type I-Fb, indicating that this may serve as a target for genetic fingerprinting [19]. CRISPR-Cas systems are likely to have an important role in the dynamics of the genomic transformation and in particular in controlling the transfer of conjugative elements. Acquisition of antibiotic resistance often results from the transfer of a mobile element encoding several resistance genes. A classical example is the genomic island (AbaR1) that contains dozens of antibiotic resistance determinants from diverse bacterial origins [10].

Genetic insertion and deletions occur in particular areas of the genome, deemed "hotspot" regions. According to a detailed comparative genomic analysis, it appears that approximately 80 "hotspot" regions only comprise 5 % of all possible loci and yet include 66 % of the accessory genome. Thus, it appears that a great deal of genetic diversity occurs in a few "hotspot" locations in the genome of *A. baumannii* [8]. Other mechanisms of genetic diversity are also at play in *A. baumannii*. Diversification can also result from allelic exchange by homologous recombination in the core genome. In *A. baumannii*, this process is estimated to affect up to a third of core gene families. Lastly, the emergence of genetic diversity within *A. baumannii* can also be due to point mutations; resistance to antibiotics can clearly result in such instances. Bacteria may undergo hypermutagenesis due to activation of error-prone DNA polymerases that can accelerate the dynamics of adaptation due to the mechanism of point mutations. These mutations in turn can affect two-component regulatory systems leading to efflux pump overexpression [20].

3 Determining Virulence, an Elusive Target in *A. baumannii*

Establishing the attributes of virulence that make *A. baumannii* such a successful human pathogen has been elusive. On the one hand, the systematic analysis of virulence has failed to distinguish a particular virulence factor distinctly linked to epidemiological and clinical outcomes. On the other hand, the complicated microbiology and nomenclature of the *Acinetobacter* spp. family make it very difficult to interpret the literature and to establish the attributes of *A. baumannii* versus other species of *Acinetobacter*. Even nonclinical isolates of *Acinetobacter* harbor some of the same virulence machinery that is found among clinical isolates of *A. baumannii*. Interestingly, although the name *Acinetobacter* is derived from the Greek term indicating "no movement," it appears that this is a misnomer. In particular, *A. baumannii* spreads rapidly over surfaces perhaps as the result of twitching motility [21].

A well-known and distinguishing characteristic of *A. baumannii* is its ability to survive in the environment; as reviewed below, this attribute also likely contributes to its dissemination [22]. Therefore, factors that permit its survival in biotic and abiotic surfaces can be considered as important attributes of virulence. *A. baumannii* is able to form highly structured microbial communities called biofilms. The factors that contribute to biofilm formation include, among others, the Csu pili [23], two-component regulatory systems such as BfmRS, and quorum-sensing systems [24, 25]. Similarly, adherence to host cells and tissues is a crucial initial stage of colonization and subsequent infection. Surface proteins such as Bap, the exopolysaccharide poly-β-1,6 *N*-acetylglucosamine, PNAG, and the autotransporter Ata play an important role in this regard [26–28]. There also is an O-glycosylation system that appears to be important for biofilm formation and virulence. Similarly, an outer membrane protein Omp 38 or ompA is involved in adhesion and in subsequent stages of cell invasion and apoptosis [29]. Paradoxically, *A. baumannii* strains

that demonstrate adherence to human epithelial cells may elicit a poor inflammatory response allowing bacteria to evade the host immune system and lead to persistence of *A. baumannii* [30].

Like any other Gram-negative bacteria, the outer membrane of *A. baumannii* contains lipopolysaccharide (LPS), a component important in the development of septic shock once *A. baumannii* enters the bloodstream. Most *Acinetobacter* LPS molecules contain an O-polysaccharide chain (O-antigen), involved in the pathogenic activity of other Gram-negative bacteria. Endotoxins of *A. baumannii* stimulate an inflammatory signal in monocytes, and responses to *A. baumannii* are dependent on Toll-like receptors, TLR2 and TLR4. Indeed, blocking the production of LPS in mice, for instance, with the use of the LPS synthesis inhibitor LPCx, effectively silences the effects of LPS on the host's immune system and facilitates the elimination of *A. baumannii* through opsonophagocytic killing, leading to improved survival [31–33].

Another component of the cell envelope of *A. baumannii*, the capsular polysaccharide, also can play a role in virulence, given its importance in mediating resistance to complement killing [34]. *A. baumannii*, similar to other Gram-negative bacteria, secrete outer membrane vesicles (OMVs) during various stages of bacterial growth and while stressed. OMVs are spherical nanovesicles composed of LPS, OMPs, lipids, select periplasmic proteins, and nucleic acids. More recently, OXA and metallo-β-lactamases were found in OMVs. Virulence factors such as OmpA have been associated with OMVs in *A. baumannii*, inducing apoptosis [35]. Other proteins considered as potential virulence determinants in *A. baumannii* include also phospholipase D and phospholipase C [36]. An interesting observation in *A. baumannii* is that ethanol may promote bacterial growth and stimulate metabolic pathways that are related to virulence [37].

4 Antibiotic Resistance in *A. baumannii*

The emergence of resistance to multiple classes of antibiotics in *A. baumannii* represents a veritable threat to public health. Because of this phenomenon, there are often only a few, at best, effective antibiotic options to treat infections caused *by A. baumannii*. In order to understand better and compare data regarding resistance to multiple classes of antibiotics, it is useful to understand the terminology commonly used to designate whether *A. baumannii* is "multidrug resistant" (MDR), "extensively drug resistant" (XDR), or "pandrug resistant" (PDR). The relevant classes of antibiotics that offer activity *against A. baumannii* but that may be inactive due to acquisition of mechanisms of resistance are aminoglycosides, carbapenems, beta-lactam/beta-lactamase inhibitor combinations (with ampicillin/sulbactam as a special category), expanded-spectrum cephalosporins, fluoroquinolones, polymyxins, folate inhibitors, and tetracyclines. When resistance or non-susceptibility occurs to more than one or more agents from three or more antibiotic categories, the organism is termed MDR; resistance or non-susceptibility to one or more agents to all but two or less categories is termed XDR; resistance or non-susceptibility to all agents in all categories is called PDR [38].

4.1 Mechanisms of Resistance to β-Lactams

The mechanisms that are implicated in the development of resistance to β-lactams in *A. baumannii* include β-lactamases and modifications in the permeability of the bacterial membrane through changes in outer membrane proteins (OMPs) or expression of efflux pumps [39].

Among the β-lactamases found in *Acinetobacter* spp., AmpC enzymes, or class C β-lactamases in the Ambler classification, are known for their ability to hydrolyze penicillins and cephalosporins and to be impervious to β-lactam–β--lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. This family of enzymes is designated as *Acinetobacter*-derived cephalosporinases or ADCs [40]. The presence of an insertion sequence (IS) such as IS*AbaI* acts as a promoter for the expression of the gene coding for ADCs.

Another group of β-lactamases of considerable clinical importance in *A. baumannii* are Ambler class D enzymes, also known as oxacillinases (OXAs). OXAs receive that designation because prototype representatives of the enzyme are able to hydrolyze oxacillin. Within *A. baumannii*, however, OXAs are characterized by their carbapenemase activity and are designated as carbapenem-hydrolyzing class D β-lactamases or CHDLs. There are five groups of OXAs relevant to *A. baumannii*: OXA-51, OXA-23, OXA-24/40, OXA-58, and OXA-143. In the case of OXA-51, its role as a resistance determinant against carbapenems and cephalosporins is greatly conditioned by the presence of insertion sequences (typically IS*Aba1*) that promotes their expression [41].

Class B metallo-β-lactamases (MBLs) are an additional group of β-lactamases of clinical relevance in *A. baumannii*. The common characteristic of MBL enzymes is that they render bacteria resistant to all β-lactams, with the exception of the monobactam aztreonam. The key feature of MBLs is the presence of a zinc moiety, which can be chelated by EDTA to inactivate the enzyme. The MBLs present in *A. baumannii* include IMP, VIM, SIM, and NDM. Interestingly, IMP and VIM enzymes, although found in *A. baumannii*, really originated in *Pseudomonas aeruginosa* from Japan and Italy, respectively. SIM, on the other hand, is a MBL native to *A. baumannii* identified originally in Korea. More

recently, NDM was identified in *Enterobacteriaceae* from travelers returning from the Indian subcontinent. Various reports have identified bla_{NDM} genes in *A. baumannii* isolates from India, Egypt, China, Germany, the Balkans, the USA, and Israel. The genetic context of bla_{NDM} in *Acinetobacter* suggests this MBL arose in this genus [42, 43]. Carbapenem resistance can occur in *A. baumannii* even in the absence of any known carbapenemase through the loss of certain outer membrane proteins, such as CarO. Other outer membrane proteins have been involved in carbapenem resistance, especially in conjunction with the expression of class C and D enzymes.

4.2 Mechanisms of Resistance to Aminoglycosides

The main mechanism of resistance to aminoglycosides among *A. baumannii* is through the expression of aminoglycoside-modifying enzymes (AMEs), such as aminoglycoside acetyltransferases, nucleotidyltransferases, and phosphotransferases. A study that analyzed *A. baumannii* isolates from a military facility in the USA uncovered the presence of the following genes coding for AMEs: *aphA6*, *aadA1*, *aadB*, *aacC1*, and *aacC2* [3]. There is an important association of AMEs with mobile genetic elements such as plasmids and class I integrons that largely accounts for their widespread dissemination [44].

Other types of AMEs have been described in *A. baumannii* from the Far East; for instance, amikacin resistance was mediated in Japan by a novel type of AME, encoded by *aac(6′)-Iad*. Similarly, *armA* has been implicated in methylation of 16S rRNA in *A. baumannii*, also resulting in resistance to amikacin, as well as gentamicin and tobramycin [45, 46]. Additionally, in *A. baumannii* aminoglycoside resistance can be mediated by expression of the AdeABC efflux pump, which is also implicated as a mechanism of resistance against fluoroquinolones, among other antibiotics [47].

4.3 Mechanisms of Resistance to Fluoroquinolones

Resistance of *A. baumannii* to fluoroquinolones is often caused by modifications in the structure of DNA gyrase secondary to mutations in the quinolone resistance-determining regions (QRDR), including the *gyrA* and *parC* genes coding for DNA gyrase and DNA topoisomerase IV. These changes result in a lower affinity for the binding of the quinolone to the enzyme–DNA complex. As mentioned, another important mechanism of resistance to fluoro quinolones is mediated by efflux systems that decrease intracellular drug accumulation. In contrast to *Enterobacteriaceae* where it is fairly prevalent, plasmid-mediated quinolone resistance (*qnr* genes) is rarely found in *A. baumannii*, with the exception of the sporadic detection of *qnrA* in Algerian and Chinese isolates [48, 49].

4.4 Mechanisms of Resistance to Polymyxins

Increasing resistance to antibiotics, especially carbapenems, has prompted the widespread use of polymyxin B and colistin as therapeutic agents to treat infections caused by *A. baumannii*. Although the majority of *A. baumannii* retain susceptibility to polymyxins, the occurrence of resistance is not rare.

The common pathway leading to resistance to polymyxins in Gram-negative bacteria is the neutralization of the negative charges of the outer membrane through lipid A modifications. The two-component regulatory system pmrA/pmrAB, upon activation, leads to lipid A modifications through the addition of phosphoethanolamine. A more drastic mechanism of polymyxin resistance in *A. baumannii* is the loss of lipopolysaccharide through mutations affecting key genes involved in the biosynthesis of lipid A, such as *lpxA*, *lpxC*, or *lpxD*. These latter changes in the structure of the outer membrane lead to profound alterations in the physiology of *A. baumannii*, leading to increased susceptibility to other classes of antibiotics and to loss of biological fitness. Heteroresistance, or subpopulations of genetically identical subclones that are more resistant than the original parent clone, also has been observed in *A. baumannii* as a result of the selective pressure exerted by polymyxins [50–55].

4.5 Mechanisms of Resistance to Tetracyclines and Tigecycline

The resistance to tetracyclines in *A. baumannii* is due to the effect of either efflux pumps or a ribosomal protection protein. There are two transposon-mediated efflux pumps: TetB facilitates the efflux of both tetracycline and minocycline, whereas TetA does not have minocycline as its substrate but only tetracycline. The ribosomal protection protein, encoded by the *tet(M)* gene, shields the ribosome from the action of tetracycline, doxycycline, and minocycline. None of the abovementioned mechanisms interfere with the activity of tigecycline, which, although related to tetracyclines, is in its own glycylcycline class [18, 56].

The role of the AdeABC efflux pump as a mechanism of resistance to tigecycline is well established. The overexpression of the *adeABC* locus correlated with an increase in the tigecycline MIC in *A. baumannii* strains. This pump confers broad substrate specificity, including tigecycline, gentamicin, levofloxacin,

and chloramphenicol. A two-component regulatory system, sensor AdeS and regulator AdeR proteins, regulates the pump and can be disrupted by the insertion sequence IS*Aba1*, among other possible mutations and insertions [18, 20, 57].

5 Infections Caused by *A. baumannii*

Infection and colonization with *A. baumannii* infections mostly affect immunosuppressed patients or those with serious underlying diseases, who are subjected to invasive procedures and treated with broad-spectrum antibiotics. Infection and colonization with *A. baumannii* are therefore more frequently found among hospitalized patients, especially those in intensive care units (ICUs) and in long-term care facilities (LTCFs).

The most common syndromes associated with *A. baumannii* are pneumonia, including hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP), and bloodstream infections associated with catheters, or from various other sources. These may include urinary tract infections, complicated skin and soft tissue infections, intra-abdominal infections, and central nervous system infections often associated with neurosurgical procedures and intracranial shunts. HAP and VAP in particular are associated with poor outcomes that are likely related to the severity of underlying illness and inappropriate initial antibiotic regimens conditioned by extensive antibiotic resistance.

There has been an ongoing controversy over whether colonization and infection with *A. baumannii* are associated with increased morbidity and mortality or if poor outcomes are due to the underlying host characteristics, the virulence of the pathogen, or the antibiotic treatment. The systematic evaluation of the available literature suggests a statistically significantly higher mortality in patients who have acquired *A. baumannii* (even colonization) [58]. Another important study indicates that infections with MDR *A. baumannii* are independently associated with prolonged hospital and ICU lengths of stay compared with the outcomes for uninfected patients and those infected with drug-susceptible *A. baumannii*. However, in this analysis, there was no difference in mortality between the groups [59]. A meta-analysis identified studies comparing mortality in patients with carbapenem-resistant *A. baumannii* vs. carbapenem-susceptible *A. baumannii*. Patients with carbapenem-resistant *A. baumannii* had a significantly higher risk of mortality, with an odds ratio of 2.2. In this analysis, patients with carbapenem-resistant *A. baumannii* were more likely to have severe underlying illness and also to receive inappropriate empirical antimicrobial treatment, which increases the risk of mortality [60].

Infections caused by *A. baumannii* play a growing role among patients with cancer. A case-control study demonstrated that acquisition of MDR *A. baumannii* among cancer patients is associated with nosocomial factors, rather than characteristics of the underlying cancer. Furthermore, *A. baumannii* in cancer patients is associated with prolonged hospitalization and increased mortality [61]. Instances of bacteremia with XDR *A. baumannii* in patients with hematological malignancy and neutropenia, however, are associated with a staggering mortality rate at 30 days of 83 % and are especially high in patients who do not receive appropriate antibiotic therapy because of the XDR phenotype [62].

Patients with solid organ transplants who become infected with *A. baumannii* that is resistant to carbapenems suffer from prolonged hospitalization, infection with other MDR organisms, allograft dysfunction and loss, and high overall infection-related mortality [63]. Especially important are VAP and other respiratory infections, which are associated with frequent recurrence in recipients of cardiothoracic transplants [64]. Their increased net state of immune suppression and exposure to healthcare may predispose patients with solid organ transplants to unusual presentations of *A. baumannii* infection. For instance, fulminant sepsis caused by carbapenem-resistant *A. baumannii* harboring the $bla_{\text{OXA-23}}$ carbapenemase gene led to the death of a patient 6 days after simultaneous kidney–pancreas transplantation. Autopsy findings revealed disseminated infection with acute mitral valve endocarditis, myocarditis, splenic and renal emboli, peritonitis, and pneumonia [65].

Although only a few cases have been described in the literature, it appears that HIV-associated *Acinetobacter* infections are associated with significant morbidity, especially when a low CD4+ cell count, neutropenia, and hospitalization are present [66]. Examination of records of patients with *A. baumannii* acquisition in an ICU in Cape Town, South Africa, revealed that patients with HIV and AIDS were more likely to have bacteremia and had higher mortality rates than non-HIV-infected patients [67]. It is interesting that similar clinical patterns of HIV-associated *A. baumannii* bacteremia are not observed in the USA.

A. baumannii became well known as a major pathogen found in personnel participating in military operations in the Middle East as part of the Global War on Terrorism, after September 11, 2001. Initially, increasing numbers of *A. baumannii* bloodstream infections were reported in patients at military medical facilities in which service members are injured in the Iraq/Kuwait region during Operation Iraqi Freedom (OIF) and in Afghanistan during Operation Enduring Freedom (OEF). Most of the cases were initially treated at Landstuhl Regional Medical Center in Germany and Walter Reed Army Medical Center (WRAMC) in the District of Columbia and later at the US Navy hospital ship Comfort, National Naval Medical Center, and Brooke Army Medical Center. Noteworthy was the high level of antimicrobial resistance [68].

Among military personnel, *A. baumannii*-associated skin and soft tissue infections presented as cellulitis, which often progressed to necrotizing infection with bullae [69]. A systematic investigation of the outbreak of *A. baumannii* among military personnel suggested that environmental contamination of field hospitals and infection transmission within healthcare facilities played a major role in the dissemination of the pathogen [70]. A detailed molecular characterization of antimicrobial resistance in *Acinetobacter* spp. from WRAMC revealed a complex genetic background, including PER-1 as well as the carbapenemases OXA-58 and OXA-23 [3].

Similarly, *Acinetobacter* has been identified as a common cause of infection among victims of natural disasters. One of the most recognized examples is the presence of MDR *Acinetobacter* among the victims of the 2005 Indian Ocean earthquake and tsunami that caused several hundred thousand fatalities. Upon evacuation to European medical centers, some of the victims were noted to have soft tissue wounds and other instances of traumatic injury where *Pseudomonas* and enterobacteria were prominently involved, as well as *Acinetobacter* [71]. The ICUs of hospitals in Turkey also saw the emergence of *Acinetobacter* infection in victims of the violent earthquake of Marmara in 1998 [72].

Of particular interest, and in contrast with its standing as a nosocomial pathogen, *A. baumannii* has been found as the cause of serious infections among community dwellers particularly in Australia and Southeast Asia. Clinical presentations are distinctly severe and include pneumonia and bacteremia complicated with acute respiratory distress syndrome and disseminated intravascular coagulopathy. Cigarette smoking, alcoholism, chronic obstructive airway disease, and diabetes mellitus are underlying factors that lead to high mortality [73, 74].

6 Epidemiology of *A. baumannii*

There is an interesting association between colonization and infection with *A. baumannii* and temperate climates. The analysis of a large database of more than 200,000 blood cultures obtained from 132 hospitals through 7 years (1999–2006) revealed that there were 51.8 % (95 % CI 41.1–63.2) more infections caused by *A. baumannii* in the summer months than in the winter months. Similarly, there is a 10 % increase in the incidence of *A. baumannii* infections for every 10 °F increase in temperature. Of note, these variations according to temperature and season exceeded those that were observed for other Gram-positive and Gram-negative pathogens [75]. The reasons behind these observations remain unclear. It has been speculated that higher temperature may facilitate growth of bacteria in the environment and increase colonization in humans.

As mentioned previously, perhaps one of the most remarkable characteristics of *A. baumannii* is its ability to survive in the environment, including hospital surfaces. This feature, together with the presence of determinants of resistance to multiple antimicrobial agents, is thought to largely explain its success as a nosocomial pathogen. Investigations exploring desiccation tolerance in *A. baumannii* demonstrated a survival of up to 27 days in simulated hospital conditions. Strain-dependent variations have been observed, but there is necessarily not an advantage among outbreak-related strains [76, 77].

There is a critical interplay between environmental contamination and patient colonization with *A. baumannii*: even when colonization is remote, the surrounding environment is frequently contaminated [78]. Conversely, patients exposed to a contaminated hospital environment have 2.77 times higher risk of acquiring *A. baumannii* than unexposed patients [79]. It is thought that patient colonization with *A. baumannii* is both an important risk factor for subsequent infection and transmission. For instance, the presence of carbapenem-resistant *A. baumannii* on surveillance cultures is associated with an eightfold higher risk of subsequent development of carbapenem-resistant *A. baumannii* infections in the ICU setting [80]. It is recognized that hospitalized patients can serve as long-term carriers of *A. baumannii*. Unfortunately, the overall sensitivity of surveillance cultures from a single site is low (30 %), and it remains so even when up to six anatomical sites are sampled (55 %). Interestingly, the mean duration of *A. baumannii* isolation has been reported to be as long as 20 months [81]. Furthermore, it is possible that aerosolization of *A. baumannii* can occur, especially linked to rectal colonization, and contribute to the transmission of this pathogen [82, 83].

The profile of patients (e.g., severe underlying comorbidities) and the types of infections (e.g., VAP) associated with *A. baumannii* means that its transmission is mostly restricted to intensive care units. Especially in the USA, however, patients residing in LTCFs demonstrate particular vulnerability to colonization and infection caused by *A. baumannii*. Therefore, LTCFs represent a new frontier in the epidemiology of this important healthcare-associated pathogen [84, 85].

Given these characteristics, the prevention and control of infections caused by *A. baumannii* becomes a particular challenge. *A. baumannii* is recognized for its ability to cause outbreaks in different healthcare settings. Outbreaks of *A. baumannii* involving closely related strains, as revealed by various discriminatory typing methods, have often been described. A common environmental reservoir may be identified as the source of such outbreaks [86]. In one particular outbreak, transmission of clonal strain of *A. baumannii* was apparently caused by dissemination during the pulsatile lavage of wounds, likely resulting in environmental

contamination [87]. In another clonal outbreak, cultures from a wound care cart, environmental and clinical cultures, were all genetically related. In this instance, patient isolation, elimination of the wound care cart, and decontamination with vaporized hydrogen peroxide resulted in effective decontamination. However, when patients colonized with *A. baumannii* reoccupied rooms, environmental contamination recurred [88].

In many other instances, the epidemiology of *A. baumannii* is much more complex and difficult to decipher. This is due to the coexistence of sporadic and epidemic clones, as well as the fluid reservoir of *A. baumannii* in patients and the environment, and therefore presents a unique challenge to the prescription and implementation of effective infection control measures. In certain locales, *A. baumannii* can attain endemic status, characterized by the presence of multiple strain types and sustained high prevalence. In order to respond to such a situation, a long-term multifaceted "bundle" approach was implemented in a Spanish hospital. The program consisted of staff education, optimization of hand hygiene, contact precautions and patient isolation, environmental cleaning, and active surveillance in select areas and periods. This bundle of interventions resulted in sustained decrease in rates of *A. baumannii* colonization and infection [89].

Understanding the transmission dynamics of MDR and XDR, *A. baumannii* requires the application of molecular tools to determine their genetic type and mechanisms of antibiotic resistance. Similarly, this information can serve to describe the temporal evolution of *A. baumannii.* Typing of DNA digests using a standardized protocol of pulsed field gel electrophoresis (PFGE) has enhanced the epidemiological investigation of outbreaks by demonstrating highly related or indistinguishable isolates, suggesting transmission from a common source or from patient to patient [90]. Additionally, the combination of PFGE, ribotyping, and amplified fragment length polymorphism (AFLP) led to the identification of certain European clones of *A. baumannii* that have also been found globally [91, 92]. MLST has permitted further characterization of the population structure, genetic diversity, and distinctness of those clones of *A. baumannii* [93]. An automated repetitive-sequence-based PCR (rep-PCR) has also been employed to type *A. baumannii* to study outbreaks and has also served to identify global clones [84, 94].

Interestingly, the application of molecular epidemiology tools to *A. baumannii* demonstrates the temporal pattern of strain replacement leading to the global predominance of three lineages, especially the erstwhile European and now international clone II, usually associated with carbapenemases OXA-23 and OXA-24/40. Due to the combination of a MDR phenotype with its successful global dissemination, *A. baumannii* belonging to these groups of strains have been designated as a "high-risk clone" [95].

7 Treatment of Infections Caused by *A. baumannii*

The treatment of infections caused by *A. baumannii* is complicated by the increasing occurrence of MDR, XDR, and PDR strains. Furthermore, clinical data of the highest quality, such as randomized controlled trials, are usually not available to support recommendations for the treatment of *A. baumannii*. Therefore, decisions regarding the antibiotic treatment of *A. baumannii* are largely based on observational studies, which are often not controlled and have selection bias as an inherent limitation. Similarly, information derived from antimicrobial susceptibility surveys is often used to guide the use of antibiotics.

The presence of cephalosporinases of the AmpC type, or ADCs, makes the choice of cephalosporins perilous. Therefore, most clinicians when confronted with a seriously ill patient infected with *A. baumannii* usually rely on a carbapenem. Imipenem as a rule demonstrates higher potency (a lower MIC) than meropenem or doripenem and therefore is the preferred carbapenem for the treatment of *A. baumannii* infections. A clinical series of 63 patients with VAP caused by *Acinetobacter* demonstrated effective treatment with imipenem in 83 % of patients. The same study also highlights ampicillin/sulbactam as an excellent alternative to imipenem with similar efficacy, albeit in a smaller number of patients [96].

In the case of infections caused by *A. baumannii*, sulbactam is considered the active beta-lactam that binds to penicillin-binding proteins (PBP2), as opposed to other situations where it serves as an inhibitor of beta-lactamases. Other combinations containing sulbactam, such as cefoperazone/sulbactam, are also used where available. In an observational study, a favorable clinical outcome occurred in 77 % of patients treated cefoperazone/sulbactam vs. 75 % in patients treated with imipenem. A small meta-analysis of four studies suggested that sulbactam-based therapies are similarly efficacious to various comparator drugs (e.g., fluoroquinolones, tetracyclines, polymyxins, carbapenems) [97, 98]. Given all these observations, sulbactam, when active, is the preferred option to treat carbapenem-resistant *A. baumannii*. Even in cases where *A. baumannii* retains susceptibility to beta-lactams, sulbactam may serve as a carbapenem-sparing alternative. Unfortunately, as MDR *A. baumannii* has disseminated widely due to the success of "high-risk clones," and a substantial fraction of carbapenem-resistant strains are also resistant to sulbactam [94].

Polymyxins are an alternative frequently relied upon for the treatment of carbapenem and sulbactam-resistant *A. baumannii*. Polymyxins have indeed become the antibiotics of "last resort" to treat infections caused by XDR *A. baumannii.* Polymyxin B and polymyxin E, better known as colistin, differ slightly in their amino acid composition, where polymyxin B features phenylalanine in place of the

D-leucine present in colistin. Polymyxins were initially developed in the 1950s, later abandoned, and then "rediscovered" in the last two decades with the emergence of MDR and XDR *A. baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Considerable progress has been made to fill gaps in the understanding of their mechanism of action, their pharmacokinetic and pharmacodynamic characteristics, and their clinical efficacy. In this regard, the critical comparison of polymyxin B and colistin has led to the notion that the former is actually a better antibiotic agent for most uses, except the treatment of urinary tract infections [99].

In the case of colistin, which is administered as the prodrug colistimethate, there is a very narrow therapeutic window, where an average concentration at steady state of 2 μg/mL is needed to achieve therapeutic targets but where a concentration of approximately 2.5 μg/mL correlates with renal toxicity [100]. Usual dosing regimens do not appear to result in sufficient initial concentrations of colistin to achieve bacterial killing, especially among patients with intact renal function (CrCl >80 mg/min) [101], and a loading dose of colistin is required to more rapidly achieve concentrations that correlate with therapeutic targets. Further caution is required with colistin monotherapy due to suboptimal exposure and the potential for the emergence of resistance [102].

These limitations support the direct coadministration of colistin for the treatment of infections occurring in certain anatomic compartments, in addition to IV therapy. For instance, the supplemental use of aerosolized colistin may offer benefit in the treatment of pneumonia, and intrathecal and intraventricular colistin may be advantageous for the treatment of central nervous system infections. A meta-analysis of observational studies describing the outcomes of patients treated with intravenous and aerosolized forms of colistin for the treatment of VAP demonstrated similar hospital mortality and nephrotoxicity in patients treated with either colistin or comparator drugs [99]. A meta-analysis and systematic review of observational studies evaluated the safety and efficacy of aerosolized colistin as an adjunctive to intravenous antimicrobials and suggested improved outcome with the additional use of aerosolized colistin [103]. Similarly, a review of the experience treating MDR and XDR *A. baumannii* ventriculitis and meningitis with intraventricular and intrathecal colistin suggests frequent successful outcomes (89 %), at the expense of not infrequent (11 %) but reversible chemical ventriculitis/meningitis [104].

An interesting opportunity to observe the utility of colistin for the treatment of *A. baumannii* bacteremia was afforded by the experience in Korea, where colistin only became available in 2006. Monotherapy with colistin was associated with similar mortality (35.5 %) than therapy with non-active drugs (38.5 %) [105]. In general, there is concern about the efficacy of colistin used as monotherapy for the treatment of bloodstream infections, and therefore combination therapy has been proposed; however, there is no consensus about its benefit. Evidence supporting combination therapy is only available from observational studies. A high-quality prospective observational study conducted in 28 Spanish hospitals did not find an association of combination therapy with improved mortality in infections caused by *A. baumannii* [106]. Unfortunately, the multiple combinations proposed and the variations in the quality of the studies preclude firm conclusions. Systematic reviews of the evidence are not definite about the merits of combination therapy with colistin and even suggest similar safety and efficacy of colistin monotherapy when compared to standard antibiotics [107, 108].

Treatment of serious infections caused by XDR *A. baumannii* with colistin monotherapy has been compared with combination therapy with colistin and rifampin in a multicenter, open-label, clinical randomized controlled trial. Although there was a significant increase in microbiologic eradication with the additional use of rifampin, no difference was observed for infection-related death (43 %) and length of hospitalization [109]. Another small study comparing colistin monotherapy with colistin and rifampin for the treatment of *A. baumannii* reported similar results [110]. It has been suggested that rifampin perhaps was not the best partner for a combination therapy regime including colistin [111].

Two randomized controlled trials are in progress to evaluate the use of colistin monotherapy vs. colistin and meropenem in combination for the treatment of *A. baumannii*, among other XDR Gram-negative bacteria (NCT01597973 and NCT0173250). Interestingly, the preliminary results of a randomized controlled trial comparing colistin versus colistin plus intravenous fosfomycin (not potent enough to be used as monotherapy) for the treatment of carbapenem-resistant *A. baumannii* infection demonstrated a significantly more favorable microbiological response, but no significant difference in important clinical outcomes such as survival, in patients who received combination therapy [112]. In contrast, a prospective multicenter observational study identified increased mortality associated with a colistin–tigecycline combination (when the MIC of tigecycline exceeded 2 mg/L) compared to the colistin–carbapenem combination [113].

Tigecycline has been used for the treatment of infections caused by carbapenem-resistant *A. baumannii*, in cases where other alternatives do not exist and to avoid the nephrotoxicity associated with the use of polymyxins. Worldwide surveillance of the in vitro activity of tigecycline against carbapenem-resistant *A. baumannii* revealed that, in 2011, 35 % of isolates displayed an MIC of 2 μg/mL or higher. Overall resistance rates to tigecycline in Latin America, among other regions, have increased [114]. Tigecycline only achieves a maximal concentration in serum of 0.6 μg/mL, raising concerns about the ability of this drug to meet the requisite PK/PD parameters to effectively treat bloodstream infections. [18]. There are only limited data supporting the

role of tigecycline to treat serious infections caused by carbapenem-resistant *A. baumannii* such as bloodstream infection or VAP [115]. An analysis of the clinical outcomes of patients with infections caused by MDR *A. baumannii* who were treated with tigecycline alone or in combination with other antibiotics, or with imipenem and sulbactam, revealed that among 386 patients, 120 patients were treated with imipenem or sulbactam, and 266 received tigecycline. Of these, 108 were treated with tigecycline alone and 158 were treated with tigecycline in combination with other agents. There were no significant differences in survival rates between the groups. However, the patients in the tigecycline group were less ill and received other agents in addition to tigecycline, whereas the patients in the non-tigecycline group were not on any agents with demonstrated activity against *A. baumannii* [116]. Clinicians may see a clearer role for tigecycline in the treatment of skin and soft tissue infections and intra-abdominal infections where carbapenem-resistant *A. baumannii* is documented as a pathogen. However, it is difficult to find clinical data supporting this choice. In general, other antibiotic options, if available, should be given as preference to treat serious infections caused by XDR *A. baumannii.*

Minocycline has received attention as a possible option for the treatment of infections caused by XDR *A. baumannii*. This representative of the tetracycline class of antibiotics displays in vitro activity against approximately 80 % of *A. baumannii* strains from a global collection [117]. Therefore, given its availability in the USA as an intravenous formulation, minocycline has been used to treat carbapenem-resistant *A. baumannii.* Clinical and microbiologic responses, according to uncontrolled observational studies, have been good. Minocycline has been administered both as monotherapy and in combination with other antimicrobials and demonstrates an acceptable side effect profile [118, 119].

8 Conclusion

We have summarized some of the major aspects of the classification, microbiology, genomics, virulence, antibiotic resistance, infection control, and therapy of *A. baumannii*, with some mention of other *Acinetobacter* species as deemed relevant. As we advance in our understanding of *A. baumannii*, we appreciate that challenges will be ever present. In the next few years, more knowledge will be uncovered revealing the complexity of *A. baumannii*, especially as the role of genetic elements such as CRISPR-Cas, siRNAs, and bacteria network theory is elucidated. We anticipate that novel therapeutic approaches such as vaccines, immunomodulators, and modification of endolysins to create artilysins that are able to pass the outer membrane and become active against *Acinetobacter* may someday be used [33, 120, 121].

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Antimicrobial Resistance in *Stenotrophomonas maltophilia*: Mechanisms and Clinical Implications

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1 Introduction

Previously known as *Pseudomonas maltophilia* [1] and *Xanthomonas maltophilia* [2], *Stenotrophomonas maltophilia* belongs to a member of the Gammaproteobacteria and is one of eight species in the genus of *Stenotrophomonas* [3, 4]. An aerobic, nonfermentative Gram-negative bacterium ubiquitous in nature, *S. maltophilia* has increasingly emerged as a global opportunistic pathogen, particularly among life-threatening infections in immunocompromised patients [5–11]. Remarkably, this microbe features high-level intrinsic resistance to a variety of antimicrobial agents, including β-lactams (even carbapenems), aminoglycosides, quinolones, macrolides, and tetracyclines regardless of its clinical and/or environmental sources [7, 10, 12]. Acquired multidrug resistance (MDR) can be readily derived after exposure of *S. maltophilia* to different antimicrobials and is rapidly emerging in clinical isolates [13]. This species possesses various molecular and biochemical mechanisms of resistance, which include the production of Ambler class A and B β-lactamases, several aminoglycoside-modifying enzymes, Qnr quinolone target protection proteins, and multidrug efflux transporters. Together with virulence factors, the MDR phenotype poses a major hurdle for therapeutic development. Trimethoprim-sulfamethoxazole and other antimicrobial combination regimes remain as the dominant therapeutics within the limited drugs available against *S. maltophilia*. However, in addition to a global emergence of resistance to trimethoprim-sulfonamides, the remaining options for combination therapies are often only based on *in vitro* antimicrobial synergy testing and/or case reports. This chapter provides an overview of the features, mechanisms, and clinical implications of antimicrobial resistance in *S. maltophilia* with an emphasis on the genetic and biochemical mechanisms of resistance.

2 *S. maltophilia*, a Global Opportunistic Pathogen

S. *maltophilia* exists in numerous aquatic and humid environments which include animals, plants, foods, and water [4, 14]. Though originally regarded as an emerging nosocomial pathogen restricted mainly to hospital settings [7], it has now expanded globally to be associated with both hospital-associated and community-acquired infections [14–16]. Its high-level MDR and propensity to colonize poses a challenge for hospital infection control practices. Risk factors for colonization and infection with this organism often include previous exposure to antimicrobial agents, intensive care unit stay, malignancy, prolonged hospitalization, mechanical ventilation, and the use of intravascular devices [7, 14, 17, 18].

Unsurprisingly, a major predisposing factor for *S. maltophilia* infection is prior antimicrobial usage, particularly the use of broad-spectrum agents [17, 18]. On the contrary, studies have also associated a decreased risk of *S. maltophilia* infections in cystic fibrosis patients using oral antimicrobials to maintain lung functions [19]. This organism is an especially obtrusive opportunistic pathogen in immunocompromised individuals, particularly those with cystic fibrosis and underlying malignancies [7, 9, 10]. A recent epidemiological study reported that *S. maltophilia* was found to be isolated in up to 9.4 % of bloodstream infection samples obtained from cancer patients [20]. Detection of *S. maltophilia* (e.g., within airways and the gastrointestinal tract) does not necessarily suggest active infection; however, a variety of infections can be caused by this organism,

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and these include, for example, respiratory tract infections, bacteremia, endocarditis, urinary tract infections, skin and soft tissue infections, bone and joint infections, gastrointestinal infections, and meningitis [7, 10, 14, 21]. *S. maltophilia* possesses numerous virulence factors [21] with strong versatility and adaption characteristics and can be part of polymicrobial infections [4, 7, 10, 14]. A systematic review of the literature indicates that the attributable mortality from *S. maltophilia* infections should not be underestimated [22]. Indeed, *S. maltophilia* was the eighth-most isolated Gram-negative organism (4.3 % [3217/74,394]) from intensive care unit patients in the USA during the period of 1993–2004 [23]. Another study ranked it as the sixth (4.4 %) and ninth (3.2 %) most frequent organism isolated from patients hospitalized with pneumonia (2009–2012) in the USA and the European/Mediterranean regions, respectively [24]. Extensively drug-resistant strains were also reported to have a strong association with higher mortality rates in patients [25]. Additionally, *S. maltophilia* is able to form biofilms, which again poses a significant challenge for effective therapeutic intervention [26, 27].

3 High-Level Intrinsic Multidrug Resistance and Emergence of Acquired Resistance

S. maltophilia is intrinsically resistant to multiple antibiotics and disinfectants [7], and both clinical and environmental isolates often display high-level MDR [6, 10, 12, 28, 29]. For instance, *S. maltophilia* isolates are resistant to nearly all β-lactams (including carbapenems) and aminoglycosides, with many isolates also showing resistance toward fluoroquinolones and tetracyclines [10, 30–33]. These characteristics further constrain the already limited therapeutic options in the treatment of *S. maltophilia* infections. Even drug combinations such as ciprofloxacin with β-lactams or aminoglycosides show limited synergistic interaction or no activity against *S. maltophilia* [34].

Table 59.1 provides antimicrobial susceptibility data available in literature [10, 32, 35, 36] as well as the drugs to which intrinsic resistance has been defined for *S. maltophilia* by the Clinical and Laboratory Standards Institute (CLSI)[37]. The strain ULA-511 included in Table 59.1 was a clinical isolate

Table 59.1 Antibacterial activity of antimicrobials against a wild-type, laboratory reference strain of *S. maltophilia* ULA-511 in comparison with MIC_{50} values for clinical isolates

Antimicrobial	MIC (μg/mL) for strain ULA-511[a]	MIC_{50} (μg/mL) for clinical isolates[b]	CLSI clinical resistance breakpoints[c]	CLSI confirmed intrinsic resistance[c]
β-Lactams				
Imipenem	512	>8–512 (>32[d])		R
Meropenem	1024	>8 to >64 (>32[d])		R
Doripenem		>32[e]		(R[e])
Cefotaxime	512	≥64		R
Ceftazidime	256	8–128 (>256[d])	≥32	(R[f])
Ceftriaxone	512	>32–256		R
Cefepime	128	16–64		(R[f])
Cefpirome	512			
Piperacillin		>256[d]		
Ticarcillin	>1024	16–512		R
Ticarcillin-clavulanate		2–128	≥128/2	
Aztreonam	>1024	>16–256 (>256[d])		R
Pirazmonam	8			
Other β-lactams[a]	>1024			R
Aminoglycosides				
Amikacin	512	>32–512		R
Gentamicin	512	>8–64		R
Kanamycin	1024			R
Neomycin	>2048			R
Netilmicin		>256[d]		
Streptomycin	256			R
Tobramycin	2048	≥16–64 (>256[d])		R
Macrolides				
Azithromycin	256	≥512[g]		R
Erythromycin	512			R
Quinolones				

(continued)

Table 59.1 (continued)

Antimicrobial	MIC (μg/mL) for strain ULA-511[a]	MIC_{50} (μg/mL) for clinical isolates[b]	CLSI clinical resistance breakpoints[c]	CLSI confirmed intrinsic resistance[c]
Nalidixic acid	16	8[h]		
Norfloxacin	16	≥16)		
Ofloxacin		0.5		
Ciprofloxain	4	0.25–>8		
Gatifloxacin		0.1–4		
Gemifloxacin	1			
Levofloxacin		0.2–2 (4[d])	≥8	
Moxifloxacin	0.5	0.06–0.5		
Sparfloxacin	0.5	0.25[h]		
Trovafloxacin	0.25–0.5			
BaYy3118	0.063			
Clinafloxacin	0.12–0.25			
Tetracyclines				
Doxycycline	0.5	1–2 (4[d])		
Minocycline	0.125	0.2–1 (2[d])		
Tetracycline	8	>8–32		R
Tigecycline	0.5	1 (2[e])	(≤2 as susceptible[i])	
Other antibiotics				
Co-trimoxazole		≤0.25 to >64 (0.5[d])	≥4/76	
Trimethoprim	16			R
Chloramphenicol	8	4–32	≥32	
D-Cycloserine	512			
Fosfomycin		128[j]		R
Novobiocin	2560			
Colistin		2[e] (8[d])	(≤2 as susceptible[e])	
Polymyxin B		1–2 (8[j])	(≤2 as susceptible[k])	
Rifampin	8	32[l]	(≤1 as susceptible[m])	
Toxicants				
Acriflavine	256			
Proflavine	>256			
Crystal violet	16			
Ethidium bromide	512			
Sodium dodecyl sulfate	3200			

[a]Data are from references [32, 35]. Other β-lactams tested included ampicillin, ampicillin-sulbactam, amoxicillin, carbenicillin, cloxacillin, panipenem, penicillin G, piperacillin, cefsulodin, and cefoperazone, all with MIC values of >1024 μg/mL

[b]Data are derived from literature in reference [10], unless otherwise indicated

[c]Clinical resistance breakpoints and intrinsic resistance (R) are recommended in reference [37]. *S. maltophilia* also displays intrinsic resistance to additional agents including β-lactams (cephalothin, cefazolin, cefuroxime, cefoxitin, and cefotetan), clindamycin, daptomycin, fusidic acid, fosfomycin, vancomycin, teicoplanin, linezolid, clarithromycin, and quinupristin-daptomycin [37]

[d]From [207]

[e][203]

[f][221]

[g][218]

[h][171]

[i][201]

[j][222]

[k][203]

[l][207]

[m]CLSI-approved susceptible breakpoint for *Staphylococcus aureus* [37] is listed as a reference. Also see [207]

from a collection at the University of L'Aquila in Italy [38] and has been used as a laboratory reference strain for studying antimicrobial resistance for over two decades [32, 35, 36, 39, 40]. It is important to note that multidrug-resistant strains can be readily selected from the exposure of susceptible *S. maltophilia* to a variety of structurally unrelated antimicrobial agents in the laboratory [32, 41, 42] or recovered from patients receiving antimicrobials, e.g., β-lactams, aminoglycosides,

fluoroquinolones, or trimethoprim-sulfamethoxazole (co-trimoxazole) [13, 43, 44]. Although trimethoprim-sulfamethoxazole is still generally the drug of choice (both used alone or in combination) to treat *S. maltophilia* infection [10], global emergence of acquired trimethoprim-sulfamethoxazole resistance has been reported [45–49]. A 2001 study [50] has shown that resistant *S. maltophilia* strains were frequently associated with pulmonary infections, independent of the geographic regions assessed (Asia Pacific, Canada, Europe, Latin America, and the USA). The rates of resistance to trimethoprim-sulfamethoxazole for 842 *S. maltophilia* isolates (1997–1999) were 2 % in Canada and Latin America and 10 % in Europe, while resistance rates to ciprofloxacin, gatifloxacin, and trovafloxacin ranged from 21 to 49 %, 2 to 15 %, and 2 to 13 %, respectively. Ceftazidime and ticarcillin-clavulanate showed higher resistance with rates ranging from 25 to 53 % and 10 to 29 %, respectively [50]. An independent German study following chronically colonized cystic fibrosis patients detected resistance rates of 17 % for trimethoprim-sulfamethoxazole, 3 % for tigecycline, 30 % for levofloxacin and moxifloaxcin, 54 % for ceftazidime, and 58 % for colistin [51]. Considering the threat from multidrug-resistant Gram-negative bacterial infections such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* implicated in ventilator-associated pneumonia or in cystic fibrosis patients [52–55], it is necessary to highlight a possible important issue regarding the selection or enrichment of *S. maltophilia* (in addition to the targeting pathogens) through carbapenem or polymyxin therapy of other Gram-negative infections because *S. maltophilia* are intrinsically resistant to carbapenems and show variable susceptibilities (in many cases, resistance) to polymyxins [56–58]. Together, the intrinsic resistance and the feature of readily acquired high-level MDR in *S. maltophilia* undoubtedly pose a major concern regarding anti-stenotrophomonal chemotherapy.

4 Molecular and Biochemical Mechanisms of Resistance

4.1 Genomic Analysis of Antimicrobial Resistance Determinants

The current massively available bacterial genomic data allow us to readily assess the presence and distribution of genetic determinants for antimicrobial resistance among bacterial species. The first whole-genome sequence data of *S. maltophilia* became available in 2008 for strain K279a, which can be considered as a wild-type strain (also used as a model laboratory organism) and has a genome size of 4.85 Mb with an average G+C content of 66 % [59]. In comparison with other high-level intrinsically resistant pathogens, this genome size is smaller than that of *P. aeruginosa* (6.3 Mb) [60] but larger than that of *A. baumannii* (3.2–3.9 Mb) [61]. Subsequent whole-genome sequencing has also ascertained several other strains including those with acquired MDR [62–65].

Genomic data provide insightful clues to the genomic features and genetic elements of resistance for *S. maltophilia*. Firstly, *S. maltophilia* strains display significant genetic heterogeneity [66, 67]. Secondly, a large number of resistance genes are identified in the genomes of *S. maltophilia*; these include genes encoding for β-lactamases, aminoglycoside-modifying enzymes, and multiple drug efflux transporters (Table 59.2; see below for details). *S. maltophilia* also contains a chromosomal *qnr* gene termed Sm *qnr* [59, 64, 68], which usually occurs on plasmids of other bacterial species [69, 70]. Mobile genetic elements such as class I integrons are also identified in the genome of multidrug-resistant isolates with similar arrangements to resistance gene cassettes [65, 71, 72]. Antimicrobial resistance genomic islands have been identified in various bacterial species such as *A. baumannii* [73] and *Salmonella* spp. [74]. A similar genomic island of ca. 40 kb was also observed in *S. maltophilia* that contained six resistance determinants (including *tetR-tetA*(A), *strA/strB*, *sul1*, *aadA2*, and *floR* genes) and conferred an MDR phenotype [75]. As expected, this island region possessed mobile genetic elements such as integrons and insertion sequences [75]. Thirdly, comparative genomic analyses between the pathogenic multidrug-resistant *S. maltophilia* and plant-associated *S. maltophilia* and *Stenotrophomonas rhizophila* strains have revealed an overall high degree of sequence similarity including genes involved in antimicrobial resistance [64] as well as strain-specific pathogenicity islands [76]. Lastly, several plasmid-borne insertion sequence common region (IS*CR*) elements, which serve as gene-capturing systems, are also found in close relation with genes conferring resistance to trimethoprim-sulfamethoxazole in *S. maltophilia* [46, 77].

4.2 β-Lactam Resistance

β-Lactamases represent the most important mechanism of β-lactam resistance in Gram-negative bacteria and hydrolyze the four-membered β-lactam ring of β-lactams [78]. There are more than 1000 naturally occurring β-lactamases reported to date, and these enzymes are divided into four classes (Ambler A, B, C, and D) on the basis of their primary amino acid sequences and catalytic mechanisms [79]. Class A, C, and D β-lactamases are serine-dependent enzymes, while class B β-lactamases are metalloenzymes [78]. As for *S. maltophilia*, two types of β-lactamases were initially reported in the early 1980s [80] and are currently known as the L1 (Ambler class B) and L2 (Ambler class A) β-lactamases, which are both constitutively produced and can be further induced [67, 81–83]. These two β-lactamases together confer resistance to virtually all β-lactam agents including carbapenems (except for several β-lactams such

Table 59.2 Biochemical mechanisms of antimicrobial resistance in *S. maltophilia*

Antibiotic class	Drug-inactivating enzymes	Drug target alteration/ protection/by-passing	Drug efflux pump/outer membrane permeability
β-Lactams	Class B metallo L1 and Class A L2 β-lactamases, and other β-lactamases (TEM-2, CTX-M, and NDM-1)		RND pumps SmeABC and SmeDEF; outer membrane permeability
Aminoglycosides	*N*-acetyltransferases: AAC(6′)-Iam, AAC(6′)-Iak, AAC(6′)-Iz and AAC(2′) *O*-nucleotidyltransferase: ANT(3′′) *O*-phosphotransferase: APH(3′)-IIc, StrA/StrB		Lipopolysaccharide alteration; ABC pump MacABC; RND SmeOP-TolC and SmeYZ
Chloramphenicol	Chloramphenicol acetyltransferase		RND pumps SmeDEF and SmeVWX; MFS pumps FloR and MfsA
Fluoroquinolones		Alterations of DNA gyrase and topoisomerase IV (although remained to be fully confirmed); Qnr pentapeptide family proteins	RND pumps SmeABC, SmeDEF, SmeIJK, and SmeVWX; ABC pump SmrA; MFS pump MfsA
Fusaric acid			ABC pump FuaABC
Macrolides	Macrolide phosphotransferase		RND pumps SmeABC, SmeDEF, and SmeOP-TolC; ABC pump MacABC; MFS pump MfsA
Polymyxins			Lipopolysaccharide alteration; ABC pump MacABC
Sulfonamides		Dihydropteroate synthase (encoded by *sul1* and *sul2*)	RND pump SmeDEF
Trimethoprim		Dihydrofolate reductase (encoded by *dhfr* or *dfrA* genes)	RND pump SmeDEF
Tetracyclines			RND pumps SmeABC, SmeDEF, SmeIJK, SmeOP-TolC, and SmeVWX; ABC pump SmrA; MFS pumps TetA, TcrA and MfsA
Tigecycline			RND pumps SmeABC and SmeDEF

as ceftazidime and ticarcillin-clavulanate which may still be active, when viewed from a clinical resistance breakpoint perspective) (Table 59.1) [13, 30, 84]. The differential contribution of these two β-lactamases toward β-lactam resistance was determined for the first time in mutants carrying chromosomal deletions of *blaL1* and/or *blaL2* genes [32]. Clinical isolates with diverse L1/L2 β-lactamases have been reported [81, 85]; yet the majority of isolates from different geographic regions showed similar profiles of β-lactamase production and phenotypes of β-lactam resistance [86]. In spite of the chromosomally encoded nature of L1 and L2 enzymes, a mega-plasmid carrying both L1 and L2 genes was also reported [87]. Additionally, low outer membrane (OM) permeability and multidrug efflux pumps also play a role in β-lactam resistance [36, 88, 89], but can be masked in detection when in the presence of L1 and/or L2 enzymes.

L1 β-lactamase. This metalloenzyme, encoded by the *blaL1* gene (also known as *blaS* gene) on the chromosome [90], belongs to class B in Ambler's classification and group 3b in functional classification [78, 91]. With an isoelectric point of ca. 7 and a monomeric molecular mass of ca. 28 kDa, this enzyme requires a divalent metal ion to exert its catalytic activity [39, 82, 92]. Divalent chelators such as ethylenediaminetetraacetic acid inhibit L1 enzyme activity. L1 β-lactamase has a broad substrate profile and hydrolyzes most β-lactams, although it still preferentially hydrolyzes carbapenems over other types of β-lactams [93]. As previously mentioned, genetic studies have identified molecular heterogeneity of the L1 enzyme among various *S. maltophilia* strains, which is largely due to amino acid sequence variation [67, 85]. This variance has led to subclassification of the L1 enzymes [87] into three sub-enzymes. Alignment of the L1 β-lactamase and class B metallo-β-lactamases with those of other bacteria has identified conserved regions within class B β-lactamases [94]. The enzyme exists as a tetramer and displays αβ/βα folding observed only in the metallo-β-lactamases [95]. Simulated binding of the substrates ampicillin, ceftazidime, and imipenem reveals direct interactions between the β-lactam carbonyl oxygen and nitrogen with zinc ions and of the β-lactam carboxylate with Ser187. Ullah et al. [95] have proposed that the catalytic mechanism of the L1 enzyme includes a nucleophilic attack

of the bridging water on the β-lactam carbonyl carbon, electrostatic stabilization of a negatively charged tetrahedral transition state, and protonation of the β-lactam nitrogen by a second water molecule coordinated by zinc ions. The direct metal-substrate interaction provides a substantial contribution to substrate binding and may explain the lack of specificity of class B β-lactamases. Therefore, it is not surprising that the L1 β-lactamase makes *S. maltophilia* highly resistant to carbapenems as well as most other β-lactams [32, 93].

The L1 enzyme, unlike the L2 enzyme (see below), is not susceptible to clinically available β-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam, indicating little application of these inhibitors against L1 β-lactamase. However, inhibition studies against the L1 β-lactamase have shown that thiol ester derivatives of mercaptoacetic acid are able to inhibit the L1 enzyme as well as other metallo-β-lactamases [40, 96–98]. A carbapenem compound with a benzothienylthio moiety at the C-2 position of 1-methylcarbapenem was shown to be a potent inhibitor of class B β-lactamases (with very low *Ki* values) including L1 enzyme [99, 100]. The flavonoids galangin and quercetin were also noted to inhibit the L1 enzyme, though the effect was not reversible by the addition of $ZnCl_2$ [101]. Two thiol-containing compounds, *N*-(2′-mercaptoethyl)-2-phenylacetamide and *N*-benzylacetyl-D-alanylthioacetic acid, were found to be competitive inhibitors of the L1 enzyme [97]. Furthermore, a peptide with a consensus sequence (i.e., Cys-Val-His-Ser-Pro-Asn-Arg-Glu-Cys) was also reported to be a specific inhibitor of the L1 enzyme [102].

L2 β-lactamase. This enzyme belongs to Ambler class A β-lactamase and Bush group 2e [78]. With a molecular mass of ca. 30 kDa and an isoelectric point of 8.4, it exists as a dimeric form [103]. The crystal structure of the L2 enzyme is also available (*doi:10.2210/pdb1o7e/pdb*). The L2 enzyme is closely related to the TEM β-lactamases with a serine active site, but it displays unusual cephalosporinase activity in which the enzyme preferentially hydrolyzes cephalosporins, including cefotaxime, but not carbapenems [104]. Consistent with class A enzymes, L2 enzymes are more sensitive to clavulanate (with IC_{50} values of <0.1 μM) than sulbactam and tazobactam (IC_{50} values are mostly >0.1–2 μM) [105]. Indeed, ticarcillin-clavulanate is among the very limited β-lactam agents active against *S. maltophilia* (Table 59.1) [106].

Regulation of L1 and L2 β-lactamase expression. β-Lactamase induction in Gram-negative bacteria is intimately linked to cell wall peptidoglycan recycling with an involvement of a complex network [107, 108]. A similar regulatory network also exists in *S. maltophilia* as detailed in this section. First, there is constitutive basal expression of both L1 and L2 enzymes, which contributes almost equally to nitrocefin hydrolysis [32]. Yet, the production of these two enzymes can be further stimulated by inducers such as various β-lactam agents (in particular by cefoxitin and imipenem, two classic AmpC β-lactamase inducers) [109]. An early study isolated three types of mutants including one with (a) constitutive overexpression of the L1 enzyme and inducible expression of the L2 enzyme, (b) overexpression of the L2 enzyme and inducible expression of the L1, and (c) constitutive overexpression of both L1 and L2 enzymes, which together suggested a possible overlapping regulatory mechanism toward L1 and 2 expression [110]. Subsequent studies have revealed the involvement of multiple gene products, AmpR, $AmpD_I$, AmpN, AmpG, MrcA, and NagZ, in the regulation of L1 and L2 expression (Fig. 59.1) [111–115].

A regulatory gene, *ampR*, is transcribed divergently from the L2 gene and encodes a LysR-type regulator (AmpR), which upon binding to anhydro-*N*-acetylmuramyl-peptides (anhMurNAc-peptides, inducing peptides) activates the expression of L1 and L2 genes (e.g., in response to β-lactam challenge) [111]. In contrast, the binding of AmpR to UDP-*N*-acetylmuramic acid pentapeptide (suppressing peptides) causes a conformation that represses β-lactamase expression. Disruption of the *ampR* gene significantly increases the susceptibility to β-lactams [111]. Degraded peptide products from cell wall peptidoglycans such as *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-peptides (GlcNAc-anhMurNAc-peptides), including GlcNAc-anhMurNAc-tripeptide, GlcNAc-anhMurNAc-tetrapeptide, and GlcNAc-anhMurNAc-pentapeptide, are transported by AmpG permease across the inner (cytoplasmic) membrane into the cytosol. AmpG is encoded from an operon comprised of *ampNG*, which is essential for induction of both L1 and L2 β-lactamases (*ampN* is a putative endonuclease gene) [113]. Inactivation of either AmpG or AmpN increases β-lactam susceptibility. Thus, mutants that do not express AmpG are unable to recycle cell wall peptides and cannot be induced by a β-lactam. AmpD, a cytosolic anhydro-*N*-acetylmuramyl-L-alanine amidase, acts as a key enzyme in balancing the concentrations of GlcNAc-anhMurNAc-peptide and UDP-*N*-acetylmuramic acid-pentapeptide within the cytosol. AmpD mutations result in the accumulation of high-level anhMurNAc-peptides even in the absence of β-lactam induction and cause β-lactamase overproduction [112]. Mutations in one of the two AmpD homologs, $AmpD_I$, are found to be involved in β-lactamase hyperexpression [112, 116]. Inactivation of another gene, *mrcA* (predicted to encode penicillin-binding protein PBP1a), causes an increase of basal L1/L2 β-lactamase activity by 100-fold [114]. Lastly, another β-lactamase expression regulatory pathway is linked to the *nagZ* gene that encodes β-*N*-acetylglucosamidase and is constitutively expressed in *S. maltophilia* [115]. NagZ produces anhydro-MurNAc-peptides and is critical for basal expression of β-lactamases [115]. Its inactivation decreases basal β-lactamase activity by 20 %; however, there are likely both NagZ-dependent and NagZ-independent pathways for the induction of β-lactamase activities. NagZ inactivation reduces cefuroxime- and piperacillin-induced β-lactamase activity but does not affect the induction by aztreonam, carbenicillin, and cefoxitin [115].

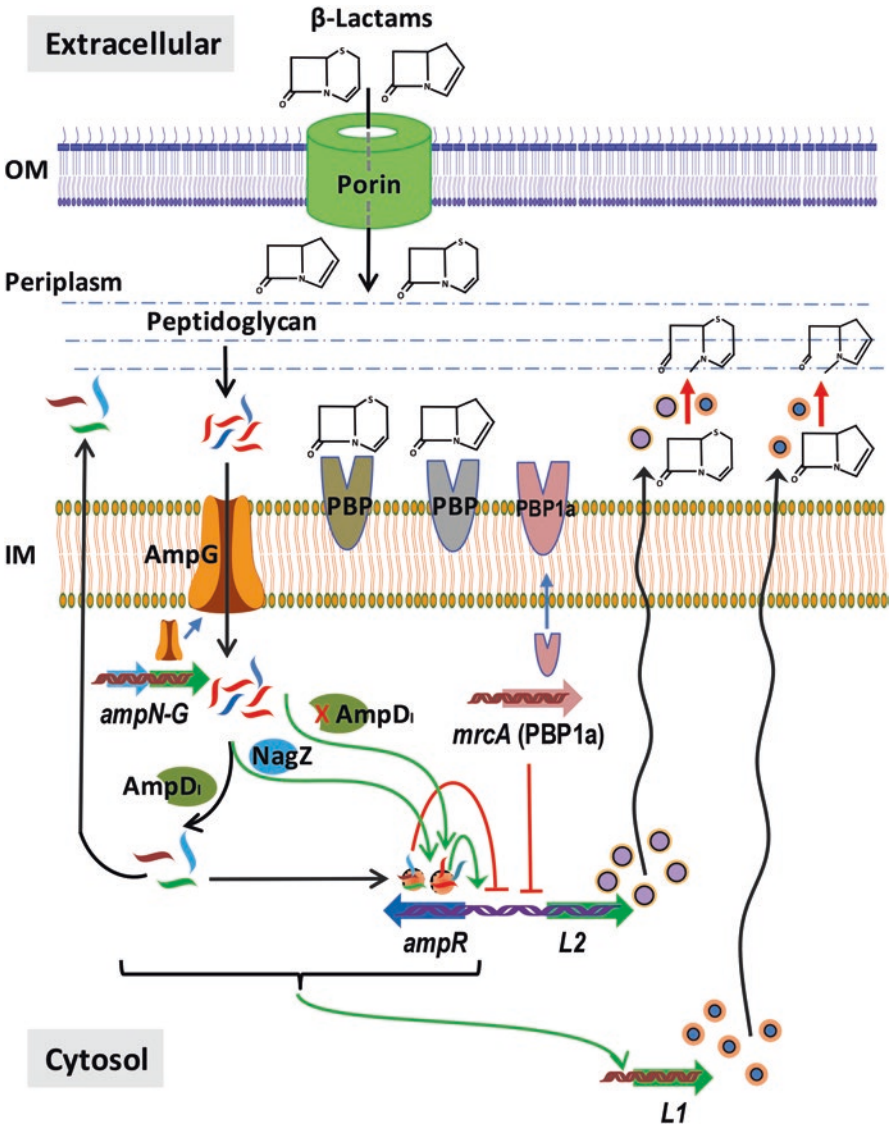

Fig. 59.1 Mechanisms involved in regulating L1 and L2 β-lactamase expression in *S. maltophilia*. There is a basal constitutive expression of *blaL1* and *blaL2* genes in wild-type strains even in the absence of β-lactams. Under normal cell wall recycling, *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-peptides are removed from the cell wall and transported into the cytoplasm via the AmpG permease. These GlcNAc-anhMurNAc-peptides are cleaved by $AmpD_I$ to generate free peptides, which are later converted into UDP-MurNAc-pentapeptides. These pentapeptides in turn interact with AmpR bound to the *ampR-blaL2* intergenic region to represses transcription of *blaL2* gene and to allow basal production of L2 β-lactamase. In the presence of β-lactam induction, β-lactams (e.g., cefoxitin and imipenem) cross the outer membrane (OM) through porins, enter the periplasm, and interact with the inner membrane (IM)-associated target penicillin-binding proteins (PBPs). There is an increase in 1,6-anhydromuropeptides, which, when bound to AmpR, convert it into a transcriptional activator to increase *blaL2* expression. Mutations in *ampDI* can inactivate $AmpD_I$ and lead to the derepression of *blaL2* expression. Expression of *nagZ* (encodes β-*N*-acetylglucosamidase) and inactivation of *mrcA* (encodes PBP1a) also enhance *blaL2* expression. Similar *blaL1* gene expression is also observed but detailed mechanisms remain to be further studied

Other β-lactamases. Due to the heterogeneity of the L1 and L2 enzymes in *S. maltophilia* [117], it was difficult to determine whether *S. maltophilia* produced additional chromosome-encoding β-lactamase(s) before its sequenced genome became available [82]. With the available L1- and L2-deficient double mutant [32], an attempt to identify additional β-lactamases via a biochemical means and to select β-lactam resistant mutants was unsuccessful (X.-Z. Li, unpublished). It is now known that there are no additional β-lactamase-encoding genes identified in the genomes of several *S. maltophilia* strains sequenced to date [59, 62–65]. Nevertheless, a gene encoding a TEM-2 β-lactamase was identified within a novel Tn*1*-/Tn*3*-type transposon in the genome of a clinical isolate. The gene was almost identical to the *blaTEM*-2 gene, representing the first example of a TEM in *S. maltophilia* [118]. Class A extended-spectrum β-lactamases of CTX-M type (e.g., CTX-M-1 and CTX-M-15) were also observed in *S. maltophilia* [119, 120]. An isolate containing *blaNDM-1* gene (that encodes another class B metallo-β-lactamase) was also reported [83, 121].

4.3 Aminoglycoside Resistance

The production of aminoglycoside-modifying enzymes represents the major mechanisms responsible for aminoglycoside resistance [122–124]. Indeed, *S. maltophilia* genome encodes multiple known and putative aminoglycoside-modifying enzymes (such as 2′ or 6′ *N*-aminoglycoside acetyltransferases [AAC; e.g., AAC(6′)-Iam] and 3′-phosphotransferases [APH]) (Table 59.2) [59], which render strains highly resistant to virtually all aminoglycosides (Table 59.1). It also contains a gene for a putative spectinomycin phosphotransferase, which explains the high-level resistance to aminocyclitol agents [59, 75]. King et al. initially demonstrated the presence of AAC(6′) [122]. Subsequently, the *aac(6′)-Iz* gene was reported to be widely distributed in *S. maltophilia* [125], and its inactivation led to an increased susceptibility to amikacin, gentamicin, netilmicin, sisomicin, and tobramycin (2- to 128-fold decrease in MIC values) [29]. Another new AAC(6′)-Iak shows 86 % identity to AAC(6′)-Iz and can acetylate amikacin, arbekacin, dibekacin, isepamicin, kanamycin, neomycin, netilmicin,

sisomicin, and tobramycin [126]. Both AAC(6′)-Iz and AAC(6′)-Iak enzymes share a similar substrate profile [126]. Disruption of the gene encoding APH(3′)-IIc increased the susceptibility to butirosin, kanamycin, neomycin, and paromomycin (4- to 32-fold MIC reduction) [127]. *S. maltophilia* was also noted to produce an *O*-nucleotidyltransferase, ANT(3″), which modifies streptomycin and spectinomycin [128].

Additionally, growth temperature can affect the compositions of the OM lipids including lipopolysaccharide (LPS), thus subsequently aminoglycoside susceptibilities (e.g., cells grown at 37 °C become more susceptible to aminoglycosides than those at 30 °C) [129–131]. In this regard, *S. maltophilia* was seen to survive exposure to aerosolized tobramycin at 16,000 μg/mL in cystic fibrosis patients; this survival was enhanced at lower temperatures [132]. Aminoglycosides are polycationic agents, and thus anionic binding sites of LPS affect the entry of aminoglycosides [128]. Inactivation of the *spgM* gene (which encodes phosphoglucomutase) results in shorter O-polysaccharide chains and a modest increase in susceptibility to gentamicin, colistin (polymyxin E), and polymyxin B [133]. Lastly, several multidrug efflux pumps MacABC, SmeOP-TolC, and SmeYZ are also involved in aminoglycoside resistance (Table 59.2) (see Sect. 4.10) [134–137]. The observation of the reduction of amikacin and gentamicin MIC values by 128-fold in SmeYZ-deficient mutants [137] shows the significance of the SmeYZ pump in aminoglycoside resistance. However, considering the presence of native aminoglycoside-modifying enzymes, we think of a need to investigate whether the genetic inactivation of *smeYZ* could affect expression of any aminoglycoside-modifying enzymes. Additionally, overproduction of SmeDEF results a fourfold reduction of the kanamycin MIC value [36].

4.4 Fluoroquinolone Resistance

S. maltophilia also exhibits high-level resistance to fluoroquinolones, though newer agents (e.g., clindafloxacin and moxifloxacin) exhibit higher activity than older compounds (Table 59.1) [31, 35, 42]. Unexpectedly, unlike many other Gram-negative bacteria, the target proteins of fluoroquinolones, topoisomerases II (also called DNA gyrase, encoded by *gyrAB*) and IV (*parCE*), are not considered as the primary targets responsible for fluoroquinolone resistance in *S. maltophilia*. For example, although sequence changes occurred in quinolone resistance-determining regions (QRDRs) of *gyrAB* and *parCE* genes of *S. maltophilia*, they were not consistently related to changes of the ciprofloxacin MIC values [138]. Additionally, no alterations in *gyrA* or *parC* were observed in other clinical isolates of varied ciprofloxacin susceptibilities [139]. A study comparing isogenic ciprofloxacin-susceptible (MICs of 0.5–4 μg/mL) and ciprofloxacin-resistant mutants (MICs of 16–128 μg/mL) did not find sequence alterations in QRDRs [140]. Instead, these aforementioned studies suggested the contribution from drug efflux pumps to fluoroquinolone resistance. SmeABC, SmeDEF, and SmeVWX pumps are, for instance, known to confer fluoroquinolone resistance in clinical isolates with high-level fluoroquinolone resistance [36, 71, 141, 142] (see Sect. 4.10). These pumps are also heavily involved in the emergence of quinolone resistance [143].

Plasmid-borne *qnr* genes are widely distributed in *Enterobacteriaceae* and encode a pentapeptide repeat family product that protects DNA gyrase and confers low-level resistance to fluoroquinolones (reviewed in Refs. [69, 70]). SmQnr, a Qnr homolog, encoded by *S. maltophilia* chromosome consists of 219 amino acid residues [68, 144] and indeed contributes to low-level intrinsic resistance to quinolones [145]. To date, approximately 60 variants of the SmQnr family have been identified in numerous isolates of varying regions [49, 146–148]. Chromosomal *qnr* genes are also present in the genomes of >90 bacteria species [69, 70]. Together, these chromosomal sources of the *qnr* genes may serve as a reservoir of plasmid-borne *qnr* genes observed in Enterobacteriaceae. Nevertheless, plasmid-borne Sm*qnr* appears unstable in *Escherichia coli* in comparison with the widely distributed plasmid-borne *qnrA* gene [149, 150]. Expression of SmQnr is negatively controlled by a transcriptional repressor, SmQnrR, whose encoding gene forms an operon with a major facilitator superfamily transporter gene (Sm*tcrA*). SmQnrR can also repress its own expression as well as Sm*tcrA* expression [151].

4.5 Resistance to Amphenicols

A gene encoding a putative chloramphenicol acetyltransferase exists within the genome of *S. maltophilia* and likely provides a mechanism of resistance to amphenicols [59]. Moreover, the amphenicol efflux exporter *floR* gene has also been identified in resistant *S. maltophilia* [46, 75]. Additional multidrug efflux pumps such as SmeDEF and SmeVWX are also involved in chloramphenicol resistance [36, 141].

4.6 Resistance to Macrolides

Though macrolide antimicrobials are mostly active against Gram-positive bacteria, certain agents (e.g., azithromycin) also possess activity toward Gram-negative bacteria [152]. However, *S. maltophilia* strains are intrinsically resistant to macrolides, a characteristic that is attributable to multiple mechanisms. Macrolides are large hydrophobic molecules and cannot effectively cross the OM barrier [153]. Their physical-chemical properties also make them good substrates of multidrug efflux pumps [36, 135, 153]. Moreover, the

S. maltophilia genome carries a gene that encodes a macrolide-inactivating enzyme, macrolide phosphotransferase [59].

4.7 Resistance to Polymyxins

Polymyxins are large (>1100 Da), cationic lipopeptide antibiotics (with a net positive charge of 4 and a fatty acid tail for polymyxin B). They target the OM of the Gram-negative bacteria by competitively displacing the divalent cations (Mg^{2+} and Ca^{2+}) from the negative-charged LPS molecules, resulting in destabilized OM membrane barrier [154]. Thus, polymyxins are strong OM perturbants and exhibit activities against many Gram-negative bacteria including nonfermentative bacilli. Their unique mode of action also explains the role of polymyxins or derivatives (e.g., polymyxin B nonapeptide) in increasing the activity of a range of anti-Gram-positive agents against Gram-negative bacteria [155]. Although the mechanisms about the variable polymyxin susceptibilities of *S. maltophilia* remain to be investigated, resistance to polymyxins in Gram-negative bacteria takes several forms, often due to the alterations in the OM. First, adaptive resistance to polymyxins occurs as a non-mutational, transient phenomenon in response to the inducing conditions as observed in *Salmonella* and *P. aeruginosa* [156]. For instance, two-component regulatory systems, PhoPQ and PmrAB, can be activated independently under magnesium starvation, leading to LPS modifications that reduce the negative charge of anionic lipid A and electrostatic attraction to polymyxins [156]. Second, mutation-mediated acquired resistance can also develop [157]. Mutations affecting PhoPQ, PmrAB, and another two-component regulatory system, ParRS, contribute to the LPS alterations and result in polymyxin resistance. For example, ParRS in *P. aeruginosa* affects expression of the LPS modification *arn* operon and of *mexXY* efflux pump gene and confers resistance to polymyxins and aminoglycosides [158]. Capsular polysaccharides can also reduce the interaction between the OM and polymyxins, producing polymyxin resistance as observed in *Klebsiella pneumoniae* [157]. In *A. baumannii*, mutations in one of several lipid A biosynthesis genes lead to the complete loss of the LPS production and yield polymyxin resistance with a >128-fold increase in MIC values of colistin and polymyxin B (as expected, with increased susceptibility to other agents including azithromycin, cefepime, and teicoplanin because of the disruption of the OM permeability barrier) [159]. In *S. maltophilia*, the LPS integrity also plays an important role against the action of polymyxins since disruption of an LPS-related gene *spgM* generates an increased susceptibility to polymyxins [133]. Moreover, the effect of growth temperature on LPS profiles in *S. maltophilia* produces a modest effect on polymyxin susceptibility (a twofold MIC decrease at 37 °C than 30 °C which is, however, in contrast to the immense effect on aminoglycoside susceptibility) [130]. Efflux pumps such as MacABC also contribute to polymyxin resistance in *S. maltophilia* [59, 135] with a four- to eightfold decrease of colistin and polymyxin B MIC values for the *macAB*-disrupted mutant [135]. Although it is speculated that the known mechanisms of polymyxin resistance in other bacteria are likely applicable to *S. maltophilia*, elucidation of polymyxin resistance in *S. maltophilia* remains an important area of research.

4.8 Resistance to Tetracyclines and Tigecycline

Tetracycline resistance in Gram-negative bacteria is mostly mediated by plasmid-encoded tetracycline-specific efflux transporters (i.e., Tet proteins) and ribosome target modifications and protection mechanisms [160]. Tigecycline, a 9-*t*-butylglycylamido derivative of minocycline (belonging to the glycylcycline class), was developed to overcome the presence of Tet pumps [161] but is still subjected to efflux by multidrug efflux transporters [153]. Although *tet(A)* is found in a genomic island containing a resistance gene cassette in *S. maltophilia* [75], the multidrug efflux pumps encoded by the chromosome are predominantly responsible for high-level intrinsic and acquired resistance to tetracyclines and tigecycline. Inactivation of multidrug efflux pump SmeDEF in a wild-type strain reduces the susceptibility to tetracyclines (tetracycline, doxycycline, and minocycline) and tigecycline (two- to fourfold MIC reduction). SmeDEF overproduction confers increased resistance to these tetracycline agents (two- to eightfold MIC increase), and its inactivation renders the mutant stain more susceptible than the wild-type strain [36]. Inactivation of another multidrug efflux pump, SmeIJK, in a mutant overproducing the SmeIJK system results in increased susceptibility to tetracycline and minocycline (fourfold MIC decrease) [134]. The TcrA pump is also involved in resistance to tetracyclines [151].

4.9 Resistance to Sulfonamides and Trimethoprim

Resistance to sulfonamides and trimethoprim is mediated by dihydropteroate synthase (encoded by a *sul* gene) and dihydrofolate reductase (encoded by a *dhfr* or *dfrA* gene), respectively, and each of these enzymes is comprised of different variants [162]. Resistance gene cassettes or genomic islands containing integrons and several resistance genes including *sul* and/or *dhfr* have increasingly been found in *S. maltophilia* strains [47, 48, 71, 163, 164], and this phenomenon explains the increasing global emergence of resistance to sulfonamides and trimethoprim in *S. maltophilia* [45, 46, 48]. In one study

from China, nearly 50 % of the 442 tested isolates were resistant to this combination agent [49]. In a report analyzing isolates from various global sources [46], 17 out 25 sulfonamide-trimethoprim-resistant isolates (MICs of >32 μg/mL) carried the *sul1* gene and a class 1 integron, while susceptible isolates (MICs of 0.5–2 μg/mL) were *sul1*-negative. Moreover, the *sul2* gene and several IS*CR* variants were also present in plasmids from the resistant isolates [46]. A recent study conducted in Korea showed the presence of *sul1*, *dfrA*, integrons, and/or IS*CR* elements in resistant isolates, and 72 % (23/32) isolates with high-level resistance to trimethoprim-sulfamethoxazole were *sul1*-positive [48]. Interestingly, epigallocatechin gallate, a component of green tea, displays activity against *S. maltophilia* (with an MIC range of 4–250 μg/mL for 18 isolates tested) due to its inhibition of dihydrofolate reductase from trimethoprim-susceptible *S. maltophilia*, and it also shows a synergistic effect with sulfamethoxazole [165]. SmeDEF overproduction yields a four- to eightfold increase of the MIC values of trimethoprim and trimethoprim-sulfamethoxazole [36, 166], suggesting a non-antifolate mechanism of resistance to sulfonamides and trimethoprim, which are also substrates of *P. aeruginosa* Mex efflux pumps [167]. Although overproduction of SmeABC results in an enhanced susceptibility to trimethoprim, the OM component SmeC may provide a function to the yet-unidentified efflux pump(s) involved in trimethoprim resistance as *smeC* inactivation has a four- to eightfold reduction of the trimethoprim MIC values [89]. Inactivation of SmeYZ decreases the MIC of trimethoprim-sulfamethoxazole by 16-fold [137].

4.10 Multidrug Resistance

The OM permeability barrier and multidrug efflux pumps interact to contribute to intrinsic and acquired MDR of *S. maltophilia* [153]. The OM consists of a LPS-containing lipid bilayer region and functions as an effective barrier in reducing influx of toxic agents including antimicrobials. Small hydrophilic agents like β-lactams may cross the OM via the water-filled porin channels, and large or hydrophobic agents require the penetration OM lipid bilayer [153]. Agents that increase OM permeability can sensitize the activity of many anti-Gram-positive agents against Gram-negative bacteria. Polymyxins act on the OM and thus increase the access of various agents to their cellular targets. In one study, polymyxin B at 0.1 μg/mL was able to enhance activities of trimethoprim-sulfamethoxazole against all 30 multidrug-resistant *S. maltophilia* isolates tested [168], consistent with the role of the OM barrier in resistance. Lactoferrin can damage the OM and increases rifampin susceptibility (with rifampin MIC values reduced by 3- to 16-fold in the presence of lactoferrin) [169]. A fragment of a cationic frog peptide, esculentin-1b, was also expected to act on bacterial membranes and was able to sensitize the activities of amikacin and colistin against *S. maltophilia* strains [170]. Additionally, alterations in OM proteins were observed in fluoroquinolone- and multidrug-resistant isolates [171]; this may contribute to reduced OM permeability and/or increased drug efflux.

On the other hand, *S. maltophilia* possesses various drug exporters (Table 59.2) that belong to one of the following superfamilies: the (1) ATP-binding cassette (ABC) superfamily, (2) drug/metabolite transporter superfamily (which includes the small multidrug resistance [SMR] family), (3) major facilitator superfamily (MFS), and (4) resistance-nodulation-cell division (RND) superfamily [153]. However, no reports have described the multidrug/oligosaccharide-lipid/polysaccharide export superfamily (that includes the drug resistance-related multidrug and toxic compound extrusion [MATE] family) in *S. maltophilia*. These drug exporters may have either a broad (polyspecific) or narrow substrate profile and actively extrude relevant drug substrate(s) out of the cell. In particular, the RND-type multicomponent efflux pumps play important roles in MDR in Gram-negative bacteria, especially in the nonfermentative bacilli including *S. maltophilia* where the OM permeability barrier is already quite effective [153].

RND pumps. Eight RND-type Sme systems and several other types of drug exporters are encoded in the chromosome of *S. maltophilia* [59]. An RND pump complex typically comprises of three components, a pump located in the inner membrane, an OM channel protein, and an accessory protein (periplasmic adaptor protein) linking the first two components [153]. Figure 59.2 shows the genetic organization of the eight RND systems including their possible regulatory genes. Each system is encoded by an operon that contains two or three genes. For several RND systems, although a gene for an OM component is absent (i.e., with *smeGH*, *smeIJK*, *smeMN*, and *smeYZ* operons), an OM channel protein encoded by other operons or genes may provide the structural part required for a functional multicomponent efflux machinery [36, 89], similar to the role of *E. coli* TolC and *P. aeruginosa* OprM in multiple RND pumps [153]. Some RND pump operons are constitutively expressed (e.g., *smeDEF*) and are responsible for intrinsic resistance. Acquired resistant mutants with RND pump overexpression can be readily selected by antibiotics (e.g., chloramphenicol, fluoroquinolones, and tetracyclines) or biocides (e.g., triclosan) [32, 141, 143, 172, 173]. The first RND pump identified in *S. maltophilia*, SmeABC, contributes to acquired MDR including resistance to trimethoprim-sulfamethoxazole [71, 89, 174]. Another RND pump, SmeDEF, is the most important RND pump with respect to its role in both intrinsic and acquired MDR [35, 36, 71, 166, 175, 176]. Inactivation of SmeDEF mostly leads to a two- to

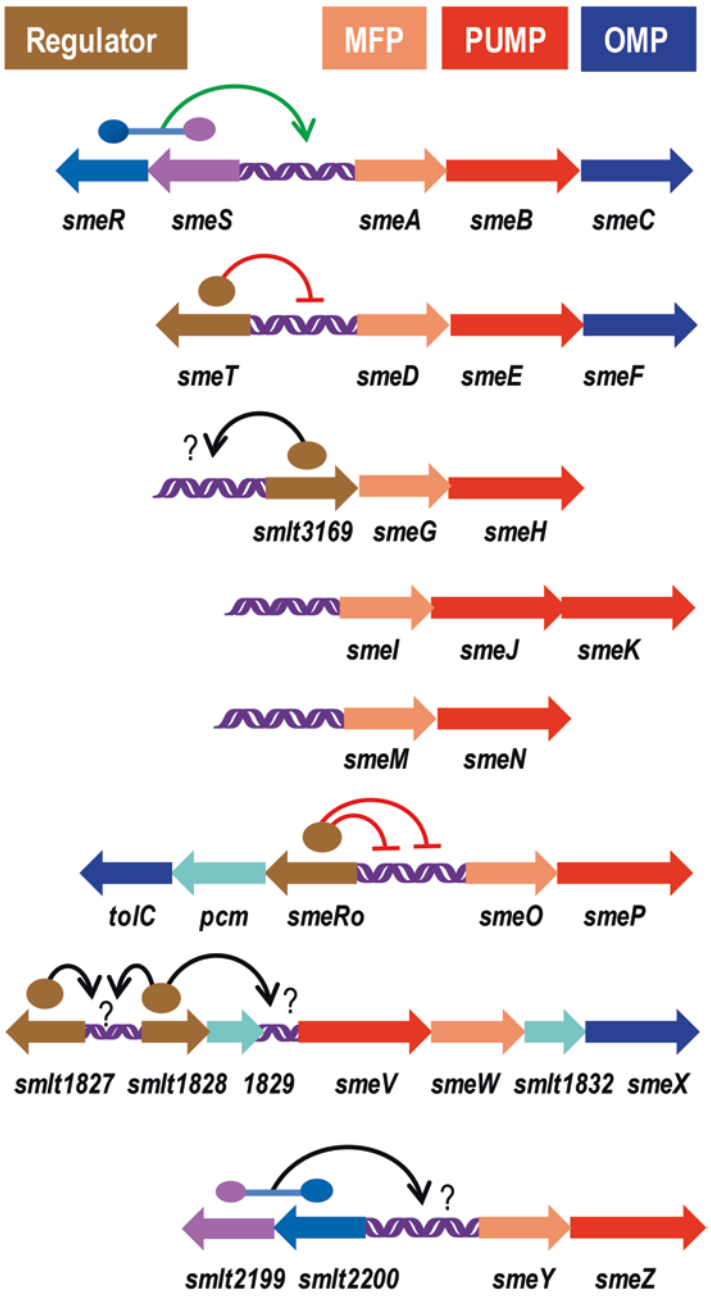

Fig. 59.2 Regulation of RND multidrug efflux system expression in *S. maltophilia*. Eight RND pump operons identified in this organism are presented (mostly in the right) with arrows showing gene transcriptional directions. The three different colors (*orange*, *red*, and *blue*) correspond to their roles as a membrane fusion protein (MFP; also called periplasmic adaptor protein)), a pump, or an outer membrane protein (OMP), respectively. Genes encoding the proven or putative regulators are shown on the left with their gene transcriptional directions indicated by arrows. While the green arrow from SmeSR represents positive regulation of *smeABC* expression, the inhibitory red lines show repression of relevant gene transcription by repressors. The role of putative regulators of relevant RND pump expression (indicated with a brown oval with a black arrow and question mark) remains to be investigated

eightfold reduction of the MIC values of fluoroquinolones, tetracyclines, tigecycline, trimethoprim-sulfamethoxazole, macrolides, chloramphenicol, novobiocin, β-lactams (in the absence of L1 and L2 β-lactamases), dyes, and detergents [36, 166]. Moreover, the SmeOP-TolC efflux system provides resistance to several antibiotics (amikacin, gentamicin, erythromycin, leucomycin, and doxycycline), carbonyl cyanide 3-chlorophenylhydrazone (a proton conductor), dyes, and detergents [136]. SmeYZ is involved in resistance to aminoglycosides and trimethoprim-sulfamethoxazole [137]. Overexpression of SmeVWX enhances resistance to chloramphenicol, quinolones, and tetracyclines but also susceptibility to aminoglycosides [141]. A simultaneous hyperexpression of SmeJK (paired pumps that form one exporter) and SmeZ pumps increases the substrate profile in clinical isolates and plays a role in resistance to ciprofloxacin and tetracyclines as well as resistance to aminoglycosides [134]. Additionally, although several efflux inhibitors including phenylalanine-arginine-β-naphthylamide (PAβN) show strong inhibitory activity against RND pumps of *E. coli* and *P. aeruginosa* [153], PAβN does not affect SmeDEF pump activity [177].

Non-RND pumps. Three ABC-type exporters are demonstrated for their involvement in resistance: (1) the

tripartite FuaABC system confers fusaric acid-inducible resistance to fusaric acid [178]; (2) MacABC provides intrinsic resistance to aminoglycosides, macrolides, and polymyxins as its inactivation yields a two- to eightfold MIC decrease [59, 135]; (3) the SmrA is linked to resistance to fluoroquinolones, tetracyclines, doxorubicin, and dyes when expressed in *E. coli* [179]. Additionally, an MFS-type pump, EmrCAB, is involved in extrusion of hydrophobic toxic agents but is not well expressed intrinsically [180]. A recent study described the induction by redox cycling agents and the activation by SoxS of another MFS-type exporter, MfsA, which mediates resistance to paraquat [181]. Lastly, a gene encoding an SMR protein was also reported [71].

Role of efflux pumps beyond drug resistance. The drug efflux pumps may also have physiological functions that go beyond drug resistance [153]. Colonization of plant roots is considered to be an original function of SmeDEF pump [182]. Native expression level of SmeDEF may provide an optimal physiological role since its overproduction results a virulence cost [183]. Inactivation of SmeIJK produces slow growth and also increases the sensitivity to membrane-damaging agents and activation of cell envelope stress response [184]. SmeYZ contributes to oxidative stress response and virulence [137]. MacABC is involved in tolerance to both oxidative and cell envelope stresses as well as biofilm formation [135].

Regulation of efflux pump expression. The presence and multiple functions of multidrug efflux transporters require a well-regulated expression of these exporters. Indeed, five of the eight RND-type pumps have regulatory genes located adjacent to the structural genes as shown in Fig. 59.2. The operons for SmeABC and SmeYZ systems are linked to two-component regulatory systems, SmeSR and Smlt2200-Smlt2199, respectively [59, 89]. SmeSR positively controls the expression of SmeABC and L2 β-lactamase [89]. A TetR family repressor [185] is each found for SmeDEF, SmeGH, and SmeOP [186, 187]. The SmeT repressor of the SmeDEF pump is the most-characterized pump regulator in *S. maltophilia*. An overlapping promoter region exists between *smeDEF* and *smeT* genes. Functioning as a dimer [187], SmeT negatively controls the expression of both *smeDEF* and *smeT*. Mutations in *smeT* such as a Leu166Gln substitution in SmeT yield an elevated production of *smeDEF* and *smeT* [186]. An *IS*1246-like element in the *smeDEF* promoter where SmeT acts was found to be responsible for SmeDEF overproduction in clinical isolates [176]. Natural flavonoids also bind to SmeT and thus derepress SmeDEF expression [182]. Additionally, the local regulator, FuaR, positively influences the expression of the fusaric acid-inducible FuaABC pump [178], and the EmrR repressor inhibits production of the EmrCAB pump [180].

4.11 Tolerance to Heavy Metals

Silver ions can affect multiple cellular processes including increased OM permeability and thus show activity against Gram-negative bacteria including *S. maltophilia* [188, 189]. A cluster of genes related to antibiotic and heavy metal resistance were observed in a clinical isolate, and these genes include *mphBM* (for a macrolide phosphotransferase) and the *cadCA* operon (that encodes a CadC regulator and CadA cadmium efflux pump) [190]. The *S. maltophilia* genome contains several heavy metal resistance genes related to arsenic, copper, and mercury resistance [59]. Indeed, attention should be paid to the high-level tolerance of *S. maltophilia* against various heavy metal salts such as cadmium, cobalt, copper, lead, selenite, tellurite, uranyl, and zinc [191]. An isolate obtained as a culture contaminant was able to grow in the presence of 500 μM $CdCl_2$, 20 mM tellurite, or 50 mM selenite or the presence of 20 μM $AgNO_3$, 50 μM $HgCl_2$, and other heavy metals. Two mechanisms were revealed, which involved the reduction of oxyanions to nontoxic elemental ions and detoxification of Cd into CdS [191]. In this case, susceptibility of *S. maltophilia* to $AgNO_3$ is comparable to the level of $AgNO_3$ susceptibility of *E. coli* [192], consistent with possible role of silver agents against multidrug-resistant Gram-negative bacteria [189].

5 Antimicrobial Therapy for *S. maltophilia* Infections

High-level intrinsic MDR poses a major challenge for the treatment of *S. maltophilia* infections, and the choice of drugs is indeed very limited. Despite the emergence of global resistance to sulfonamides/trimethoprim, the antifolate sulfonamide/trimethoprim combination regimen still constitutes the major active antimicrobial against *S. maltophilia* [9, 10, 193]. Susceptibility to other agents may likely be unpredictable [9]. The drugs currently recommended from the CLSI [37] for antimicrobial susceptibility testing only include trimethoprim-sulfamethoxazole, ceftazidime, ticarcillin-clavulanate, chloramphenicol, levofloxacin, and minocycline (Table 59.1). Thus, even though susceptibility testing conducted with other antimicrobial agents, the lack of their interpretative criteria may not provide sufficient guidance for the selection of drugs against *S. maltophilia*. In this regard, the understanding of pharmacodynamics and pharmacokinetics of relevant antimicrobial agents under specific administration routes and dosages will be important for guiding the selection of the antimicrobials.

In clinical settings, antimicrobial combination therapies are often considered for treating *S. maltophilia* infections. However, in many cases, data are often generated from *in vitro* antimicrobial synergy studies, and their clinical efficacy

remains to be fully investigated via clinical trials. Trimethoprim-sulfamethoxazole remains the empirical or the first-line choice for *S. maltophilia* infections [7, 14, 193–195]. This combinational agent is also used together with other agents. For instance, the combination of trimethoprim-sulfamethoxazole and ciprofloxacin was able to cure a case of pediatric *S. maltophilia* meningitis [196].

Ticarcillin-clavulanate combination was another major choice of the therapy [10, 14, 195] but is not commercially available (discontinued by the manufacturer in the late 2014). Certain other β-lactam-β-lactamases inhibitor combinations including aztreonam-clavulanate, ticarcillin-sulbactam, piperacillin-tazobactam, ampicillin-sulbactam, ceftazidime-clavulanate, cefoperazone-sulbactam, cefepime-clavulanate, and ceftolozane-tazobactam do not show a good activity against *S. maltophilia* [194, 197]. It is noted that many of these combinations are, however, not commercially available. Additionally, an attention should be paid to carbapenems to which *S. maltophilia* are intrinsically resistant (Table 59.1), and carbapenems are not suitable for treating *S. maltophilia* infections [56].

Fluoroquinolones have been a drug option. A study showed that the therapy with levofloxacin, ciprofloxacin, or trimethoprim-sulfamethoxazole for *S. maltophilia* infections (mostly pulmonary) produced similar effectiveness results (with 52–61 % clinical success rates) [44]. Based on the 30-day mortality rates and adverse drug events, a retrospective study suggested levofloxacin as an alternative regimen to trimethoprim-sulfamethoxazole in treating *S. maltophilia* bacteremia [43]. Another retrospective study confirmed the use of fluoroquinolone agents in combination with trimethoprim-sulfamethoxazole [198]. Moxifloxacin or levofloxacin is suggested as second-line treatment options [195]. Subinhibitory concentrations of moxifloxacin and other fluoroquinolones decrease adhesion and biofilm formation of *S. maltophilia* [26, 199].

Minocycline has a good activity against *S. maltophilia* (Table 59.1). Similarly, tigecycline is a candidate for clinical investigations with respect to *S. maltophilia* infections (Table 59.1) [200, 201]. The MIC_{50} and MIC_{90} values of tigecycline for 120 isolates were, respectively, 0.5 and 1.5 μg/mL in a study conducted in Spain [200]. Another study reported tigecycline MIC_{50} and MIC_{90} values of 2 and 4 μg/mL, respectively, for 903 isolates from 2006 to 2010 in Taiwan (with an MIC range of 0.03–16 μg/mL) [202]. Yet, a French study showed higher MIC_{50} and MIC_{90} values of 2 and 8 μg/mL, respectively, for 72 isolates [203]. This agent was compared with trimethoprim-sulfamethoxazole in treating *S. maltophilia* infections with no significant differences in mortality and clinical response rates between the two treatment regimens [204]. A high doubled tigecycline dosage regimen was reported to be successful in treating *S. maltophilia* bacteremia [205].

Despite the high rates of insusceptibility to numerous agents overall, the literature has provided various examples of assessing antimicrobial combinations against multidrug-resistant isolates. A review article published in 2008 conducted a literature analysis of therapeutic options for *S. maltophilia* infections beyond trimethoprim-sulfamethoxazole [206]. This study found that: (1) 20 of 49 cases (41 %) were treated with ciprofloxacin alone or in combination with other antibiotics (with a cure/improvement rate of 90 %); (2) 12 of 49 cases (25 %) were treated with ceftriaxone- or ceftazidime-based regimens (with a cure/improvement rate of 75 %); (3) 6 of 49 cases (12 %) were treated with ticarcillin- or ticarcillin-clavulanate-based regimens (with a cure/improvement rate of 67 %). Other 11 patients received various antimicrobials including aminoglycoside-based regimens, carbapenems, levofloxacin, chloramphenicol, aztreonam, minocycline, and other β-lactams [206]. This study also indicated the lack of clinical trials for these therapeutic combination options as a major limitation. Another study [207] describes the *in vitro* testing of 517 combinations which included levofloxacin, ceftazidime, ticarcillin-clavulanate, piperacillin-tazobactam, aztreonam, chloramphenicol, minocycline, tobramycin, and trimethoprim-sulfamethoxazole for their activities against 80 respiratory isolates from cystic fibrosis patients, showing that the most synergistic combination was ticarcillin-clavulanate plus aztreonam (92 % synergistic), followed by ticarcillin-clavulanate plus colistin (40 %), and ticarcillin-clavulanate plus levofloxacin (19 %). A case report revealed that chloramphenicol and rifampin were the only agents active against an isolate which was recovered from the urinary device of a patient with myelofibrosis, and this patient had been exposed to treatments with levofloxacin, amoxicillin-clavulanate, ceftazidime, and piperacillin-tazobactam [208].

Polymyxins are among the limited "last-resort" antibiotics for treating multidrug-resistant Gram-negative infections such as those caused by multidrug-/carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae* [53, 209]. However, *S. maltophilia* isolates display variable susceptibility to polymyxins, rendering the challenge for using colistin in treating *S. maltophilia* infections. In one study, the colistin MIC range, MIC_{50}, and MIC_{90} values of 0.01–32, 2, and 32 μg/mL, respectively, were reported for 72 isolates that were from France in 2008–2009 [203]. In another study, all 17 tested isolates from Singapore in 2004 were resistant to colistin with MIC_{50} of 128 μg/mL [210]. A case report showed an extensively drug-resistant isolate from a burnt, septicemia patient to be only susceptible to colistin [211]. Although colistin is not an agent for *in vitro* susceptibility testing of *S. maltophilia* [37], colistin has, nevertheless, been tested in combination with other agents for activity against *S. maltophilia*. Colistin in combination with trimethoprim-sulfamethoxazole, tigecycline, or rifampin showed a synergistic effect against *S. maltophilia* [168, 212]. However,

little information is available on intravenous administration of colistin for therapy of *S. maltophilia* infections. There were only a few retrospective studies that described the limited numbers of *S. maltophilia* isolates in colistin therapy of infections associated with multidrug-resistant Gram-negative bacteria [213, 214]. A report suggests using the combination regimens that include colistin, fluoroquinolones, or tigecycline [215]. A case study described the successful treatment of recurrent *S. maltophilia* ventilator-associated pneumonia with intravenous doxycycline and aerosolized colistin [216].

Additionally, azithromycin-trovafloxacin combinations did not show a measurable activity against *S. maltophilia* [217]. However, azithromycin-trimethoprim-sulfamethoxazole, azithromycin-ceftazidime, or clarithromycin-ceftazidime combination produced *in vitro* synergistic or additive effects [218]. The lipoglycopeptide telavancin plus colistin was also synergistic against colistin-susceptible *S. maltophilia* [219]. A triple combination comprising of a siderophore monobactam (BAL19764), a class C β-lactamase inhibitor (a monobactam), and a clavulanate had a BAL19764 MIC_{90} value of 2 μg/mL against 12 isolates [220]. To recapitulate, despite extensive *in vitro* combination testing and retrospective clinical case reports, well-designed clinical trials including microbiological and clinical outcomes are often lacking for the combinatorial therapeutic options.

6 Conclusions

S. maltophilia is an important nosocomial opportunistic pathogen that poses a great challenge for antimicrobial therapy due to its high-level broad intrinsic resistance. This resistance phenotype can be explained by the available genome data and various biochemical studies, which show the presence of resistance determinants against major clinically relevant antimicrobial agents. The intrinsic resistance feature is further complicated by the global emergence of resistance to the primary choice of drugs, trimethoprim-sulfamethoxazole, as well as to other agents. Susceptibility/resistance phenotype of clinical isolates can be unpredictable; thus, it is important to conduct antimicrobial susceptibility testing with suspected *S. maltophilia* infections. Nevertheless, the interpretative susceptibility breakpoints for *S. maltophilia* are only available for a limited number of agents. Therapeutic options often require a combination of agents which display variable activities against *S. maltophilia*. It is worrisome that there is a lack of data from clinical trials since most therapeutic recommendations are only generated from *in vitro* antimicrobial synergy data and clinical case reports. Additionally, the characteristic of *S. maltophilia* with intrinsic resistance to carbapenems and variable susceptibilities to polymyxins requires clinical investigation of the effect of these "last-resort" antibiotics on the emergence of *S. maltophilia* infections during their therapy of infections associated with other multidrug-resistant Gram-negative bacteria. Given the nosocomial, opportunistic nature of *S. maltophilia* (especially affecting immunocompromised patients), hospital infection control and hygiene practices play an important role in minimizing bacterial infections and should not be underestimated. Intervention measures should focus on preventing transmission of *S. maltophilia* within healthcare facilities and reducing the predisposing risk factors that enhance *S. maltophilia* colonization in patients, particularly with at-risk populations.

7 Addendum in Proof

Outer membrane vesicles from *S. maltophilia* were shown to contain both L1 and L2 β-lactamases and to increase β-lactam resistance in *S. maltophilia* as well as in *P. aeruginosa* and *Burkholderia cenocepacia* [223]. Regulation of Sm*qnr* expression by SmQnrR was suggested to be strain-specific [224]. Genetic inactivation of *smlt2199-smlt2200-encoded* two-component regulatory system ($SmeS_yR_y$) yielded an increased susceptibility to aminoglycosides but a decreased susceptibility to multiple antibiotics including chloramphenicol, ciprofloxacin, macrolides, and tetracycline [225]. This differential susceptibility phenotype is attributable to the reduced expression of SmeYZ pump and elevated expression of SmeDEF pump [225]. Two-component regulatory system PhoPQ also affects antimicrobial susceptibility with a PhoP mutant showing increased membrane permeability and reduced expression of SmeZ pump [226]. Disruption of the MFS-type efflux pump, MfsA, led to increased susceptibility to aminoglycosides, chloramphenicol, erythromycin, fluoroquinolones, rifampicin, tetracycline, and two first-generation β-lactam agents (4- to 16-fold decrease in MIC values) [227]. Increased prevalence (39%) of resistance to trimethoprim-sulfamethoxazole in *S. maltophilia* has been reported [228], highlighting the importance of antimicrobial susceptibility testing in selection of antimicrobials against *S. maltophilia* infections. A genetic assay named loop-mediated isothermal amplification (LAMP) was recently shown to provide a helpful tool for monitoring the spread of sulfonamide resistance genes, dihydropteroate synthase *sul1* and *sul2* genes [229]. Finally, a new clinical case study from the USA discussed several potential antimicrobial combinations for the treatment of a renal transplant patient with blooddstream *S. maltophilia* infection by showing the utility of a novel drug combination, ceftazidime-avibactam and aztreonam, although the latter remains to be further studied for its safety and efficacy [230].

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Antimicrobial Resistance of *Shigella* spp., Typhoid *Salmonella*, and Non-typhoid *Salmonella*

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Herbert L. DuPont and Jean M. Whichard

1 Introduction

At the end of the nineteenth century, the two great forms of dysentery were identified: sporadically occurring amoebic dysentery (amebiasis) and bacillary dysentery (shigellosis) that tended to produce outbreaks of diarrheal disease. Since first identified, *Shigella* spp. have been shown to be important causes of morbidity worldwide and for epidemic strains of *S. dysenteriae* type 1 (Shiga bacillus) major causes of mortality in tropical endemic regions.

Enteric or typhoid fever is a striking syndrome of fever with abdominal symptoms and signs associated with bacteremic salmonellosis. If untreated, typhoid fever may progress to life-threatening complications in the second week of illness including perforated intestine and intestinal hemorrhage. The serotypes of *Salmonella* responsible for typhoidal illness are Typhi, Paratyphi A, Paratyphi B, and Paratyphi C.

Non-typhoidal strains of *Salmonella* are important causes of usually self-limiting foodborne gastroenteritis in healthy children and adults, although at extremes of age and when infection occurs in certain very susceptible hosts, illness is complicated by the presence of fever and systemic toxicity due to systemic or bacteremic infection which may be associated with disease complications.

Humans and primates are the reservoir for shigellosis and typhoid *Salmonella* infections, and the widespread use of antibacterial drugs in human medicine is most relevant for emergence of antibacterial resistance among those diseases. In these cases, self-medication and purchase of antibacterial drugs without a prescription are commonly practiced in many areas of the developing world. In industrialized regions, antibiotic use for viral infections and other conditions for which antibiotics are not indicated contribute to a rising rates of resistance. For non-typhoid *Salmonella* strains, animals serve as the major microbial reservoir, and the use of antibiotics in animals provides selective pressure that contributes to selection of antibacterial-resistant strains that can infect humans that come into contact with animals or food animal products.

This review looks at the current state of antibacterial resistance among shigellae and salmonellae and focuses on current guidelines of antimicrobial therapy in the setting of changing resistance.

2 Burden and Importance of *Shigella* and *Salmonella*

2.1 *Shigella*

The annual number of *Shigella* diarrhea and dysentery cases has been estimated to be 165 million leading to approximately one million deaths in the developing world [1]. *S. dysenteriae* 1 (the Shiga bacillus) characteristically has a more severe outcome and can produce widespread and severe epidemics. In the USA, it is estimated that we have approximately 500,000 cases of shigellosis each year, but only very few cases caused by *S. dysenteriae* 1 [2]. *Shigella* strains continue to be important causes of travelers' diarrhea in international visitors and military populations [3, 4]. Shigellosis is uniquely pathogenic among bacterial pathogens resulting in common person-to-person spread because of low dose required for illness [5]. Infection results from oral exposure to human feces containing *Shigella*, directly or in the form of contaminated food or water or exposure to contaminated recreational waters. Shigellosis can spread among young children in daycare, in settings where hand hygiene is inadequate, and outbreaks have occurred in men

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who have sex with men (CDC [6] *Shigella*—Shigellosis. 29 Sept 2014). Strains of *Shigella* should be suspected as a potential etiologic agent in patients with sporadically occurring dysentery where many stools of small volume are passed that contain gross blood and mucus. The illness tends to be clinically striking and may persist for a week or longer if untreated.

2.2 Typhoid *Salmonella*

Buckle et al. estimated that there were approximately 26.9 million cases of typhoid fever in the world in 2010 [7]. Crump et al. [8] have estimated a conservative case fatality rate of 1 % among patients suffering from typhoid fever The infectious dose of typhoid *Salmonella* is moderately high [9] explaining the rarity of person-to-person spread and the need for a food or water vehicle for disease transmission. Typhoid fever is particularly endemic in the Indian subcontinent, Southeast Asia, Africa, and South America. The disease is striking, and patients with typhoid fever often present themselves to medical centers for evaluation. It is one of the most important febrile conditions among international travelers to endemic areas. Blood cultures should always be obtained in travelers with fever following return from endemic tropical regions to evaluate for the presence of typhoid fever.

2.3 Non-typhoid *Salmonella*

Non-typhoid salmonellosis causes approximately 1.0 million cases of domestically acquired foodborne disease in the USA leading to an estimated 19,000 hospitalizations and nearly 400 deaths [2, 10]. A surprisingly high incidence of the organism is seen in young infants less than 1 year of age. This appears to be related to a reduced number of organisms needed for development of gastroenteritis in this age group plus household exposure to the organism from common in-home cross contamination.

3 Patterns of Susceptibility of *Shigella* spp. by Geography

The first antibiotic shown to be effective in shortening shigellosis was ampicillin [11]. In children with severe shigellosis, orally absorbable ampicillin was shown to be superior to orally administered nonabsorbable neomycin with both drugs showing similar levels of in vitro susceptibility. This study provided indirect evidence that drug absorption was required for mucosally invasive shigellosis. With the subsequent widespread use of ampicillin for therapy of bacterial diarrhea in the 1970s and 1980s, ampicillin resistance occurred widely [12] leading to the search for other drugs to treat this severe form of diarrhea and dysentery. Nelson et al. [13] demonstrated that trimethoprim-sulfamethoxazole (TMP/SMX) was active in vitro and showed that the drug shortened clinical shigellosis in infected children. Soon after this study in pediatric shigellosis, adults with endemic shigellosis were shown to have improvement in their clinical disease by administration of the drug [14], and DuPont et al. [15] showed TMP/SMX was active in shortening the duration of travelers' diarrhea due to strains of *Shigella* during short-term stay in Mexico. During the 1980s, TMP/SMX remained active against isolated strains of *Shigella* in the USA, Europe, Latin America, and Asia [12], while in the 1990s identified enteric bacterial pathogens including strains of *Shigella* began to lose their susceptibility to TMP/SMX with rates of resistance reaching 50–94 % throughout the world. In the USA, we initially found TMP/SMX resistance among persons returning from international travel after visiting regions showing resistance [16]. The National Antimicrobial Resistance Monitoring System (NARMS) found that 43.3 % of the *Shigella* isolates tested from patients in the USA in 2012 were resistant to TMP/SMX [17].

One of the first drugs to be successfully used to treat TMP-/SMX-resistant shigellosis was nalidixic acid, a quinolone available in pediatric suspension form and with in vitro activity against enteric bacterial pathogens [16]. The drug possessed a potential for quinolone toxicity in children limiting widespread use. Mecillinam (pivamdinicillin) was further evaluated and found to have value in the treatment of shigellosis in Bangladesh [18] for susceptible and more resistant forms of *Shigella*. Resistance to nalidixic acid became common, presumably through general use, particularly with strains of *S. dysenteriae* 1 [19]. With the availability of the newer fluoroquinolones, beginning with norfloxacin (NF) followed by ciprofloxacin (CF) and levofloxacin (LF), the outcome of treatment of shigellosis in adults was immediately improved.

In the USA, 4.5 % of the *Shigella* tested by NARMS in 2012 were resistant to nalidixic acid, and 2.0 % were resistant to the fluoroquinolone (FQ) ciprofloxacin [17]. *Shigella* that are nalidixic acid resistant usually show resistance or decreased susceptibility to fluoroquinolones because the main mechanism of resistance, topoisomerase mutations, affects efficacy of fluoroquinolones as well as nalidixic acid. However, mechanisms that confer resistance to fluoroquinolones but not nalidixic acid have also been identified [20]. In recent years in Asia, nalidixic acid resistance has reached very high levels for *S. flexneri* and *S. dysenteriae* strains with these strains typically showing fluoroquinolone resistance [21, 22]. A clonal epidemic of antimicrobial susceptible strain of *S. dysenteriae* 2 has been seen in Bangladesh [23].

Antimicrobial susceptibility of strains of *Shigella* has related to the general use of antimicrobials in the population, as well as by the pathogen species causing illness. *S. flexneri*

Table 60.1 Changing susceptibility of *Shigella* spp.

Region	Amp	TMP/SMX	Nalidixic acid	Fluoroquinolone	Azithromycin	References
USA	18 → 26	0 → 43	Low → 5	Low → 2	4	[17]
Europe, Middle East	18 → 10–77	6 → 64–95	Low → 0–49	Low → 0–4	4	[29–34]
Latin America	15 → 43–100	0 → 27–100	0	0 → 0	Low → ?	[35–38]
Asia	10 → 43–100	10 → 63–98	Low → 59–100	0 → 12–82	Low → 17–49	[39–47]
Africa	37 → 12–60	6 → 25–99	Low → 0–7	Low → 0–7	?	[48–51]

Initially from 1970s to 1980s until the 2000s–2010s by Region of the World
[a]Antimicrobial resistance is currently highest for strains of *S. dysenteriae* 1 with high rates also seen in strains of *S. flexneri* and low rates for *S. sonnei* isolates

showed greater resistance to prevalent drugs than *S. sonnei* [24], and *S. dysenteriae* 1 showed the highest degree of resistance compared with other serotypes [16]. Over time in endemic areas such as Bangladesh, nalidixic acid resistance has become clinically important with the drug no longer being helpful in the management of Shiga dysentery [16].

Given the high levels of resistance to traditional antimicrobial agents, physicians have turned to azithromycin (AZ) for treatment of shigellosis. Early reports of azithromycin-resistant *Shigella* surfaced in the late 1990s in Vietnam and Thailand [25]. Azithromycin treatment failure in a pediatric outbreak of *Shigella sonnei* in France in 2007 was attributed to a plasmid-borne macrolide phosphotransferase gene, *mphA* [26]. This gene was first found in *Shigella sonnei* isolated from patients in the USA in 2005 [27]. It has also since been found in *Shigella flexneri* and occasionally *Shigella boydii*. Outbreaks have occurred in men who have sex with men, and a 2014 study found a high proportion of HIV coinfection among men infected with *Shigella* that showed decreased susceptibility to azithromycin [28]. Clinical interpretive criteria for azithromycin are lacking for *Shigella*, and more data on the clinical implications of *Shigella* that show azithromycin non-susceptibility are necessary before CLSI can establish those clinical interpretive criteria.

In Table 60.1 a world region summary of antimicrobial susceptibility data for isolated *Shigella* strains in various published studies is provided.

4 Enteric Fever Due to Strains of *Salmonella* Typhi and *S.* Paratyphoid

Since the 1940s typhoid fever has been managed in the developing world with chloramphenicol. The drug was inexpensive and effective in shortening the illness. In the 1970s, chloramphenicol resistance among typhoid *Salmonella* strains emerged in the Indian subcontinent and in Mexico [52] leading to the successful evaluation of other antimicrobial agents, including ampicillin and then trimethoprim-sulfamethoxazole for therapy of typhoid fever [53]. Beginning with the 1980s, multidrug-resistant strains of typhoid *Salmonella* emerged in Asia and Europe. The plasmid-encoded resistance identified not only was directed to chloramphenicol but to ampicillin and trimethoprim-sulfamethoxazole complicating therapy and leading to increased mortality [54]. Worldwide occurrence of *Salmonella* Typhi resistance to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole has continued to increase.

Nalidixic acid resistance among enteric *S.* Typhi strains was shown to be an important predictor of intermediate resistance for the fluoroquinolone and represented an indication to administer higher doses of fluoroquinolones for successful treatment [55]. Fluoroquinolone resistance has been documented among isolated strains of typhoid *Salmonella* [56, 57], although it has not yet become important or widespread [55, 58]. Most fluoroquinolone-resistant *S.* Typhi strains have been shown to have point mutations in the genes encoding DNA gyrase or DNA topoisomerase IV enzymes located within bacterial chromosomes [59]. Fluoroquinolones act on *GyrA* and at higher concentrations on *ParC*, with point mutations leading to reduced susceptibility to ofloxacin, ciprofloxacin, and to gatifloxacin [60]. A few years ago, the Clinical and Laboratory Standards Institute updated clinical interpretation of resistance among invasive strains of *Salmonella* based on more recent clinical outcomes and microbiological data. The updated definition of clinical resistance captures isolates with lower minimum inhibitory concentration results (≥1 μg/mL) or disk diffusion zone diameters (≤20 mm) [61].

In the USA where most cases of typhoid fever occur secondarily to international travel, particularly to the Indian subcontinent, multidrug resistance rates rose from <1 % to >10 % in the 1990s [62, 63]. Resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole rose to 18.6 % in 2006 and subsequently declined. In 2012, 9.2 % of the *Salmonella* Typhi isolates obtained from patients in the USA were resistant to all three of these drugs [17].

In Europe, multiresistance became even more common in recent years with nearly one third of isolates showing reduced fluoroquinolone susceptibility indicating a need for higher doses when treated with this class of drugs [64]. Of relevance, in some areas with an increasing resistance of

S. Typhi strains to fluoroquinolones, a concomitant decrease in resistance to chloramphenicol has been found [65], which could influence future treatment recommendations in developing countries where the cost differential between fluoroquinolones and chloramphenicol is great. In a study carried out in Nepal, multiresistant and extended-spectrum β-lactamase producing enteric fever strains were more commonly classified in the laboratory as Paratyphi A than Typhi [66]. A study of Paratyphi isolates from India also demonstrated nalidixic acid resistance and a reduced susceptibility to ciprofloxacin [67]. Almost all *Salmonella* Paratyphi A isolates obtained from humans in the USA are resistant to nalidixic acid [17], and infection has been associated with travel to South Asia [68]. Susceptibility testing of isolated typhoid and paratyphoid *Salmonella* is important in managing enteric fever patients to identify the optimal therapy and to prevent delayed recovery and treatment failures.

5 Gastroenteritis due to Non-typhoid *Salmonella*

During the 1970s and 1980s, strains of non-typhoid *Salmonella* were shown to have variable susceptibility to ampicillin, tetracycline, and TMP/SMX [12]. In more recent years, resistance to ampicillin, TMP/SMX, chloramphenicol, aminoglycosides, chloramphenicol, and sulfonamides has become widespread throughout the world related at least in part to the potential of these bacterial pathogens for horizontal transfer of resistance mediated by plasmids, transposons, or integron cassettes. Just as with other enteric pathogens, the use of traditional antimicrobial agents for treatment of invasive salmonellosis has largely been replaced by other drugs, in this case fluoroquinolones and extended-spectrum cephalosporins. Extended-spectrum cephalosporins are particularly important for treating pediatric infections.

There is evidence for the non-typhoid *Salmonella* that dissemination of multidrug resistance is secondary to both local antimicrobial use with local selection of resistance strains as well as by more widespread dissemination of resistant clones of *Salmonella* amplified within livestock and other animal populations [69–71]. Resistant *Salmonella enterica* serovars isolated from different institutions may show the same genetic lineage supporting the concept of clonal spread [72]. Retail meats can be shown to harbor antibacterial-resistant strains of *Salmonella* supporting current recommendations that national surveillance for antimicrobial-resistant *Salmonella* should include the monitoring of retail foods and that restrictions of the use of antibiotics important in human medicine should be imposed for all food animals [73].

Rates of fluoroquinolone resistance among strains of non-typhoid *Salmonella* were shown to increase between 1995 and 1999 in travelers returning to the USA. Ciprofloxacin resistance increased from 4 % to 24 % for various travel destinations, while for those returning from Thailand, the increase was even greater with increase in resistance rates being shown to rise from 6 % to 50 % [74].

Resistant *Salmonella enterica* serovars may be isolated from poultry [75], and shell eggs have been found to harbor resistant *Salmonella*, including strains resistant to nalidixic acid, with the resistance pattern showing serotype dependence [76]. Isolation of quinolone-resistant *Salmonella* from retail meats purchased in the USA has been very rare, and among the four food animals (cattle, chickens, swine, and turkeys) under surveillance at slaughter by NARMS, cattle have been the most frequent source of nalidixic acid-resistant *Salmonella* (between 1 % and 3 % from 2009 to 2011) [77]. Enteritidis is the most frequent serotype of quinolone-resistant *Salmonella* isolated from humans in the USA [17], and quinolone-resistant infections in humans in the USA are associated with foreign travel [78].

Of the many serotypes of *Salmonella*, serovar Typhimurium is the most resistant to antibacterial drugs. Multidrug-resistant definitive type (DT) 104 *S.* Typhimurium has emerged worldwide and has appeared in the USA, DT 104 strains are responsible for about one third of infections in the USA. They are characteristically resistant to ampicillin, chloramphenicol, tetracycline, streptomycin, sulfamethoxazole, and kanamycin [79]. Bacteriophage typing can identify various DT 104 types belonging to multiple serotypes but with similar mechanisms of integron gene resistance that may confer resistance to aminoglycosides, trimethoprim, and β-lactam drugs [73]. *Salmonella* phage type DT104 harbors a genomic island called *Salmonella* genomic island 1 (SGI-1) containing an antibiotic gene cluster conferring multidrug resistance [80]. The CDC-sponsored FoodNet program demonstrated that human acquisition of DT 104 in the USA was related to prior receipt of an antimicrobial agent during the 4 weeks preceding illness onset [79].

Between 1998 and 2002, multidrug-resistant *Salmonella* Newport emerged as an important public health problem in the USA [81]. This strain is particularly important because it is not only resistant to the drugs seen with DT 104 strains, but it is resistant to the third-generation cephalosporin, ceftriaxone, which is the treatment of choice for systemic pediatric salmonellosis. Instead of the chromosomal multidrug resistance seen in *Salmonella* Typhimurium DT104, this multidrug resistance in *Salmonella* Newport is attributed to a plasmid that carries several resistance genes including one or more copies of the bla_{CMY-2}β-lactamase gene [82, 83]. Resistance in these strains appears to be encouraged by the use of antibacterial drugs in livestock with resultant spread to humans [81, 84]. The spread from bovine sources to humans may also be facilitated by human use of antibacterial drugs [85]. Currently, *S.* Newport is the third most common *Salmonella* serotype in the USA [17] having increased in importance fivefold from 1998 to 2001 [81].

Ceftriaxone-resistant *Salmonella* Heidelberg emerged more recently in the USA. Ceftriaxone resistance among this serotype isolated from humans in the USA rose to over 20 % in 2009 [17], but this emergence has been smaller in magnitude than the increase in serotype Newport because Heidelberg is a less frequently isolated serotype in humans than Newport [86]. Infections are mainly associated with poultry, a common reservoir for this serotype. Unlike cephalosporin-resistant *Salmonella* Newport, cephalosporin-resistant members of this serotype generally do not exhibit the other non-β-lactam resistances because the types of plasmids circulating in these two serotypes are different.

Ciprofloxacin-resistant *Salmonella* Kentucky was isolated from French patients who had traveled to northeast and eastern Africa in the early 2000s [87]. This was also seen in England and Wales and Denmark in travelers returning from African countries or the Middle East [88], and most of the isolates were resistant to several other drugs. The global spread of the predominant sequence type (ST) of ciprofloxacin-resistant *Salmonella* Kentucky, ST 198, was described in 2013 [89]. These strains contain *gyrA* mutations; most contain SGI1 or variants of SGI1, many contain β-lactamase genes including those that confer resistance to extended-spectrum cephalosporins, and a few contained genes associated with resistance or decreased susceptibility to carbapenems ($bla_{\text{VIM-2}}$ and $bla_{\text{OXA-48}}$). Poultry are the main reservoir, but these strains have also been isolated from contaminated meats, seafood, and spices and from other animals [89].

When compared with susceptible strains, multidrug-resistant strains of *Salmonella* are more likely to produce severe infection and mortality [90] and to lead to hospitalization [91]. There is evidence that antimicrobial-resistant *Salmonella* are not only able to resist the effect of antibiotics to which they show low susceptibility, but they may be more virulent than susceptible strains causing more prolonged and more severe illness than their antibiotic-susceptible counterparts [92]. Comorbid conditions of the host may affect susceptibility to salmonellosis and influence outcome. In one study in Ethiopia, the *Salmonella* isolates from patients with HIV infection showed greater resistance to antimicrobials than those from HIV negative controls [93]. It will be important to monitor the incidence of antimicrobial-resistant *Salmonella* in human populations as well as the food supply to help predict the evolution of antimicrobial resistance in human infections [94, 95].

6 Current Therapeutic Recommendations

In Table 60.2, recommendations for therapy of the various bacterial enteric infections considered herein are summarized.

6.1 *Shigella*

The fluoroquinolones have become the mainstay of therapy for adult patients with shigellosis. Most cases should be treated with antibacterial drugs for 3 full days, although for many persons with milder forms of shigellosis caused by species other than *S. dysenteriae* type 1, single-dose treatment may be effective [96, 97]. Single-dose therapy with azithromycin appears to be effective in treating many forms of shigellosis [98]. Azithromycin or one of the third-generation cephalosporins should currently be considered the drug of choice for treatment of pediatric shigellosis. Strains of *S. sonnei* resistant to third-generation cephalosporins have been encountered [99]. While azithromycin is an important form of therapy for shigellosis, there may be challenges with the interpretation of in vitro susceptibility testing of *Shigella* isolates using Etest and disk diffusion [100].

Table 60.2 Recommended therapy of shigellosis and salmonellosis based on current susceptibility patterns

Condition	Children		Adults	
Shigellosis	Azithromycin[b]	5 mg/kg/day for 3 days	Norfloxacin (NF), or ciprofloxacin (CF), or levofloxacin (LF), or azithromycin (AZ)	NF 400 mg bid, CF 500 mg bid, or LV 500 qd for 3 days or AZ 1000 mg in a single dose
Typhoid fever	Ceftriaxone	50 mg/kg/day in two IV doses/day for 7–10 days	CF, LF, or other fluoroquinolone (FQ) or azithromycin (AZ)	FQ given in full doses for 7–10 days
				AZ 500 mg qd for 7 days
Salmonellosis (a febrile, nontoxic condition in healthy host)	Fluid therapy and observation		Fluid therapy and observation	
Salmonellosis (When a febrile, or toxic condition, or in special host[a])	Treat for bacteremia as typhoid fever or with azithromycin (AZ) for cephalosporin-resistant strains	See above for ceftriaxone dosing, AZ 10 mg/kg/day in single daily dose for 7 days	Treat for bacteremia as typhoid fever[c]	

[a]Extremes of age (<3 months, >65 years of age), sickle cell anemia, inflammatory bowel disease, hemodialysis, receiving systemic corticosteroids or anticancer or anti-immunity drugs, AIDS (for AIDS or immunocompromised, continue therapy for at least 2 weeks)
[b]Ciprofloxacin can be safely given for 3 days to older children, although not approved for bacterial diarrhea
[c]Immunocompromised patients may need prolonged treatment

One concern in the therapy of Shiga dysentery due to *S. dysenteriae* 1 is the development of hemolytic uremic syndrome (HUS), an association which has been reported [101]. The relationship of antimicrobial therapy to the development of HUS in Shiga dysentery was studied by Bennish and coworkers [102] who found in one small study that treatment of Shiga *Bacillus* dysentery did not predispose to HUS and stools in treated subjects showed a reduction of Shiga toxin.

6.2 Typhoid Fever

While the drug of choice for three decades, chloramphenicol, has been used more sparingly in recent years due to the emergence of resistance, high relapse rate, and failure to eradicate intestinal carriage of the organism [9]. Resistance to chloramphenicol, ampicillin, and TMP/SMX has been seen worldwide for strains of *Salmonella* Typhi. Fluoroquinolones remain active in vitro, render high concentrations in bile and macrophages, and can also be given for shorter courses. For adults in regions where it is not prohibitively expensive, the fluoroquinolones represent the current treatment of choice [58, 63]. The recommended duration of fluoroquinolone use in adults with typhoid fever is 7–10 days, and the drug should be given orally as soon as oral medications can be taken. Ceftriaxone (1–2 g per day) for 7–10 days is effective in adults with typhoid fever, and the third-generation cephalosporins represent the treatment of choice for pediatric typhoid fever [103]. Oral cefixime and oral azithromycin remain alternatives for resistant strains [104].

6.3 Non-typhoid Salmonellosis

Over the years multidrug-resistant strains of non-typhoid *Salmonella* have emerged producing dilemmas in therapy of human infections. Fortunately, most cases of non-typhoid salmonellosis represent mild to moderate self-limiting gastroenteritis. In a subset of patients, bacteremia or other systemic infection including meningitis may occur which explains the nearly 600 deaths in the USA each year associated with intestinal salmonellosis. In conditions in which *Salmonella* bacteremia and systemic infection should be suspected and where antimicrobial therapy should be initiated empirically, it must include patients with *Salmonella* gastroenteritis in the following patient groups: (1) extremes of age (<3 months and >65 years of age); (2) undergoing regular hemodialysis; (3) receiving high-dose steroids; (4) with the presence of AIDS or cancer or receipt of anticancer drugs that alter immunocompetence; (5) and with the presence of inflammatory bowel disease or sickle cell disease. In these cases antibiotics are given for 7–10 days to treat bacteremic disease rather than a localized enteric infection. In immunocompromised persons with cancer or AIDS and possible systemic salmonellosis, the antibiotics are given for at least 2 weeks with some needing therapy even longer. The antimicrobials given to patients with bacteremic disease or possible bacteremic disease do not shorten nonsystemic intestinal disease and may encourage the emergence of resistant forms with transient shedding [105] without decreasing post-diarrhea shedding of *Salmonella* [106].

The treatment of choice for therapy of systemic non-typhoid *Salmonella* infection in adults is a fluoroquinolone, given orally when it can be taken by that route. The fluoroquinolones remain active against strains of *Salmonella* encountered in the USA [94]. For children, parenteral third-generation cephalosporins should ordinarily be used for systemic salmonellosis. With the occurrence of cephalosporin-resistant non-typhoid *Salmonella* from animal populations, new treatments are needed for children.

Since most non-typhoid *Salmonella* strains now are multidrug resistant, in vitro susceptibility testing should routinely be performed with isolated strains while empirically employed.

7 General Comments in regard to Enteric Pathogen Resistance

Non-*Salmonella enterica* pathogens, including *Shigella*, typhoid *Salmonella*, and *Campylobacter jejuni*, characteristically develop polyclonal resistance in response to local antibiotic use patterns. The occurrence of important clonal spread of non-typhoid *Salmonella* strains has facilitated widespread distribution of antimicrobial resistance resembling the problem of methicillin-resistant *Staphylococcus aureus*, which has spread within the community from a hospital reservoir. In studying clonal spread of non-typhoid *Salmonella*, multiple genetic typing procedures may be needed for epidemiologic study as single gene characterization may give an incomplete epidemiologic picture [107]. Although less important from a public health standpoint, multiclonal spread can occur for *Salmonella enterica* strains [108].

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Antimicrobial Resistance in *Vibrio*

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1 Introduction

This chapter addresses antimicrobial resistance in a genus—*Vibrio*—that contains over 100 species, of which at most 15 are known to be pathogenic in man [1, 2]. *Vibrio* species are facultative anaerobic gram-negative bacilli. With the exception of *Vibrio cholerae,* all are halophilic (salt loving) [1]. Several *Vibrio* species that were only rarely associated with human disease have recently, based on phylogenetic analysis, been reclassified into other genera and will not be considered in this chapter [3, 4].

Pathogenic vibrios cause both intestinal and extraintestinal illnesses. The best known and most common of these intestinal illnesses is cholera. *Vibrio* infections, potentially requiring antimicrobial therapy, fall into three distinct clinical syndromes: cholera caused by either *Vibrio cholerae* O1 or O139 and rarely other *V. cholerae* serogroups; less severe non-cholera diarrhea caused by non-O1 or O139 *V. cholerae* or other *Vibrio* species; and soft tissue infections and sepsis caused by halophilic, marine vibrios. Infections with *V. cholerae* O1 or the currently much less frequently identified O139 serogroup, both of which can produce the secretagogue cholera toxin, occur almost exclusively in poor countries where access to clean water and proper sanitation is uncommon.

Vibrio cholerae serogroups in addition to *V. cholerae* O1 and O139 have been associated with intestinal infection and diarrhea [5–7], as have other *Vibrio* species, including *Vibrio parahaemolyticus* [8–10] and *Vibrio mimicus* [11, 12]. Occasionally, these other *V. cholerae* serogroups and *Vibrio* species may have the genetic capacity for the elaboration and production of cholera toxin [7, 13, 14] and thus can cause a cholera-like illness. More commonly, they cause less severe diarrhea. These occur wherever marine or seafood exposure takes place. Cholera and non-cholera diarrhea occur in otherwise healthy hosts, and most commonly in children in endemic areas.

Skin and soft tissue infections, including necrotizing fasciitis and sepsis, are the other clinical syndromes caused by vibrios [15]. These are most common in immunocompromised hosts, especially those with hepatic impairment. The most common pathogen associated with soft tissue infections and septicemia is *Vibrio vulnificus* [16–19]. *V. vulnificus* is also the *Vibrio* infection with by far the highest associated mortality rate [1, 17].

The primary reservoir for all vibrios is marine or estuarine waters—usually in tropical and subtropical areas, but occasionally in temperate regions as well [20]. *V. cholerae,* because it is not halophilic, may also inhabit freshwater (or at least non-saline waters). These inland waters are often heavily polluted with human waste that may serve as the nidus for outbreaks of cholera [21]. Thus, the frequent occurrence of cholera outbreaks inland from ocean coasts or estuaries. Infections can occur from exposure to "fresh" water, food containing *V. cholerae*, or from person-to-person transmission.

Vibrios replicate best in water temperatures between 20 and 30 °C [22]. Global warming has changed both the geographic distribution and seasonality of coastal marine water temperatures in the 20–30 °C interval. These changes in ocean temperatures, rather than simply better case ascertainment, are believed to be partially responsible for the increases in *Vibrio* infections, especially with non-cholera vibrios, which have been observed in recent years in the United States and Europe [1, 17, 23, 24].

The pathogenesis and epidemiology of these three distinct clinical syndromes caused by vibrios—cholera, non-cholera diarrhea, or wound infection and sepsis—differ substantially and affect the need for antimicrobial therapy and the development of antimicrobial resistance.

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Acquired multidrug resistance to *V. cholerae* O1 and O139 is now common and firmly established wherever infections occur. Acquired resistance in *V. cholerae* O1and O139 is primarily from acquisition of transmissible genetic elements, including conjugative plasmids, integrons, or integrative conjugative elements that carry genes encoding resistance. Circulating strains can both gain and lose resistance during the course of an epidemic, and surveillance of resistance patterns is essential. Because onset of disease is rapid, and disease can be rapidly fatal without appropriate fluid and antimicrobial therapy, antimicrobials should be administered empirically to patients with clinical cholera based on the known prevalence of resistance. In addition, cholera is usually treated in settings where isolation of the infecting organism and susceptibility testing are not routinely available. Thus surveillance programs that monitor resistance and report to peripheral clinics where cholera patients are cared for, are essential for the management of this disease.

Resistance is not as common in halophilic vibrios as it is in *V. cholerae*. Although there are a number of agents that remain active in vitro against these organisms, because of the relative rarity of infections, and the absence of clinical trials, choice of therapy is predicated upon in vitro and animal studies, and limited clinical experience.

Cholera caused by infection with *V. cholerae* O1 or O139 is by far the most common of all illnesses resulting from infection with vibrios. Cholera results from ingestion of water or food contaminated with *V. cholerae* O1 or O139. Infection is confined to the intestinal lumen where the elaboration of cholera toxin results in a profound watery diarrhea.

Cholera is a voluminous diarrhea that occurs in pandemics (there have been seven pandemics to date), can be periodically epidemic, and is endemic in its historic cradle in the Ganges Delta and elsewhere in the Indian subcontinent. Cholera is often fatal if rapid access to effective therapy is not available [25]. There are an estimated 1.3 billion persons at risk of infection in 69 countries where cholera is endemic [26]. Infections are almost entirely confined to poor countries lacking basic hygiene, good sanitation, and access to potable water. Both endemic disease and epidemics that can affect tens or hundreds of thousands of persons in previously non-endemic areas (particularly refugee camps) occur [27, 28]. Infections occur in all ages, but in endemic areas infections disproportionately affect the young [29, 30].

The most recent (2014) annual worldwide summary of cholera from the World Health Organization (WHO) reported 190,549 cases of cholera and 2231 deaths from cholera in 42 countries [31]. These numbers are clearly a gross underestimate of the actual burden of disease. For instance, research institutes in India and Bangladesh that extensively study cholera (and publish results in the international scientific literature) do not report any cases of cholera to the WHO and have not done so for many years. A more accurate estimate is that there are approximately 20 times more cholera cases than what is reported to the WHO (thus 2,800,000 actual cases) and that deaths are approximately 45 times greater than what is reported (91,000 actual deaths) [26].

Because of the large number of *V. cholerae* O1 or O139 infections and its distinctive clinical presentation, it has been possible to conduct numerous randomized controlled trials to identify agents effective in its treatment. This has provided a solid base of evidence for determining optimal antimicrobial therapy [32–42]. The large number of infected persons has also provided extensive information on resistance patterns, usually obtained as part of systematic longitudinal surveys at research centers devoted to the study of enteric infections, or during outbreak investigations [43–84] (Table 61.1).

Diarrhea resulting from infections with *V. cholerae* serogroups other than O1 or O139 or with other *Vibrio* species (most commonly *Vibrio parahaemolyticus*) is, unlike cholera, indistinct enough clinically, and sufficiently sporadic, that it has precluded randomized clinical trials of antimicrobial therapy [1, 8–10, 85–87]. Thus, the efficacy of antimicrobial therapy for non-cholera diarrhea caused by vibrios other than *V. cholerae* O1 or O139 is uncertain.

Most cholera infections occur in isolated areas of developing countries that lack access to basic diagnostic microbiologic facilities. For most patients with cholera, the infecting organism is not isolated, and antimicrobial susceptibility is not determined. In any case, antimicrobial treatment is required early in the course of illness if it is to be useful and cannot await the 48–72 h required to isolate the infecting organism and to determine its in vitro susceptibility. Cholera is a disease that strikes rapidly (patients can purge a volume of water equal to their body weight in 24 h). By the time the organism is isolated and antimicrobial susceptibility determined, the patient will be either dead or in better health. Antimicrobial treatment thus has to be empiric, based upon the known prevalence of resistance in circulating strains of *V. cholerae* O1 or O139.

In contrast, *Vibrio* infections causing fasciitis, tissue necrosis, or sepsis result from ingestion of contaminated seafood or inoculation through the skin by injury while in contaminated waters or while handling seafood [1, 2, 11, 17, 19, 22, 88], are locally and systemically invasive [16, 89, 90], generally occur sporadically and in relatively small numbers [90–92], and disproportionately affect the elderly and the immunocompromised, especially those with cirrhosis [19, 90, 92, 93]. Infections are most commonly reported from rich and medium-income countries, perhaps because ascertainment is difficult in poor countries. As with non-cholera *Vibrio* species causing diarrhea, there have been no randomized trials that define the best antimicrobial therapy, and reports on patterns of resistance are based upon small numbers of clinical isolates or surveys of environmental isolates

[94–101]. In contrast to cholera patients, patients with invasive vibrio infections are more likely to be cared for in hospital settings where a definitive microbiologic diagnosis and ascertainment of antimicrobial resistance can be done [16].

This chapter will discuss each of these clinical syndromes—cholera, non-cholera diarrhea, and wound infection and sepsis—in turn.

2 Cholera Caused by *V. cholerae* O1 or O139

2.1 Geographic Spread and Epidemiology of Resistance

Tetracycline was the first antimicrobial agent systematically evaluated for the treatment of cholera [40–42]. It soon established itself as the drug of choice for treating this disease. For the first two decades of its use—until the late 1970s—reported resistance to tetracycline was rare [102]. Resistance to other agents used for cholera treatment—including ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole—was also infrequent. In a report on 1109 isolates of *V. cholerae* O1 obtained from patients in the Philippines in 1969, only 11 (1.0%) demonstrated resistance to drugs in use for treatment [103]. In a report of 1156 strains from Asia, Africa, and Europe reported on in 1976, only 27 (2.7%) were resistant to one of the drugs tested—tetracycline, ampicillin, chloramphenicol, or a sulfa agent—all drugs then used to treat cholera [102].

By the end of the 1970s, however, plasmid-mediated multiple drug resistance to tetracycline, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole was being commonly reported from *V. cholerae* O1 strains isolated in Asia and Africa [104–107]. The conjugative group C plasmid acquired by *V. cholerae* contained genes encoding for type II dihydrofolate reductase, a ß-lactamase, and other resistance mechanisms.

Since then multiple antimicrobial resistance has been a characteristic feature of *V. cholerae* O1 worldwide, from Africa [44, 70, 74, 78, 79, 83, 84, 108–110], Asia [48–50, 52, 53, 56, 58–60, 63, 64, 66, 71, 72, 80, 82, 111–114], Europe [115, 116], South and Central America and the Caribbean [47, 117–119], and Oceania [77] (Table 61.1). The spread of resistance was facilitated by the transfer of the resistance plasmid between circulating strains of *V. cholerae* and from other *Enterobacteriaceae*.

V. cholerae O139 was first identified as a cause of clinical cholera in 1992, when it caused large epidemics of severe diarrhea in Bangladesh and subsequently in other Asian countries [120]. This was the first non-O1 serogroup of *V. cholerae* to produce cholera toxin and to cause epidemic cholera. The epidemic strain evolved from a *V. cholerae* El Tor O1 strain that had acquired the O139 antigen-encoding genes following horizontal gene transfer from a donor strain and recombination with the El Tor O1 chromosome [121, 122]. The O139 epidemic strain also differed from endemic O1 strains by its resistance to trimethoprim-sulfamethoxazole, streptomycin, and furazolidone. Resistance to these agents was conferred by a novel transmissible genetic element—the SXT "constin." Constin was an acronym of its properties—conjugative, self-transmissible, and integrating. And SXT because the constin incorporated genes conferring resistance to sulfamethoxazole and trimethoprim [123, 124]. This SXT constin, or variants thereof with different resistance genes, has now been found in *V. cholerae* O1 and other organisms [81]. After rapid spread in the decade after its identification as a cause of cholera, *V. cholera* O139 has largely disappeared, with only scattered cases in Asia, having been reported since 2000 [51, 125].

Resistance in both *V. cholerae* O1 and O139 is not easily predictable in advance of a cholera outbreak. In endemic areas there may be multiple clones of either *V. cholerae* O1 or O139 circulating simultaneously, and these different clones may have differing antimicrobial susceptibilities [126–130]. In non-endemic areas, outbreaks usually result from introduction of a single strain of *V. cholerae* O1 or O139 [131], and most initial infections will be due to organisms with identical antimicrobial resistance profiles [131]. Over time, however, these epidemic strains may acquire (or lose) antimicrobial resistance. Isolates obtained from patients later in the epidemic may differ in antimicrobial resistance when compared to initial isolates [44, 132–135]. One report suggests that in endemic areas, screening environmental isolates using selective enrichment with a combination of antibiotics helps identify *V. cholerae* strains with new antibiotic resistance profiles that are likely to become epidemic [136]. But this hypothesis has yet to be confirmed with sequential observations.

Antimicrobial resistance in *V. cholerae* O1 and O139 is encoded by a number of mobile genetic elements [137]—plasmids [44, 106, 116, 132, 138–141], integrons [116, 134, 135, 141–145], and integrative conjugative elements or constin [123, 124, 143–146]—which can be acquired from other *V. cholerae*, including non-O1 or O139 serogroups which are in the aquatic environment where *V. cholerae* O1 and O139 reside, and from other gram-negative bacteria in the gut [117] (Table 61.2). These mobile elements are not stable [124]. With changing antimicrobial pressure and other ecological changes, resistance can be acquired and resistant strains quickly propagate, or resistance genes can be lost and susceptibility reestablished. Such ecological pressure also enhances the selection of isolates with chromosomal mutational changes in antimicrobial gene targets or antimicrobial efflux pump mechanisms [147, 148]—such as *V. cholerae* O1 isolates with diminished susceptibility, and clinical resistance, to the fluoroquinolones [36, 62, 149–152] (Table 61.2).

Table 61.1 Recent reports of resistance of *V. cholerae* O1 and O139 clinical isolates to antimicrobial agents potentially useful in the treatment of cholera

Author and reference	Location, country	Year isolates obtained	Number of isolates tested	Percent of isolates resistant										
				AMP or AMX	AZM	CEP	CHL	CIP or NOR	DOX	ERY	FUR	NAL	SXT	TET
Vibrio cholerae O1														
Chander [59]	Chandigarh, India	1999–2007	277	34		0	3	2			85		88	5
Das [112]	Delhi, India	2001–2006	584	100		0	6	12			100	100	88	0
Mandomando [74]	Maputo Province, Mozambique	2002–2004	77	12			58/29					4/1	97	97/2
Kingston [72]	Chennai, India	2002–2005	41	32		7	10	0	2	68		97	92	0
Wang [50]	Multiple provinces, China	2002–2010	109	3	0	0	2	2	0			46	38	11
Mwansa [157]	Lusaka, Zambia	2004	150	100		0	0/100	0/100		0/100			100	0
Ngandjio [78]	Multiple sites, Cameroon	2004–2005	352	6		0	99	0				0	100	0
Roychowdhury [82]	Kolkata, India	2004–2005	135	79				10			100	100	89	7
Rajeshwari [111]	Delhi, India	2005	40	83		3		5				90		15
Rahmani [80]	Multiple provinces, Iran	2005–2007	107				99						97	
Ahmed [52]	Dhaka, Bangladesh	2005–2008	5934					0		31/57			99	61
Smith [83]	Omusati and Kunene districts, Namibia	2006–2007	9	0		0	0	0		0/100		0	100	0
Balaji [53]	Tamil Nadu province, India	2006–2009	31	100			3	32		29		97	90	
Saidi [110]	Nyanza, Kenya	2007–2008	80	0		1/3	0/99	0				4/96	100	0
Chomvarin [63]	Northeast Thailand	2007–2008	84	5			1	2		2/92			95	76/12
Das [64]	Delhi, India	2007–2009	238			2	1	37			100	100	89	17
Borkakoty [58]	Assam, India	2007–2010	40	23		8		8					100	40
Ranjbar [48]	Karaj, Iran	2008	70	100		7		0	55	65	91	100	96	28
Karki [113]	Nepal	2008–2009	57	18/9				0		0/32	100	100	100	0
Islam [108]	Four sites, Zimbabwe	2008–2009	31		0			0			100	84	100	0
Ismail [70]	Multiple provinces, South Africa	2008–2009	716	2		1	42	0		25		100	100	2
Mandal [158]	Puducherry, India	2008–2010	154	64		2		3			77			17
Quilici [79]	Multiple sites, Nigeria and Cameroon	2009	19	0/100			0/100	0				100	100	0
Murhekar [77]	Multiple districts, Papua New Guinea	2009–2011	305	76			3	1		38/55		<1	3	10/31

Jain [56]	Solapur, India	2010	41	0		0	0	0	0	0		100	100	0
Kar [71]	Odisha, India	2010	35	100	0		100	0		100	100	100	100	100
Roy [60]	Karnataka, India	2010	18	100	6/11	28/17	17	6/17				100	100	22/44
Sjolund-Karlsson [47]	Haiti	2010–2011	122		0			0			100	100	100	0
Das [65]	Four cities in Bangladesh	2010–2011	811		23			1		96			95	34
Smith [84]	Togo	2010–2012	42	0		0	0	0		0		91	100	0
Smith [84]	Democratic Republic of Congo	2011	36	0		0	0	0		5		18	97	0
Smith [84]	Guinea	2012	125	1		0	6	0		0		0	99	0
Smith [84]	Ivory Coast	2012	28	0		0	10	0		0		100	97	0
Dixit [66]	Three sites, Nepal	2012	22	0	0			0		0		100	100	0
Shrestha [49]	Kathmandu, Nepal	2012	22	0		18	9	9		91		100	100	0
Smith [84]	Mozambique	2012–2013	26	100		100	97	0		0		100	97	48
Khan (unpublished)	Bangladesh	2014–2015	478		0			0		0/100			100	99
Vibrio cholerae O139														
Yu [51]	China	1993–2009	290	73	50	0	67	6	14	94		83	91	83
Kingston [72]	Chennai, India	2002–2004	10	10						70		90		10

Abbreviation for antimicrobial agents: *AMP* ampicillin; *AMX* amoxicillin; *AZM* azithromycin; *CEP* cephalosporin agent, including cefepime, cefixime, cefotaxime, ceftazidime, ceftriaxone, or cefalothin; *CHL* chloramphenicol; *CIP* ciprofloxacin; *DOX* doxycycline; *ERY* erythromycin; *FUR* furazolidone; *NAL* nalidixic acid; *NOR* norfloxacin; *SXT* trimethoprim-sulfamethoxazole; *TET* tetracycline

Norfloxacin was the fluoroquinolone agent used for susceptibility testing by Wang, Mwansa, Rajeshwari; all others used ciprofloxacin as one of the fluoroquinolone agents evaluated

Dual values in a cell indicate resistant/intermediate susceptibility

All studies included in the table used the agar disc-diffusion method to determine susceptibility, with the exception of the studies by Wang, Rahmani, and Borkakoty which used broth-dilution MIC testing and studies by Smith, Islam, Ismail, and Quilici, which used the Etest

Acquisition and loss of resistance genes have been clearly illustrated by the experience with *V. cholerae* O139 infections in Asia. After the initial epidemics of cholera caused by *V. cholerae* O139 in the first half of the 1990s, this pathogen largely disappeared as a cause of diarrhea. When infections again reappeared in the latter part of the decade, the organism had lost its resistance to trimethoprim-sulfamethoxazole, one of its original defining characteristics [43, 126, 153–155]. This was as a result of the antibiotic resistance gene cluster in the SXT constin having been deleted [124]. Paradoxically, the clones of *V. cholerae* O1 that emerged after the *V. cholerae* O139 epidemic had subsided (*V. cholerae* O1 as a cause of cholera virtually disappeared during the height of the O139 epidemic in the Indian subcontinent) were resistant to trimethoprim-sulfamethoxazole as a result of acquisition of the SXT constin encoding resistance to trimethoprim-sulfamethoxazole [124]. Multidrug-resistant isolates of *V. cholerae* O1 containing the SXT element have now spread widely to other continents [134]. The most dramatic (and the most highly publicized) example of this spread is the introduction of *V. cholerae* O1 with multiple drug resistance into Haiti by UN staff from South Asia [156]. Within 2 years of the inadvertent introduction of this multiple resistant strain of *V. cholerae* O1, more than 600,000 persons had developed cholera, and more than 7000 had died from cholera [27]. This was in a country with a population of approximately 10,000,000.

What is the current status of antimicrobial resistance in *V. cholerae* O1 and O139*?* Making broad generalizations is difficult. Resistance patterns vary geographically because circulating strains in any area are likely to have evolved from parent strains that may have been the source of the initial epidemics, either acquiring or losing resistance genes. This results in a great variety of resistance phenotypes in areas where cholera is endemic or epidemic, as seen in Table 61.1, which summarizes recent reports from more than 20 countries of the resistance profile of *V. cholerae* O1 or O139.

There is no easily available and up-to-date source tracking resistance patterns. The published literature presents findings that even at the time of publication are likely to be—because of the time required for data collation, manuscript writing, and the publication process (even assuming electronic publication)—2 or more years old. In addition, reports are likely to be weighted to reporting resistance, which is presumably more publication worthy, than reporting the absence of resistance, thus providing a skewed picture of actual resistance patterns. The Weekly Epidemiological Record published by the World Health Organization is the one publication that regularly contains updates on cholera outbreaks as part of its "outbreak news" feature, but these reports most often do not contain information on antimicrobial susceptibility patterns.

In the first edition of this chapter 10 years ago, we noted that there was no available online source where resistance patterns for V. *cholerae* can be reported and the results obtained in a timely fashion. We remain unaware of such a resource currently. There have been efforts to coordinate activities for cholera prevention, control, and treatment. One such example is the Africa Cholera Surveillance Network—Africhol (http://africhol.org/)—funded by the Gates Foundation, which has published continent-wide reports of resistance [84]. But despite the increase in electronic communication in cholera-endemic sub-Saharan Africa—where the population of almost 1 billion persons has 500 million cell phone subscriptions (a 25-fold increase in the last 15 years)—access to contemporaneous information on resistance is not easily available, especially in rural areas. Even in Bangladesh, where cholera remains endemic and annually epidemic and the International Centre for Diarrhoeal Disease Research (icddr,b) treatment center maintains routine surveillance of resistance patterns of diarrheal pathogens isolated from patients, access to this information by healthcare workers in primary care settings remains problematic.

The current picture of resistance in *V. cholerae* O1 remains disconcerting (Table 61.1). Multidrug-resistant *V. cholerae* O1 is now commonplace in all areas where cholera is endemic. At the icddr,b treatment center, where approximately 12,000 patients with cholera caused by *V. cholerae* O1 and 450 with *V. cholerae* O139 were provided care in 2015 (relatively low numbers by historical standards [159]), virtually all isolates were resistant in vitro to trimethoprim-sulfamethoxazole and to tetracycline. Most had only intermediate susceptibility to erythromycin (and presumably to azithromycin as well), and virtually all isolates had diminished susceptibility and diminished clinical response to the fluoroquinolones [33, 160].

Fluoroquinolone in vitro susceptibility does not always equate to clinical efficacy. All *V. cholerae* O1 isolates at the icddr,b appear to be susceptible to fluoroquinolones when tested by either the disc-diffusion method (zone of inhibition >21 mm) or the agar dilution method (minimum inhibitory concentrations below the threshold level of <1 μg/mL that is used for defining resistance) [33, 161]. Globally, most *V. cholerae* O1 isolates remains susceptible to the fluoroquinolones when using these standard threshold levels (Table 61.1) with only occasional resistance being reported [60, 64].

From 1994 to 2012, however, the MIC [90] to ciprofloxacin of *V. cholerae* O1 increased 20-fold, from 0.012 to 0.250 μg/mL [33, 36]. At the same time isolates became frankly resistant to nalidixic acid, an earlier quinolone, with the MIC [90] for nalidixic acid increasing from 32 to ≥256 μg/mL. Isolates resistant to nalidixic acid by disc diffusion had a median ciprofloxacin MIC of 0.190 μg/mL compared to 0.002 μg/mL for nalidixic acid-susceptible isolates. Importantly, the rate of clinical success of single-dose

ciprofloxacin treatment of patients infected with nalidixic acid-resistant isolates was only 18 %, compared to 94 % for the treatment of patients with nalidixic acid-susceptible isolates [33]. Clearly, as with *Salmonella* [162] and *Neisseria gonorrhoeae* [163], applying the in vitro susceptibility breakpoints for the fluoroquinolones for *V. cholerae* O1 does not predict in vivo response to these agents during cholera.

In vitro susceptibility thresholds are derived in part from expected tissue and serum concentration of the antimicrobial agent and the relationship between those concentrations and the MIC for the pathogen under consideration. For cholera, the important determinant of efficacy is concentration in the gut lumen—which is the site of infection. Given high fluid volumes during cholera, gut concentrations of drug are quickly diluted. With single-dose therapy, concentrations of ciprofloxacin in the gut lumen can fall below the MIC of strains of *V. cholerae* with diminished susceptibility within 24 h of ciprofloxacin administration, thus explaining the relative lack of efficacy of single-dose therapy in these patients. Three-day therapy regimens with ciprofloxacin may be more effective than single-dose regimens in patients infected with strains of *V. cholerae* with diminished susceptibility, but clinical response is still not optimal [33, 164].

The decreased susceptibility to the fluoroquinolones among *V. cholerae* is invariably associated with frank resistance to nalidixic acid, and results from a single mutation in the *gyr*A gene coding the enzyme—DNA gyrase—which is the target for the quinolones [165]. Further mutations can then lead to frank resistance among the fluoroquinolones.

Resistance is not fixed. With diminished antimicrobial pressure, there is hope that susceptible isolates may again establish themselves, as both resistance plasmids and resistance genes within the SXT element are unstable [67]. In addition to the example of loss of resistance genotypes in *V. cholerae* O139, the experience at the icddr,b is that resistance to tetracycline fluctuates. Resistance rates among *V. cholerae* O1 reached over 80 % in the early 1990s, disappeared by the latter part of that decade, only to return in 2004, and then fluctuated greatly from year to year [81, 160]. All of the *V. cholerae* O1 isolates from 2006 to 2011 contained the SXT element, presumably still harboring sulfamethoxazole and trimethoprim resistance genes, thus accounting for their continued resistance to those two drugs. Fluctuations in tetracycline resistance were then due either to the loss of the tetracycline resistance genes from the SXT element or the transfer and then loss of the plasmid coding for tetracycline resistance.

The picture is not entirely grim. As can be seen in Table 61.1, in Africa most isolates remain susceptible to erythromycin [84], though it is not clear if they have diminished susceptibility to this agent and to azithromycin given that susceptibility thresholds for erythromycin in *V. cholerae* infections have not been well established [161]. Virtually all African isolates were susceptible to ampicillin, though there is very limited data on the efficacy of ampicillin in the treatment of cholera [32, 166]. The gut luminal concentration of ampicillin in relation to the MIC of *V. cholerae* O1 is not likely to be very high, which does not augur well for efficacy. Many isolates remain susceptible to chloramphenicol, but there is reluctance to reintroducing this drug into routine use given its known (if rare) hematopoietic toxicity.

But in some locations, the problem of resistance is exceedingly grim. In Mozambique, isolates from 2012 and 2013 were almost uniformly resistant to ampicillin, ceftriaxone, chloramphenicol, nalidixic acid, and sulfamethoxazole-trimethoprim. Half were resistant to tetracycline. This leaves only azithromycin as a known effective agent, or using a fluoroquinolone despite its lesser activity and poorer clinical response when used to treat strains that are resistant to nalidixic acid (Table 61.3). In a four-site study from 2010 to 2011 in Bangladesh, virtually all *V. cholerae* isolates were resistant to erythromycin and sulfamethoxazole-trimethoprim, a third were resistant to tetracycline, and a quarter were resistant to azithromycin (the latter based upon susceptibility breakpoints for *Staphylococcus*—breakpoints for azithromycin versus *V. cholerae* have not been established) [161]. Only 1 % were overtly resistant to ciprofloxacin, and though neither nalidixic acid susceptibility testing nor fluoroquinolone MICs determination was done, these isolates most likely had diminished susceptibility to the fluoroquinolones (Table 61.2).

2.2 Clinical Significance

Antimicrobial use in cholera is adjunctive therapy, rather than essential for cure. Because *V. cholerae* O1 and O139 are noninvasive and self-limited infections, the infectious process is in itself not lethal, i.e., there are no infection-induced inflammatory changes leading to cell death and tissue destruction. The lethal consequence of *V. cholerae* O1 and O139 is related to cholera toxin production and the intestinal fluid loss resulting from the effect of toxin and the effects of hypovolemia and shock on organ function. Replacement of fluids—either orally (for patients with mild disease) or both orally and intravenously (for patients with more severe cholera)—is lifesaving [185].

Antimicrobials can, however, reduce the volume of diarrhea by half to two-thirds, and duration of diarrhea by half or more [37, 185, 186] (Table 61.3). Without antimicrobial therapy, patients severely ill with cholera will purge approximately 750 mL per kg body weight after presenting for care; with effective antimicrobial therapy fluid loss can be reduced to 250 mL per kg body weight [37, 41, 42, 186]. In a 50 kg person, a 500 mL per kg difference amounts to 25 l during the course of their stay for treatment. With effective antimi-

Table 61.2 Mechanisms of resistance to antimicrobial agents commonly used in the treatment of *V. cholerae* O1 or O139

Agent	Period when resistance first commonly emerged	Genetic determinants and mechanism of resistance	Comment
Ampicillin and other ß-lactams	1970s	Conjugative plasmids, integrons, or integrative conjugative elements (ICEs) expressing ß-lactamases, including extended-spectrum ß-lactamases [167]	Although strains of *V. cholerae* resistant to ampicillin have waxed and waned in prevalence over the years, and limited surveys suggest that resistant strains are currently uncommon (Table 61.1), ampicillin is not routinely used for the treatment of cholera. Often surveys of resistance, and clinical laboratories that test *V. cholerae* from cholera patients, do not routinely include determination of ampicillin susceptibility, thereby lessening the likelihood that it will be used in clinical practice. It is also not one of the agents recommended by the World Health Organization for treatment of cholera [168]
Macrolides—erythromycin and azithromycin	1990s and 2000s	Multidrug efflux systems [169, 170] carried on conjugative plasmids, integrons, or integrative conjugative elements (ICEs)	Azithromycin, because of its higher concentrations at the site of infection and its inherently greater activity against the bacterial target, may retain activity even when there is diminished susceptibility to erythromycin. Resistance thresholds for these agents have not been well defined when used to treat *V. cholerae* O1 or O139 infections
Nalidixic acid	1990s	The presence of a single mutation in the quinolone resistance-determining region of *gyrA* which encodes the quinolone target enzyme DNA gyrase usually results in high-level resistance to nalidixic acid. Such a change can confer diminished susceptibility, but not frank resistance, in the fluoroquinolones [171]. High levels of resistance to fluoroquinolones require additional mutations—either in *gyrA* or *parC,* which also encodes a fluoroquinolone target enzyme, DNA topoisomerase	Resistance to nalidixic acid can be used as a screening test for identifying isolates with diminished susceptibility, but not frank resistance, to fluoroquinolones [33]. Infection with such isolates usually results in diminished clinical response to treatment with a fluoroquinolone [33]
Fluoroquinolones	1990s	Chromosomal mutations in genes *gyrA* and *parC* that encode the fluoroquinolone target enzymes DNA gyrase and DNA topoisomerase, respectively. Sequential mutations in these genes can incrementally decrease susceptibility to the fluoroquinolones [165]. These mutations can increase the MIC [50] by 100-fold, while isolates still remain susceptible using current threshold definitions (growth inhibited by ≤1 μg/mL of the fluoroquinolone) [33], or they can code for frank resistance (growth inhibited by <4 μg/mL). The transferable genetic element *qnrVC3* facilitates the selection of higher-level resistance mutations [172].The role of efflux pumps in resistance in O1 and O139 serogroups is uncertain [147]	Unlike with other antimicrobial agents, the direction of resistance in the fluoroquinolones has been a one-way street—only getting worse. To date, there has not been a reversion to increased susceptibility in areas where strains of *V. cholerae* O1 with diminished susceptibility have been identified. Of note is that strains with diminished susceptibility to fluoroquinolones but that are still considered susceptible using the threshold criteria of the Clinical Laboratory Standard Institute for either disc-diffusion or broth-dilution testing methods [161] are clinically resistant to fluoroquinolones, especially if used in a single dose [33]

Tetracycline	1970s	Conjugative plasmids that contain genes for multidrug resistance efflux pumps [173]	Tetracycline resistance was one of the first commonly identified occurrences of resistance in *V. cholerae* O1 [104, 105]. This is in large part because it had been the first antimicrobial agent commonly used for the treatment of cholera [174], and presumably ecological pressure resulted in selection of resistant strains. Tetracycline resistance is unstable, and susceptible strains can reemerge following periods when resistant strains have predominated
Trimethoprim-sulfamethoxazole	1970s	Conjugative plasmids, integrons, or integrative conjugative elements (ICEs) [104, 106, 124, 175, 176]. These transferable elements contain genes encoding variants of the dihydropteroate synthase or dihydrofolate reductase enzymes that are essential for bacterial folic acid synthesis. These variants are resistant to the effects of sulfamethoxazole and trimethoprim [177, 178]	As with other resistance determinants that are carried on transmissible elements, the carriage of these elements in *V. cholerae* is unstable. Thus resistance is not fixed. Susceptible strains can arise after resistant strains have emerged and have, for a period, become the predominant circulating strains

Table 61.3 Options for antimicrobial treatment of cholera in adults and children

Drug	Dose	Adult dose	Pediatric dose
Ampicillin	Multiple	Not evaluated	12.5 mg/kg body weight every 6 h for 3 days [166]
	Single	Not evaluated	Not evaluated
Azithromycin	Multiple	Not evaluated	10 mg/kg body weight daily for 3 days [179]
	Single	1 g [36]	20 mg/kg body weight [35]
Ciprofloxacin	Multiple	500 mg every 24 h for 3 days [180, 181]	Not evaluated
	Single	1 g [34]	20 mg/kg body weight [37]
Doxycycline	Multiple	100 mg twice (q 12 h) on day 1, then 100 mg once on days 2 and 3 [39]	2 mg/kg body weight twice (q 12 h) on day 1, then 100 mg once on days 2 and 3 [39]
	Single	300 mg [38]	4 mg/kg body weight [38, 39]
Erythromycin	Multiple	500 mg every 6 h for 3 days [180]	12.5 mg/kg body weight every 6 h for 3 days [182]
	Single	Not evaluated	Not evaluated
Tetracycline	Multiple	500 mg every 6 h for 3 days [180]	12.5 mg/kg body weight every 6 h for 3 days [166]
	Single	1 g [183]	Not evaluated
Trimethoprim-sulfamethoxazole	Multiple	160 mg of trimethoprim and 800 mg of sulfamethoxazole every 12 h for 3 days [184]	10 mg/kg trimethoprim and 50 mg/kg sulfamethoxazole per kg body weight every 12 h×3 days [182]
	Single	Not evaluated	Not evaluated

Resistance to all of the agents listed in the table is common. Selection of an agent for treatment of cholera depends on knowing the contemporaneous susceptibility pattern of *V. cholerae* in the locale where the infection occurred

crobial therapy, the duration of diarrhea is reduced from a mean of slightly less than 4 days to slightly less than 2 days.

The reduction in fluid losses, and the consequent reduction in fluid replacement needs, has important consequences for management of patients. In inexperienced hands, management of fluid replacement in severely dehydrated cholera patients can be problematic, with health care providers often not giving sufficient fluids, resulting in unnecessary mortality. In experienced hands, mortality in cholera should be 0.2 % or less [46]. Cholera mortality, overall, however, remains much higher than that [31, 187]. During epidemics, especially at the beginning of epidemics, mortality rates can be very high. Mortality was almost 50 % during a large outbreak of multiple drug-resistant *V. cholerae* O1 infection in Rwandan refugee camps [28]. At the beginning of the cholera epidemic following the earthquake in Haiti in 2010, the mortality rate exceeded 5 % [188], subsequently reducing to 1 % as international assistance came from the United States (a 2-h flight away) and other donor countries [27].

Inexperienced or overwhelmed staffs have difficulty judging the magnitude of fluid replacement required, most often underestimating the volume needed. Operational constraints—too many patients, too few staff, few if any trained staff, and lack of supplies—are also major impediments to successful treatment of patients with cholera. These problems are exacerbated by the fact that cholera is most common where capacity is most limited. By reducing fluid requirements and duration of illness, effective antimicrobial therapy can greatly reduce the logistic constraints of treating patients with severe cholera. In the end, effective antimicrobial therapy reduces not only the cost of treatment, but can substantially affect mortality.

Successful treatment of cholera has been complicated by the acquisition by the El Tor (named after the quarantine camp for returning Hajis in Egypt where it was first identified in 1905) biotype of *V. cholerae* O1 of genes coding for cholera toxin of the classical biotype of *V. cholerae* [189]. The El Tor biotype is the cause of the seventh pandemic of cholera, which began in 1961 in Indonesia and subsequently spread to all continents except Antarctica and continues to the current day (by definition—the beginning and ending of pandemics having somewhat more arbitrary bookends than even economic recessions). El Tor infection was less likely to produce severe dehydrating diarrhea than infection with the classical biotype—presumably because of lesser expression and lower virulence of the El Tor cholera toxin [190]. With the acquisition of cholera toxin of the classical biotype, infections with this variant El Tor strains are now producing more severe disease, with a higher fatality rate [110, 191]. This variant *V. cholerae* O1 El Tor biotype has now spread from Bangladesh, where it was first identified [189], to other parts of Asia [58, 192, 193], Africa [110, 194], and the Americas [195].

Treatment of cholera is empiric. In cholera-endemic areas or during cholera outbreaks, any adult with severe watery diarrhea is assumed to have cholera [185]. Standard therapy is replacement of fluids—orally if the patient is not dehydrated or intravenously if the patient is severely dehydrated

or has a high rate of purging (>5 mL/kg body weight/h) [196]. All patients with dehydration or a high purging rate should also be treated with an antimicrobial, with the choice of agent guided by preexisting knowledge of the susceptibility pattern of circulating strains [196, 197] (Table 61.3).

2.3 Laboratory Diagnosis of Resistance

Laboratory diagnosis of resistance in individual patients for the purposes of selecting an antimicrobial agent is not useful. The benefit of antimicrobial treatment is evinced when treatment is provided early in the course of illness, rather than after the 48–72 h required for isolation and susceptibility testing of the infecting strain of *V. cholerae* O1 and O139. Thus, the choice of antibiotic for treatment must be based on the knowledge of the pattern of resistance of circulating strains. Susceptibility testing should be used to determine the resistance profile at the beginning of a cholera outbreak and to monitor the resistance profile of circulating strains as part of surveillance of endemic or epidemic disease [198].

Disc-diffusion testing on agar plates is the most commonly used means for determining susceptibility [161, 198–200]. Disc-diffusion testing has the advantage of simplicity, low cost, and reproducibility, all crucial concerns when conducting antimicrobial susceptibility testing in the impoverished settings where cholera occurs. These settings often lack even the most basic diagnostic capacity, and field laboratories usually have to be established during epidemics so that isolation and susceptibility testing can be conducted, or the samples are sent to regional laboratories. These conditions preclude the use of more sophisticated automated systems, no matter how reliable they might be in other settings [161, 198, 201]. In any case, none of these automated systems is approved for use in *V. cholerae*. And although there have been numerous initiatives to develop a rapid diagnostic test for cholera, including point-of-care tests, none have attempted to identify antimicrobial resistance [202].

There are limitations to the use of the disc-diffusion method. The Clinical and Laboratory Standards Institute's (CLSI) interpretive standards for testing of *V. cholerae* are based on limited clinical information and often derived from other organisms [161]. Disc-diffusion testing of doxycycline does not accurately predict clinical response [34]. Patients infected with *V. cholerae* strains that are susceptible to doxycycline in vitro but resistant to tetracycline will not respond to doxycycline administration [34]. Therefore, tetracycline susceptibility testing, rather than doxycycline susceptibility testing, should be used when doxycycline is being considered for treatment of *V. cholerae* O1 or O139 infections. Disc-diffusion and broth-dilution susceptibility standards for testing of erythromycin or azithromycin are extrapolated from activity against *Staphylococcus* [161]. It is important, therefore, to determine clinical response when the macrolides are used for treatment, rather than solely relying on interpretive breakpoints, and adjust treatment protocols accordingly. The latter is easier said than done, as systematic evaluation of clinical response is difficult during the tumult and chaos surrounding a cholera epidemic, or even in endemic settings where cholera is treated.

Disc-diffusion breakpoints for fluoroquinolones are based upon activity against other *Enterobacteriaceae,* with a proposed zone of inhibition for susceptible organisms of ≥21 mm for ciprofloxacin [161]. The zone of inhibition is equivalent to an MIC of <1 μg/mL [161]. These in vitro definitions of susceptibility do not correlate with clinical response. Patients infected with *V. cholerae* O1 strains that were susceptible to ciprofloxacin using the proposed ciprofloxacin susceptibility disc-diffusion breakpoint, but that had ciprofloxacin MICs of 0.250 μg/mL when determined using the Etest, did not respond to ciprofloxacin treatment [33, 36]. Thus ciprofloxacin disc-diffusion results cannot be used for determining susceptibility in *V. cholerae* O1 and O139. And treatment with a fluoroquinolone of infections caused by organisms that have intermediate susceptibility to ciprofloxacin—defined as a zone of inhibition of 18–20 mm, or an MIC of 2 μg/mL [161]—will commonly result in clinical failure, even if a multidose, rather than single dose, therapy regimen is used [33].

Because fluoroquinolones are an important option for treatment of cholera and other enteric infections, it is important to have some method of determining susceptibility that is predictive of clinical response. One option is to use nalidixic acid susceptibilities—and consider all isolates resistant to nalidixic acid in disc-diffusion testing to also be clinically resistant to the fluoroquinolones [33]. The current CLSI guidelines do not contain interpretive standards for nalidixic acid activity against *V. cholerae*, but the CDC and WHO suggest that a zone of inhibition of <19 mm denotes resistance [198, 203].

Another option for determining fluoroquinolone susceptibility is to use the Etest to determine MICs. The Etest, though more expensive than disc-diffusion testing, has the same advantages as disc-diffusion testing of simplicity and reliability for use in developing country settings. Strains with an MIC to ciprofloxacin of ≥0.250 μg/mL should be considered clinically resistant [36]; strains with an MIC 0.002–0.025 μg/mL, though most commonly resistant to nalidixic acid by the disc-diffusion method, will have at least an intermediate clinical response to ciprofloxacin [33, 37, 204]; strains with a ciprofloxacin Etest MIC of ≤0.002 μg/mL will be susceptible to nalidixic acid by disc-diffusion testing and will be fully responsive to ciprofloxacin therapy.

Although the CLSI standards susceptibility testing of *V. cholerae* to erythromycin or azithromycin are based on experience with *Staphylococcus* [161], our experience is that isolates with an Etest MIC of ≤0.750 μg/mL to erythromycin

or an azithromycin Etest MIC of ≤0.125 μg/mL are clinically responsive to these agents [35, 36]. The erythromycin Etest for *V. cholerae* has shown good correlation with agar diffusion testing [205], but the in vitro susceptibility threshold suggested in that study—16 μg/mL—is in excess of the level at which a clinical response could be expected [205].

2.4 Treatment Alternatives

Most circulating strains of *V. cholerae* O1 remain susceptible to at least one antimicrobial agent known to be effective in the treatment of cholera (Table 61.3). Having no alternative agent in hand, however, is not a comfortable position to be in. The last new agent evaluated for the treatment of cholera is azithromycin, which we evaluated in 1999 [35]. Since then, there have not to our knowledge been any new antimicrobial agents identified for the treatment of cholera, nor could we identify any ongoing studies of antimicrobial agents on registers of clinical trials (www.clinicaltrials.gov). A drug to be useful in cholera must be active in vitro, attain high-concentrations in the gut lumen, be orally administered, be safe for use in children, and be inexpensive. No class of antimicrobial agents not already evaluated for use for treating cholera meets those criteria—even if cost is not considered.

Agents that appeared promising when we wrote the previous version of this chapter a decade ago—the nonabsorbable antimicrobial agent rifaximin [206], virstatin, a transcriptional regulator governing expression of cholera toxin and toxin-coregulated pilus [207, 208]—have not panned out.

There are suggestions that drugs that show in vitro resistance may still be effective when used clinically. A nonrandomized study of tetracycline showed some efficacy even in patients whose isolates were resistant in vitro [209]. Although single-dose ciprofloxacin was ineffective in the treatment of *V. cholerae* O1 infections with an increased MIC to ciprofloxacin [36], retrospective observations suggest that multiple-dose therapy may have some efficacy [33].

In one study supplemental zinc provided a very modest reduction in diarrhea duration and volume [210]. This is a somewhat surprising finding because zinc has most often not proved beneficial in the treatment of diarrhea of other etiologies [211] and because the biologic plausibility for such an immediate effect of zinc (within 48 h in a disease that does not involved tissue destruction) is problematic.

The mainstay of cholera treatment remains fluid replacement. If infections occur with strains that have become resistant to all currently known effective agents (a realistic possibility), there will be a commensurate increase in the need for intravenous fluids for patients with cholera. Given the immense logistic challenges that already occur in treating cholera, this will add an additional burden that may, for some patients, result in death.

2.5 Infection Control Measures

The most effective means of avoiding the problem of antimicrobial resistance in *V. cholerae* is to prevent cholera by the provision of potable water and improved sanitation [212]. Unfortunately, the majority of the world still lacks access to clean and safe water, and 1/3 of the global population lack basic sanitation facilities [213]. Both of course should be considered an essential human right, but investment in water and sanitation provision—both by local governments and international and binational funding agencies—remains inadequate [213].

Efforts for the provision of potable water from central water reticulation systems have proved problematic in rural areas of developing countries. There have been substantial efforts to dig tube wells, but maintenance and unintended consequences (high levels of arsenic in some tube wells in the Indian subcontinent, for instance) have proven to be stumbling blocks to these programs [214].

An alternative, or supplemental, solution for the provision of potable water has been efforts to sterilize water after it is collected but before it is ingested. These efforts have included using locally available materials—such as the cloth that is used for making *saris*—to filter out the copepods and other marine life that carry *V. cholerae* [215]. Other methods include the use of narrow-mouth (to prevent continued contamination) water containers to which chlorine is added [216] or the use of ceramic water filters [217]. All such methods recognize that centralized systems to provide potable water remain a distant aspiration for much of the world's population, especially those living in rural areas of poor countries. And the reality that methods for providing safe drinking water have to be inexpensive (for instance boiling water is too expensive for most persons lacking clean water) and adapted to poor, rural conditions.

The continued development and increased utilization of cholera vaccines are perhaps the most promising advances in cholera control since the previous edition of this chapter. Cholera vaccines have been under development and study for more than 100 years [204]. There have been incremental advances in their efficacy and in their simplicity of administration and storage, making them more appropriate for use in the poor regions of the world where cholera most often occurs. Both the level and duration of protection provided are less than ideal [218, 219]. Currently they are mostly used during epidemics in emergency situations—such as refugee camps or after natural disasters. A stockpile of cholera vaccine has been developed for use in emergencies [220], and this may ameliorate the severity of some of the worst cholera outbreaks during disasters and civil strife, such as the epidemics that occurred in Rwanda and Haiti [27, 28]. The routine use of vaccines where cholera is endemic probably awaits development of vaccines that provide longer-lasting immunity.

There are suggestions (but as of yet little empiric evidence) that antimicrobial therapy of patients with cholera might reduce cholera incidence by reducing secondary cases. Recent studies have suggested that *V. cholerae* organisms shed by patients with cholera are "hyper-infective" (have a much lower ID_{50}) when compared to environmental isolates [221]. Such transient hyper-infectivity of *V. cholerae* shed by patients may help explain the predilection of *V. cholerae* to cause explosive epidemics [222]. Modeling suggests that antimicrobial treatment of all patients with moderate or severe disease—who are shedding large volumes of cholera stool (which contains 10^{12} organisms per liter)—may itself reduce cholera death by 12% by reducing the number and severity of secondary cases [223]. When provided along with clean water and cholera vaccines, the combined effect on reducing the number of persons infected and dying during epidemics would be even greater [223].

But it remains unclear how the provision of expanded antimicrobial treatment would actually work to reduce secondary cases of cholera. Patients with moderate or severe cholera seeking care are usually kept at treatment centers or hospitals until their diarrhea has abated—whether or not they have received antimicrobials. The greatest risk for secondary transmission is likely to be before they have come to a facility for care, and it is unclear how antimicrobial treatment would be provided at home. Hospitals themselves can be a nidus of infection—either through nosocomial transmission [224, 225] or by untreated waste from medical facilities leading to exposure in surrounding communities [226, 227]. Presumably more comprehensive antimicrobial treatment of patients in treatment facilities might lessen the risk of secondary transmission occurring in or adjacent to these facilities. But the more obvious (but not always attainable) approach to these problems would be to enhance infection control and treatment of waste.

Because of the high rate of secondary cases in households (up to 50% of household contacts of a person with cholera also become infected), it has also been suggested that prophylactic antimicrobial therapy be administered to household members. A number of studies of this approach have reached varied conclusions: that multidose, multiday prophylaxis, but not short-course antimicrobial prophylaxis, can reduce transmission and secondary cases [228]; that excretion of *V. cholerae* in household contacts can be reduced [229]; that prophylaxis reduces the severity but not the incidence of secondary infections [230]; that prophylaxis transiently delays but does not prevent excretion in household contacts [231]; or that prophylaxis has minimal effect on secondary infections [232]. It is difficult to directly compare the results of these studies because they were conducted under different conditions using different drug regimens. Current guidelines do not recommend the use of antimicrobials for prophylaxis, considering the evidence to support their use is too weak [197, 233]. And the analyses to date—either for increasing the spectrum of patients treated or for prophylactic use—have not accounted for the potential impact of the expanded use of antimicrobials on selecting for resistant *Vibrio cholerae* (or other pathogens for that matter) [223].

3 Diarrheal Disease Caused by Vibrios Other Than *V. Cholerae* O1 or O139

3.1 Geographic Spread and Epidemiology of Resistance

Even more so than for *V. cholerae* O1 or O139, there is no systematic reporting or surveillance for resistance among vibrios causing non-cholera diarrhea.

Vibrio parahaemolyticus is the most common cause of non-cholera *Vibrio*-associated diarrhea, and reports on resistance are most common for this species. This includes a spate of reports on a recent increase in cases due to a distinct serotype that has spread globally [234]. Reports are especially common from Asia, perhaps because ingestion of raw or undercooked seafood can be a cultural norm, general levels of sanitation are poor, and the disease is more common there [9, 10, 85, 87, 235–237]. Infections can occur, however, wherever exposure to marine environments and seafood occur [8, 17, 18, 86]. Ampicillin and streptomycin resistance appears to be a common feature of most recently isolated strains of *V. parahaemolyticus* globally, with most strains remaining susceptible to trimethoprim-sulfamethoxazole, tetracycline, and the quinolones [9, 85, 86, 235, 238].

Diarrhea has been less commonly associated with infection with other *Vibrio* species, including *Vibrio vulnificus*, *Vibrio fluvialis*, *Vibrio mimicus*, and non-O1, non-O139 serogroups of *V. cholerae* [1]. The latter can rarely have virulence attributes—such as the capacity to produce cholera toxin—most commonly associated with *V. cholerae* O1 and O139, and result in clinical cholera [7]. Reports on resistance of these organisms are very sporadic, with many reports including environmental isolates, which may or may not be representative of isolates from patients [5, 101, 239–241].

3.2 Clinical Significance

With the exception of the uncommon non-O1, non-O139 *V. cholerae* isolate that produces cholera toxin, the self-limited diarrhea produced by these vibrios is modest in comparison to cholera. Infection with these organisms does not produce a clinical syndrome that would distinguish it from many other causes of diarrhea not requiring antimicrobial therapy, including viral infections. In the absence of a rapid diagnostic test that would allow for identification of the cause of

diarrhea as a non-cholera vibrio, empiric therapy would result in the overtreatment of many patients with diarrhea caused by organisms that do not require antimicrobial therapy. The only exception may be in foodborne outbreaks, where identification of an outbreak strain may allow presumptive treatment of subsequent infections. But even if infection with a non-cholera vibrio could be assumed or presumed, there have been no controlled studies demonstrating the benefit of antimicrobial therapy in diarrhea caused by non-cholera vibrios.

3.3 Laboratory Diagnosis of Resistance

The most recent (2015) CSLI guidelines for susceptibility testing of infrequently identified bacteria include guidelines for testing of non-cholera *Vibrio* species [161]. Both broth-dilution MIC and disc-diffusion testing are now considered appropriate and acceptable for determining susceptibility of these organisms, using standard media (cation-adjusted Mueller-Hinton broth or Mueller-Hinton agar, respectively) [161]. But as with *V. cholerae* O1 or O139, the scarcity of information on the clinical and bacteriologic response to antimicrobial agents means that the thresholds for susceptibility for these agents are often extrapolated from other organisms. The new guidelines continue to rely on extrapolation from other *Enterobacteriaceae* or *Staphylococcus* in the case of azithromycin. The use of derived breakpoints for these "orphan" organisms recognizes that because of their infrequent isolation, it was not possible to adhere to all the rigorous requirements for establishing breakpoints, especially correlation of the breakpoints with clinical response [242].

3.4 Treatment Alternatives

As with most watery diarrheas, the mainstay of treatment remains the replacement of fluids—orally for most patients or intravenously if dehydration is severe.

3.5 Infection Control Measures

Most infections are associated with poor food hygiene—either ingestion of undercooked seafood, lack of adequate hygienic measures to ensure that other foods are not fecally contaminated by a person with infection, or fecal-oral transmission from person to person. Standard hygienic practices could prevent most infections. But as noted in the section above on cholera, the absence of access to clean water and adequate sanitation continues to remain an obstacle to good hygienic practices in poor countries. In rich countries the emphasis has to be on limiting the risk from seafood that is improperly handled or consumed. This often boils (literally) down to the competing demands of the gourmand and sportsman versus the pesky (and often resented and ineffective) nagging of public health authorities.

4 Tissue-Invasive Disease and Septicemia Caused by Vibrios Other Than *V. cholerae* O1 or O139

4.1 Geographic Spread and Epidemiology of Resistance

Summarizing resistance patterns for vibrios causing invasive disease or septicemia is complicated by a number of factors. These include the relative rarity of their isolation from humans, the equal rarity of reports in the literature on susceptibility, the scarcity of reports on mechanisms of resistance, the diversity of geographic sites in which they are isolated, the absence for the most part of human-to-human spread and therefore the absence of a single strain causing multiple infections, and the absence until recently of standardized methods for susceptibility testing.

Tissue-invasive infections with non-cholera vibrios—especially *Vibrio vulnificus*, the *Vibrio* species most commonly causing tissue-invasive disease—are of greater concern than gastrointestinal infections with non-cholera vibrios because tissue-invasive infections are often lethal without effective antimicrobial therapy. Although the clinical syndromes of soft tissue infection and sepsis with these organisms may include diarrhea in the constellation of symptoms these patients have, it is the former (tissue invasions and sepsis) rather than the latter (diarrhea) that is the concerning feature of this illness.

Unlike *V. cholerae* O1 or 139, where acquired resistance is now widespread, resistance among halophilic (non-cholera) tissue-invasive vibrios appears to be less common. Most reports suggest that *V. vulnificus* is usually susceptible to the fluoroquinolones [89, 94–96, 100, 238, 243, 244], cefotaxime and other third-generation cephalosporins [89, 95, 96, 100, 238, 244, 245], trimethoprim-sulfamethoxazole [96, 100, 238, 243], tetracycline or minocycline [89, 95, 96, 100, 238, 244, 245], and imipenem [89, 96, 238]. Resistance to ampicillin was common at the time the clinical syndromes with these organisms were first characterized in detail [98, 99, 243, 246]. Resistance or intermediate susceptibility (MIC ≥ 1 μg/mL) to ampicillin remains common, as it does to first- or second-generation cephalosporins [89, 96, 100, 244, 245]. The addition of clavulanate to ampicillin or amoxicillin reduces the MIC of strains that are frankly resistant to the β-lactam, but only to an MIC [90] of 4 μg/mL [96]. Gentamicin has also shown at best intermediate activity against *V. vulnificus*, with a reported MIC [50] of 2 μg/mL

and MIC [90] of 4 μg/mL in two studies [89, 95], and MIC values double that in an earlier study [243].

Studies have also reported on the susceptibility of *V. alginolyticus*. For the most part the pattern of resistance is similar for that reported for *V. vulnificus* [96, 100, 247]. Reports on other tissue-invasive *Vibrio* species are even scarcer.

4.2 Clinical Significance

Unlike the noninvasive diarrheal disease caused by *V. cholerae* O1 and O139 or non-cholera vibrios, antimicrobial therapy is essential for survival in tissue-invasive *Vibrio* infections.

There are, as far as we are aware, no controlled studies of antimicrobial therapy for tissue-invasive *Vibrio* disease. Choice of therapy, therefore, is based upon relatively small series of patients, clinical experience, and extrapolation from in vitro or animal studies [89, 94, 95, 245, 248–250].

Because patients with invasive disease caused by non-cholera vibrios are often immunocompromised, and the disease can fulminate (illness onset within 36 h of exposure, 50 % or higher fatality rate in most series), antimicrobial therapy must be started when infection is first suspected based upon clinical presentation and epidemiologic profile (marine exposure in a patient with liver disease or is otherwise immunocompromised), and then adjusted based on subsequent laboratory findings. Drug regimens suggested based upon in vitro and animal studies include ciprofloxacin and cefotaxime [95], single-agent treatment with tetracycline [248], single-agent fluoroquinolone therapy [94], and a combination of cefotaxime and minocycline, which was synergistic in vitro [251, 252].

In recent years the largest series of patients reported in the literature have come from Taiwan. In general, the approach has been to treat with two drugs which are known to have good in vitro activity against non-cholera vibrios. One series reporting on 93 patients who received a variety of antimicrobial regimens suggested that the lowest fatality rate was achieved with a combination of a third-generation cephalosporin and tetracycline or a congener [16]. Patients treated with a first- or second-generation cephalosporin and an aminoglycoside did noticeably worse [16]. Another retrospective report of 89 patients with necrotizing fasciitis caused by *V. vulnificus* found that a combination of a third-generation cephalosporin and minocycline, or a fluoroquinolone and minocycline, did better than patients who received only a third-generation cephalosporin [253]. These studies, like all uncontrolled retrospective studies, are subject to the limitations inherent to such studies—selection of treatment regimens by severity of illness, changing antimicrobial regimens over time, and differences in provider care.

The current CDC treatment recommendations are doxycycline (100 mg PO/IV twice a day for 7–14 days) combined with a third-generation cephalosporin (e.g., ceftazidime 1–2 g intravenously or intramuscularly every 8 h), or a fluoroquinolone as a single agent [250]. Because of concerns about the putative toxicity of fluoroquinolones and doxycycline in children, the CDC recommends trimethoprim-sulfamethoxazole plus an aminoglycoside for this age group. Given the severity of the disease, the recommendation seems misplaced. Fluoroquinolones appear to be safe when used for limited periods in children, and tooth staining with tetracyclines in children is both not inevitable and not resulting in substantial long-term morbidity. Given the lethal nature of tissue-invasive *Vibrio* infections, the risk-benefit ratio for the use of these agents tilts heavily to benefit. In any case, invasive *Vibrio* infections in children are even more infrequent than they are in adults.

4.3 Laboratory Diagnosis of Resistance

As with *V. cholerae* O1 or O139 and non-cholera vibrios causing diarrhea, more complete recommendations for both disc-diffusion and agar dilution MIC testing now exist for tissue-invasive vibrios [161]. And as with *V. cholerae*, the susceptibility breakpoints are in large part extrapolated from the experience with other organisms.

4.4 Treatment Alternatives

Surgical treatment (incision and drainage, debridement of necrotic tissue, fasciotomies, or amputations) when required is an important adjunct to antimicrobial therapy in tissue-invasive disease [16, 88, 89, 249]. Other crucial supportive measures include maintenance of blood pressure (septic shock is a common manifestation of infection, especially in immunocompromised hosts) and measures to control disseminated intravascular coagulation.

4.5 Infection Control Measures

Patients with liver disease are at high risk for *V. vulnificus* septicemia. In the United States, raw seafood, especially oysters, is the most common vehicle of transmission [97]. Efforts should be made to warn all persons who are at high risk of disease to avoid eating raw or undercooked seafood. Posted warnings in restaurants or seafood shops are often not noticed by those at risk, either because they are not conspicuous or they are in a language that is not understood by the person at risk (English language signs for high-risk Hispanic persons with liver disease in the United States, for instance) [254].

In at least one report from Japan, many healthcare providers in endemic areas were also unaware of the risk to persons with hepatic disease from eating raw or undercooked seafood [255]. Thus, in addition to warnings in restaurants and food shops, it is important that the healthcare workers provide advice to patients who would be at increased risk of contracting invasive *Vibrio* infections. Other precautions include the use of gloves for all persons handling seafood commercially and warnings for sports fisherman or others involved in marine activities of the risk of infection if they have an open wound or abrasion or have a penetrating injury (such as with a fishhook). Given the ubiquity of such risk when fishing, it may be best to advise all persons with liver disease or who are immunocompromised to avoid such activities.

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Antimicrobial Resistance in *Helicobacter* and *Campylobacter*

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1 Introduction

Helicobacter and *Campylobacter* are Gram-negative spiral flagellated bacteria that inhabit and cause diseases of the gastrointestinal tract. Despite early microscopic observations of "*Vibrio*-like" organisms in blood, stool, and gastric contents, the role of these two genera in infectious disease was established in relatively recent times.

Campylobacter was first generally accepted as an important fecal pathogen in the 1970s, when improvements in culture methods made it feasible to systematically study the role of *Campylobacter* in diarrheal diseases [1]. Today, it is recognized as one of the leading causes of foodborne gastroenteritis in the USA and worldwide, with *Campylobacter jejuni* and *Campylobacter coli* being the most commonly isolated species [2]. It is also the most common antecedent microbial infection associated with Guillain-Barré syndrome [3]. *Campylobacter* are enteric commensals in several animal hosts, which include various avian and mammalian species from which most human infections are thought to originate.

Helicobacters can also be found in the intestinal tract of animals and humans, but the most important species, *Helicobacter pylori*, is essentially present in the stomach of humans where it induces gastritis [4]. Despite the fact that they commonly persist in the human stomach asymptomatically, infection by *Helicobacter pylori* is the most important risk factor for peptic ulcers and gastric MALT lymphoma in some subjects as well as for gastric adenocarcinoma [5].

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Other gastric Helicobacters have been described in various animal species. The recognition of the importance of *Campylobacter* and *Helicobacter* in human illness has sparked intense research over the past 30 years into the epidemiology, microbiology, and treatment of diseases caused by these organisms. Each pathogen presents unique and fascinating challenges for intervention and control. While we await future advances in vaccines and other preventive measures, the clinical management of both pathogens relies on the availability of effective antimicrobial agents.

2 *Helicobacter pylori*

Approximately 80 % of adults in developing countries are infected with *H. pylori*, whereas fewer than 30 % are infected in industrialized countries [6]. Poor socioeconomic status is the major risk factor for infection [6]. Large families, small houses, lack of hygiene facilities, and poor education can favor transmission. Although the mode of transmission of *H. pylori* is not definitely proven, oral-oral or fecal-oral are the most probable routes of transmission and occur primarily in early childhood [7]. Family members, and particularly mothers [8, 9], play an important role in transmitting this infection. There is very little evidence for existence of viable sources of *H. pylori* outside the human host.

The standard clarithromycin-based triple therapy used to eradicate *H. pylori* in patients with symptoms is comprised of two antibiotics, most often clarithromycin (Cla) and amoxicillin (Amx), and a proton pump inhibitor (PPI) [10]. It was originally successful in 80–90 % of patients [11], but Cla resistance emerged and is currently the first cause of treatment failure [12]. Antibiotic resistance of *H. pylori* varies widely by geographic regions and among subpopulations within a region. As a result, alternative treatment regimens have been developed. Other antibiotics also used in combination include levofloxacin (Lvf) (as a fluoroquinolone) [13] or rifabutin (Rif) (as a rifampin) [14] or nitrofurans [15, 16]. The current recommendations for treatment [17] include

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bismuth-based quadruple therapy, sequential therapy [18], and non-bismuth quadruple therapy [19].

Relapses of *H. pylori* infection are lower in developed than developing countries, with averages of 2.7 and 13 %, respectively, according to a meta-analysis of studies where the patients were followed for 24–60 months [20]. In developed countries, these relapses occur most often during the first year following eradication treatment and have been found to be essentially recurrences of the original infection. In contrast, during the following years, reinfections are mostly identified based on molecular typing. However, it is difficult to differentiate recurrence from reinfection, since some individuals may harbor genotypically different strains of *H. pylori* simultaneously. In addition, one could be reinfected by the same strain from the same source.

There is no "gold standard" for *H. pylori* diagnosis. The test most commonly used is histopathology which has the added value of showing the status of the gastric mucosa, but this technique is very much dependent on the expertise of the pathologist and on the quality of the biopsy specimen. Among the other invasive tests used, rapid urease test allows a quick and simple diagnosis but lacks sensitivity, and culture has the important advantage of allowing susceptibility testing of all antibiotics but is demanding for transport and for lab procedures. Molecular methods have been developed using PCR, real-time PCR, and FISH. These methods allow, in particular, the detection of point mutations associated with clarithromycin resistance. The noninvasive tests most often used are the urea breath test, serology, and stool antigen tests [21].

2.1 In Vitro Antimicrobial Susceptibility Testing and Interpretive Criteria for *H. pylori*

In the USA, the Clinical and Laboratory Standards Institute (CLSI) recognizes only the agar dilution susceptibility testing method for *H. pylori* [22]. This method requires a cell suspension equivalent to a 2.0 McFarland standard, Mueller-Hinton plates containing 5 % aged (>2 months) sheep blood, and incubation for 72 h in a microaerobic atmosphere at 35 °C ± 2 °C. *H. pylori* ATCC 43504 is the quality control (QC) strain. Currently, there are QC ranges for Amx, Cla, metronidazole (Mtz), and tetracycline (Tet), but interpretive criteria are established only for Cla (resistant breakpoint ≥ 1 μg/mL) [22]. While reproducible, this method is labor intensive and not amenable to regular testing of small numbers of clinical isolates. There is a need for a more rapid and affordable method for routine laboratory use.

In Europe, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) also validated an agar dilution method with a few differences. Mueller-Hinton plates contain 10 % horse blood, the suspension has an opacity equivalent to a McFarland 4 standard, and the QC strains are CCUG 38770, 38771, and 38772 for Amx, Cla, and Mtz, respectively. The interpretative criteria for resistance are Cla, >0.5 mg/L; Amx, 0.12 mg/L; Tet, Lvf, and Rif, 1 mg/L; and Mtz, 8 mg/L (Table 62.1 [23]).

A variety of other methods have been examined for their suitability for testing antimicrobial susceptibility of *H. pylori*. These include a simplified version of the agar dilution method which tests only an antibiotic concentration equivalent to the resistant breakpoint, disk diffusion, the Epsilometer test (Etest, AB bioMérieux, Solna, Sweden), and broth dilution. Disk diffusion is generally not considered a good choice for slow-growing organisms such as *H. pylori,* since the antibiotic gradient decays over time. However, a study in France showed a good correlation between disk diffusion and agar dilution for testing macrolides, given the important MIC differences between susceptible and resistant strains. The cutoff value was established at 22 mm for Cla [24].

There are now other methods derived from the disk diffusion methods such as the Etest which was the pioneer, but others have followed, e.g., Minimum Inhibitory Concentration Evaluators (M.I.C.E.™, Oxoid, Basingstoke, Hants, UK). Etest uses a predefined stable gradient of 15 antibiotic concentrations on a plastic strip using dry chemistry technology.

Table 62.1 Proposed breakpoints for *Helicobacter* susceptibility testing

Antimicrobial agent	Organization	MIC (μg/mL)		
		S	*I*	*R*
Amoxicillin[a]	EUCAST	≤0.12	–	>0.12
Clarithromycin[a,b]	CLSI	–	–	≥1
	EUCAST	≤0.25	–	>0.5
Metronidazole[a]	EUCAST	≤8	–	>8
Rifampicin	EUCAST	≤1	–	≥1
Tetracycline[a]	EUCAST	≤1	–	≥1
Levofloxacin	EUCAST	≤1	–	≥1

[a]Standardized testing methods with quality control ranges available for *H. pylori* ATCC43504
[b]Only breakpoint validated by clinical outcome data. All others are based on ECOFFs and/or the presence of known resistance determinants

While Etest is relatively expensive in comparison to other susceptibility testing methods, it is much simpler than agar dilution and thus is often preferred in clinical settings. Several studies have evaluated the Etest relative to the agar dilution reference method for testing *H. pylori*. These studies suggest that, in general, the Etest correlates well with agar dilution except for Mtz for which the Etest generates higher MIC values [25]. Even when using agar dilution, Mtz susceptibility testing results have shown a lack of reproducibility including intra-laboratory testing [25]. The reason has not been clearly established but it may be linked to the lack of control of the redox potential. Other reasons could be the existence of hetero-resistant subpopulations [26] or infection by multiple strains which may occur in 15–20 % of patients in developed countries [27]. Broth dilution has the advantage of being adapted to automatization, the limitation being the difficulty to grow *H. pylori* in a liquid medium.

The limit of phenotypic methods lies essentially in the need to culture *H. pylori* which requires specific conditions to maintain its viability during transport, and takes time, a minimum of 3 days but up to 8–10 days if few bacteria are present. For these reasons, molecular methods detecting specific mutations associated with resistance have been developed, essentially for Cla for which *H. pylori* resistance was the main factor of failures of the triple therapy recommended all over the world since 1997 [28]. Another resistance important to detect is to fluoroquinolones, which can also be determined by molecular methods [29, 30]. The strong points are that gastric biopsies can be transported without specific requirements and that the test can be performed in a few hours. Details of these methods will be provided after a description of the resistance mechanisms.

2.2 Resistance to Specific Antimicrobials

Below we shall consider the different mechanisms of resistance to specific antimicrobials used to treat *H. pylori*-associated diseases.

2.2.1 Macrolide Resistance

Macrolides bind to the 50S subunit of bacterial ribosomes and interfere with protein synthesis by inhibiting the elongation of peptide chains [31, 32].

Cla is the macrolide of choice to treat *H. pylori* infection because of its bacteriological and pharmacological properties. Its metabolite 14OH Cla is also active [33]. When present, macrolide resistance concerns all of the drugs in the group. It is due to point mutations in the 23S rRNA gene [31, 34, 35], and not to adenine methylation like in the MLSb resistance type. The most common point mutations within domain V of the peptidyl transferase loop of 23S rRNA are adenine (A) to guanine (G) transition, rarely A to cytosine (C) transversion, at either of the two nucleotides position 2142 and 2143 in *H. pylori* coordinates (based on the determination of the transcription start site of *H. pylori* 23S rRNA) [35], corresponding to positions 2058 and 2059, respectively, using *Escherichia coli* coordinates. These mutations lead to a decrease in Cla binding to ribosomes. Other much less common mutations have been observed in the 23S rRNA, but their role in resistance has not been formerly established.

The A2142G mutation appears to be more frequent in *H. pylori* strains with an MIC >64 mg/L (65 %) than in those with an MIC <64 mg/L (35 %).

There are two rRNA operons on the *H. pylori* genome, but usually the mutations are found in both; heterozygotia is rare. These mutations appear spontaneously at a rate evaluated between 3.2×10^{-7} and 6×10^{-8}. This rate may be dependent on the inflammatory status at the mucosal level. The resistant mutants are then selected by administration of the macrolides [36]. The so-called primary resistance of *H. pylori* against macrolides is due to previous consumption of macrolides for diverse infections, notably respiratory tract infections. It varies widely according to the use of macrolides in the area or country. In Europe it varies from 5.6 to 36.6 % [37]. Unfortunately, there are no studies indicating the current rate for Cla resistance in the USA, but logically it should exceed the threshold of 15–20 %. The secondary resistance is that observed after treatment for *H. pylori* infection. It is normally in the range of 60–70 % [38].

Resistant mutants may remain in the stomach, depending on the cost of the mutation on the ability of the bacteria to grow and divide, i.e., the fitness of the strain [39]. Some studies showed that the mutations for Cla resistance had a low impact on the fitness of the strain, but other studies showed the contrary.

2.2.2 Fluoroquinolone Resistance

The fluoroquinolones (FQs) act via the inhibition of DNA gyrase and topoisomerase IV in DNA synthesis [29]. FQs are not generally used for primary eradication of *H. pylori*, but may be used in rescue treatment regimens when other antibiotics fail [40–43]. Resistance to FQs may be acquired rapidly; therefore, this group of agents should be used with caution for the treatment of *H. pylori*. In addition, FQs are not recommended for use in children. Primary resistance of *H. pylori* isolates to FQs varies from 4 to 28 % in Europe [37]. Secondary resistance is in the range of 50 % [44, 45].

In *H. pylori* strains, only the DNA gyrase is present and is comprised of two subunits encoded by the *gyr*A and *gyr*B genes. FQ resistance in *H. pylori* is essentially due to various mutations in the quinolone resistance-determining region (QRDR) of *gyr*A, the gene that encodes the A subunit of DNA gyrase [46, 47] as described for other bacteria [48]. Several types of base substitutions, usually resulting in a single amino acid change at Asp91 or Asn87 in the QRDR of

*gyr*A, are associated with an increase in MIC and with cross-resistance to other FQs [47]. However, there are FQ-resistant strains which do not harbor these mutations and for which the resistance mechanism remains to be elucidated.

The FQ essentially used is levofloxacin (Lvf) because it leads to good success rates when the strain is susceptible. In contrast, ciprofloxacin (Cip) is not recommended because of its low success rate [49]. Primary resistance to FQ is highly dependent on the FQ consumption in the area or country and varies widely. Secondary resistance is very common after failure of FQ-based eradication therapy. FQs with a higher activity at low pH, like sitafloxacin [50] and finafloxacin, may lead to better results. At this stage sitafloxacin is only available in the Far East, and finafloxacin is not yet available.

2.2.3 β-Lactam Resistance

β-Lactams interfere with cell wall peptidoglycan biosynthesis, resulting in lysis of replicating cells [51]. The only β-lactam used to treat *H. pylori* infection is amoxicillin (Amx) [52]. *H. pylori* strains for which Amx resistance has been well documented are extremely rare. One is the Hardenberg strain with a stable Amx resistance (MIC, 8 mg/L), reported in the Netherlands, isolated from a patient treated with multiple courses of Amx for a respiratory infection [53]. Mutations in the *pbp*1A gene have been found associated with this resistance, especially Ser414Arg [53]. The same results have been obtained by serial cultures of an Amx susceptible strain, with progressively increasing concentrations of Amx. In another study, the genes coding for PBP2 and PBP3 have been incriminated. Another type of resistance was described in Italy and the USA, but this resistance phenotype was unstable, i.e., it was lost after freezing of the strains [54]. This resistance could be due to a mosaicism of the C-terminal end of PBP1A.

When Amx resistance is found in the context of multiresistance, the mechanism is essentially a decrease in membrane permeability [55].

In the literature there are reports indicating relatively high prevalence of *H. pylori* resistance to Amx. When there is not a detailed analysis of the strains, we think that the results must be interpreted with caution because it could be a false resistance.

2.2.4 Tetracycline Resistance

Tetracycline inhibits protein synthesis by binding reversibly to the 16S rRNA in the 30S ribosomal subunits, blocking the binding of aminoacyl-tRNA, and thus stopping the synthesis of the growing peptide chains [56, 57].

In *H. pylori,* triple mutations in both copies of the 16S rRNA genes, ^{965}AGA^{967} to ^{965}TTC^{967} (*E. coli* numbering), were determined to be responsible for high-level Tet resistance [58, 59]. Single and double mutations at nucleotides 965 to 967 result in lower levels of Tet resistance [60, 61]. Decreased binding of Tet to *E. coli* ribosomes with nucleotide substitutions in positions 965 to 967 of the 16S rRNA has been demonstrated [62].

One study showed 16S rRNA mutations in only 54 % of Tet-resistant *H. pylori* isolates, while the remainder showed decreased Tet uptake [61]. A role for efflux was demonstrated by Li et al. who showed that inactivation of a Tet efflux homologue abrogated inducible Tet resistance [63].

While Tet-susceptible isolates of *H. pylori* show modal MICs of 0.5 μg/mL, Tet-resistant isolates show a wide range of MICs. The reason for this wide range of Tet MICs has not yet been clearly explained. Site-directed mutagenesis in *H. pylori* using limited (seven) substitutions within the triplet mutation suggested that single- and double-base-pair mutations mediate only low-level Tet resistance (MIC, 1–2 μg/mL) but also decrease growth rates in the presence of Tet [58]. This study thus offers a possible explanation for the prevalence of the ^{965}TTC^{967} mutation observed in clinical Tet-resistant isolates of *H. pylori*.

The prevalence of Tet resistance is usually low [37]. However, high levels have been described in Korea [64] and Brazil [65].

Further studies are necessary to understand exactly (1) how the mutations found in the Tet-resistant *H. pylori* are selected in vivo, (2) the reason for the presence of a wide variation in MICs in *H. pylori* isolates which contain similar 16S rRNA mutations, and (3) the possible importance of genes other than the 16S rRNA mutation which may contribute to Tet resistance.

2.2.5 Rifamycin Resistance

Resistance of *H. pylori* to rifamycins and its derivatives, especially rifampin (Rif) which is used in *H. pylori* rescue treatments, results from the inability of these compounds to bind to the β-subunit of RNA polymerase, which is encoded by *rpoB* [66–69].

A study performed on laboratory-induced mutants showed the presence of mutations at codons 524, 525, and 585 of the *rpo*B gene, i.e., at the same positions described for *Mycobacterium tuberculosis* and *E. coli.*

The prevalence of primary resistance to rifamycins is very low [37]. It concerns essentially strains isolated from subjects previously treated for tuberculosis [69].

2.2.6 Nitroimidazole Resistance

Nitroimidazoles include such compounds as metronidazole (Mtz) and tinidazole. Mtz is a prodrug that is reduced to a hydroxylamine derivative that damages DNA and appears to cause cell death by nicking DNA [70]. Nitroimidazoles, in general, and Mtz, in particular, were among the first groups of antibiotics to be used for the treatment of *H. pylori* [71].

Bacterial resistance to nitroimidazole compounds appears to be due to an inability to reduce the prodrug [52]. Mutations in *rdx*A, which encodes an oxygen-insensitive NADPH nitroreductase, resulted in Mtz resistance [72–74]. Later, Kwon et al. [75] and Jeong et al. [76] independently demonstrated that *frx*A, which codes for an NAD(P)H-flavin oxidoreductase, a paralog of RdxA, can also be involved in Mtz resistance. They showed that inactivation of *rdx*A alone resulted in moderate Mtz resistance (MIC, 16–32 μg/mL), whereas single mutations in both *rdx*A and *frx*A conferred higher levels of resistance (MIC >64 μg/mL). There is still controversy concerning the exact role of *rdx*A and *frx*A in Mtz resistance [77–79]. It has been suggested that other genes may play a role in Mtz resistance. Mutations in *rec*A [80] and *fdx*B (encoding a ferredoxin-like protein) [81], repression of pyruvate oxidoreductase (POR) and α-ketoglutarate oxidoreductase [82], and decreased transcription of *rdx*A and *for* (ferredoxin oxidoreductase) and possibly *por* and *fdx*B have all been implicated in Mtz resistance in *H. pylori* [83]. Therefore, diverse mutations in *H. pylori* may result in Mtz resistance. In addition, an efflux mechanism has been observed [84].

Primary resistance to Mtz is widespread. It is in the range of 30 % in developed countries and 70 % or more in developing countries because of the common use of this drug for parasitic infections. It increases after treatment failure.

2.2.7 Nitrofuran Resistance

The nitrofurans include furazolidone, nifuratel, and nitrofurantoin. Nitrofurans function through multiple mechanisms by binding to a variety of proteins. While none of these agents is commonly used in primary eradication of *H. pylori,* they may be used when primary treatment fails [15, 16]. The susceptibility of *H. pylori* to these antibiotics is not commonly tested; however, primary resistance to nitrofurans appears to be very seldomly found [85]. In other bacteria, resistance is associated with reduced nitrofuran reductase [86]. As yet, no studies have been published on the mechanisms of resistance to nitrofurans in *H. pylori*.

2.3 Molecular Methods of Detection

As was previously indicated, the genetic basis of antibiotic resistance in *H. pylori* involves essentially point mutations on the chromosome which can easily be detected by molecular tests. The most important clinical concerns relate to Cla resistance, and therefore a large number of molecular methods have been developed to detect this resistance, most being PCR based on the 23S rRNA gene.

Historically, PCR-restriction fragment length polymorphism was the first to be applied [31]. *H. pylori* specific primers were designed, and the amplicons obtained were submitted to restriction enzymes. Indeed, the point mutations associated with Cla resistance induced new restriction sites recognized by *Bsa*I or *Bsb*I, for example, leading to two bands instead of one [31]. This method has been surpassed by real-time PCR which gives a quicker result without the need to manipulate the amplicons produced, which is in itself a source of PCR contamination.

There are several formats of real-time PCR using either the SYBR Green I fluorophore or a biprobe in order to apply the fluorescence resonance energy transfer (FRET) principle. The amplification is performed directly on gastric biopsy samples and is followed by a melting curve analysis of the amplicons. The melting temperature is different between the wild-type strain and the mutants because of mismatches [87].

This method has even been applied to detect *H. pylori* and its resistance on stool specimens [88], but because of the difficulty to obtain purified DNA from stools, its sensitivity is not optimal. Kits using this technique are currently available.

There is also the possibility to detect *H. pylori* and its Cla resistance without DNA amplification by fluorescence in situ hybridization (FISH). Two probes with different labeling are used; one targeting the 16S rDNA to detect *H. pylori* and the other targeting the 23S rDNA to detect the mutations. This method can be applied on histological preparations [89].

When compared to phenotypic methods, molecular methods, especially those based on real-time PCR, lead to better results (1) for detection of *H. pylori*, compared to culture or histology, and (2) for detection of resistance, compared to the standard antibiogram. Indeed, real-time PCR allows a better detection when there is a mixture of susceptible and resistant organisms in a population. However, this heteroresistance may not be clinically significant. A study showed that resistance detected by Etest was a better predictor of the clinical outcome than PCR, since the extra hetero resistance detected could be eradicated [90]. Other studies are needed.

2.3.1 Fluoroquinolones

Real-time PCR based on melting curve analysis of the amplicons has also been applied to the detection of mutations associated with *H. pylori* resistance to Lvf. However, the results are more difficult to interpret because a number of silent mutations can be present in the QRDR [30]. For this reason a DNA strip test has been proposed. This method includes two steps: first, a multiplex PCR which allows an amplification of the relevant parts of the *gyr*A gene (for FQ resistance) as well as those concerning the 23S rRNA gene (for macrolide resistance) and, second, hybridization of the amplicons with biotin-labeled oligodeoxynucleotides immobilized on a strip and then visualized by a streptavidin-alkaline phosphatase reaction.

This test is commercially available (GenoType HelicoDR, Hain Lifescience GmbH, Germany). Its sensitivity and specificity were found to be satisfactory for both macrolide and FQ resistance [91].

2.3.2 Tetracycline

A PCR-RFLP was first developed using the Hinf1 restriction enzyme [65]. Later two real-time PCR were also proposed [92, 93], but, given the rarity of Tet resistance, they are rarely used.

2.3.3 Other Antibiotics

To detect the *rpo*B mutation associated with Rif resistance, no specific test has been developed, but it is possible to amplify and sequence the gene.

For Mtz, because a number of mutations present on different genes appear to be involved in resistance, there is no possibility to use a simple molecular test.

No molecular test has been developed to detect Amx resistance.

2.4 Clinical Significance of Resistance

H. pylori treatments are all complex. They are comprised of three or four drugs. The standard triple therapy consists of Cla, a PPI, and Amx or Mtz. Quadruple therapies containing bismuth salts or not are also used. Antimicrobial resistance is the first cause of failure of eradication therapies. However, this resistance does not concern all antibiotics at the same level. There is a clinical impact if the prevalence of resistance is high and if, in the case of resistance, there is a high rate of failure. In this respect, Cla is mainly concerned. We reviewed the clinical trials performed between 1999 and 2003 with the Cla-based triple therapy where Cla susceptibility was determined [94]. With PPI-Cla-Amx, the rate of success decreased by 70 % (from 87.8 % when the strain was Cla susceptible to 18.3 % when it was resistant). The same scenario was published later on by Fischbach et al. (66 % reduction) [95]. With PPI-Cla-Mtz, the decrease was only 47 %, i.e., from 97 % to 50 %. More recent studies confirmed these data. In contrast, in the same clinical trial review, the impact of Mtz resistance was less important: a decrease of 25 %, from 97 % to 72.6 % for PPI-Cla-Mtz [94] (35 % reduction in Fischbach et al. trial) [95] and from 89.4 % to 64.4 % for PPI-Amx-Mtz. When metronidazole is used in a quadruple therapy including bismuth such as the drug Pylera® as the 3-in-1 capsule containing bismuth subcitrate-Tet-Mtz administered with a PPI, only a 5 % decrease in success was observed for Mtz-resistant strains [96].

The other antibiotic for which the clinical significance is important is Lvf, as is pointed out in the few studies where susceptibility testing was performed. A 14-day treatment of PPI-Amx-Lvf led to 97.3 % eradication for FQ susceptibility strains but only 34.5 % for FQ-resistant strains in China [97]. In contrast, resistance to the other antibiotics is so rare that their impact cannot be determined.

3 *Campylobacter jejuni/coli*

Most cases of *Campylobacter* enteritis are sporadic in nature, occurring in individuals or small groups [98]. In infected humans, gastroenteritis is usually indistinguishable from that caused by other enteric bacterial pathogens such as *Salmonella* and *Shigella* [99]. In the USA, most studies to determine risk factors have identified consumption of unpasteurized milk, or contaminated food, especially poultry [100]. *Campylobacter* colonize a wide variety of mammalian and avian species. Among food animals, *C. jejuni* is most often isolated from chickens and cattle, while *C. coli* is more commonly isolated from pigs and turkeys [101]. Although the organisms can be transmitted directly to humans from farm animals [102] and pets [103–106], undercooked or mishandled fresh poultry meat is considered one of the main sources of infection [100, 107–112]. Therefore, interventions have focused on reducing the prevalence of *Campylobacter*-positive poultry flocks destined for human consumption or the freezing or further processing of meats derived from colonized birds [107].

Depending on host factors, inoculum size, and strain virulence, symptoms follow 1–7 days after ingesting the organism. In the only reported study with human volunteers [113], the infectious dose for some strains was as low as 500 organisms. Symptoms usually consist of diarrhea (with or without blood) with severe abdominal pain and fever. Headache, myalgia, and nausea are also common. Extraintestinal infections include cholecystitis, pancreatitis, hepatitis, bacteremia, and peritonitis [114]. Intestinal symptoms usually resolve within 3–7 days, and primary treatment consists of fluid and electrolyte replacement. As with other types of bacterial gastroenteritis, campylobacteriosis is usually self-limiting. Antimicrobial chemotherapy may be necessary only in cases of severe, relapsing, or invasive illness [99].

A number of different laboratory approaches have been examined for isolating *Campylobacter* on primary culture medium. Most clinical laboratories employ selective culture methods optimized for the recovery of *C. jejuni* and *C. coli*, which requires incubation in a microaerobic atmosphere (5 % O_2, 10 % CO_2, and 85 % N_2) at elevated temperatures (42 °C). Primary culture usually includes a medium containing one or more antimicrobial agents (e.g., cefoperazone) to inhibit competing enteric flora. Using this approach, Gram-stained smear showing small curved or spiral bacilli from typical *Campylobacter* colonies is a very reliable presumptive diagnosis. Additional chemical and genetic methods can be used to confirm the identification and determine species.

When antimicrobial therapy is indicated, erythromycin (Ery) or one of the newer macrolides such as Cla or azithromycin (Azi) is currently considered the drug of choice for treating culture-confirmed cases of campylobacteriosis [115]. Because symptoms are indistinguishable from salmonellosis and other diarrheal illness, a FQ is often given empirically in

adults [99]. In some countries, Tet and doxycycline (Dox) have been used therapeutically, but resistance to these agents can be common. Serious systemic infections are often treated with an aminoglycoside or a carbapenem [114, 116]. Although often used for other diarrheal disease, third-generation cephalosporins have not proven effective for treating *Campylobacter* infections, other than bacteremia due to *Campylobacter fetus* [117]. Clindamycin and tigecycline show potent in vitro activity and may prove valuable for treating infections.

3.1 In Vitro Antimicrobial Susceptibility Testing and Interpretive Criteria

The relatively recent recognition of *Campylobacter* as a common cause of diarrhea, the self-limiting nature of most infections, and the fastidious growth requirements of the organism all contributed to a delay in developing standardized in vitro susceptibility testing methods for this organism. In the absence of formal multi-laboratory trials, laboratories used an array of methods that differed in testing conditions and often lacked proper quality control parameters. The need for a standardized method became acute when surveillance studies began reporting rising resistances [118] that rendered empirical therapy less reliable.

A method based on agar dilution was the first testing process formally standardized, which consisted of quality control parameters for five antimicrobial classes [119]. A broth microdilution susceptibility testing method for *Campylobacter* was later developed, with quality control ranges for 14 antimicrobial agents [120]. This method requires testing in Mueller-Hinton broth supplemented with 2–5 % lysed horse blood and incubation in a humid atmosphere of 10 % CO_2 and 5 % O_2. Testing can be done at either 36–37 °C/48 h or 42 °C/24 h, the latter for testing thermotolerant species [119]. It is important that testing be done using a well-controlled gas mixture and constant temperature, since not all isolates will grow at incubation temperatures of 35 °C or 43 °C and not all commercially available gas-generating systems produce consistent results [120]. A similar method is published by the EUCAST using similar testing conditions and materials [121].

Other methods have been used to measure antimicrobial susceptibility. Disk diffusion testing is an attractive method because of its flexibility, convenience, and cost. The EUCAST recently published a disk-based testing method for *Campylobacter* that has quality control parameters for three antibiotics, Cip, Ery, and Tet [122]. A variation on the standard disk diffusion susceptibility testing is the use of commercially available antibiotic test disks to screen for resistance. This approach uses the lack of a zone of inhibition (growth up to the edge of the 6 mm disk) as an indicator of acquired resistance. The method works very well to predict resistant to Cip (5-μg disks) and Ery (15-μg disk) in *C. jejuni* [22] the drugs of choice for treating *Campylobacter* infections. The CLSI is currently reviewing a standard disk diffusion method with zone diameter interpretive criteria for Ery, Cip, and Tet.

As with *Helicobacter*, the Etest® method has been used to measure antibiotic susceptibility in *Campylobacter*. The Etest® is convenient and has the advantage of providing MIC values over a wide range (15 $\log_2$ dilutions). Using incubation at 36 °C, it has been observed that, for many agents, the Etest® endpoints fall one or more dilutions above or below those observed using agar dilution [123, 124]. The two methods compare favorably for some drugs, with a reported overall MIC agreement between Etest® and agar dilution ranging from 62 % [123] to 83 % [125]. The Etest® works well to predict strains above or below the clinical breakpoint, but is not consistent for monitoring drifts in MICs to detect emerging trends.

3.2 Interpretation of Susceptibility Testing Methods

There are two general approaches used to interpret antimicrobial susceptibility testing results, one for clinical purposes (clinical breakpoints) and one for monitoring purposes (epidemiological cutoff values, ECOFFs or ECVs). In order to establish clinical breakpoints, three data sets are normally required: (1) data on population MIC distributions generated using validated in vitro methods, (2) information on the pharmacokinetic/pharmacodynamic properties of the drug at the site of infection under specific dosing conditions, and (3) clinical outcome data on drug efficacy. Because controlled clinical studies are largely lacking, clinical breakpoints have not been formally established for *Campylobacter*. As noted above, formal clinical breakpoints are only established for a single anti-*Helicobacter* agent, clarithromycin.

In the absence of clinical outcome data, both the CLSI and EUCAST have resorted to ECOFFs for interpreting *Campylobacter* susceptibility data. ECOFFs are based only on MIC (or zone diameter) distributions. The standard ECOFF method distinguishes wild-type from non-wild-type populations, where the breakpoint is set at the highest MIC value of the susceptible population [126].

Both the CLSI and EUCAST publish ECOFFS (Table 62.2) along with in vitro susceptibility testing methods. Using population MIC data, the CLSI established tentative MIC breakpoints for resistance to Cip (MIC ≥ 4 μg/mL), Ery (MIC ≥ 32 μg/mL), doxycycline (MIC ≥ 8 μg/mL), and Tet (MIC ≥ 16 μg/mL) by defining resistance as the lowest MIC of the resistant population [22]. These values are highly correlated with the presence of known acquired resistance determinants and were established to help guide therapy.

Table 62.2 Interpretive criteria for *Campylobacter* susceptibility testing

Antimicrobial agent[a]	Organization	Disk content (μg)	Zone diameter (mm)[b]			MIC (μg/mL)		
			S	*I*	*R*	*S*	*I*	*R*
Erythromycin	CLSI	15	>6	>6	6	≤8	16	≥32
	EUCAST, *C. jejuni*	15	≥20	–	<20	≤4	–	>4
	EUCAST, *C. coli*	15	≥24	–	<24	≤8	–	>8
Ciprofloxacin	CLSI	5	>6	>6	6	≤1	2	≥4
	EUCAST	5	≥26	–	<26	≤0.5	–	>0.5
Tetracycline	CLSI	–	–	–	–	≤4	8	≥16
	EUCAST	30	≥30	–	<30	≤2	–	>2
Doxycycline	CLSI	–	–	–	–	≤2	4	≥8

[a]Erythromycin can be used to determine susceptibility to azithromycin (CLSI and EUCAST) and clarithromycin (CLSI, EUCAST), and tetracycline can be used to determine susceptibility to doxycycline (EUCAST)
[b]According to CLSI, no zone of inhibition (growth up to the edge of a 6-mm disk) indicates acquired resistance to macrolides and ciprofloxacin. Appearance of any zone of inhibition would require MIC determination for accurate categorization of susceptibility

ECOFFs are intended to identify non-wild-type bacteria for surveillance purposes, whereby MIC breakpoints are based on the highest MIC of the susceptible population. EUCAST publishes ECOFFS breakpoints for 14 antimicrobial agents including Cip (MIC>0.5 μg/mL), Ery (MIC>4 μg/mL), and Tet (MIC>2 μg/mL). These values are intended to identify strains with reduced susceptibility, which may include organisms still responsive to antibiotic therapy (i.e., not clinically resistant).

It should be noted that the EUCAST has decided to offer *Campylobacter* ECOFF values as clinical breakpoints in the absence of clinical outcome data [127]. While this lack of harmonization in determining and classifying breakpoints is being addressed by the CLSI and EUCAST, and although the breakpoints are many dilutions apart, it is important to note that when the two approaches are applied to the same *Campylobacter* data set, they match very well in categorizing strains with acquired resistance traits. This is because members of this genus generally exhibit broadly bimodal MIC distributions, with few intermediate phenotypes, for most antibiotics.

3.3 Clinical Significance of Resistance

Campylobacter enteritis is usually a self-limiting disease where treatment often consists of fluid and electrolyte replacement. Antimicrobial therapy is indicated for patients with high severe or relapsing enteritis, fever, or extraintestinal infections. In these cases, resistant strains limit therapeutic options.

There is conflicting evidence that antimicrobial resistance causes adverse health outcomes in patients with *Campylobacter* infections. The first report by Smith et al. [128] calculated that, among subjects treated with a quinolone, the median duration of diarrhea was 7 days if the causative strain was susceptible vs. 10 days if it was resistant. Engberg et al. [129] also observed a longer duration of illness in patients with a quinolone-resistant *C. jejuni* infection (median 13.2 days), compared to patients infected with a susceptible strain (median 10.3, $p=0.01$). Based on the analysis of 3471 patients with *Campylobacter* infections, quinolone resistance was associated with a sixfold increased risk of invasive illness or death within 30 days of infection [130]. Comparing infections caused by quinolone-resistant and quinolone-susceptible strains, one study estimated a 2-day increase in duration of diarrhea caused by resistant strains (9 vs. 7 days) [131]. This difference was greater among subjects who did not take antidiarrheal medications or antimicrobial agents (12 vs. 6 days). In contrast, Wassenaar et al. found no difference in duration of disease between cases caused by FQ-resistant and FQ-susceptible infections [132]. In a UK study of 653 patients, no association was detected in a cohort study of resistant and susceptible infections acquired abroad [133].

There are plausible biologically reasons to think that resistance and virulence might be linked, such as through the action of efflux pumps that have both antibiotics and bile components as substrates [134]. Hypothetically, increased bile resistance increases the survivability of the organism as it transits the upper gastrointestinal tract to colonize distal sites in the gut and cause illness, as it is a known colonization factor for *Campylobacter* [135]. In addition, cell culture assays showed that certain FQ-resistant mutants of *C. coli* and macrolide-resistant mutants of *C. jejuni* had higher rates of invasion, and in some cases cytotoxicity, of Caco-2 cells than their isogenic parental strain [134, 136]. The links between resistance and virulence are poorly understood, and further work is needed to understand the clinical importance of this phenomenon.

3.4 Resistance to Specific Antimicrobials

The genetic elements underlying *Campylobacter* resistance include the common chromosomal and plasmid-borne mechanisms present in other bacteria, namely, target-site modification, structural gene mutation, enzymatic inactivation, and energy-dependent drug efflux. Resistance to the major and relevant antimicrobial drug classes is presented below.

3.4.1 Macrolides

Macrolides are considered a primary treatment for *Campylobacter* infections. In many other countries, resistance is uncommon in *C. jejuni* with approximately 1–2 % of human isolates showing Ery MICs ≥8 μg/mL in the USA [2]; however, higher rates have been reported in other countries [137–140]. Resistance to macrolides (and other antimicrobials) is usually higher in *C. coli*, where resistance to Ery resistance ranges from 3 to 9 % in the USA [2].

In *C. jejuni* and *C. coli*, macrolide resistance is caused by target-site mutations and efflux. As in other bacteria, macrolide resistance results from target-site mutations in two positions of domain V (peptidyl transferase region) of the 23S rRNA genes. *Campylobacter* contains three copies of the rRNA gene; evidence suggests that at least two copies must be mutated to cause resistance [141]. Ribosomal gene mutations are present only in isolates with Ery MICs ≥32 μg/mL [142], supporting the use of 32 μg/mL as an MIC breakpoint denoting clinical resistance. Nucleotide changes at positions A2074 and A2075 are most common, corresponding in *E. coli* to positions 2058 and 2059 [143]. An A2075G transition is the most frequent mutation observed in clinical strains [141, 144–146]. It is usually present in all three copies of the 23S rRNA gene and can confer high MICs (>128 μg/mL) [141]. In vitro transformation experiments demonstrated that these mutations are readily transferred and stably incorporated into the chromosomes of susceptible *C. jejuni* and *C. coli* strains [141, 147]. Ribosomal mutations in *Campylobacter* can confer cross-resistance to tylosin, Azi, and Cla. Ribosomal mutations imparting Ery resistance also impact susceptibility to tylosin and Azi, but the MICs to those latter drugs are not always equivalent to those of Ery [148].

The only example of an extrachromosomal macrolide resistance determinant in *Campylobacter* is a plasmid-encoded rRNA methylase (*erm*). Until recently, this determinant had been found only in *C. rectus* [149]. A report by Wang et al. [150] examined 1554 *Campylobacter* from human and food-producing animal sources in China and found *erm*(B) in 58 strains, 57 of which were *C. coli* with 40 % on plasmids. This finding implies that the epidemiology of macrolide resistance might change in *Campylobacter*, with the possibility of horizontal spread.

It is known that efflux plays a role both in baseline Ery susceptibility levels and acts synergistically with other factors in elevated MICs conferring clinical resistance [142, 151]. The first report of a multidrug efflux system in *Campylobacter* was made by Charvalos et al. [152] using *C. jejuni* mutants selected on pefloxacin and on cefotaxime. The MDR phenotype included β-lactams, quinolones, chloramphenicol (Chl), and Tet, in addition to macrolides, but the genes were not identified.

In resistant clinical isolates, studies by Lin et al. [153] and by Pumbwe and Piddock [154] identified an efflux system encoded by the *cmeABC* locus. CmeB is related to multidrug transporters of the resistance nodulation and cell division (RND) superfamily, which includes AcrB in *E. coli* and MexB in *Pseudomonas*. This pump extrudes a variety of structurally unrelated antimicrobials, as well as detergents and dyes, and is widespread in *C. jejuni* and *C. coli* [155]. CmeABC also confers resistance to bile and is consequently required for intestinal colonization in chickens [156]. Inactivation of *cme* yielded a 4- to 16-fold reduction in Ery MICs in wild-type susceptible strains [153, 157]. Overexpression of *cmeB* also confers resistance to ampicillin, Chl, and Tet. A second macrolide efflux phenotype was revealed by exposure to the efflux pump inhibitor Phe-Arg-ß-naphthylamide (PAßN). This compound increased Ery susceptibility to wild-type levels in intermediately susceptible strains and to a lesser degree in resistant strains. Furthermore, it made a wild-type isolate hypersusceptible [151]. Further characterization of this phenotype confirmed that this pump was independent of *cmeB* [158]. Ge et al. examined ten putative *Campylobacter* efflux pumps, including CmeB and CmeF, which were identified based on sequence homology. Using site-directed mutagenesis, they found that only *cmeB* influenced susceptibility to Chl, Ery, nalidixic acid, and Tet [159]. Recent studies by Xia et al. [160] have taken a genomics approach to understanding macrolide resistance in *C. jejuni*. This work identified the upregulation of various other efflux pumps and other adaptive responses to macrolide exposure that will open new avenues to understanding macrolide resistance in *Campylobacter*.

3.4.2 Fluoroquinolones

In contrast to the relatively low and stable incidence of macrolide resistance to date, FQ-resistant *C. jejuni* has emerged in many countries over the past two decades [118, 143, 161, 162]. This rise has been attributed in part to the use of FQs (sarafloxacin and enrofloxacin) in poultry medicine. Endtz et al. reported that the emergence of FQ resistance in human *C. jejuni* infections in the Netherlands coincided with the approval of enrofloxacin in poultry in 1987 [161]. In Minnesota from 1992 to 1998, the number of quinolone-resistant infections increased from 1.3 to 10.2 %. In only 2 years after approval of the poultry FQ sarafloxacin in 1995, Cip^R among *Campylobacter* in Minnesota had doubled. Part

of this increase was attributed to the acquisition of resistant strains from poultry meats [128]. In a study examining *C. jejuni* infections among patients treated at Philadelphia-area hospitals, Nachamkin et al. reported Cip^R rising from 8.3 % in 1996 to 40.5 % in 2001 [163].

Among human *C. jejuni* isolates submitted to the CDC, Cip^R rose from 0 % in 1989–1990 [118] to 21.6 % in 2005 and was detected in about 25 % of isolates in 2012 [2]. In 2012, NARMS data also showed that 16.4 % of *C. jejuni* isolated from retail chicken breast samples were Cip^R [164]. The epidemiological and microbiological associations of Cip resistance *Campylobacter* in humans to selection in the poultry production environment prompted the Food and Drug Administration to withdraw approval of FQs in poultry [165], which became effective in September 2005.

Cip^R in *Campylobacter* results from a single topoisomerase mutation in *gyrA*, similar to that seen in *H. pylori*, but unlike *Salmonella* and *E. coli*, in which two mutations are required for clinical levels of Cip resistance [166]. The sufficiency of a single mutation in *Campylobacter gyrA* does not appear to be the case for all FQs. A study by Ruiz et al. [167] showed that moxifloxacin resistance required double topoisomerase mutations (Ile86, Asn90), suggesting that the efficacy of this newer FQ may be less subject to compromise by *gyrA* mutations. For Cip, however, the most common mutation associated with high-level MICs (≥32 μg/mL) is a substitution of Ile at Thr86 [168–171]. Mutations at Asp90 and Ala70 [168, 172] impart intermediate levels of Cip resistance (MICs 1–4 μg/mL). A small number of *Campylobacter* with resistance to nalidixic acid but not to Cip have been associated with a Thr86Ala substitution in *gyrA* [173]. Other *gyrA* mutations have been detected, but their respective contributions to quinolone resistance have not been measured [174]. No changes in GyrB have been associated with FQ resistance, and *C. jejuni* lacks the *parC* gene encoding topoisomerase IV [175]. The requirement of only a single-base change for high-level Cip MICs helps explain the rapid evolution of Cip^R in *Campylobacter* from animals [176, 177] and humans [178] exposed to FQs, as well as the widespread occurrence of Cip^R in retail raw meats [164] and human clinical isolates [2].

Multidrug efflux pumps, including CmeB, contribute to baseline levels of FQ susceptibility in *Campylobacter*. Wild-type susceptible isolates of *Campylobacter* display higher Cip MICs (0.125–0.5 μg/mL) than do wild-type strains of other Gram-negative enterics such as *E. coli* and *Salmonella* (MIC, 0.015–0.06 μg/mL). This intrinsic resistance appears to result from the constitutive expression of *cmeB* [153, 154]. Inactivation of *cmeB* by site-directed mutagenesis lowered Cip MICs in susceptible isolates to levels in the range for *E. coli* and *Salmonella* [153, 154]. Similarly, in resistant strains (also containing *gyrA* mutations), inactivation of the c*meABC* operon reduced Cip MICs near to that of wild-type isolates [179]. These findings show that, as with macrolide resistance [148], *cmeB* functions cooperatively in isolates with target-site mutations to maintain acquired high-level FQ MICs in *Campylobacter*. The expression of *cmeB*, and perhaps of *cmeF* as well as other uncharacterized loci [157], likely also contributes to acquired quinolone/multidrug resistance.

A putative efflux pump encoded by the *cmeG* gene has also been shown to play a role in both Cip and multidrug resistance in *C. jejuni* [180]. Insertional inactivation of *cmeG* caused a fourfold reduction in Cip MICs compared to the wild-type parental strain. Complementation in *trans* restored susceptibility to near wild-type levels and led to an eightfold increase in Cip MICs when *cmeG* was overexpressed.

3.4.3 Tetracycline

Tetracycline is considered as a second-line treatment for *Campylobacter*. It is used mainly in developing regions due to its low cost and low toxicity. Resistance to tetracycline has risen in many countries, making this class of antimicrobials less attractive for therapy. In Canada, Tet^R has increased from 7 to 9 % in 1980–1981 [181] to 43–68 % in 1998–2001 [182], with more recent resistant strains also showing even higher MIC values [183]. In the USA from 1997 to 2002, Tet^R ranged from 38 to 48 % [184]. In some countries, the proportion of resistant isolates is much higher [185, 186].

While efflux plays a role [159], Tet^R is mainly due to ribosomal protection mediated by the *tet*(*O*) gene product [187]. Tet(O) confers resistance by allosterically displacing tetracycline from its primary binding site on the ribosome [188, 189]. The *tet*(*O*) gene is prevalent in *Campylobacter,* worldwide, and is also present in various Gram-positive species. Alleles of *tet*(*O*) in *C. jejuni* usually impart MIC levels of tetracycline ranging from 32 to 128 μg/mL, but mutations in *tet*(*O*) can lead to MICs as high as 512 μg/mL [183].

The *tet*(*O*) gene is usually plasmid-borne [190] but may be located on the chromosome [191]. Two large self-transmissible Tet^R plasmids were sequenced by Batchelor et al. [192], one from *C. jejuni* and one from *C. coli*, which isolated on separate continents about 20 years apart. Both plasmids had mosaic sequence structures, with gene signatures suggesting origins in various commensal and pathogenic bacteria, including *H. pylori*. Remarkably, the two plasmids were 94.3 % identical at the DNA sequence level and are widespread in plasmid-containing Tet^R *Campylobacter* isolates [192]. Other plasmid vehicles, ranging in size up to 100 kb, also carry Tet^R determinants [190]. More recent whole genome sequencing data confirm *tet(O)* as the sole determinant for tetracycline resistance in *Campylobacter* to date [193].

3.4.4 Aminoglycosides

The genetic determinants that cause aminoglycoside resistance are well known and diverse in numerous bacteria. In *Campylobacter*, kanamycin resistance is due to the presence of the *aphA-3* gene [194], usually located on large plasmids (40 to >100 kb) that often carry *tet*(*O*) as well [195]. Integrons also have been identified in *Campylobacter* [196, 197], which in one report were found to be common (16.4 %) in isolates from different sources and to contain the aminoglycoside-modifying enzyme encoded by *aadA2* [198]. Spectinomycin/streptomycin resistance due to adenylyltransferases encoded by *aadA* and *aadE* has been associated with plasmids from human clinical isolates [199]. Resistance to the aminoglycoside streptothricin has been linked to the *sat4* gene product in animal and clinical isolates from Europe [200]. In isolates recovered from a poultry production house, integrons carrying the *aacA-4* gene were detected in isolates resistant to tobramycin and gentamicin [196].

In the USA, gentamicin resistance began a rapid upward trend mainly in *C. coli*, beginning in 2008 and peaking in human and retail chicken isolates in 2010 at 11.3 % and 12.8 %, respectively. Whole genome sequencing of two *C. coli* strains revealed an array of plasmid-borne resistances for gentamicin, kanamycin, streptomycin, streptothricin, and tetracycline on the pTet plasmid backbone [201]. GenR was conferred by a phosphotransferase encoded by *aph(2")-Ig*, along with *tet(O)*, *aad9*, *hph*, *aadE*, *sat4*, and *aphA*-3 [201].

3.4.5 Other Resistances

Most *Campylobacter* strains are resistant to β-lactam antimicrobials, with over 80 % of *C. jejuni* carrying β-lactamases [202]. *C. jejuni* are resistant to cefamandole, cefoxitin, and cefoperazone. Most isolates also are resistance to cephalothin and cefazolin, and resistance is variable for cefotaxime, moxalactam, piperacillin, and ticarcillin [203]. The most active β-lactam agents include ampicillin, amoxicillin, cefpirome, and imipenem [202]. Meropenem also shows good activity against *Campylobacter* [204] and has been recommended as a treatment option [205, 206].

Campylobacter are generally resistant to trimethoprim and sulfonamides, through mechanisms common to other bacteria. Trimethoprim resistance in *C. jejuni* is due to the chromosomal presence of acquired trimethoprim resistance-associated dihydrofolate reductase gene cassettes (*dfr1*, *dfr9*) [207]. Sulfonamide resistance in *Campylobacter*, as in other bacteria, results from mutations in dihydropteroate synthase [208], while streptomycin resistance is linked to the *rpsL* gene [209]. Chl resistance is rare in *Campylobacter* and results from acetylase activity encoded by *cat* genes [210]. In vitro selection identified a novel point mutation (G2073A) in all three copies of the 23S rRNA genes, which conferred resistance to Chl and florfenicol [211].

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Antimicrobial Resistance of Anaerobic Bacteria

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1 Introduction: Infections Caused by Anaerobic Bacteria

Infections caused by anaerobic bacteria are common and may be serious and life-threatening. Anaerobes are the predominant components of the bacterial flora of normal human skin and mucous membranes [1] and they are a common cause of bacterial infections of endogenous origin. Because of their fastidious nature, they are difficult to isolate from infectious sites and are often overlooked. Their isolation requires appropriate methods of collection, transportation, and cultivation of specimens [2–5]. Treatment of anaerobic bacterial infections is complicated by the relatively slow growth of these organisms (which makes diagnosis in the laboratory only possible after several days), by the frequent polymicrobial nature of the infection, and by the growing resistance of anaerobic bacteria to antimicrobial agents.

Failure to direct therapy against anaerobic organisms often leads to clinical failures. The inadequate isolation, identification, and subsequent performance of susceptibility testing of anaerobes from an infected site can prevent detection of antimicrobial resistance. Therefore, correlation of the results of in vitro susceptibility and clinical and bacteriological response can be difficult or impossible [1, 3, 6]. This discrepancy occurs because of a variety of reasons. Individuals may improve without antimicrobial or surgical therapy and others can get better because of adequate drainage. In some instances of polymicrobial infection, eradication of the aerobic component may be adequate, although it is well established that it is important to eliminate the anaerobic pathogens [2, 7–14].

Reasons that may lead to failure in therapy include: variation in duration, severity, and extent of infection; lack of surgical drainage, or poor source control; patient age, nutritional status, and comorbidities; impaired host defenses; antimicrobial poor penetration and low levels at the site of infection; enzymatic inactivation of antimicrobials; low pH at the infection site; and inaccuracies in the susceptibility testing procedure.

Despite all of these factors, a correlation between the antimicrobial resistance of the anaerobic pathogens and poor clinical outcome has been reported in several retrospective studies [7–9]. There are a number of studies showing that inappropriate therapy will directly affect clinical outcome [10–15].

Microbiological semi-quantitation of all of the infecting flora is important; it is not necessary to eliminate all of the infecting organisms because reduction in counts or modification of the metabolism of certain isolates alone may be sufficient to achieve a good clinical response. Synergy between two or more infecting organisms, which is a common event in anaerobic infections, may confuse the clinical picture.

A prospective study of *Bacteroides* bacteremia reported the adverse clinical outcomes in 128 patients who were treated with an antibiotic to which the organism was resistant [14]. Clinical outcome was correlated with results of in vitro susceptibility testing of the isolates recovered from blood and/or other sites, and was determined by three endpoints: mortality at 30 days, clinical response (cure vs. failure), and microbiological response (eradication vs. persistence). The mortality rate among those who received inactive treatment (45 %) was higher than among patients who received active therapy (16 %; $P=0.04$). Clinical failure (82 %) and microbiological persistence (42 %) were higher for those who received inactive therapy than for patients who received active therapy (22 % and 12 %, respectively; $P=0.0002$ and 0.06, respectively). In vitro activity of agents directed at *Bacteroides* spp. reliably predicts outcome (specificity 97 %, and positive predictive value 82 %). The authors conclude that the antimicrobial susceptibility testing should be performed for patients whose blood specimens yield *Bacteroides* spp. [14].

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Table 63.1 Anaerobic infections for which susceptibility testing is indicated

1. Serious or life-threatening infections (e.g., brain abscess, bacteremia, or endocarditis)
2. Infections that failed to respond to empiric therapy
3. Infections that relapsed after initially responding to empiric therapy
4. Infections where an antimicrobial will have a special role in the patients' outcome
5. When an empirical decision is difficult because of absence of precedent
6. When there are few susceptibility data available on a bacterial species
7. When the isolate(s) is often resistant to antimicrobial
8. When the patient requires prolonged therapy (e.g., septic arthritis, osteomyelitis, undrained abscess, or infection of a graft or a prosthesis)

These findings emphasize that it is important to perform susceptibility testing to isolates recovered from selected cases to guide therapeutic choices. Susceptibility testing should be performed to organisms recovered from sterile body sites, those that are recovered in pure culture, and isolates that are clinically important and have variable or unique susceptibility (Table 63.1). The standardization of testing methods by the Clinical Laboratory Standards Institute (CLSI) (Wayne, PA) allows for comparison of resistance trends among various laboratories [15–17]. Organisms that should be considered for individual isolate testing include highly virulent pathogens for which susceptibility cannot be predicted, such as *Bacteroides*, *Prevotella*, *Fusobacterium*, and *Clostridium* spp., *Bilophila wadsworthia*, and *Sutterella wadsworthensis*.

The routine susceptibility testing of all anaerobic isolates is very time-consuming and is not cost-effective. However, susceptibility testing should be performed for epidemiological and survey purposes for a limited and selected number of anaerobic isolates. Antibiotics tested should include penicillin, a beta-lactam plus a beta-lactamase (BL) inhibitor combination, clindamycin, metronidazole, and a carbapenem (i.e., imipenem, meropenem, or ertapenem). If needed, ancillary susceptibility can be performed for cefoxitin, tigecycline, and moxifloxacin that have approved anti-anaerobe indications.

Antimicrobial resistance among anaerobes has consistently increased in the past 30 years and the susceptibility of anaerobes to antimicrobials has become less predictable. The most commonly isolated antibiotic-resistant anaerobes are members of the *Bacteroides fragilis* group [18]. Resistance to several antimicrobial agents by *B. fragilis* group species and other anaerobic Gram-negative bacilli (AGNB) has increased over the past decade [15–17, 19–22]. Resistance has also increased among other anaerobes such as *Clostridium* spp. that were previously very susceptible. This increase makes the choice of appropriate empirical therapy even more difficult. Resistance patterns have been monitored through national and local surveys, but susceptibility testing of anaerobic bacteria at individual hospitals is rarely done [20].

2 Susceptibility Patterns of Anaerobic Bacteria

The increase in antibiotic resistance among anaerobes generated extensive studies of the mechanisms of resistance and resistance-gene transfer. These investigations brought about more insight into the causes of the rapid development of resistance. The observed resistance patterns to different antibiotics vary among the different groups of organisms as variations in the mechanisms of resistance exist.

2.1 Antimicrobial Resistance of Specific Anaerobic Species

Resistance among some anaerobes and especially *B. fragilis* group to all classes of antimicrobials has increased significantly over the last few decades [23–26]. Ongoing in vitro surveillance studies in the USA have reported significant increases in resistance among the *B. fragilis* group strains since the 1980s [27, 28]. These studies observed that there are unpredictable variations between medical center in susceptibility patterns. Variations in clindamycin activity were observed amongst different Chicago area hospitals [29]. Geographic area, sources of isolates, or quality control (QC) reading variations may also cause discrepancies even when the same methodology is employed. Caution must therefore be used in extrapolating survey report susceptibility data to an individual patient.

All anaerobes are resistant to aminoglycosides and sulfamethoxazole-trimethoprim. Chloramphenicol resistance is extremely rare although there is clustering of MICs around the breakpoint for some strains. When resistance is detected, it is due to inactivation of the drug by nitroreduction or acetyltransferase; this agent is also rarely used clinically in the USA due to potential hematopoietic toxicity.

2.1.1 *Bacteroides fragilis* Group

The *B. fragilis* group has 23 species; *B. fragilis* generally being the most susceptible to antimicrobials, although greater than 95 % are resistant to penicillin mostly due to BL production. A survey of 5223 *B. fragilis* group isolates from ten geographically US medical centers analyzed the trends from 1997 to 2004 using the reference agar dilution method [28]. The species isolated were *B. fragilis* (52.1 % of isolates), *B. thetaiotaomicron* (18.7 %), *B. ovatus* (10.4 %), *B. vulgatus*

(5.9 %), *Parabacteroides distasonis* (5.2 %), *B. uniformis* (3.2 %), and other species (4.5 %). Unexpectingly, the study found an increased susceptibility over the study period with decreases in geometric mean MICs for imipenem, meropenem, Piperacillin/tazobactam, and cefoxitin. *B. fragilis* was more susceptible to antimicrobials than the other species, while *P. distasonis* was the most resistant to beta-lactams. There was, however, an increase in geometric mean MICs to clindamycin and moxifloxacin for some isolates.

Of the ureidopenicillins, piperacillin is the most active against the *B. fragilis* group, even though susceptibility has declined from approximately 90–70 % over the study period [28]. Beta-lactam-resistant penicillins (e.g., oxacillin, nafcillin) and first-generation cephalosporins are not active against these organisms. Beta-lactam/BL inhibitor (BL/BLI) combinations, such as ampicillin/sulbactam, amoxicillin/clavulanate, ticarcillin/clavulanate, and piperacillin/tazobactam, are effective against nearly all *B. fragilis* group strains, with <2 % resistance [30, 31]. A study from Taiwan [25], however, reported 48 % resistance to ampicillin/sulbactam of *B. fragilis* group isolates. A European survey [26] observed a 10 % resistance of *Bacteroides* species to both amoxicillin/clavulanate and piperacillin/tazobactam.

Cefoxitin and cefotetan are generally active against *B. fragilis* but the latter is much less effective against the other members of the *B. fragilis* group [13, 26, 32]. Clindamycin resistance of about 40 % is reported against *Bacteroides* spp. worldwide [25, 28, 30, 31]. Chloramphenicol, metronidazole, tinidazole, and the carbapenems (imipenem, ertapenem, doripenem, and meropenem) are generally active against all members of the *B. fragilis* group [28, 33] although imipenem- and metronidazole-resistant strains have been recovered [25, 34]. A recent report from Taiwan [15] found the rates of nonsusceptibility to imipenem and meropenem was 7–12 % for *B. fragilis* and 3–7 % for *B. thetaiotaomicron*. Only five clinical cases of metronidazole resistance have been reported worldwide so far [34].

Resistance to fluoroquinolones in *B. fragilis* group species and other anaerobes is increasing. Trovafloxacin was approved for therapy of anaerobic infections in 1994 but is no longer used because of toxicity concerns. Moxifloxacin is also approved for intra-abdominal and skin and soft tissue anaerobic infections and can have good in vitro activity against *B. fragilis* and a broad range of other anaerobes but less so against *B thetaiotaomicron* [8, 28, 35]. Several studies reported an increase of *B. fragilis* resistance [29, 32, 36].

2.1.2 *Prevotella* and *Porphyromonas*

Prevotella and *Porphyromonas* species are more susceptible to antimicrobials than the *B. fragilis* group. Resistance due to BL production is 50 % in the USA and 94 in Europe [2, 37] and Taiwan [25], and to piperacillin, cefoxitin, and cefotetan ranges from 10 to 30 % [30, 38]. About 8–17 % of *Porphyromonas* spp. strains produce BL [37, 39]. Both genera are uniformly susceptible to carbapenems, metronidazole, and chloramphenicol, although clindamycin resistance has been observed in a few of strains [40].

2.1.3 Other Anaerobic Gram-Negative Bacilli

Penicillin resistance in *Fusobacterium nucleatum* has increased in children due to BL production and related to exposure to antimicrobial agents [41, 42]. A European survey reported 11 % of *Fusobacterium* species to produce BL [26]. More than 90 % of *Fusobacterium* spp. are susceptible to cephalosporins and cephamycins [30, 31]. Four percent of fusobacterium spp. were "nonsusceptible" to imipenem and 7 % to meropenem in a study from Taiwan [25]. *Bilophila wadsworthia* frequently produces BL, but is generally susceptible to clindamycin, cefoxitin, BL/BLI combinations, carbapenems, and metronidazole. *Sutterella wadsworthensis* may demonstrate resistance to clindamycin, piperacillin, and/or metronidazole.

2.1.4 Gram-Positive Organisms

Non-spore forming Gram-positive bacilli. The *Eubacterium* group, *Actinomyces*, *Propionibacterium*, and *Bifidobacterium* are generally susceptible to beta-lactam antimicrobials. *Lactobacillus* spp. exhibit wide species variably in susceptible patterns to cephalosporins and other agents; penicillin and ampicillin are frequently active [43]. There are no breakpoints for vancomycin and anaerobes. However, it has very good in vitro activity against all *Propionibacterium* spp., *Actinomyces* spp., *Eubacterium* group species, anaerobic Gram-positive cocci, and some *Lactobacillus* spp. vancomycin is much less effective against *L. casei* and several other species [43]. Linezolid, daptomycin, and telavancin also possess excellent in vitro activity against most anaerobic Gram-positive species. Most non-spore forming Gram-positive rods are resistant to metronidazole. Moxifloxacin has good activity against *Actinomyces* species, including *A. odontolyticus* and *A. viscosus*, *Pseudoramibacter alactolyticus*, and *Eubacterium limosum* and a variety of lactobacilli with MIC_{90}s <2 μg/mL (36.37). Most *Eggerthella lenta* and *Lactobacillus plantarum* were susceptible to moxifloxacin, there was strains variability and resistance in some isolates.

2.1.5 Clostridia

Clostridium perfringens is generally susceptible to most anti-anaerobic antimicrobials, as well as fluoroquinolones [44]. However, *Clostridium clostridioforme* and *C. innocuum* and *C. difficile* have variable susceptibility [31, 43, 45] and can resist clindamycin, fluoroquinolones, and beta-lactams, but not metronidazole. *C. difficile* is universally susceptible to metronidazole and vancomycin although there has been some MIC creep [19]; The MICs of *C. innocuum* to vancomycin is 8–32 μg/mL [43].

2.1.6 Gram-Positive Cocci

These Gram-positive cocci are very susceptible to all beta-lactams, BL/BLI, cephalosporins, carbapenems, chloramphenicol, and metronidazole [30, 31, 43]. Fluoroquinolone and clindamycin resistance is increasing among skin and soft tissue infections isolates [19]. Streptococci *milleri* group are always resistant to metronidazole.

3 Susceptibility Testing and Their Interpretation

The antibiograms of anaerobes have become increasingly unpredictable and multidrug-resistant clinical isolates are emerging confounding the assumption of foolproof empirical anti-anaerobic therapy [5, 46, 47]. Resistance to even the most effective antimicrobials such as BL/BLI, carbapenems, and metronidazole is documented [46, 48, 49]. Furthermore, there are clear differences in the geographic patterns of resistance, and resistance patterns in various hospitals within the same city [28]. Multidrug-resistant *B. fragilis* group strains have been increasingly reported [6, 11, 14, 22, 50]. Suboptimal therapy was found to select for emergence of antibiotic resistance and induce transfer of resistance determinants and more isolates are manifesting multiple resistance [21, 46].

These factors emphasize the need for antimicrobial susceptibility testing of anaerobes as well as periodic surveillance antimicrobial susceptibility studies to detect geographic or temporal trends. In the last few decades, testing methodologies used have been standardized. The most appropriate susceptibility testing method may differ depending on whether the test is performed for a specific isolate in a hospital laboratory (or by a commercial laboratory) or whether surveillance testing is performed at a hospital or reference laboratory [51].

3.1 Standardization of Testing

The US Clinical and Laboratory Standards Institute (CLSI) evolved from a voluntary consensus organization in 1967 to become a World Health Organization Collaborating Center for Clinical Laboratory Standards and Accreditation. CLSI has standardized the anaerobic susceptibility testing and has published documents for anaerobic susceptibility testing (also called M11) [16]. CLSI policy does not allow it to advocate any commercial technique; it presents two reference methods (agar dilution and broth microdilution) and underscores that other methods such as gradient techniques (generally referring to Etest®) or commercial broth microdilution plates can be used as long as equivalence to the reference methodology are established. CLSI presently recommends the broth microdilution method only for the testing of *B. fragilis* group because many other anaerobes will not consistently grow well in broth media.

Surveillance studies that are done in reference laboratories throughout the world commonly use the CLSI method (see below). The most recent document, M11-A8 was published in 2012 [16]. The CLSI reference standard is not for testing single isolates; it provides a standard against which other methods can be measured.

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has its own breakpoints which are not always identical to those of CLSI [52]. The EUCAST does not specify a testing method for anaerobes. Most susceptibility studies from Europe use CLSI methodology, although breakpoint interpretation is often based on EUCAST recommendations and which lead to differences in reported resistance rates.

Testing methods from Argentina [53] and Japan [54] have been published, which are based on CLSI methodology. European surveillance studies have also used CLSI methodology and frequently include both CLSI and EUCAST breakpoints [4, 51]. Some reports refer to other methods documents; a recent German multicenter study used a specific German testing methodology [47]. The differences between different methods may seem trivial; however in instances where minimal inhibitory concentrations (MICs) cluster around breakpoint values, minimal changes in MICs (due to differences in media, inoculum or endpoint reading method) may generate a perceived significant differences in resistance rates.

3.2 Surveillance Tests for Specific Hospitals or Geographic Regions

Surveillance tests have been performed for several decades by groups worldwide and illuminate overall general trends [18, 22, 25, 28, 47, 55]. However, the data do not necessarily reflect the patterns of specific patients or hospitals. Because of this, CLSI recommends that hospitals conduct at least annual surveillance antimicrobial susceptibility testing to find out their local patterns. The numbers and choice of species of strains tested should reflect the frequency with which they are recovered. At least 50–100 strains should be tested to get an accurate pattern of local isolates, which should include isolates from different body sites. It is recommended that at least 20 isolates of *Bacteroides* spp. and ten from other frequently isolated genera should be tested. The strains should be sent to a reference laboratory for testing if the expertise is not available in the hospital clinical laboratory.

Reference laboratories may use the CLSI-approved methods for the antimicrobials and adjust the tested antimicrobials to reflect the hospital's formulary. Ideally the test should

include at least one agent from each antimicrobial class, even if it is not part of the hospital's formulary.

The results of the surveillance study should be recorded so that trends in emerging resistance may be recognized. If routine surveillance testing cannot be performed, hospitals should summarize their antimicrobial susceptibility test results and generate a hospital-specific antibiogram that can be used if needed. A 2008 survey of clinical hospital laboratories in the USA [20] found that less than half of the laboratories did any anaerobic testing, either in-house or testing sent to an outside laboratory.

3.3 Testing in a Clinical Setting

Susceptibility testing may not be needed for many routine clinical isolates. The CLSI suggests testing isolates from brain abscess, blood, endocarditis, osteomyelitis, arthritis, and infection of prosthetic devices or vascular grafts (Table 63.1). Also, any organism recovered from normally sterile body sites should be tested (if they are not contaminants). Also isolates from patients undergoing long-term therapy, from a therapy failure or in a case in which the therapeutic decisions will be influenced by the results, should be tested.

Organisms to test should include those that are likely to be the most resistant (such as *B. fragilis* group species) or highly virulent (certain *Bacteroides*, *Prevotella*, *Fusobacterium*, *Clostridium*, *Bilophila*, and *Sutterella*), especially if their susceptibility patterns are not predictable. Antimicrobials to be tested should include those on the hospital formulary, and those considered or used for therapy.

Recent surveys of anaerobic susceptibility testing illustrate that only 21 % (21/98) of hospital laboratories performed anaerobic susceptibility testing in-house [20]. This is a sharp decline from earlier rates: in 1990, 70 % performed susceptibility testing [56] and 33 % in 1993 [40]. Blood isolates were always tested when testing was performed. Isolates from sterile body sites were tested by 85 % (17/20) of laboratories and selected surgical wound isolates by 14/20 (70 %). Most hospital laboratories used the Etest® (62 %; 13/21) for susceptibility testing, while only 17 % of reference laboratories used it. Since almost two-thirds of laboratories do not perform testing, the clinicians often chose therapy based on manufacturers' information, FDA's indications, published studies, or their clinical judgment [20].

The majority of commercial labs use Etest® methodology for performing anaerobic susceptibility on isolates sent to them for testing. This testing method is especially suitable for testing one or a few isolates against multiple agents (as long as that agent is available on an Etest® strip). At present, there is no commercially available readymade broth microdilution panels that are "FDA approved" for clinical diagnostic use. Thus, a clinical laboratory would have the option of either using the FDA-approved Etest®, using a noncommercial panel with CLSI-approved methodology, or sending the isolates to a commercial or reference laboratory for testing.

3.4 Testing in a Research or Reference Laboratory

3.4.1 Agar Dilution

Agar dilution requires the incorporation of diluted concentrations of the antimicrobials into a nutrient agar medium followed by the plating standardized number of bacterial cells to the surface of the plate. Plates are read following 48 h of growth by comparing the growth of different strains in the series and the MIC is designated as the lowest antimicrobial concentration that inhibits growth. The CLSI method specifies using control strains including *B. fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Clostridium difficile* ATCC 700057.

3.4.2 Broth Microdilution

In this assay, a polystyrene tray wells is filled with small volumes of serial twofold dilutions of different antibiotics. The drugs and concentration ranges needed can be tailored in trays that are made in-house. The panels can be prepared and frozen until use [16].

3.4.3 Etest®

The Etest® (©AB BIODISK, bioMerieux) has become the most popular test for testing individual isolates. An individual isolate is suspended in broth or saline and swabbed onto a Brucella blood agar plate. The Etest is a plastic strip with a predetermined antimicrobial concentration gradient on one side and an interpretative MIC scale on the other. The MIC is read as that concentration where the elliptical zone of inhibition intersects the strip. The Etest® correlates well with the reference procedure; there are some discrepancies for certain drugs [57–63].

3.4.4 Spiral Gradient Endpoint (SGE) System

The Autoplate 4000 (Advanced Instruments, Inc., Boston, MA) deposits a specific amount of antimicrobial stock solution in a spiral pattern on a 150 mm agar plate, generating a concentration gradient that decreases radially from the center to the edge of the plate. After the antimicrobials are allowed to diffuse, the isolates are plated with an automated inoculator or manually streaked from the center to the edge of the plate. After incubation, endpoints of growth are marked and the distance is measured in millimeters from the center of the plate to the point where growth ceases. A computer software program determines the concentration of drug from the radius of growth and the molecular weight

(i.e., diffusion characteristics) of the antimicrobial agent. This procedure is compared favorably with standard agar dilution [64–66]. Also, it can detect any tendency for spontaneously resistant mutants that may develop (i.e., colonies that grow beyond the "endpoint").

3.5 Commercially Available Testing

There are several commercially available test panels available in the USA through Thermo Fisher Scientific, Oxoid and Sensititre Trek. However, if the panels contain antimicrobials that are not approved by the FDA for use in anaerobic infections, the panel is not approved by the FDA for clinical diagnostic use. In practice, most hospitals that use microbroth panels order panels that reflect their needs based on hospital formulary and drug used and not on FDA approval.

Specialty Laboratories in the USA (operated by Quest Diagnostics Inc.) provides testing services for 6 antimicrobials (Cefoxitin, Penicillin, Clindamycin, Piperacillin/Tazobactam, Metronidazole, and Imipenem) using Etest® methodology. Focus Diagnostics (also a subsidiary of Quest Diagnostics Inc.) and Mayo Medical Laboratories (Rochester, Minn.) offers routine testing using Etest. Six to nine drugs are tested routinely, depending on the organism tested. For *B. fragilis* group organisms, ampicillin/sulbactam, clindamycin, imipenem, meropenem, metronidazole, and piperacillin/tazobactam are included in the panel. The addition of penicillin, cefoxitin, and cefotetan may be ordered for testing *Clostridium*. Other drug testing can be custom ordered (depending on the availability of the Etest® strip).

3.5.1 Beta-Lactamase Test

Anaerobes can be tested for the presence of the enzyme BL using a chromogenic cephalosporin test such as nitrocefin disks. These are colorimetric tests that are easy to perform and results can be read within 5–30 min. Because most *B. fragilis* group isolates generate BL, testing for BL production is generally not recommended for this group. Other isolates that have less predictable patterns and certain anaerobes include some *Clostridium*, *Fusobacterium,* and *Prevotella*. Isolates with positive BL test should be considered resistant to penicillin and ampicillin. A negative test does not necessarily predict susceptibility to these agents, as some anaerobes are resistant to beta-lactam antimicrobial agents through other mechanisms.

Increased activity of efflux pumps and changes in penicillin-binding proteins have been shown to affect MICs of BL for many *Bacteroides* isolates. However, systematic surveys of these mechanisms have not been conducted, so the percentage of strains that have or utilize these mechanisms is not known [67, 68].

3.6 Factors Contributing to Variability in MIC Results

Technical variability among laboratories was a major factor in variability in MIC in the past [68] as laboratories used different media, different inocula sizes, and may have read results after different incubation times [68]. However, since the CLSI (formerly NCCLS) extensively revised these procedures, they were adopted by virtually all testing laboratories across the world. Consequently, most technical variability among laboratories has been reduced. Variable breakpoints do not effect the individual MIC for a certain strain, but will alter the percent of strains reported as susceptible or resistant in surveillance reports. Most studies adhere to CLSI breakpoints, but some EUCAST breakpoints are different. Many studies recognize these differences and report results with both breakpoints.

Another factor that can affect survey antibiograms' results include the particular composition of the groups of strains included. The bacterial species of a particular genus may possess different susceptibility patterns. Studies of different proportions of the various *B. fragilis* group species may reflect different antibiograms for the *B. fragilis* group as a whole, because members of the group have variable susceptibilities. The source of isolates included in the report (i.e., normal flora, clinical infection) can also influence the resistance profile of the entire species and should be taken into consideration when the survey is evaluated.

The most common cause of variability in MIC reports is the variation in interpretation of what the MIC is in instances where endpoints are not very clear. A margin of error (usually + one twofold dilution) exists for any of the susceptibility techniques. The MICs for a large percentage of *B. fragilis* group strains cluster within one twofold dilution range of the breakpoint for some antimicrobial agents. All testing methods exhibit clustering of the breakpoint and this is a characteristic feature of the organism–drug interaction. When a MIC is near the breakpoint, an organism may be called susceptible on one occasion and called resistant when retested. Because of this, in the case of single isolates, it is helpful to know the MIC of a drug for the strain as well as the established breakpoint, rather than just the laboratory determination. Even though variabilities in results may exist between survey studies, they provide useful information on trends and patterns in antimicrobial susceptibility.

Large survey studies can detect changes in susceptibility due to specific resistance mechanisms. These initially induce relatively modest changes in resistance rates that may quickly increase as the resistance determinant disseminate. This can pinpoint the relevant mechanisms of resistance and help monitor, understand and perhaps even control these shifts by making recommendations based on the molecular traits of the pathogen.

3.7 Detection of Resistance Using Molecular Methods

These detection methods are limited at present to research laboratories. The commonest molecular techniques are PCR amplifications to identify *nim* genes responsible for metronidazole resistance or *cfiA*-type genes that confer resistance to carbapenems.

3.8 Investigational Rapid Tests

It is hoped that simple molecular or multiplex PCR tests, that would determine the actual or potential resistance of an organism to multiple antibiotics would be developed. Future tests could measure many genetic determinants that confer drug resistance including enzymes that confer resistance to carbapenems (e.g., *cfiA*), metronidazole (*nim*), chloramphenicol (*cat*), erythromycin (*erm*), tetracycline (*tet*), or quinolones (changes in *gyr* or *parC* genes). Pumbwe et al. [69] described a multiplex PCR test that detected multiple resistance determinants in *B. fragilis* isolates and predicted likely resistance patterns. Unfortunately, the presence of systems of multidrug efflux pumps may not permit a definitive determination of a resistance profile by molecular techniques. Such multidrug resistance of multidrug efflux pumps was observed in aerobes, and may be operative in anaerobes as well. A 16 homologs of tripartite efflux pumps of the resistance nodulation division (RND) family have been described (Bme 1–16) in *B. fragilis* and are important in conferring multidrug resistance [46, 70–73]. Pump activity related to resistance has also been found in *Clostridium* [74–76]. Several multidrug-resistant isolates seem to exhibit significantly increased efflux pump activity. Because genes for efflux pumps are present in all bacterial strains, a PCR test to detect the gene would always be positive. It is possible that the levels of efflux pump genes transcribed and expressed are important. Currently, the only way to measure these in clinical isolates is to quantitatively identify and sequence RNA transcripts, which is time consuming and impractical.

3.9 Resistance to Antimicrobial Agents Effective Against Anaerobic Bacteria

Table 63.2 illustrates the antimicrobial effective against anaerobic bacteria and their efficacy against both aerobic and anaerobic bacteria. Many of the older antimicrobial agents do not have an FDA-approved indication for treatment of anaerobic infection(s), and many of the newer agents have only limited number of indications for anaerobic infections. However, many of these agents are administered for the treatment of anaerobic infections without an FDA indication. Tables 63.3 and 63.4 illustrate the resistance of *B. fragilis* group and other anaerobes to antimicrobial agents [77].

3.10 Beta-Lactam Antibiotics

Penicillin G is the drug of choice whenever the infecting organism is susceptible to this agent in vitro. Most *Clostridium* strains (with the exception of some strains of *Clostridium ramosum*, *Clostridium clostridioforme,* and *Clostridium innocuum*) and *Peptostreptococcus* spp. are susceptible to penicillin. Most *B. fragilis* group are resistant to penicillin G, and it should not be used for the treatment of

Table 63.2 Antimicrobial agents effective against mixed infection[a]

	Anaerobic bacteria		Aerobic bacteria	
Antimicrobial agent	Beta-lactamase-producing anaerobic Gram-negative bacilli	Other anaerobes	Gram-positive cocci	Enterobacteriaceae
Penicillin[b]	0	+++	+	0
Chloramphenicol[b]	+++	+++	+	+
Cephalothin	0	+	++	+/–
Cefoxitin	++	+++	++	++
Carbapenems	+++	+++	+++	+++
Clindamycin[b]	++	+++	+++	0
Ticarcillin	+	+++	+	++
Amoxicillin + clavulanate[b]	+++	+++	++	++
Piperacillin + tazobactam	+++	+++	++	++
Metronidazole[b]	+++	+++	0	0
Moxifloxacin	++	++	++	+++
Tigecycline	++	+++	+++	++

[a]Degrees of activity: 0 to +++
[b]Available also in oral form

Table 63.3 Susceptibility of Gram-negative anaerobic bacteria

	% Susceptible to[a]						
Anaerobe	<50	50–69	70–84	85–95	>95		
B. fragilis	PEN[b]	CFP	MOX	CTT	PIP	FOX	SIT
	CIP	CTX	CRO	ZOX	AMC	BIA	LVX
	FLE	CAZ	CLR	CLI	SAM	IPM	OFX
	LOM	SPX		MIN	CPS	MEM	TVA
	AZM				TZP	CHL	MND
	ERY				TIM	CLX	
	ROX						
	TET						
Other *B. fragilis* group[c]	PEN	CFP	LVX	AMC	SAM	IPM	SIT
	CTX	CTT	CLR	PIP	CPS	MEM	TVA
	CAZ	MOX	CLI	FOX	TZP	CHL	MND
	CRO	OFX		ZOX	TIM	CLX	MIN
	CIP	SPX			BIA		
	FLE						
	LOM						
	AZM						
	ERY						
	ROX						
Other *Bacteroides* spp.	FLE	CIP	PEN	CTT	PIP	CTX	CLZ
	LOM	TET	MOX	CAZ	AMC	FOX	SIT
			OFX	CRO	SAM	ZOX	LVX
			SPX	CLR	TIM	BIA	TVA
			AZM	ERY	CFP	IPM	MND
				ROX	CPS	CHL	CLI
				MIN			
Prevotella spp.	FLE	TET	CIP	CRO	PIP	ZOX	CLX
	LOM		OFX	AZM	AMC	BIA	SIT
			SPX	CLR	SAM	IPM	TVA
			MIN	ERY	TZP	MEM	MND
				ROX	TIM	CHL	CLI
					FOX		
Porphyromonas spp.	FLE	TET		CIP	PIP	IPM	SPX
	LOM			CLR	AMC	MEM	TVA
				CLI	FOX	CHL	MND
				ERY	ZOX	CLX	AZM
				ROX	CRO	SIT	MIN
					BIA		
F. nucleatum	FLE			CIP	PIP	BIA	OFX
	LOM			AZM	AMC	IPM	SPX
	CLR				TZP	MEM	TVA
	ERY				TIM	CHL	CLI
	ROX				FOX	CLX	MND
					ZOX	SIT	MIN
					CRO	LVX	TET
F. mortiferum and *F. varium*	FLE	CIP	CLI	AMC	PIP	IPM	SIT
	LOM	SPX	TET	ZOX	TZP	MEM	TVA
	AZM	TEM		CRO	TIM	CHL	MND
	CLR				FOX	CLX	MIN
	ERY				BIA		
	ROX						

(continued)

Table 63.3 (continued)

Anaerobe	% Susceptible to[a]						
	<50	50–69	70–84	85–95	>95		
Other *Fusobacterium* spp.	FLE		CAZ	PIP	PEN	IPM	MND
	LOM		MOX	AMC	SAM	MEM	CLI
	CLR		CIP	TIM	TZP	CHL	MIN
	ERY		SPX	CPS	FOX	CLX	TET
	ROX		AZM	CTX	BIA	SIT	
				CTT			
				ZOX			
				CRO			

[a]The order of listing of drugs within percent susceptible categories is not significant. According to the NCCLS-approved breakpoints (M11-A3), using the intermediate category as susceptible. AMC, amoxicillin/clavulanate; AZM, azithromycin; BIA, biapenem; CAZ, ceftazidime; CFP, cefoperazone; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; CLR, clarithromycin; CLX, clinafloxacin; CPS, cefoperazone/sulbactam; CRO, ceftriaxone; CTT, cefotetan; CTX, cefotaxime; ERY, erythromycin; FLE, fleroxacin; FOX, cefoxitin; IPM, imipenem; LOM, lomefloxacin; LVX, levofloxacin; MEM, meropenem; MIN, minocycline; MND, metronidazole; MOX, moxalactam; OFX, ofloxacin; PEN, penicillin; PIP, piperacillin; ROX, roxithromycin; SAM, ampicillin/sulbactam; SIT, sitafloxacin; SPX, sparfloxacin; TEM, termafloxacin; TET, tetracycline; TIM, ticarcillin/clavulanate; TVA, trovafloxacin; TZP, piperacillin/tazobactam; ZOX, ceftizoxime

[b]NCCLS approved breakpoint in 4 μg/mL. However, the breakpoint should probably be lowered to 1 μg/mL, which will considerably lower the values for % susceptible. For example, at 1 μg/mL, no strains of the *B. fragilis* group were susceptible

[c]Excluding *B. fragilis*

Table 63.4 Susceptibility of Gram-positive anaerobic bacteria

Anaerobe	% Susceptible to[a]						
	<50	50–69	70–84	85–95	>95		
Peptostreptococcus spp.	LOM	FLE	CIP	LVX	PEN	CTT	MEM
		TET	OFX	CLI	PIP	FOX	CHL
		ROX	AZM	MIN	AMC	CAZ	CLX
			CLR		SAM	ZOX	SIT
			ERY		TZP	CRO	SPX
					TIM	BIA	TVA
					CFP	IPM	MND
					CPS		
C. difficile[b]	FOX	CLI		CRO	AMP	TZP	CLX
	ZOX	MIN		BIA	PIP	TIM	SIT
	CIP	TET		CHL	TIC	CTT	TVA
	FLE	AZM			AMC	IPM	MND
	LOM	CLR			SAM	MEM	
	SPX	ERY					
		ROX					
C. ramosum	CIP	SPX	FOX	AMP	AMC	ZOX	SIT
	FLE	MIN		PIP	TZP	IPM	MND
	LOM	TET		SAM	TIM	CLX	
	AZM			CHL			
	CLR			TVA			
	ERY			CLI			
	ROX						
C. perfringens		TET	MIN	LOM	AMP	ZOX	SPX
				CLI	PIP	BIA	TVA
					TIC	IPM	MND
					SAM	CHL	AZM
					AMC	CIP	CLR
					TZP	CLX	ERY
					TIM	SIT	ROX
					CTT	FLE	

(continued)

Table 63.4 (continued)

Anaerobe	% Susceptible to[a]						
	<50	50–69	70–84	85–95	>95		
Other *Clostridium* spp.	CAZ	CFP	LVX	MOX	AMX	TIC	CLX
	FLE	CTX	OFX		AMP	SAM	SIT
	LOM	FOX	SPX		CAR	AMC	TVA
		ZOX	CLI		PEN	BIA	MND
		CRO	TET		PIP	IPM	MIN
		CIP				CHL	
		AZM					
		CLR					
		ERY					
		ROX					
Nonspore-forming Gram-positive rod	FLE	CIP	CFP	CTT	PEN	FTX	CLI
	LOM	OFX	MOX	FOX	PIP	ZOX	CLX
		MND	SPX	CRO	AMC	BIA	SIT
			TET	CPS	SAM	IPM	LVX
				TVA	TZP	MEM	MIN
				AZM	TIM	CHL	
				CLR			
				ERY			
				ROX			

[a]The order of listing of drugs within percent susceptible categories is not significant. According to the NCCLS approved breakpoints (M11-A3), using the intermediate category as susceptible. AMP, ampicillin; AMX, amoxicillin; TIC, ticarcillin, see Table 63.2 footnote for other antimicrobial agents

[b]Breakpoint is used only as a reference point. *C. difficile* is primarily of interest in relation to antimicrobial induced pseudomembranous colitis. These data must be interpreted in the context of level of drug achieved in the colon and impact of agent on indigenous colonic flora

infections caused by these organisms. Other strains that may be resistant to penicillins are growing numbers of AGNB, such as the pigmented *Prevotella* and *Porphyromonas* spp., *Prevotella oralis*, *Prevotella bivia*, *Bacteroides disiens*, strains of clostridia, *Fusobacterium* spp. (*Fusobacterium varium* and *Fusobacterium mortiferum*), and microaerophilic streptococci. Some of these strains show MIC of 8–32 units/mL of penicillin G. In such instances, administration of very high dosages of penicillin G (for non-BL producers) may eradicate the infection.

Clinical experience with penicillin G in the management of bacterial infections caused by susceptible anaerobes has been good. Penicillin, ampicillin, and amoxicillin (AMX) generally are equally active, but the semisynthetic penicillins are less active. Methicillin, nafcillin, and the isoxazolyl penicillins (oxacillin, cloxacillin, and dicloxacillin) are ineffective against *B. fragilis* group, have unpredictable activity, and frequently are inferior to penicillin G against anaerobes [78].

Penicillin, ampicillin, and amoxicillin are of limited utility due to the production of BLs by many oral and most intra-abdominal anaerobes. Clavulanate, sulbactam, and tazobactam are BL inhibitors that resemble the nucleus of penicillin but differ in several ways. They irreversibly inhibit BL enzymes produced by some *Enterobacteriaceae*, staphylococci, and BL-producing *Fusobacterium* spp. and AGNB [78–80]. When used in combination with a beta-lactam antibiotic (such as ampicillin/sulbactam, amoxicillin/clavulanate, and piperacillin/tazobactam) they are effective in treating anaerobic infections caused by BL-producing bacteria (BLPB).

Beta-lactam/BL inhibitor combinations (BL-BLIs) are appropriate choices for mixed aerobic–anaerobic infections as they have good activity against the majority of anaerobes. While 89 % of *B. fragilis* are susceptible to ampicillin/sulbactam, 98 % are susceptible to piperacillin/tazobactam [80] compared to 86 % and 92 % respectively, for *B. thetaiotaomicron* isolates. The Infectious Diseases Society of America (IDSA) has removed ampicillin/sulbactam from the recommended agents list for treatment of intra-abdominal infections because of the increased *E. coli* resistance worldwide even though it has maintained good activity against *B. fragilis* group and other anaerobes [81]. AMX-C is the drug of choice for human and animal bite wound infections [82], especially when anaerobes may be involved. Piperacillin/tazobactam is an appropriately agent for serious intra-abdominal infections as it has maintained good activity against the majority of anaerobic bacteria [80].

The *semisynthetic penicillins*, the carboxy-penicillins (carbenicillin and ticarcillin), and ureidopenicillins (piperacillin, azlocillin, and mezlocillin), generally are given in large quantities to achieve high serum concentration. These

agents are effective against *Enterobacteriaceae* and posses good activity against most anaerobes in these concentrations. However, up to 30 % of the *B. fragilis* group are resistant to these agents [83].

Many anaerobes produce cephalosporinases and therefore as a class, cephalosporins have very limited efficacy [81, 83]. The activity of *cephalosporins* against the BL-producing AGNB is variable. The spectrum of activity of the first-generation cephalosporins against anaerobes is similar to penicillin G, although on a weight basis, they are less active. Most strains of the *B. fragilis* group and many *Prevotella*, *Porphyromonas,* and *Fusobacterium* spp. are resistant to cephalosporins by virtue of cephalosporinase production [84]. The enzyme has little or no activity against the second-generation cefoxitin (a cephamycin) which is the most effective cephalosporin against the *B. fragilis* group. However, efficacy may vary by geographic location and is generally directly related to cefoxitin clinical use. It is relatively inactive against most of *Clostridium* species including *C. difficile*, with the exception of *C. perfringens* [6, 7, 84].

Studies performed in the 1980s found cefoxitin to be effective in eradication of anaerobic infections [85–87]. It has frequently been used for surgical prophylaxis at body sites that evolve exposure to mucus membrane. Third-generation cephalosporins have improved activity against *Enterobacteriaceae*, but with the exception of moxalactam, they are not as active against *B. fragilis* as cefoxitin.

Currently about 85 % of *B. fragilis* group isolates are susceptible to cefoxitin but the other group's species are more resistant [17]. Cefotetan is less effective than cefoxitin against *B. fragilis* group.

The *B. fragilis* group includes more than 20 *Bacteroides* spp. that were promoted to a genus level [88]. Among the group, *B. fragilis* accounts for 40–54 % of the *Bacteroides* isolates recovered from all infections [4, 89–91]. *B. thetaiotaomicron*, a member of the *B. fragilis* group, accounts for 13–23 % of the isolates, and other members of the *B. fragilis* group account for 33–39 %. The antimicrobial susceptibility of some members of the *B. fragilis* group varies, especially to the second- and third-generation cephalosporins. *B. fragilis* is the most susceptible, and *B. thetaiotaomicron* and *Parabacteroides distasonis* generally are more resistant [27, 92].

The *cephamycins*, cefoxitin and cefotetan are often used for surgical prophylaxis for abdominal surgery and for the treatment of aspiration pneumonia. Recently, the IDSA has removed cefotetan from the recommended list of agents used for intra-abdominal infections because of its poor activity against *B. fragilis* group and documented clinical failures [93–95].

The *carbapenems* (Imipenem, meropenem, doripenem, and ertapenem) have excellent activity against anaerobes [96]. *Imipenem*, a thienamycin, is a beta-lactam antimicrobial active against a broad variety of aerobic and anaerobic Gram-positive and Gram-negative organisms including multiresistant species such as *Pseudomonas aeruginosa*, *Serratia* spp., *Enterobacter* spp., *Acinetobacter* spp., and enterococcus [32, 97]. It has also excellent activity and low MIC against BL-producing AGNB including *B. fragilis* group. It is also effective against most Enterobacteriaceae with about 5–15 % of *Pseudomonas* spp. resistance [98].

Imipenem is poorly absorption from the gastrointestinal tract, reaches high plasma concentration after intravenous administration, is minimally metabolized, and is renally excreted. In the kidney, imipenem is metabolized by breakage of the BL bond in the proximal tubular cells. This results in low urinary excretion of the active drug, which can impair its ability to inhibit some urinary pathogens. To overcome the renal metabolism of imipenem, it is combined at a 1:1 ratio with an inhibitor of the renal dipeptidase, cilastatin. This increases the urinary excretion of the active agent and its half life in the serum. Imipenem is an effective single agent for the treatment of mixed aerobic–anaerobic infections.

Meropenem is a carbapenem with a broad spectrum of activity against aerobic and anaerobic organisms, similar to that of imipenem. Imipenem is more activity against staphylococci and enterococci, but meropenem possesses greater coverage of aerobic and facultative Gram-negative bacteria such as *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Providencia*, *Morganella*, *Aeromonas*, *Alcaligenes*, *Moraxella*, *Kingella*, *Actinobacillus*, *Pasteurella*, and *Haemophilus* spp. [99, 100]. Meropenem is effective in treating abdominal infections, meningitis, community-acquired and nosocomial pneumonia, and neutropenic fever [101].

Ertapenem is a newer 1-beta-methyl carbapenem, stable to dehydropeptidase. It manifests a broad antibacterial spectrum for penicillin-susceptible *Streptococcus pneumoniae*, *Streptococcus pyogenes*, methicillin-sensitive *Staphylococcus aureus*, *Haemophilus influenzae*, *Moraxella catarrhalis*, *Escherichia coli*, *Citrobacter* spp., *Klebsiella* spp., *Serratia* spp., *Proteus* spp., *C. perfringens*, *Fusobacterium* spp., *Peptostreptococcus* spp., and AGNB [102]. It is indicated for treatment of complicated intra-abdominal and skin structure infections, including diabetic foot infections without osteomyelitis, and acute pelvic infections including postpartum endomyometritis, septic abortion, and postsurgical gynecologic infections. In comparison to other carbapenems, it has a long half-life of 4.5 h and is given in a single daily dose. It is not active against *P. aeruginosa*, *Enterococcus* spp., and *Acinetobacter* spp.

Doripenem, a synthetic 1-beta-methyl carbapenem, is the newest available carbapenem. Its antimicrobial spectrum resembles those of meropenem and imipenem [97]. It possesses excellent in vitro activity against streptococci, methicillin-susceptible staphylococci, *Enterobacteriaceae* (including extended-spectrum BL-producing strains), *P.*

aeruginosa, *Acinetobacter* spp., and *B. fragilis* group. It is not active against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and most Gram-negative bacilli that are resistant to meropenem or imipenem [97]. Doripenem has been approved in the USA for use in treatment of complicated intra-abdominal infection and complicated urinary tract infection.

Two recent reports have observed the development of some carbapenem resistance among anaerobic bacteria [22] ranging from 1.1 to 2.5 % in a multicenter US survey but higher in a small number of isolates from Taiwan [25].

Anaerobes manifest three major resistance mechanisms to beta-lactam antibiotics: inactivating enzymes, mainly BLs, which include penicillinases and cephalosporinases; low affinity penicillin-binding proteins (PBPs); and decreased permeability through alterations in the porin channel [67]. All *B. fragilis* group species are generally resistant to penicillins (average 90 %), piperacillin (25 %) cefoxitin (25 %), cefotetan (30–85 %), and third-generation cephalosporins [27, 28, 86].

3.10.1 Beta-Lactamase Production

The production of BLs is the most common mechanism of resistance to beta-lactam antibiotics in anaerobes, especially among the *B. fragilis* group and *Prevotella* spp. [103]. The cephalosporinases are most often of the 2e class type and can be inhibited by BL inhibitors (e.g., clavulanic acid, sulbactam, tazobactam). Each individual cephalosporin may have either a class or specific BL enzyme that is able to inactivate it.

BL hydrolyzes the cyclic amide bond of the beta-lactam nucleus, causing its inactivation. There are a variety of BLs produced by different organisms which can be exoenzymes, inducible or constitutive, and genetically they can be of either chromosomal or plasmid origin [104]. There are different classifications of the enzymes. A classification based on amino acid sequence has been proposed by Ambler [105], and one based upon substrate of inhibition profiles, molecular weight, and isoelectric points was created by Richmond and Sykes [106].

Most *B. fragilis* group produce constitutive BLs that are primarily cephalosporinases [107]. More than 97 % of *Bacteroides* isolates in the USA and 76 % in Great Britain produce BLs [108]. Of the non-*fragilis* AGNB 2/3 produce BLs [51, 109]. Pigmented *Prevotella* and *Porphyromonas*, *Prevotella bivia*, *Prevotella disiens*, and *Fusobacterium nucleatum* produce primarily penicillinases [109].

Carbapenem resistance in *B. fragilis* is related to *cfiA*- or *ccrA*-encoded class B metallo-BL. Although not all *cfiA*-positive *B. fragilis* strains are carbapenems resistant, they are all capable of becoming resistant to these antibiotics through acquisition of an appropriate insertion sequence (IS) element for full expression of the *cfiA* gene, which can lead to treatment failure. The presence of the *cfiA* gene, as well as associated IS elements, can be determined by a PCR method [110–112]. Two new studies used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify division II type strains of *B. fragilis* that possess the *cfiA* gene [113, 114].

Carbapenemases act against the carbapenems as well as all beta-lactam antimicrobials. Although they are usually chromosomally mediated, a plasmid-mediated metallo-BL has been reported in Japan [115]. Carbapenem resistance is present in <1 % of US isolates, and in up to 3 % of *Bacteroides* strains harbor one of the genes which is expressed at a very low level. BL inhibitors are unable to inactivate carbapenemases which are a zinc metalloenzymes, encoded by either *ccrA* or *cfiA* genes of *B. fragilis* group [116].

A study of the molecular characterization of imipenem-resistant *cif*A-positive *B. fragilis* strains noted that the *cfiA* genes of 10 of the 15 evaluated strains were upregulated by insertion sequence (IS) elements while 5 others did not contain an IS insertion but produced carbapenemase [116]. These observations illustrate that some isolates possessed novel inactivation mechanisms suggesting that more than one mechanism of inactivation exists. A study from Taiwan observed increased carbapenem resistance in *B. fragilis* group and some *Prevotella* spp. [25].

Clostridium, *Porphyromonas*, and *Fusobacterium* strains have also been found to BLs. The BLs producing *Fusobacterium* and *Clostridium* spp. are generally inhibited by clavulanic acid [117]. Resistance to beta-lactams through changes in the OMP/porin channels, efflux pumps, and decreased PBP affinity [118] are less well studied.

The carbapenems and the combinations of BL/BLIs inhibitors have maintained their excellent antibacterial activity against anaerobes. The combination agents of amoxicillin/clavulanate (AMX-C), ampicillin/sulbactam, ticarcillin/clavulanate, and piperacillin/tazobactam are generally very active against members of the *B. fragilis* group [28]. However, variation in susceptibility occurs among species, and many non-BL producing *P. distasonis* have elevated MICs at or close to the susceptible breakpoint [119]. *B. fragilis* group resistance rates for piperacillin/tazobactam is generally <1 % [28]. However, resistance of *P. distasonis* to ampicillin/sulbactam has increased to 20 % in 2002–2004 but continued to be low for the species of other *B. fragilis* group.

The carbapenems continue to be very effective against all members of the *B. fragilis* group, and resistance is rare at <0.1 % [28, 29, 119]. Geometric mean MICs for imipenem and meropenem for *P. distasonis*, *B. thetaiotaomicron*, and *Bacteroides ovatus* are onefold dilution lower than those for ertapenem [28]. In a study of pediatric intra-abdominal infections [85] all *Bacteroides* isolates produced BL and were susceptible to carbapenems and BL/BLIs. Cefoxitin had poor activity against *B. thetaiotaomicron* isolates.

Beta-lactams are usually active against non-*B. fragilis* group isolates and resistance to them is generally low. However, more

than half of *Prevotella* spp. may also produce BLs. A multicenter survey [32] found penicillin resistance to be 9% for *Fusobacterium* spp., 21% for *Porphyromonas* spp., and 6% for *Peptostreptococcus* spp. That survey found no resistance to cefoxitin, cefotetan, BL/BLI combinations, and carbapenems. An exception was 4% *Peptostreptococcus* spp. and 5% *Porphyromonas* spp. resistance to ampicillin-sulbactam. BLs were noted in several *Prevotella and Porphyromonas* spp. recovered from pediatric intra-abdominal infections.

3.10.2 Penicillin-Binding Proteins

Penicillin binding to the PBPs determines whether a beta-lactam antimicrobial will be effective. Maintaining PBPs function in the final stage of cell wall synthesis is essential for bacterial growth. Beta-lactams work by successfully competing for binding to the active site of the essential PBP, thus causing cell death. Three to five PBPs can be found in *Bacteroides* strains: a PBP 1 complex with one to three different enzymes, PBP 2, and PBP 3. These PBPs are most likely similar to the high-molecular-weight PBPs present in aerobic Gram-negative bacteria. It may be possible that other low-molecular-weight PBPs also exist, but the number of these proteins vary among strains, and are probably not essential for bacterial growth [120].

Alteration in PBP are not a major mechanisms of resistance in anaerobes as the binding of most beta-lactam agents to PBP 1 complex and PBP 2 is adequate. An exception are the monobactams (i.e., aztreonam) who are not active against *B. fragilis* because they do not have good affinity for their PBPs [121]. Decreased affinity of cephalosporins for PBP 3 was demonstrated in *B. fragilis* G-232 recovered in Japan [122]. Cefoxitin resistance in some *Bacteroides* strains has also been attributed to decreased binding to the PBP 1 complex or the PBP 2 [123, 124]. This resistance was also inducible in vitro [125].

3.10.3 Permeability

Increased BL production was associated with decreased permeability in Gram-negative bacteria. Permeability factors can vary among strains of *B. fragilis* [126–128], and in certain *B. fragilis* strains, resistance was associated with both reduced permeability and BL production [128]. Cefoxitin resistance correlated with a decrease in outer-membrane permeability and the loss of an outer-membrane protein with a molecular size of 49–50 kid [124].

Studies of pore-forming proteins of *Bacteroides*, *Porphyromonas*, and *Fusobacterium* spp. identified and cloned outer-membrane proteins from these AGNB. The absence of at least one outer-membrane protein was associated in some strains with resistance to ampicillin/sulbactam [129].

Selective pressure similar to that observed for many aerobic species most likely also plays a role in the development and selection of resistance to beta-lactams. Although the prevalence of resistance of anaerobes to beta-lactams has increased, several of these antibiotics are still clinically useful. However, their utilization should be determined according to the local resistance patterns or the susceptibility of individual isolates.

3.11 Chloramphenicol

Chloramphenicol, a bacteriostatic agent, is active against most anaerobic bacteria but is rarely used in the USA [3, 84]. Resistance to this agent is rare, although it has been reported in some *Bacteroides* spp. [29] strains. One must be aware that MICs of chloramphenicol often cluster around the susceptibility break point. Although several failures to treat anaerobic infections, including bacteremia, with chloramphenicol have been documented [130], this drug has been used for over 65 years for treatment of anaerobic infections. Chloramphenicol was regarded for many years as the drug of choice for treatment of serious anaerobic infections when the nature and susceptibility of the causative organism(s) are unknown and in central nervous system infections (CNS). However, the agent has potential significant toxicity. Fatal aplastic anemia with chloramphenicol is estimated to occur in approximately one per 25,000–40,000 patients treated. This complication is not related to the reversible, dosage-dependent leukopenia. Other side effects include the potentially fatal "gray baby syndrome" when administered to neonates, hemolytic anemia in individuals with G6PD deficiency, and optic neuritis in patients who take chloramphenicol for a prolonged time.

Serum level measurements are often indicated for infants, young children, and sometimes for adults, because of their wide variations [131]. The usual goal is the therapeutic levels of 10–25 μg/mL. Levels above 25 μg/mL can cause reversible bone marrow suppression, and levels of 40–200 μg/mL have been associated with the gray syndrome in neonates or encephalitis in adults [131].

Chloramphenicol distributes throughout the body fluids and tissues, with a mean volume distribution of 1.4 L/kg [131]. The drug possesses a unique property of lipid solubility to enabling its penetration across lipid barriers. It consistently achieves high concentrations in the CNS, even in the absence of inflammation. Cerebrospinal fluid levels with or without meningitis, usually are one-third to three-fourths the serum concentrations. Brain tissue levels may be substantially higher than serum levels [132].

3.11.1 Chloramphenicol Resistance

Even though no resistance of anaerobic bacteria against chloramphenicol has been noted [133–136], clinical failures using this drug have been reported [137]. The absence of resistance can be explained by the infrequent clinical use of this agent.

Bacteroides spp. possess two unique classes of chloramphenicol resistance genes that produce resistance through drug inactivation, either by nitroreduction at the p-nitro group on the benzene ring [138] or by acetylation [139, 140]. Resistance through acetylation is transferable and associated with a 39.5-kb plasmid, pRYC3373 [139].

3.12 Macrolides: Erythromycin, Azithromycin, Clarithromycin

The macrolides, which cause little toxicity, have moderate to good in vitro activity against anaerobes other than *B. fragilis* group and fusobacteria [84]. Macrolides are active against pigmented *Prevotella* and *Porphyromonas*, microaerophilic streptococci, Gram-positive non-spore-forming anaerobic bacilli, and certain clostridia. They are less effective against *Fusobacterium* and *Peptostreptococcus* spp. [141]. They show relatively good activity against *C. perfringens* and poor or inconsistent activity against AGNB.

Clarithromycin is the most active macrolide against Gram-positive oral flora anaerobes, including *Actinomyces* spp., *Propionibacterium* spp., *Lactobacillus* spp., and *Bifidobacterium dentium*. Azithromycin is slightly less active than erythromycin against these species [141]. Azithromycin is, in general, the most active macrolide against AGNB: *Fusobacterium* spp., *Bacteroides* spp., *Wolinella* spp., and *Actinobacillus actinomycetemcomitans*, including those resistant to erythromycin. Clarithromycin has similar activity to erythromycin against most AGNB [142].

Erythromycin-resistant organisms can emerge during therapy [143, 144]. Erythromycin is effective in the treatment of mild to moderately severe anaerobic soft tissue infections when combined with adequate debridement or drainage of infected tissue. Phlebitis can develop in about one-third of those receiving intravenous erythromycin.

Five genes conferring macrolide-lincosamide-streptogramin (MLS) resistance have been identified in anaerobes, including erm(B), erm(C), erm(F), erm(G), and erm(Q). In contrast, no genes coding for MLS-resistant efflux proteins or inactivating enzymes have been described in anaerobic species [144].

3.13 Clindamycin

Clindamycin has a broad spectrum of activity against anaerobic bacteria and has proven its efficacy in past clinical trials. It is used for dental infections, especially for penicillin allergic patients and for aspiration pneumonia. Clindamycin hydrochloride is rapidly and almost completely absorbed from the gastrointestinal tract [145–147]. It penetrates well into body tissues and fluids, including saliva, sputum, respiratory tissue, pleural fluid, soft tissues, prostate, semen, bones, and joints [148].

3.13.1 Clindamycin Resistance

Clindamycin resistance is conveyed by a macrolide-lincosamide-streptogramin (MLS) type 23S methylase, generally encoded by one of several *erm* genes which are regulated and expressed at high levels [149].

B. fragilis resistance to clindamycin is increasing worldwide and varies by region. Clindamycin is no longer recommended as empiric therapy for intra-abdominal infections [22, 28, 29, 81]. A recent study (1997–2004) found 19.3 % of 2721 *B. fragilis* group isolates, 29.6 % of *P. distasonis*, 33.4 % of *B. ovatus*, 33.3 % of *B. thetaiotaomicron*, and 35.6 % of *B. vulgatus* strains to be clindamycin resistant. This is a significant increase compared to only 3 % clindamycin resistance in 1987 [27]. A study of pediatric intra-abdominal isolates found clindamycin resistance in only 6 % of *B. fragilis* isolates compared to 80 % for *B. thetaiotaomicron* and 45 % for other *B. fragilis* group strains [85].

Resistance has also increased, for many non-*Bacteroides* anaerobes. Up to 10 % resistance was noted for *Prevotella* spp., *Fusobacterium* spp., *Porphyromonas* spp., and *Peptostreptococcus* spp., with higher rates for some *Clostridium* spp. (especially *C. difficile*) [98]. *Propionibacterium acnes* isolates have also become more resistant to clindamycin and this has been associated with prior therapy for acne [150].

Clindamycin has lost some of its activity against anaerobic Gram-positive cocci, and *Prevotella* spp., although its activity against *Fusobacterium* and *Porphyromonas* spp. remains good [83].

Other resistant anaerobes are several species of clostridia, especially *C. difficile.* Approximately 20 % of *C. ramosum* are resistant to clindamycin, as are a smaller number of *C. perfringens* [83].

3.14 Metronidazole and Tinidazole

These nitroimidazoles have similar in vitro efficacy against anaerobes. Metronidazole has excellent in vitro activity against most obligate anaerobes, including *B. fragilis* group, other species of Bacteroides, fusobacteria, and clostridia [28]. Only six strains of *B. fragilis* group were ever reported to be clinically resistant and associated with therapeutic failure [21].

Anaerobic Gram-positive nonsporulating bacilli are commonly resistant while anaerobic Gram-positive cocci are rarely resistant. Microaerophilic streptococci, *P. acnes*, and *Actinomyces* spp. are almost uniformly resistant [151]. Aerobic and facultative anaerobes are usually very resistant.

Over 90 % of anaerobes are susceptible to less than 2 μg/mL of metronidazole [84].

Because of metronidazole lack of activity against aerobic bacteria, an antimicrobial effective against these organisms (e.g., beta-lactam, cephalosporin, fluoroquinolone) need to be added when treating a polymicrobial aerobic–anaerobic infection.

Gastrointestinal side effects are frequent and include nausea, vomiting, metallic taste, anorexia, and diarrhea. Other adverse reactions to metronidazole are infrequent and include CNS toxicity, such as ataxia, vertigo, headaches, and convulsions. Peripheral neuropathy is associated with prolonged use of the agent. Tinidazole may be better tolerated in individuals with gastrointestinal side effects caused by metronidazole. Other adverse reactions include reversible neutropenia, phlebitis at intravenous infusion sites, and drug fever. Metronidazole is generally well tolerated.

Some studies in mice [152] have shown possible mutagenic activity associated with administration of large doses of metronidazole. However, the drug has generally been administered for the lifetime of the animal, a situation that may not be relevant for humans. Other experiments [153] have illustrated that administration of metronidazole to rats and hamsters does not induce any pathology. Furthermore, evidence of mutagenicity was never found in humans despite metronidazole use for over two decades [154]. Because of safety concerns, the FDA approved the use of metronidazole for the treatment of serious anaerobic infections only in adults.

Clinical experiences in adults [155] illustrated metronidazole's efficacy in the treatment of anaerobic infections, including CNS infections [156]. Safety data during pregnancy are contradictory and more data on the safety of metronidazole in pregnancy are needed. The non-teratogenicity of metronidazole is difficult to prove, but the existing information indicates no major risks and or need to terminate pregnancies in those receiving the drug [157].

3.14.1 Metronidazole Resistance

Metronidazole resistance is usually attributed to nitroimidazole reductase (*nim*) resistance gene. This gene codes for an enzyme that converts 4- or 5-nitroimidazole to 4- or 5-aminoimidazole (thus avoiding the formation of toxic nitroso radicals that are essential for antimicrobial activity.) *Nim* homologs are present in both Gram-positive and -negative genera of aerobic and anaerobic bacteria and Archaea, suggesting that the *nim* gene family is ancient and widespread. The *nim* genes are often found on mobilizable plasmids and pose a significant threat to the continuing utility of 5-Ni drugs, including metronidazole [158].

Polymerase chain reaction (PCR) can detect the presence of the *nim* gene. This was first described using the universal primers NIM-3 and NIM-5 [159] followed by restriction analysis to identify the specific *nim* type [160]. Nine *nim* genes were described in *B. fragilis* (*nim A-I*) and an additional *nimI* gene was described in *Prevotella* [161, 162]. However, increasing numbers of metronidazole-resistant isolates are recovered that do not possess any of the *nim* A-H genes. Also, metronidazole resistance could be induced in *nim*-negative strains by exposure to sub-MIC concentrations of metronidazole [162, 163].

Resistance to metronidazole among *B. fragilis* group has rarely been observed [24, 25]. Resistant *B. fragilis* group isolates carry one of nine known *nim* genes [*nim* A-I] on either the chromosome or on a mobilizable plasmid that seems to encode a nitroimidazole reductase that converts 4- or 5-nitroimidazole to 4- or 5-aminoimidazole, thus preventing the formation of toxic nitroso residues necessary for the agents' activity. These *nim* genes were observed in 50/206 (24 %) of *Bacteroides* spp. isolates and resulted in MICs of 1.5 to >256 μg/mL for metronidazole, including 16 isolates with MICs >32 μg/mL [162]. These findings suggested incomplete mobilization of *nim* gene associated resistance. It was speculated [162] that other mechanisms of resistance can occur and that prolonged exposure to metronidazole may select them. The mechanism of metronidazole resistance for non-*Bacteroides* anaerobes is unknown. Resistance of Gram-positive organisms that are not strict anaerobes is frequent, mostly for *P. acnes* and *Actinomyces* spp.

3.15 Tetracyclines

Tetracycline is currently of limited use for treating anaerobic infection because of the development of resistance to it by all types of anaerobes including *Bacteroides* and *Prevotella* spp. Resistance to *P. acnes* has been related to previous use [150]. Only about 45 % of all *B. fragilis* strains presently are susceptible to tetracycline [84]. The newer tetracycline analogues, doxycycline and minocycline, are more effective than the parent compound. Because of the significant resistance to these agents, they are useful only when susceptibility tests can be performed or in less severe infections in which a therapeutic trial is possible. The use of tetracycline is not recommended before 8 years of age because of the adverse effect on teeth.

Tigecycline is the first glycylcyclines antibiotic approved. Glycylcyclines are tetracycline class antibiotics containing a glycylamido moiety attached to the 9-position of a tetracycline ring; tigecycline is a direct analog of minocycline with a 9-glycylamide moiety. It is active against both aerobic Gram-negative and Gram-positive bacteria, anaerobes, and some drug-resistant pathogens [164]. These include MRSA, penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant enterococci, *Acinetobacter baumannii*, BL-producing strains of *H. influenzae* and *M. catarrhalis*,

and extended-spectrum BL-producing strains of *E. coli* and *Klebsiella pneumoniae*. In contrast, MICs for *Pseudomonas* and *Proteus* spp. are significantly elevated. It is effective against *Streptococcus anginosus* group (includes *S. anginosus*, *S. intermedius*, *and S. constellatus*), *B. fragilis*, *B. thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides vulgatus*, *C. perfringens*, *C. difficile*, and *Parvimonas micra* (*Peptostreptococcus micros*) [38]. Resistance of members of the *B. fragilis* group is 3.3–7.2 % [28].

3.15.1 Tetracycline Resistance

Four tetracycline efflux genes were identified in anaerobes: *tet*(*B*), *tet*(*K*), *tet*(*L*), and *tetA*(*P*). There are five genes confirming ribosomal protection proteins, *tet*(*M*), *tet*(*O*), *tetB*(*P*), *tet*(*Q*), *tet*(*W*), and *tet*[32], have been found in anaerobes. Three enzymes which inactivate tetracycline; *tet*(*X*), *tet*(*X*1), and *tet*(*Q*); have been found in *Bacteroides* [144, 165].

The mechanism of tetracycline resistance in *Bacteroides* spp. is through changes or shielding of the target site. The *tetQ* gene encodes a protein that makes the ribosomal protein synthesis resistant to tetracyclines' inhibitory effects [166–168]. The DNA sequences of several *Bacteroides tetQ* genes have been discovered [167, 168]. The TetQ is 40 % homologous with TetM and TetO proteins and may represent a new class of ribosomal protection proteins [167, 168].

DNA cross-hybridization demonstrated that a *tetQ*- or *tetQ*-related gene is found in most tetracycline-resistant *Bacteroides* isolates [166]. However, other mechanisms (such as tetracycline efflux) or other classes of ribosomal protection proteins may also assist in tetracycline resistance because some tetracycline-resistant isolates do not contain *tetQ* DNA sequences. This possibility is supported by the identification of a tetM-related determinant in some tetracycline-resistant isolates of *Bacteroides ureolyticus* [169].

C. perfringens harbors two tetracycline resistance genes—the *tetA*(*P*) and *tetB*(*P*) genes that create an operon that encodes two unrelated proteins which conveys resistance by two unique mechanisms [170]. The *tetA*(*P*) gene generates a tetracycline efflux pump, and the *tetB*(*P*) creates a protein generating ribosomal resistance [170].

Bacteroides spp. can harbor two additional genes related to tetracycline resistance that may not contribute to clinical resistance. The oxidation of tetracycline is through product of the *tetX* gene that is active only in aerobic conditions [171–173]. Another gene encodes a protein that produces tetracycline efflux in *Bacteroides* but is not able to produce tetracycline resistance in *E. coli* [174, 175].

The *tetQ* resistance gene is inducible [167, 176] and transferable [177, 178]. The tetracycline resistance is transferred by conjugation mediated through the tetracycline resistance transfer element [176, 179, 180]. The frequency of transfer is generally very low except when the organisms are preexposed to tetracycline [181, 182]. Controlled of the transfer is by a prokaryotic two-component regulatory system [178, 180]. The two regulatory genes, *rteA* and *rteB*, are located in the *tetQ* operon downstream from the *tetQ* gene [180], and their expression is enhanced by the presence of tetracycline.

RteA, the cytoplasmic membrane protein component is encoded by the *rteA* gene, and the RteB is encoded by the *rteB* gene [180]. RteB takes part in the transfer and mobilization of the tetracycline resistance transfer element. An additional gene, *rteC*, that produces RteC, may participate in the self-transfer of tetracycline resistance [176].

RteA and RteB also control the transfer of unlinked chromosomal elements called nonreplicating *Bacteroides* units (NBUs) [166, 181, 182]. Even though most NBUs do not contain an identifiable phenotype, a cefoxitin-hydrolyzing, BL gene (*cfxA* [54]) can be present on an NBU [183]. The transfer of the cefoxitin-hydrolyzing BL is enhanced by pretreatment with tetracycline [183, 184].

The transfer elements of tetracycline resistance are chromosomally located, are similar to the conjugal transposon *Tn916* in *Enterococcus faecalis* [166, 185–187], the rarer large (70–80 kbp) [179], and they often contain other resistance genes (e.g., *ermF*) [188].

Tetracycline resistance is common among *Bacteroides* and *Prevotella* species and many other anaerobic bacteria, limiting its clinical utility [167]. Several encoding tetracycline resistance genes have been found in several anaerobes, which encode protective proteins leading to protection of the ribosomes. Tetracycline resistance and the inducible transfer of resistance determinant can take place after exposure to low levels of this agent. The emergence of tetracycline resistance in *P. acnes* has been correlated with previous tetracycline therapy [150].

Tigecycline has been approved by the FDA for treatment of complicated skin and soft tissue infections including those caused by *B. fragilis* and intra-abdominal infections including those due to *B. fragilis*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, *C. perfringens,* and *Ps. micros* [189, 190]. In the study that compared tigecycline's efficacy in the treatment of abdominal infections tigecycline to imipenem-cilastatin, sepsis/shock developed in six tigecycline treated patients compared to two imipenem treated patients [190].

Tigecycline is considered to be effective against anaerobic bacteria [38, 191], and has a low rate (5.5 %) of resistance against *B. fragilis* group [22]. Jacobus et al. [191] found that 90 % of 831 *B. fragilis* group isolates were susceptible to <8 μg/mL of tigecycline and that *P. distasonis* isolates were the most resistant. Snydman et al. [28] observed that 4.7 % of *B. fragilis*, 3.6 % of *B. thetaiotaomicron*, 5.8 % of *B. ovatus,* and 3.2 % of *B. distasonis* showed resistance to tigecycline. Goldstein et al. [38] found all 164 Gram-positive anaerobes and 228/232 Gram-negative anaerobes to be susceptible to <1 μg/mL of tigecycline.

3.16 Fluoroquinolones

The first-generation fluoroquinolones are inactive against most anaerobes. However, several newer quinolones possess significant anti-anaerobic activity. Quinolones with low activity against anaerobes include ciprofloxacin, ofloxacin, levofloxacin, fleroxacin, pefloxacin, enoxacin, and lomefloxacin. Agents with intermediate antianaerobic activity include sparfloxacin and grepafloxacin [192]. Trovafloxacin, gatifloxacin, and moxifloxacin are effective against most anaerobes [59]. The use of trovafloxacin has been restricted because of hepatotoxicity. Quinolones with the greatest in vitro activity against anaerobes include clinafloxacin and sitafloxacin [193].

Moxifloxacin has been evaluated and approved by the FDA as single agent therapy in intra-abdominal infections in adults [81, 94] and is active against intra-abdominal anaerobic isolates [36, 194]. However, because of increasing fluoroquinolone resistance in both *B. fragilis* group [19, 22, 36] and *E. coli*, it has limited use in intra-abdominal infections [81].

A pooled analysis of 4 randomized clinical trials (2000–2010) assessed the comparative efficacy of moxifloxacin in complicated intra-abdominal infections in 745 microbiologically evaluable cases and evaluated its efficacy against *B. fragilis* [194]. Of pre-therapy anaerobes from moxifloxacin-treated patients, 561 (87.4 %) were susceptible at ≤2 mg/L, 34 (5.3 %) were intermediate at 4 mg/L, and 47 (7.3 %) were resistant at ≥8 mg/L. Moxifloxacin had similar clinical success rates against all anaerobes including those isolated from patients infected with *B. fragilis* (158 [82.7 %] of 191 patients), *B. thetaiotaomicron* (74 [82.2 %] of 90 patients), and *Clostridium* spp. (37 [80.4 %] of 46 patients). The overall clinical success rate for all anaerobes was 82.3 %. For all anaerobes combined, the clinical success rate was 83.1 % (466 of 561 patients) for an MIC of ≤2 mg/L, 91.2 % (31 of 34 patients) for an MIC of 4 mg/L, 82.4 % (14 of 17 patients) for an MIC of 8 mg/L, 83.3 % (5 of 6 patients) for an MIC of 16 mg/L, and 66.7 % (16 of 24 patients) for an MIC of ≥32 mg/L. This data suggests that moxifloxacin can be used for anaerobic intra-abdominal infections provided that the patient has mild or moderate disease and has not been recently exposed to a fluoroquinolone therapy. Moxifloxacin can be an alternative agent in the highly penicillin allergic patient.

The use of the quinolones is restricted in growing children because of their potential adverse effects on the cartilage. The main concerns with expanding the use of fluoroquinolones to treat anaerobic infections have been the increasing resistance in *B. fragilis* group as well as anaerobic Gram-positive cocci and the impact of these antibiotics on the growing incidence of *C. difficile*-associated disease [193].

3.16.1 Fluoroquinolone Resistance

Bacteroides spp. resistance to fluoroquinolone can be caused by either an alteration in efflux of the antibiotic or a mutation in the quinolone resistance determining region (QRDR) of the gyrase A gene (*gyrA*) from single or multiple mutations [8]. Both mechanisms can cause high-level resistance.

A study [195] of 4434 *B. fragilis* group isolates obtained from 12 US medical centers between 1994 and 2001 found that fluoroquinolones resistance was species and source of isolation dependent. *B. vulgatus* isolates from decubitus ulcers were the most resistant (71 %). Moxifloxacin resistance rates ranged from 17 % for *B. fragilis* recovered from the female genitourinary tract to 52 % isolated from blood culture (moxifloxacin MIC breakpoint, 4 μg/mL).

A recent US survey [28] illustrated that 27 % of *B. fragilis*, 26 % of *B. thetaiotaomicron*, 38 % of *B. ovatus*, and 55 % of *B. vulgatus* were moxifloxacin resistant. A study of strains recovered from intra-abdominal infections (2001–2004) found moxifloxacin resistance in 13 % of both of *B. fragilis* and *B. thetaiotaomicron* [194]. Overall 86 % (303/363) of all *B. fragilis* group and 417/450 of all other anaerobic genera and species, including *Fusobacterium*, *Prevotella*, *Porphyromonas*, *C. perfringens*, *Eubacterium*, and *Peptostreptococcus* spp., were susceptible to <2 μg/mL of moxifloxacin. AS study of 179 respiratory tract anaerobes identified a single resistant strain of *C. clostridioforme* [196]. A study of 550 anaerobes recovered from intra-abdominal and diabetic foot infections reported that 97 % were susceptible to moxifloxacin [59]. A study from Taiwan of nosocomial infections and bacteremias observed that 90 % of *B. fragilis* isolates were susceptible to moxifloxacin [25]. In contrast, a report from Europe [26] found 15 % fluoroquinolone resistance with geographic variations from 7 % in southern Europe to 30 % in northern Europe. Factors that may account for these variations include differences in susceptibility that depend on the sources of isolation and local antimicrobial utilization patterns. Supportive of this theory was that 41/42 *B. fragilis* group strains isolated from pediatric intra-abdominal infections were susceptible to moxifloxacin, which is infrequently utilized in children [85].

Fusobacterium canifelinum, isolated from cat and dog bite wound infections, is intrinsically resistant to fluoroquinolones because of Ser79 replacement with leucine and Gly83 replacement with arginine on *gyr*A [197].

Moxifloxacin has been approved by the FDA for the treatment of complicated skin and skin structure infections including those caused by *B. fragilis* and for mixed intra-abdominal infections due to *B. fragilis*, *B. thetaiotaomicron*, *Peptostreptococcus* spp., and *C. perfringens*.

3.17 Aminoglycosides

Anaerobes are resistant to all aminoglycosides because these agents do not reach their target site in these bacteria. Of interest is that anaerobic bacteria do not inactivate aminoglycosides and that in a cell-free system both streptomycin and gentamicin are able to bind and inhibit protein synthesis in both *B. fragilis* and *C. perfringens* ribosomes [198].

The uptake of aminoglycosides involves a two-step process: an energy-independent and an energy-dependent one. The energy necessary for the energy-driven phase of drug uptake is obtained from an oxygen- or nitrogen-dependent electron transport system. Strictly anaerobes do not possess this electron transport system and are therefore incapable of importing aminoglycosides [199, 200]. This is supported by the fact that aminoglycosides do not accumulate inside either *B. fragilis* or *C. perfringens* [36].

3.18 Other Agents

In vitro data is available for several antimicrobials. Bacitracin is active against pigmented *Prevotella* and *Porphyromonas* spp. but is not effective against *B. fragilis* and *Fusobacterium nucleatum* [84]. Vancomycin and daptomycin are active against all Gram-positive anaerobes, but is inactive against AGNB [201]. Quinupristin/dalfopristin possesses antibacterial activity against *C. perfringens*, *Lactobacillus* spp., and *Peptostreptococcus* spp. [202]. Linezolid is effective against *Fusobacterium nucleatum*, other *Fusobacterium* spp., *Porphyromonas* spp., *Prevotella* spp., and *Peptostreptococcus* spp. [141]. Minimal clinical experience has, however, been gained in the treatment of anaerobic bacteria using these agents.

4 Transfer of Antibiotic Resistance

Anaerobic bacteria are capable of acquiring and disseminating by conjugation a variety of mobile DNA transfer factors, many of which harbor antibiotic resistance genes. These organisms are the main component of the normal human gastrointestinal flora, contribute to polymicrobial infections including abscesses, and can survive in hypoxic/anoxic environments. All these environments can provide conditions for the rapid disseminate of antibiotic resistance determinants.

The transfer of resistance genes has been observed in the *B. fragilis* group and in *Prevotella*, *Clostridium*, and *Fusobacterium* spp. [203]. Bacterial conjugation, which is the dominant mechanism in the *Bacteroides*, is the most common transmission method of antibiotic resistance genes in anaerobes. The resistance genes are situated in DNA transfer factors that contain sometimes mobile transposons, plasmids, and chromosomal elements [149, 204]. These elements can be small harboring only the genes needed for initiation of DNA transfer. The actual transfer of the DNA from one cell to another cell requires a mating connector bridge that is encoded by much larger transferable conjugative transposons [149]. Two sets of biochemical processes are needed for successful horizontal transmission of the transmissible DNA. One process forms a DNA protein complex (called the relaxosome), comprising transfer factor-encoded mobilization proteins that is assembled on the origin of transfer (*oriT*), and results in the formation of a single-stranded nick that creates the transferred molecule. The nicked DNA is then unwound and is transmitted from the donor to the recipient cell. This process occurs during conjugation alongside with the restoration to the double-stranded form in both cells. One to three mobilization proteins are required for adequate relaxosome formation in *Bacteroides* spp. with mobilization proteins specific for their cognate *oriT*s.

The second process needed for transfer is the formation of the mating or conjugal apparatus. This apparatus is a proteinaceous structure that spans the donor and recipient cell membranes and facilitates the transfer of DNA and has not been well characterized among the anaerobes. It is believed to be encoded by the transfer region of conjugative transposons. Genes that possibly encode this apparatus have been found on a conjugative transposon called cTnDOT [205], and the formation of a pilus-like cell-surface appendage is required for the conjugation [206].

Members of the *B. fragilis* group that resist tetracycline are likely to harbor conjugative transposons. CTnDOT, which is the most thoroughly investigated conjugative transposon, contains a tetracycline resistance determinant and genes whose by-products are involved in the formation of the mating bridge [207].

Anaerobic conjugative transposons are mobile genetic elements that are also called Tet elements. The name entails their ability to harbor a tetracycline resistance gene that confers ribosomal protection [208]. These elements encode the conjugative transfer apparatus that assembles at the interface of donor and recipient cells and forms the physical conduit through which DNA containing antibiotic resistance genes is transferred from cell to cell [205, 206]. Exposure of the bacteria to a low, subinhibitory concentration of tetracycline seems to upregulate the expression of transfer apparatus proteins in *Bacteroides* spp. [175]. This exposure increases the conjugative transfer frequency of the intracellular Tet element and the other coresident mobile [209]. Multiple unrelated transfer factors that may carry different antibiotic resistance genes can therefore be transferred during conjugation. This may result in the rapid rise in stable antibiotic resistance among the different bacterial genera of anaerobic bacteria [210]. A conjugative transposon named CTnGERM1

that carries an erythromycin resistance gene and was previously identified only in Gram-positive bacteria was also found in *Bacteroides* spp. [211]. Based on hybridization and DNA sequence analyses, it is assumed that Gram-positive bacteria are likely to be the origin of this transposon. This phenomenon of transposon transfer can be demonstrated in the laboratory, where resistance determinants can be efficiently transferred by conjugation within *Bacteroides* spp. and from *Bacteroides* spp. to *E. coli* and other unrelated bacteria.

Animal bacterial flora may also be a source of resistant anaerobic bacteria as transfer factors can also be transmitted from ruminal animals to humans [212]. Human colonic bacterial flora may acquire resistance determinants from animal sources. The extensive use of antibiotic in livestock has generated an increase in the spread of resistant determinants among ruminal gut flora, many of which may also be acquired by humans.

5 The Role of Beta-Lactamase Producing Bacteria in Mixed Infections

Penicillins have been the agents of choice for the therapy of a variety of anaerobic infections at different anatomical locations (Table 63.8). However, within the last 50 years, an increased resistance to these drugs has been observed, especially in AGNB (*Bacteroides fragilis* group, Pigmented *Prevotella* and *Porphyromonas*, *Prevotella bivia*, and *Prevotella disiens*) and *Fusobacterium* spp. [2, 3, 42].

BLPB may have an important clinical role in infections. Not only can these organisms cause the infection, but they may also have an indirect effect through their ability to produce the BLs. BLPB may not only survive penicillin therapy but also may protect other penicillin-susceptible bacteria from penicillins by releasing the free enzyme into their environment [213].

Anaerobic BLPB were isolated in a variety of mixed infections. These include respiratory tract, skin, soft tissue, and surgical infections and other infections. The clinical in vitro and in vivo evidence supporting the role of these organisms in the increased failure rate of penicillin in eradication of these infections and the implication of that increased rate on the management of infections is discussed below.

5.1 Mixed Infections Involving Anaerobic BLPB

Anaerobic BLPB can be isolated from a variety of infections in adults and children, sometimes as the only isolates and sometimes mixed with other flora (Table 63.5). Table 63.6 summarizes our experience in the recovery of these organisms from skin and soft tissue infections [214–223], upper respiratory tract [224–236], lower respiratory tract [237–240], intra-abdominal [241–243], obstetric and gynecologic [244], and miscellaneous infections [245–248].

The rate of isolation of these organisms varies in each infection entity (Table 63.6) [248]. BLPB were present in 288 (44 %) of 648 patients with *skin and soft tissue infections*,

Table 63.5 Infections involving beta-lactamase-producing bacteria (BLPB)

Infections	Predominant BLPB
Respiratory tract	
Acute sinusitis and otitis	*H. influenzae*, *M. catarrhalis*
Chronic sinusitis and otitis	*S. aureus*, anaerobic Gram-negative bacilli
Tonsillitis	*S. aureus*, anaerobic Gram-negative bacilli
Bronchitis, pneumonia	*H. influenzae*, *M. catarrhalis*, *L. pneumophila*
Aspiration pneumonia, lung abscesses	*S. aureus*, anaerobic Gram-negative bacilli, *Enterobacteriaceae*
Skin and soft tissue	
Abscesses, wounds, and burns in the oral areas, paronychia, bites	*S. aureus*, pigmented Prevotella and Porphyromonas
Abscesses, wounds, and burns in the rectal area	*E. coli*, *B. fragilis* group, *P. aeruginosa*
Abscesses, wounds, and burns in the trunk and Extremities	*S. aureus*, *P. aeruginosa*
Obstetric and gynecologic	
Vaginitis, endometritis, salpingitis, pelvic inflammatory disease	*N. gonorrhoeae*, *E. coli*, *Prevotella* spp.
Intra-abdominal	
Peritonitis, chronic cholangitis, abscesses	*E. coli*, *B. fragilis* group
Miscellaneous	
Periapical and dental abscesses	pigmented Prevotella and Porphyromonas
Intracranial abscesses	*S. aureus*, anaerobic Gram-negative bacilli
Osteomyelitis	*S. aureus*, anaerobic Gram-negative bacilli

Anaerobic Gram-negative bacilli = Bacteroides, prevotella and porphyromonas

Table 63.6 Recovery rate of anaerobic BLPB from various sites [248]

Infection	No. patients with BLPB/total no. patients (%)	Total no. of BLPB	Pigmented *Prevotella* and *Porphyromonas* spp.	*P. oralis*	*P. oris* and *buccae*	*B. fragilis* group	*Bacteroides* and other anaerobic Gram-negative bacilli
Skin/subcutaneous	288/648 (44 %)	332	19/87	2/9	2/3	75/75	8/63
	% of patients[b]		7 %	1 %	0.6 %	26 %	3 %
Upper respiratory	262/514 (51 %)	344	73/191	19/45	2/14	52/52	3/98
Tract	% of patients		28 %	7 %	1 %	20 %	1 %
Pulmonary	81/137 (59 %)	104	13/59	0/1	1/9	29/29	0/11
	% of patients		16 %	0 %	1 %	36 %	0 %
Surgical	104/113 (92 %)	113	0/26			102/102	5/23
	% of patients		0 %			98 %	5 %
Other infections	16/57 (28 %)	17	6/24	2/7		4/4	1/10
	% of patients		37 %	12 %		25 %	6 %
All patients	744/1469 (51 %)	910	111/387	23/62	5/26	262/262	17/205
3 %	% of patients		15 %	3 %	1 %	35 %	2 %

[a]Number of strains producing beta-lactamase/total number of strains
[b]Number of patients with the specific BLPB/total number of patients with BLPB

75 % harbored aerobic and 36 % had anaerobic BLPB. The infections in which BLPB were most frequently recovered were vulvovaginal abscesses (80 % of patients), perirectal and buttock abscesses (79 %), decubitus ulcers (64 %), human bites (61 %), and abscesses of the neck (58 %). The predominant BLPB were *S. aureus* (68 % of patients with BLPB) and the *B. fragilis* group (26 % of patients with BLPB).

BLPB were found in 262 (51 %) of 514 patients with *upper respiratory tract infection* (URTI); 72 % had aerobic BLPB and 57 % had anaerobic. The infections in which these organisms were most frequently recovered were adenoiditis (83 % of patients), tonsillitis in adults (82 %) and children (74 %), and retropharyngeal abscess (71 %). The predominant BLPB were *S. aureus* (49 % of patients with BLPB), pigmented *Prevotella* and *Porphyromonas* (28 % of patients with BLPB) and the *B. fragilis* group (20 % of patients with BLPB).

BLPB were isolated in 81 (59 %) of 137 children with *pulmonary infections*; 75 % had aerobic BLPB, and 53 % had anaerobic BLPB. The largest number of patients with BLPB was found in patients with cystic fibrosis (83 % of patients), followed by pneumonia in intubated patients (78 %) and lung abscesses (70 %). The predominant BLPB was *B. fragilis* group (36 % of patients with BLPB), *S. aureus* (35 % of patients with BLPB), pigmented *Prevotella* and *Porphyromonas* spp. (16 % of patients with BLPB), *P. aeruginosa* (14 % of patients with BLPB), *K. pneumoniae* (11 % of patients with BLPB), and *E. coli* (10 % of patients with BLPB).

BLPB were recovered in 104 (92 %) of 113 patients with *surgical infections*; 5 % of the patients had aerobic BLPB and 98 % had anaerobic BLPB (Table 63.3). The most predominant BLPB was the *B. fragilis* group (98 % of patients with BLPB).

BLPB were recovered in 16 (28 %) of 57 patients with *miscellaneous infections*, which included periapical and intracranial abscesses and anaerobic osteomyelitis; 25 % had aerobic BLPB and 80 % had anaerobic BLPB. The rate of recovery of BLPB was not significantly different in these infections. The most frequently recovered BLPB were pigmented *Prevotella* and *Porphyromonas* spp. (37 % of patients with BLPB), *S. aureus* and *B. fragilis* groups (25 % each of patients with BLPB).

Pelvic inflammatory disease (PID) is a polymicrobial infection [249] involving in most cases numerous isolates, including *N. gonorrhoeae*, *Chlamydia trachomatis*, *Enterobacteriaceae*, and AGNB (*B. fragilis*, *P. bivius*, and *P. disiens*). All of the above organisms (except for *C. trachomatis*) are capable of producing BL. In a summary of 36 studies published from 1973 to 1985, Eschenbach found BLPB in 1483 (22 %) of 6637 specimens obtained from obstetric and gynecologic infections [249]. The predominant BLPB were *Enterobacteriaceae*, *S. aureus*, *B. fragilis* group and pigmented *Prevotella* and *Porphyromonas* spp. The increase in the failure rate of penicillin in eradicating these infections is an indirect proof of their importance [244, 250, 251].

We have recovered 2052 isolates from 736 patients with obstetrical and gynecological infections [244]. Of these isolates, 355 (17 %) were BLPB, 211 (59 %) were anaerobes, and 144 (41 %) were aerobes and facultative. These BLPB were recovered from 276 (37 %) of all 736 patients. The most frequently recovered BLPB were *Bacteroides* spp. Among them *B. fragilis* group accounted for 129 (36 %) of all 355 BLPB. Ninety-nine percent of *B. fragilis* group were BLPB. Others were *P. bivia* (49 of 151 isolates, or 32 %, were BLPB), *P. disiens* (6 of 17, or 35 %), and *P. melaninogenica*

(23 of 110, or 21 %). *S. aureus* was the second most common BLPB isolated in 21 % of patients.

5.2 Production of Beta-Lactamase by Anaerobic Gram-Negative Bacilli in Clinical Infections

B. fragilis group has been known to be capable of producing BL. These organisms are the predominant anaerobic Gram-negative bacilli present in intra-abdominal infections [242] and anaerobic bacteremias [252]. Within the last decade, however, other AGNB previously not recognized as capable of producing BL have acquired this ability. These include the pigmented *Prevotella* and *Porphyromonas* (*P. intermedia*, *P. melaninogenica*, *Porphyromonas asaccharolytica*, and *Porphyromonas gingivalis*), *Prevotella oralis* and *Prevotella oris-buccae* (all are the most common AGNB in respiratory tract infections), and *Prevotella disiens* and *Prevotella bivia* (the most prominent AGNB in pelvic and other obstetrical and gynecological infections) [250].

All 262 isolates of *B. fragilis* group that we recovered from our patients produced BL (Table 63.6). These isolates accounted for 29 % of the BLPB and were isolated in 35 % of the patients with BLPB. *B. fragilis* was recovered in 98 % of patients with BLPB with surgical infections, in 36 % of those with pulmonary infections, in 26 % of those with skin and soft tissue infections, and in 20 % of those with URTI.

One-hundred eleven of 387 (29 %) pigmented *Prevotella* and *Porphyromonas* spp., which accounted for 12 % of BLPB, were isolated in 15 % of the patients with BLPB. The highest frequency of recovery of BL-producing pigmented *Prevotella* and *Porphyromonas* spp. isolates was found in URTI (38 % of all pigmented *Prevotella* and *Porphyromonas* spp. isolates); the isolates were recovered in 28 % of patients with URTI, mostly in those with recurrent tonsillitis and chronic OM. In pulmonary infections 22 % of the pigmented *Prevotella* and *Porphyromonas* spp. isolates produced BL, and they were isolated in 16 % of the patients. Although 22 % of the isolates of the pigmented *Prevotella* and *Porphyromonas* spp. produced BL in skin and soft tissue infections, these organisms were isolated only in 7 % of patients with these infections, mostly in those that were in close proximity or originated from the oral cavity.

Although 37 % of isolates of *P. oralis* produced BL, they were isolated in 3 % of the patients. Smaller percentages of *P. oris-buccae* and other AGNB were also detected. Their distribution among the infectious processes was similar to the distribution of pigmented *Prevotella* and *Porphyromonas* spp.

Penicillin resistance through production of BL is increasingly seen in the genus *Fusobacterium*. This is most commonly seen in *F. nucleatum*, but also in other member of the genus such as in *Fusobacterium varium* and *Fusobacterium mortiferum* [253, 254]. Since *Fusobacterium* spp. is predominant in oral infection, it is not surprising that their presence was associated with failure of therapy of respiratory infections [255].

5.3 Evidence for Indirect Pathogenicity of Anaerobic BLPB

The production of the enzyme BL is an important mechanism of indirect pathogenicity of aerobic and anaerobic bacteria that is especially apparent in polymicrobial infection. Not only are the organisms that produce the enzyme protected from the activity of penicillins, but other penicillin-susceptible organisms can also be shielded. This protection can occur when the enzyme BL is secreted into the infected tissues or abscess fluid in sufficient quantities to break the penicillin's beta-lactam ring before it can kill the susceptible bacteria [256–260] (Fig. 63.1). Clinical and laboratory studies will be described that provide support for this hypothesis.

5.3.1 In Vivo and In Vitro Studies

Animal studies demonstrated the ability of the enzyme BL to influence polymicrobial infections. Hackman and Wilkins showed that penicillin-resistant strains of *B. fragilis*, pigmented *Prevotella* and *Porphyromonas* spp., and *P. oralis* protected a penicillin-sensitive *Fusobacterium necrophorum* from penicillin therapy in mice [261]. Brook et al. [256–260], using a subcutaneous abscess model in mice, demonstrated protection of group A beta-hemolytic streptococci (GABHS) from penicillin by *B. fragilis* and *P. melaninogenica*. Clindamycin or the combination of penicillin and clavulanic acid (a BL inhibitor), which are active against both GABHS and AGNB, were effective in eradicating the infection. Similarly, BL-producing facultative bacteria protected a penicillin-susceptible *P. melaninogenica* from penicillin

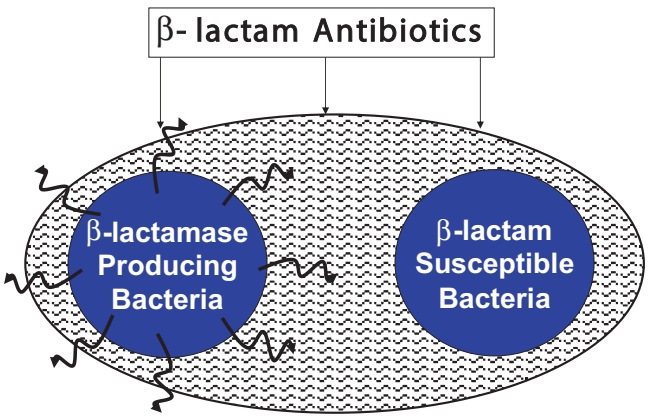

Fig. 63.1 Protection of penicillin-susceptible bacteria from penicillin by beta-lactamase-producing bacteria

[257]. O'Keefe et al. [262] demonstrated inactivation of penicillin-G in an experimental *B. fragilis* infection model in the rabbit peritoneum.

In vitro studies have also demonstrated this phenomenon. A 200-fold increase in resistance of GABHS to penicillin was observed when it was inoculated with *S. aureus* [258]. An increase in resistance was also noted when GABHS was grown with *Haemophilus parainfluenzae* [259]. When mixed with cultures of *B. fragilis* the resistance of GABHS to penicillin increased 8500-fold [263].

BL in clinical infections: Several studies demonstrate the activity of the enzyme BL produced by anaerobic bacteria in polymicrobial infections. De Louvois and Hurley [264] demonstrated degradation of penicillin, ampicillin, and cephaloridine by purulent exudates obtained from 4 of 22 patients with abscesses. Studies by Masuda and Tomioka [265] demonstrated BL activity in empyema fluid. Most infections were polymicrobial and involved both *K. pneumoniae* and *P. aeruginosa.*

The presence of the enzyme BL in clinical specimens was also reported. Bryant et al. [266] detected strong enzyme activity in 4 of 11 pus specimen obtained from 12 patients with polymicrobial intra-abdominal abscess or polymicrobial empyema.

We measured BL activity in 40 (55 %) of 109 abscesses [241]. One hundred BLPB were recovered in 88 (77 %) specimens. These included all 28 isolates of *B. fragilis*, 18 of 30 pigmented *Prevotella* and *Porphyromonas* spp., 42 of 43 *S. aureus*, and 11 of 14 *E. coli.*

We detected the presence of BL activity in 46 of 88 (55 %) ear aspirates that contained BLPB [251]. We were also able to find BL activity in ear aspirates of 30 of 38 (79 %) children with chronic otitis media [267], in 17 of 19 (89 %) ear aspirates of children with acute otitis media who failed AMX therapy [268], and in 12 sinus aspirates (three acute and nine chronic infection) of the 14 aspirates that contained BLPB. The predominant BLPBs in acute sinusitis were *H. influenzae* and *M catarrhalis*; those in chronic sinusitis were *S. aureus*, *Prevotella* spp., *Fusobacterium* spp., and *B. fragilis* (Table 63.7) [269].

A study investigated the monthly changes in the rate of recovery of aerobic and anaerobic penicillin-resistant bacteria in the oropharynx of children [270]. Each month over a period of year, 1993, 30 children who presented with URTI were studied. The maximal total number of aerobic and anaerobic BLPB and number of patients with BLPB was in April (60 % of patients) and the lowest was in September (13 %). A gradual increase of BLPB and penicillin-resistant *S. pneumoniae* occurred from September to April, and a slow decline took place from April to August. These changes correlated directly with the intake of beta-lactam antibiotics. The study was reported over the following year with similar results. The crowding and the increased use of antibiotics that are more common in the winter might have also contributed to the spread of BLPB. Monitoring the local seasonal variation in the rate of BLPB may be helpful in the empiric choice of antimicrobials. Judicious use of antimicrobials may control the increase of BLPB.

Table 63.7 Beta-lactamase detected in four patients with chronic sinusitis aspirates [269]

Beta-lactamase detected in chronic sinusitis aspirates					
		Patient no.			
	Organism	1	2	3	4
	Staphylococcus aureus (BL +)		+		+
	Streptococcus pneumoniae	+			
	Peptostreptococcus spp.	+			+
	Propionibacterium acnes	+			
	Fusobacterium spp. (BL +)		+		+
	Fusobacterium spp. (BL −)		+		+
	Prevotella spp. (BL +)			+	
	Prevotella spp. (BL −)	+	+	+	
	Bacteroides fragilis group (BL +)	+			+
	Beta-lactamase activity in plus	+	+	+	+
	BL+ = beta-lactamase-producing bacteria				

Clinical studies illustrating failure of penicillins due to anaerobic BLPB: The recovery of penicillin-susceptible bacteria mixed with BLPB in patients who have failed to respond to penicillin or cephalosporin therapy suggests the ability of BLPB to protect a penicillin-susceptible or cephalosporin-susceptible organism from the activity of those drugs.

Selection of BLPB following antimicrobial therapy may account for many of the clinical failures after penicillin therapy. Heimdahl et al. [263] described five adults with clinical failures after penicillin therapy associated with the isolation of anaerobic BLPB. In a study of 185 children with orofacial and respiratory infections who failed to respond to penicillin, BLPB were recovered in 75 (40 %) [271]. The predominant BLPB were *S. aureus*, pigmented *Prevotella* and *Porphyromonas* spp., *B. fragilis* group, and *P. oralis.*

Increased failure rate of penicillins in the therapy of pelvic inflammatory disease (PID) has also been noticed and these agents are no longer recommended for this infection. Treatment failure has been noticed in as many as 33 % of patients and increased frequency of abscess formation has been observed [272]. Therapy with penicillin, either alone or with an aminoglycoside or tetracycline, failed in 15–25 % of cases [251]. This increased failure rate may be due to the increased resistance to penicillin of anaerobic Gram-negative bacilli and *Neisseria gonorrhoeae* as well as that of the *Enterobacteriaceae* involved in PID.

The URTI in which the phenomenon of indirect pathogenicity was most thoroughly studied is recurrent tonsillitis due to GABHS. Penicillin was considered the drug of choice for the therapy of this infection. However, the frequently reported inability of penicillin to eradicate GABHS is of

concern. GABHS persists in the pharynx despite treatment with intramuscular penicillin in 21 % of the patients after the first course of therapy and in 83 % of the remainder of the patients after retreatment [273]. Two randomized, single-blind, trials illustrated that either oral penicillin V or intramuscular penicillin failed to eradicate GABHS in pharyngitis in 35 % children treated with oral penicillin V and 37 % of intramuscular penicillin [274].

Various theories have been offered to explain this penicillin failure. One theory is that repeated penicillin administration results in a shift in the oral microflora with selection of BL-producing strains of *Haemophilus* spp., *S. aureus*, *M. catarrhalis*, and AGNB [258, 259, 263, 271, 275, 276]. It is possible that these BLPB can protect the GABHS from penicillin by inactivation of the antibiotic.

Clinical evidence supporting the ability of a BLPB to protect a penicillin-susceptible pathogen was reported in numerous studies [258, 259, 277].

The role of anaerobic BLPB in persistence of GABHS was suggested by Brook et al. [233, 234] who studied core tonsillar cultures recovered from children and young adults suffering from recurrent tonsillitis. One or two strains of aerobic and/or anaerobic BLPB were recovered in over 3/4 of the tonsils. The anaerobic BLPB included strains of *B. fragilis* group, pigmented *Prevotella* and *Porphyromonas* spp., and *P. oralis*, while the aerobic bacteria were *S. aureus*, *Haemophilus* spp., and *M. catarrhalis*. This observation was confirmed by Reilly et al. [278], Chagollan et al. [279], and Tuner and Nord [280]. Assays of the free enzyme in the tissues demonstrated its presence in 33 of 39 (85 %) tonsils that harbored BLPB, while the enzyme was not detected in any of the 11 tonsils without BLPB [281].

Tuner and Nord [282] and Brook and Gober [283] have demonstrated the rapid emergence of aerobic and anaerobic BLPB following penicillin therapy. Tuner and Nord [282] studied the emergence of BLPB in the oropharynx of ten healthy volunteers treated with penicillin for 10 days. A significant increase in the number of BL-producing strains of *Bacteroides* spp., *F. nucleatum*, and *S. aureus* was observed. BL activity in saliva increased significantly in parallel to the increase of BLPB.

Brook and Gober [283] isolated BLPB in 3 of 21 (14 %) children prior to penicillin therapy, and in 10 of 21 (48 %) following one course of penicillin. These organisms were also isolated from household contacts of children repeatedly treated with penicillin, suggesting their possible transfer within a family. The organisms were members of the pigmented *Prevotella* and *Porphyromonas* spp., *S. aureus*, *M. catarrhalis*, and *H. influenzae.* In a study of 26 children who received 7 days' therapy with penicillin, prior to therapy 11 % harbored BLPB in their oropharyngeal flora [284]. This increased to 45 % at the conclusion of therapy, and the incidence was still 27 % 3 months later. These data suggest that it is easy to induce BL production in the upper respiratory tract. Following penicillin therapy, these patients became colonized with BLPB.

Certain groups of children are at greater risk for developing penicillin-resistant flora. The daily administration of amoxicillin chemoprophylaxis selected for colonization with aerobic and anaerobic BLPB in all 20 children studied by Brook and Gober [285].

An association has been noted between the presence of BLPB even prior to therapy of acute GABHS tonsillitis and the outcome of 10-day oral penicillin therapy [286]. Of 98 children with acute GABHS tonsillitis, 36 failed to respond to therapy. Prior to therapy, 18 isolates of BLPB were detected in 16 (26 %) of those cured and following therapy 30 such organisms were recovered in 19 (31 %) of these children. In contrast, prior to therapy, 40 BLPB were recovered from 25 (69 %) of the children who failed, and following therapy, 62 such organisms were found in 31 (86 %) of the children in that group.

A correlation was noted between the rate of recovery of BLPB in healthy children and the rate of AMX failure to eradicate GABHS pharyngo-tonsillitis. Brook and Gober obtained pharyngo-tonsillar cultures from 228 children with GABHS PT, treated with AMX for 10 days, and 663 healthy children [287]. AMX failed to eradicate GABHS from 48 of the 228 treated children (21 %). AMX failure rate varied from month to month; it was high between October and May (22–32 %), with the exception of April (11 %), and low between June and September (8–12 %). BLPB were recovered from 226 of 663 (34 %) well children. The rate of recovery of BLPB varied; it was also high between October and May (40–52 %), with exception of April (23 %), and the lowest between June and September (10–12 %). Prior to their treatment, BLPB were recovered from 26 of the 48 (54 %) children who eventually failed AMX therapy, and from 28 of the 180 (16 %) who did not fail ($p < 0.001$). A high failure rate of penicillins in eradication of GABHS in pharyngo-tonsillitis can serve as sensitive indicator for a high prevalence rate of BLPB in the community.

Roos et al. [288] observed high levels of BL in saliva reflecting colonization with numerous BLPB. These investigators also demonstrated that patients with recurrent GABHS tonsillitis had detectable amounts of BL in their saliva compared to patients with tonsillitis that did not recur.

5.4 Therapeutic Implications of the Presence of BLPB

The presence of BLPB in mixed infection warrants administration of drugs that will be effective in eradication of BLPB as well as the other pathogens. The high failure rate of penicillin therapy associated with the recovery of BLPB in a

growing number of cases of mixed aerobic–anaerobic infections highlights the importance of this therapeutic approach [263, 270].

One infection in which this therapeutic approach has been successful is recurrent tonsillitis [273, 288–301]. Antimicrobial agents active against BLPB as well as GABHS were effective in the eradication of this infection. Studies demonstrated the superiority of lincomycin [289–292], clindamycin [293–298], AMX-C [302], and penicillin plus rifampin [299, 300], over penicillin alone. The superiority of these drugs compared to penicillin is due to their efficacy against GABHS, *S. aureus* as well as AGNB.

Over 83 % of the adenoids in children with chronic adeno-tonsillitis are colonized with aerobic and anaerobic BLPB [303]. The existence of BLPB within the adenoids core may explain the persistence of many pathogens, including *S. pneumoniae*, where they may be shielded from the activity of penicillins. The effect on the adenoid bacterial flora of 10 day therapy with either AMX, AMX-C [304], or clindamycin [305] prior to adenoidectomy for recurrent OM was recently studied. The total number of isolates and bacteria per gram of tissue were lower in those treated with any of the antibiotics. However, the number of potential pathogens and BLPB was lower in those treated with AMX-C [304] and clindamycin [305] as compared to AMX and controls ($P<0.001$).

A similar study evaluated the effects of AMX-C and AMX therapy on the nasopharyngeal flora of 50 children with acute otitis media [306]. After therapy, 16 (64 %) of the 25 patients treated with AMX and 23 (92 %) of the 25 patients treated with AMX-C were considered clinically cured. A significant reduction in the number of both aerobic and anaerobic isolates occurred after therapy in those treated with either agent. The number of all isolates recovered after therapy in those treated with AMX-C was significantly lower (60 isolates) than in those treated with AMX (133 isolates, $P<0.001$). The recovery of known aerobic pathogens (e.g., *S. pneumoniae*, *S. aureus*, GABHS, *Haemophilus* spp., and *M. catarrhalis*) and penicillin-resistant bacteria after therapy was lower in the AMX-C group than in the AMX group ($P<0.005$).

The superiority of AMX-C and clindamycin over AMX in eradicating penicillin-susceptible pathogens such as *S. pneumoniae* and GABHS may be due to their activity against aerobic and anaerobic BLPB. The elimination of both potential pathogenic and nonpathogenic BLPB may be beneficial, as these organisms might "shield" penicillin-susceptible pathogens from penicillins. This phenomenon might explain the survival of penicillin-susceptible bacteria such as *S. pneumoniae* in children treated with AMX.

Two studies compared the efficacy of clindamycin to penicillin in the therapy of lung abscesses [307, 308]. Clindamycin was superior to penicillin in treating the infection. The superiority of clindamycin over penicillin was postulated to be due to its ability to eradicate the BL-producing anaerobic Gram-negative bacilli present in lung abscess.

Antimicrobials effective against anaerobic BLPB (ticarcillin-clavulanate or clindamycin) were superior to an antibiotic without such coverage (ceftriaxone) in the therapy of aspiration or tracheostomy-associated pneumonia in children (93 % vs. 46 %, $p<0.05$) [309].

5.5 Antimicrobial Therapy of Anaerobic Infections

The recovery from an anaerobic infection depends on prompt and proper management. The principles of managing anaerobic infections include neutralizing bacterial toxins, preventing bacterial proliferation by changing the environment, and hampering bacterial spread into healthy tissues.

Toxin neutralization by specific antitoxins may be employed, especially in infections caused by *Clostridium* spp. (tetanus and botulism). Controlling the environment is achieved by debriding of necrotic tissue, draining the pus, improving circulation, alleviating the obstruction, and increasing the tissue oxygenation. In many cases surgical therapy is the most important and sometimes the only form of treatment required, whereas in others it is an adjunct to a pharmacologic approach. Without drainage the infection may persist despite antimicrobial therapy and serious complications can develop. The primary role of antimicrobials is in limiting the local and systemic spread of the organism.

Because anaerobic infection is often polymicrobial and is caused by aerobic and anaerobic organisms, antimicrobials that are effective against both components of the infection should be administrated. When such therapy is not given, the infection may persist, and serious complications may occur [2, 3, 310]. A number of factors should be considered when choosing appropriate antimicrobial agents: They should be effective against all target organism(s), induce little or no resistance, achieve sufficient levels in the infected site, have minimal toxicity, and have maximum stability and longevity.

When selecting antimicrobials for the therapy of mixed infections, their aerobic and anaerobic antibacterial spectrum and their availability in oral or parenteral form should be considered (Tables 63.2 and 63.8). Some antimicrobials have a limited range of activity. For example, metronidazole is active against only anaerobic bacteria and therefore cannot be administered as a single agent for the therapy of mixed infections. Other antimicrobials, such as carbapenems, tigecycline, and the combinations of BL/BLIs, possess a broader spectrum of activity against aerobic and anaerobic bacteria.

Selecting antimicrobial agents is simplified when a reliable culture result is available. However, this may be particularly difficult in anaerobic infections because of the difficulties in obtaining appropriate specimens. For this reason, many

Table 63.8 Antimicrobial recommended for the therapy of site-specific anaerobic infections

	Surgical		
	Prophylaxis	Parenteral	Oral
Intracranial	1. Penicillin	1. Metronidazole[a]	1. Metronidazole[a]
	2. Vancomycin	2. Chloramphenicol	2. Chloramphenicol
Dental	1. Penicillin	1. Clindamycin	1. Clindamycin, amoxicillin + CA
	2. Erythromycin	2. Metronidazole[a], chloramphenicol	2. Metronidazole[a]
Upper respiratory tract	1. Cefoxitin	1. Clindamycin	1. Clindamycin, amoxicillin + CA
	2. Clindamycin	2. Chloramphenicol, metronidazole[a]	2. Metronidazole[b]
Pulmonary	NA	1. Clindamycin[b]	1. Clindamycin[e]
		2. Ticarcillin + CA, ampicillin + SU[c], A carbapenem	2. Metronidazole[b], amoxicillin + CA
Abdominal	1. Cefoxitin	1. Cefoxitin[c], metronidazole[c]	1. Metronidazole[e], Amoxicillin + CA
	2. Clindamycin[c]	2. A carbapenem, piperacillin-tazobactam, tigecycline,	2. Clindamycin[e]
Pelvic	1. Cefoxitin	1. Cefoxitin[c], clindamycin[b]	1. Clindamycin[c]
	2. Doxycycline	2. piperacillin-tazobactam[c], ampicillin + SU[c], metronidazole[c]	2. Amoxicillin + CA[c], metronidazole[c]
Skin and soft tissue	1. Cefazolin[d]	1. Clindamycin, cefoxitin	1. Clindamycin, amoxicillin + CA
	2. Vancomycin	2. Metronidazole + Vancomycin	2. Metronidazole + linezolid
		3. Tigecycline	
Bone and joint	1. Cefazolin[d]	1. Clindamycin, a carbapenem	1. Clindamycin
	2. Vancomycin	2. Metronidazole + vancomycin, piperacillin-tazobactam	2. Metronidazole + linezolid
Bacteremia with BLPB	NA	1. A carbapenem, metronidazole	1. Clindamycin, metronidazole
		2. Cefoxitin, ticarcillin + CA	2. Chloramphenicol, amoxicillin + CA
Bacteremia with non- BLPB	NA	1. Penicillin	1. Penicillin
		2. Clindamycin, metronidazole, cefoxitin	2. Metronidazole, chloramphenicol, clindamycin

NA, not applicable; CA, clavulanic acid; SU, sulbactam
[a]Plus a penicillin
[b]Plus a macrolide (i.e., erythromycin)
[b]sulbactam
[d]In location proximal to the rectal and oral areas use cefoxitin
[e]Plus a quinolone (only in adults)

patients are treated empirically on the basis of suspected, rather than established pathogens. Fortunately, the types of anaerobes involved in many anaerobic infections and their antimicrobial susceptibility patterns tend to be predictable [2, 3]. However, some anaerobic bacteria have become resistant to antimicrobial agents, and many can develop resistance while a patient is receiving therapy [118, 283].

Anaerobic bacteria have always been resistant to aminoglycosides and trimethoprim-sulfamethoxazole. Resistance among some anaerobes has increased significantly over the past three decades. The potential for growing resistance of anaerobes to antimicrobials is especially noted with penicillins, fluoroquinolones, clindamycin, and cephalosporins. Chloramphenicol is rarely used in the United States and resistance is very rare and when present it is due to its inactivation by acetyltransferase.

Aside from susceptibility patterns, other factors influencing the choice of antimicrobial therapy include the pharmacologic characteristics of the various drugs, their toxicity, their effect on the normal flora, and bactericidal activity [2, 3]. Although identification of the infecting organisms and their antimicrobial susceptibility may be needed for selection of optimal therapy, the clinical setting and Gram stain preparation of the specimen may indicate the types of anaerobes present in the infection as well as the nature of the infectious process.

Antimicrobial therapy for anaerobic infections usually should be given for prolonged periods because of their tendency to relapse. This may range from 3 weeks to 3 months depending on the site and severity of the infection.

Because anaerobic bacteria generally are recovered mixed with aerobic organisms, selection of proper therapy becomes more complicated. In the treatment of mixed infection, the

choice of the appropriate antimicrobial agents should provide for adequate coverage of most of the pathogens, aerobic and anaerobic. Some broad spectrum antibacterial agents possess such qualities, while for some organisms additional agents should be added to the therapeutic regimen.

6 Choice of Antimicrobial Agents

The available parenteral antimicrobials in most infections (Tables 63.2 and 63.8) are clindamycin, metronidazole, chloramphenicol, cefoxitin, a penicillin (i.e., ticarcillin, ampicillin, piperacillin) and a BL inhibitor (i.e., clavulanic acid, sulbactam, tazobactam), and a carbapenem (i.e., imipenem, meropenem, ertapenem). An agent effective against Gram-negative enteric bacilli (i.e., aminoglycoside) or an antipseudomonal cephalosporin (i.e., cefepime) are generally added to clindamycin, metronidazole, and, occasionally, cefoxitin when treating intra-abdominal infections to provide coverage for these bacteria. Penicillin can be added to metronidazole in the therapy of intracranial, pulmonary, and dental infections to cover for microaerophilic streptococci, and *Actinomyces*. A macrolide (i.e., erythromycin) is added to metronidazole in upper respiratory infections to treat *S. aureus* and aerobic streptococci. Penicillin is added to clindamycin to supplement its coverage against *Peptostreptococcus* spp. and other Gram-positive anaerobic organisms.

Doxycycline is added to most regimens in the treatment of pelvic infections for chlamydia and mycoplasma. Penicillin is still the drug of choice for bacteremia caused by non-BLPB. However, other agents should be used for the therapy of bacteremia caused by BLPB.

Because the duration of therapy for anaerobic infections, which are often chronic, is generally longer than for infections caused by aerobic and facultative anaerobes, oral therapy is often substituted for parenteral therapy. The agents available for oral therapy are limited and include clindamycin, amoxicillin/clavulanate, chloramphenicol, and metronidazole.

Clinical judgment, personal experience, safety, and patient compliance should direct the physician in the choice of the appropriate antimicrobial agents. The length of therapy generally ranges between 2 and 4 weeks, but should be individualized depending on the response. In some cases, such as lung abscesses, treatment may be required for as long as 6–8 weeks, but can often be shortened with proper surgical drainage.

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Mycobacteria: Tuberculosis

64

Akos Somoskovi and Max Salfinger

1 Introduction

In 2014, there were 9.6 million incident tuberculosis (TB) cases, equivalent to 133 cases per 100,000 population, with 1.5 million TB deaths. Patients with infectious pulmonary TB, which is mainly caused by *M. tuberculosis* and to a lesser degree *M. bovis* and *M. africanum*, are the main sources of transmission of the disease. Their timely diagnosis (including detection of drug resistance) and prompt treatment have four goals:

1. To cure the patient by killing the rapidly multiplying pathogens
2. To prevent transmission
3. To prevent development of drug resistance
4. To sterilize the infected tissue from dormant bacteria to prevent relapse

Non-pulmonary TB cases are usually not infectious to others. In 2014, there were an estimated 1.2 million new HIV-positive TB cases (12 % of all TB cases) [1].

Drug resistance is often divided into two different types. *Acquired drug resistance* (or drug resistance among previously treated cases) develops in a patient who has received or is currently receiving treatment due to interruptions in therapy or an inadequate therapeutic regimen. *Primary* (*or initial*) *drug resistance* is a resistance in newly diagnosed TB cases, which have previously not received anti-TB treatment, i.e., they have been infected with a resistant strain of bacteria. Multidrug resistance (MDR), i.e., resistance to at least rifampin (RIF) and isoniazid (INH), is a problematic form of resistance. High MDR-TB rates are often used as a marker of contemporary weaknesses in the TB control program as they reflect problems with TB treatment and active transmission of resistant cases. Early detection of drug resistance allows the use of appropriate treatment regimens for patients, which has an important impact on improved TB control. Extensively drug-resistant TB (XDR-TB) is defined as resistance to INH and RIF, plus resistance to any fluoroquinolone (FQ) and at least one of three injectable second-line anti-TB drugs.

Acquired drug resistance is the result of inadequate, incomplete, or poor treatment quality that allows the selection of mutant resistant strains. If drug-susceptible TB is treated with a regimen exclusively based on a single effective TB medicine, there is a risk that bacteria with drug-resistant mutations will be selected and multiply further during the course of treatment, eventually becoming the dominant strain. If a person infected with a strain, initially resistant to a specific drug, is treated with that drug plus a new additional drug, then there is a risk of developing resistance to the additional drug. Stepwise additions of drugs may eventually lead to more severe patterns of drug resistance and eventually to untreatable forms of TB [2].

A person has been infected with a drug-resistant TB strain that has primary drug resistance. Transmission of drug-resistant TB occurs in the same way as transmission of drug-susceptible TB. High prevalence of drug-resistant TB in the community increases the risk of drug-resistant TB exposure in the community. Undiagnosed, untreated, or poorly treated drug-resistant TB contributes to sustained high drug-resistant TB prevalence, as well as high proportions of infectious drug-resistant TB cases among the community [2].

The World Health Assembly, convened annually by the World Health Organization (WHO), passed a resolution in May 2014 approving the new post-2015 Global TB Strategy with its ambitious targets (Table 64.1). The "End TB Strategy" (2016–2035) aims to end the global TB epidemic, with targets to reduce TB deaths by 95 % and to cut new cases by 90 % between 2015 and 2035 and to ensure that no family is burdened with catastrophic expenses due to TB. It sets interim milestones for the years 2020, 2025, and 2030 [3].

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Table 64.1 2015 global tuberculosis strategy framework

Vision	A world free of tuberculosis
	– Zero deaths, disease, and suffering due to tuberculosis
Goal	End the global tuberculosis epidemic
Milestones for 2025	– 75 % reduction in tuberculosis deaths (compared with 2015)
	– 50 % reduction in tuberculosis incidence rate (less than 55 tuberculosis cases per 100,000 population)
	– No affected families facing catastrophic costs due to tuberculosis
Targets for 2035	– 95 % reduction in tuberculosis deaths (compared with 2015)
	– 90 % reduction in tuberculosis incidence rate (less than 10 tuberculosis cases per 100,000 population)
	– No affected families facing catastrophic costs due to tuberculosis
Principles	
1	Government stewardship and accountability, with monitoring and evaluation
2	Strong coalition with civil society organizations and communities
3	Protection and promotion of human rights, ethics, and equity
4	Adaptation of the strategy and targets at country level, with global collaboration
Pillars and components	
1	Integrated, patient-centered care and prevention
A.	Early diagnosis of tuberculosis including universal drug-susceptibility testing and systematic screening of contacts and high-risk groups
B.	Treatment of all people with tuberculosis including drug-resistant tuberculosis and patient support
C.	Collaborative tuberculosis/HIV activities and management of comorbidities
D.	Preventive treatment of persons at high risk and vaccination against tuberculosis
2	Bold policies and supportive systems
A.	Political commitment with adequate resources for tuberculosis care and prevention
B.	Engagement of communities, civil society organizations, and public and private care providers
C.	Universal health coverage policy and regulatory frameworks for case notification, vital registration, quality and rational use of medicines, and infection control
D.	Social protection, poverty alleviation, and actions on other determinants of tuberculosis
3	Intensified research and innovation
A.	Discovery, development, and rapid uptake of new tools, interventions, and strategies
B.	Research to optimize implementation and impact and promote innovations

2 Epidemiology

Since 1994, the WHO and the International Union Against Tuberculosis and Lung Disease Supranational Reference Laboratories Network have been a driving force in strengthening national and central level laboratories globally. The network, comprising more than 30 laboratories covering all six WHO regions, is also instrumental in supporting antimicrobial resistance surveys, providing quality assurance through proficiency testing and validating antimicrobial susceptibility test (AST) data [2].

In 2014, globally, an estimated 3.3 % of new cases (Fig. 64.1) and 20 % of previously treated cases have MDR-TB (Fig. 64.2); these levels have remained virtually unchanged in recent years. In 2014, there were an estimated 480,000 new cases of MDR-TB worldwide and approximately 190,000 deaths from MDR-TB. More than half of these patients were in India, China, and the Russian Federation. XDR-TB had been reported by 105 countries by 2015. An estimated 9.7 % of people with MDR-TB have XDR-TB [1].

The 22 high-burden countries that have been given highest priority at the global level since 2000 (listed in Table 64.2) accounted for 83 % of all estimated incident cases worldwide. The six countries that stand out as having the largest number of incident cases in 2014 were India, Indonesia, China, Nigeria, Pakistan, and South Africa [1].

The proportions of new and previously treated TB cases with MDR-TB are shown for the 27 high MDR-TB burden countries in Table 64.3. Eastern European and Central Asian countries continue to have the highest levels of MDR-TB. Among new cases, the proportions with MDR-TB were highest in Belarus, Estonia, Kazakhstan, Kyrgyzstan, the Republic of Moldova, the Russian Federation, Ukraine, and Uzbekistan. Among previously treated TB cases, the proportions with MDR-TB were highest in Belarus, Estonia, Kazakhstan, Kyrgyzstan, the Republic of Moldova, Tajikistan, Ukraine, and Uzbekistan. In the Russian Federation, even though the average proportion of previously treated cases with MDR-TB does not exceed 50 %, the proportion is well above 50 % in several federal subjects [1].

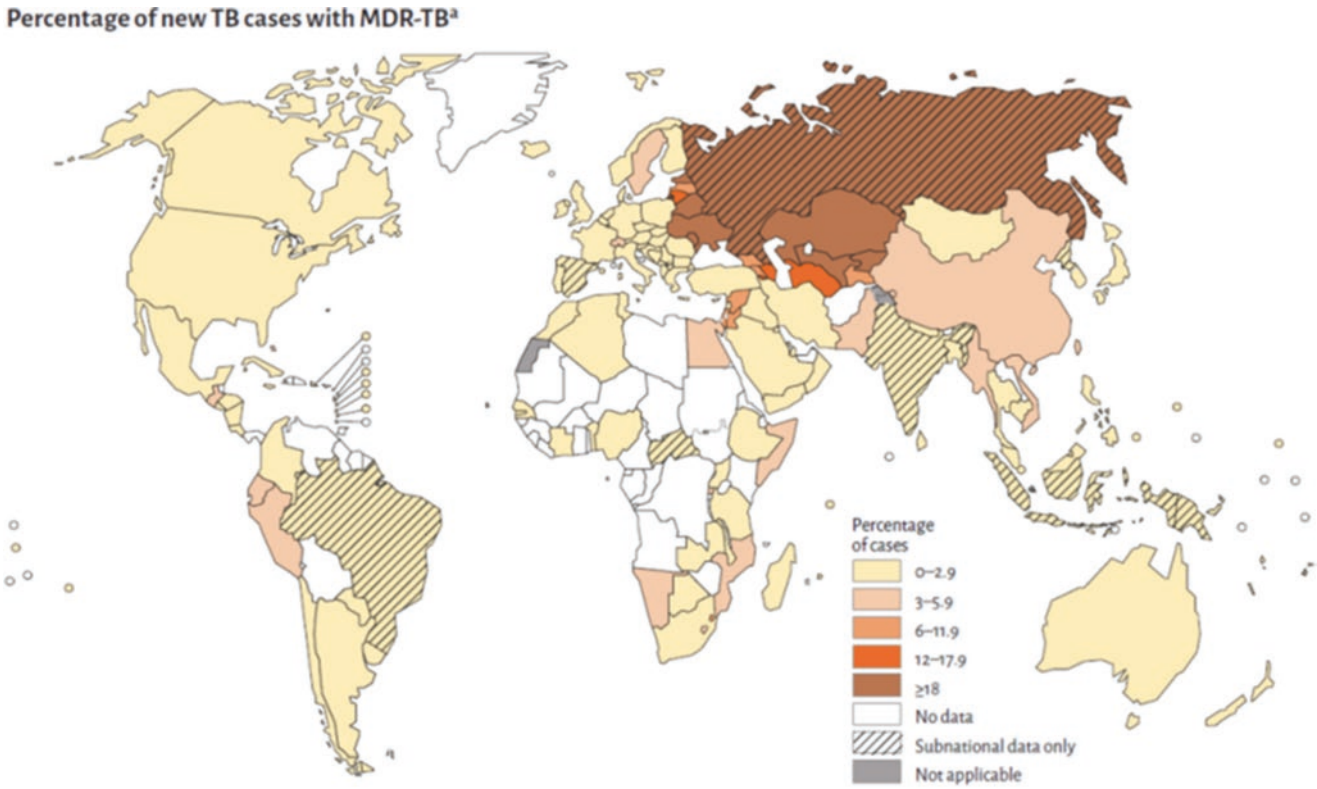

Fig. 64.1 Percentage of new TB cases with MDR-TB

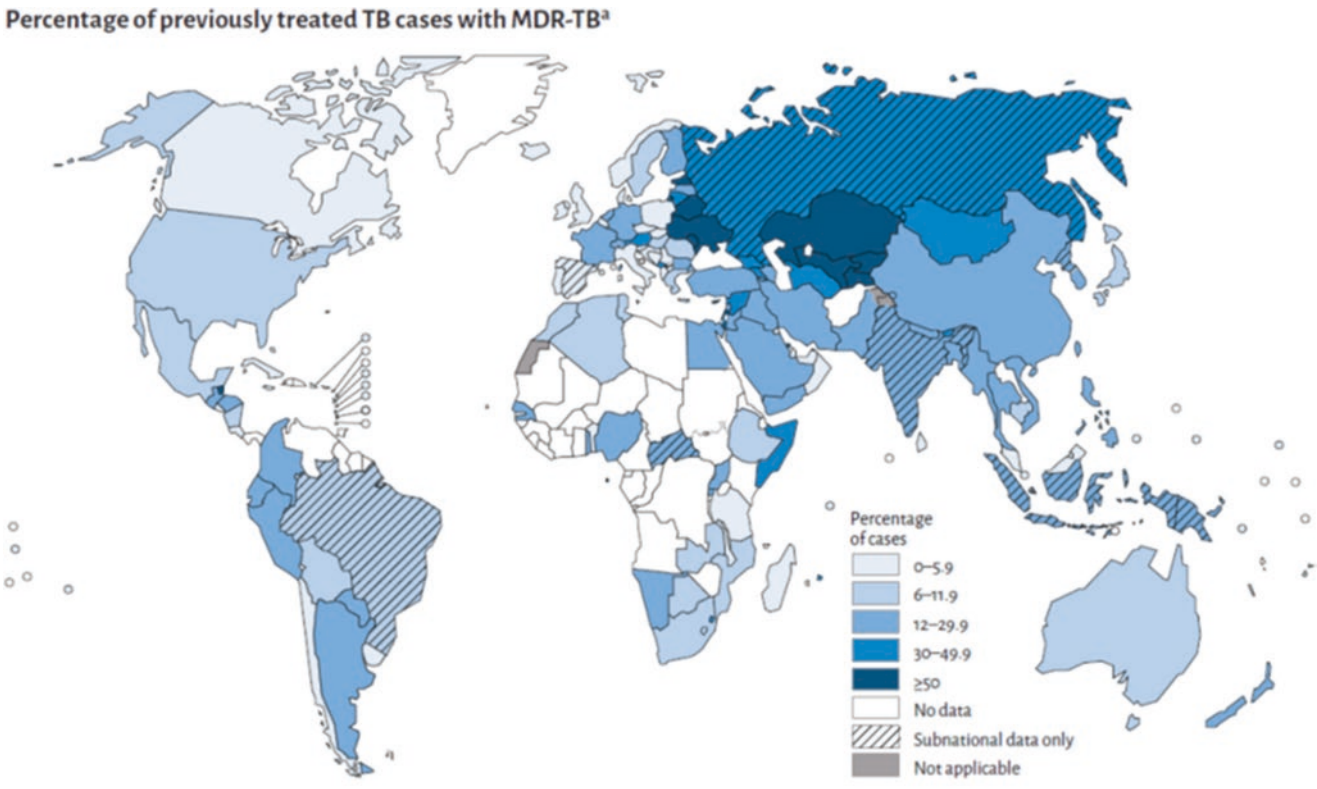

Fig. 64.2 Percentage of previously treated TB cases with MDR-TB

Table 64.2 Estimated epidemiological burden of TB, 2014

	Population	Mortality[b]		HIV-positive TB mortality		Prevalence		Incidence		HIV-positive incident TB cases	
Afghanistan	31,628	14	10–18	<0.1	0–0.1	110	56–180	60	53–67	0.3	0.2–0.4
Bangladesh[c]	159,078	81	59–110	0.2	0.1–0.2	640	340–1000	360	320–410	0.6	0.4–0.7
Brazil	206,078	5.3	4.9–5.7	2.4	1.8–3.2	110	51–180	90	86–95	16	14–17
Cambodia	15,328	8.9	6.3–12	0.8	0.6–1.0	100	87–120	60	54–66	1.8	1.6–2.0
China	1,369,436	38	37–40	0.7	0.5–0.9	1,200	1100–1400	930	860–1000	13	11–16
DR Congo	74,877	52	38–68	6.3	5.0–7.7	400	210–640	240	220–270	34	27–42
Ethiopia	96,959	32	22–43	5.5	4.4–6.8	190	160–240	200	160–240	19	15–23
India	1,295,292	220	150–350	31	25–38	2,500	1700–3500	2 200	2000–2300	110	96–120
Indonesia	254,455	100	66–150	22	13–32	1 600	1300–2000	1 000	700–1400	63	41–90
Kenya	44,864	9.4	6.7–12	8.1	6.4–10	120	64–190	110	110–110	40	38–42
Mozambique	27,216	18	12–26	37	29–45	150	80–240	150	120–180	85	65–110
Myanmar	53,437	28	20–37	4.1	3.3–5.1	240	190–310	200	180–220	19	15–24
Nigeria	177,476	170	91–280	78	53–110	590	450–740	570	340–870	100	59–160
Pakistan	185,044	48	11–110	1.3	0.8–1.9	630	530–740	500	370–650	6.4	4.4–8.7
Philippines	99,139	10	9.0–11	<0.1	0–0.1	410	360–470	290	250–320	2.5	2.0–3.2
Russian Federation	143,429	16	15–16	1.1	0.8–1.3	160	70–270	120	110–130	5.5	4.5–6.6
South Africa	53,969	24	22–26	72	58–89	380	210–590	450	400–510	270	240–310
Thailand	67,726	7.4	3.9–12	4.5	2.3–7.4	160	110–220	120	61–190	15	7.8–24
Uganda	37,783	4.5	3.2–6.1	6.4	5.0–8.1	60	33–95	61	53–69	28	24–32
UR Tanzania	51,823	30	13–54	28	15–43	270	110–510	170	80–290	62	29–110
Vietnam	92,423	17	11–23	1.9	1.3–2.5	180	76–330	130	110–150	7	5.7–8.5
Zimbabwe	15,246	2.3	1.4–3.4	5.2	3.2–7.8	44	24–71	42	29–58	25	17–35
High-burden countries	**4,552,704**	**940**	**790–1100**	**320**	**280–360**	**10,000**	**9200–12,000**	**8 000**	**7500–8500**	**930**	**850–1 000**
AFR	963,361	450	350–560	310	270–350	3200	2800–3600	2700	2400–3000	870	790–950
AMR	981,613	17	16–18	6	5.2–6.8	350	270–440	280	270–290	36	34–38
EMR	635,745	88	43–150	3.2	2.6–4.0	1000	880–1200	740	610–890	12	10–15
EUR	907,279	33	33–34	3.2	2.7–3.7	440	330–560	340	320–350	20	18–21
SEAR	1,906,087	460	350–570	62	51–74	5400	4400–6500	4000	3700–4400	210	180–240
WPR	1,845,184	88	81–95	4.9	4.2–5.7	2100	1900–2400	1600	1500–1600	31	28–35
Global	**7,239,269**	**1100**	**970–1300**	**390**	**350–430**	**13,000**	**11,000–14,000**	**9600**	**9100–10,000**	**1200**	**1100–1300**

Best estimates are followed by the lower and upper bounds of the 95 % uncertainty interval

[a]Numbers for mortality, prevalence, and incidence, in thousands; shown to two significant figures. Totals (HBCs, regional and global) are computed prior to rounding

[b]Mortality excludes deaths among HIV-positive TB cases. Deaths among HIV-positive TB cases are classified as HIV deaths according to ICD-10 and are shown separately in this table

[c]For Bangladesh, a joint reassessment of estimates of TB disease burden will be undertaken following completion of the national TB prevalence survey

Table 64.3 Estimated proportion of TB cases that have MDR-TB globally and for 27 high MDR-TB burden countries and WHO regions

	Estimated % of new TB cases with MDR-TB[a]	95 % confidence interval	Estimated % of re-treatment TB cases with MDR-TB[a]	95 % confidence interval
Armenia	9.4	7.0–12	43	38–49
Azerbaijan	13	10–16	28	22–37
Bangladesh	1.4	0.7–2.5	29	24–34
Belarus	34	32–36	69	66–72
Bulgaria	2.3	1.3–3.8	23	17–31
China	5.7	4.5–7.0	26	22–30
DR Congo[b]	2.2	0.3–4.1	11	6.2–16
Estonia	19	14–27	62	42–79
Ethiopia	1.6	0.9–2.8	12	5.6–21
Georgia	12	10–13	39	35–44
India	2.2	1.9–2.6	15	11–19

(continued)

Table 64.3 (continued)

	Estimated % of new TB cases with MDR-TB[a]	95 % confidence interval	Estimated % of re-treatment TB cases with MDR-TB[a]	95 % confidence interval
Indonesia	1.9	1.4–2.5	12	8.1–17
Kazakhstan	26	25–27	58	57–59
Kyrgyzstan	26	23–31	55	52–58
Latvia	8.2	5.8–11	30	21–40
Lithuania	14	12–16	49	43–55
Myanmar	5.0	3.1–6.8	27	15–39
Nigeria	2.9	2.1–4.0	14	10–19
Pakistan	3.7	2.5–5.0	18	13–23
Philippines	2.0	1.4–2.7	21	16–29
Republic of Moldova	24	21–26	62	59–65
Russian Federation	19	14–25	49	40–59
South Africa	1.8	1.4–2.3	6.7	5.4–8.2
Tajikistan	8.1	6.9–9.4	52	47–57
Ukraine	22	20–24	56	50–61
Uzbekistan	23	18–30	62	53–71
Vietnam	4.0	2.5–5.4	23	17–30
High MDR-TB burden countries	**3.8**	**2.2–5.4**	**22**	**13–31**
AFR	2.1	0.5–3.7	11	6.7–16
AMR	2.4	1.3–3.5	11	6.5–16
EMR	3.2	2.3–4.1	18	12–25
EUR	15	10–20	48	43–53
SEAR	2.2	1.9–2.6	16	14–18
WPR	4.4	2.5–6.3	22	18–25
Global	**3.3**	**2.2–4.4**	**20**	**14–27**

AFR African Region, *AMR* Region of the Americas, *EMR* Eastern Mediterranean Region, *EUR* European Region, *SEAR* Southeast Asia Region, *WPR* Western Pacific Region

[a]Best estimates are for the latest available year

[b]The estimates for DR Congo are indirect estimates based on data from countries in the same epidemiological region

Levels of drug resistance among new cases remain low (<3 %) in many parts of the world, including in almost all countries in the Region of the Americas, most African countries where antimicrobial resistance surveys have been conducted, most of the Southeast Asia Region, most of Western Europe, and several countries in the Western Pacific Region [1].

2.1 Data from the United States

For the first time since 1992, the number of US TB cases (9,557) reported increased over the previous year by 1.6 %. Despite this slight increase in case count, the TB incidnce rate per 100,000 persons has remained relatively stable at approximately 3.0 since 2013. Since the 1992 TB resurgence peak in the United States, the number of TB cases reported annually has decreased by 64 % [4].

In foreign-born persons, the percentage of primary INH resistance declined from 12.1 % in 1993 to 10 % in 2015. In US-born persons, the percentage decreased from 6.7 % in 1993 to 4.2 % in 2007 but has increased since then to 6.4 % in 2015 (Fig. 64.3a). From 1996 to 2015, the percentage of primary MDR-TB cases has fluctuated between 1.3 and 0.9 % (Fig. 64.3b). Since 1996, the percentage of US-born patients with primary MDR-TB has remained below 1 %. However, of the total number of reported primary MDR-TB cases, the proportion occurring in foreign-born persons increased from 25 % (103 of 407) in 1993 to 86 % (63 of 73) in 2015 (Fig. 64.3c). One case of XDR-TB was reported in 2015, and the most reported in a single year was 10 in 1993. No cases were reported in 2003 and 2009, and no apparent trend exists in the number of cases over time (Fig. 64.3d) [4].

2.2 Europe

Fifteen of the world's 27 countries with a high MDR- and XDR-TB burden are in the WHO European Region (Fig. 64.4). With the dissolution of the Soviet Union in the early 1990s, TB and MDR-TB case rates began to increase in the newly independent states, largely due to the ensuing

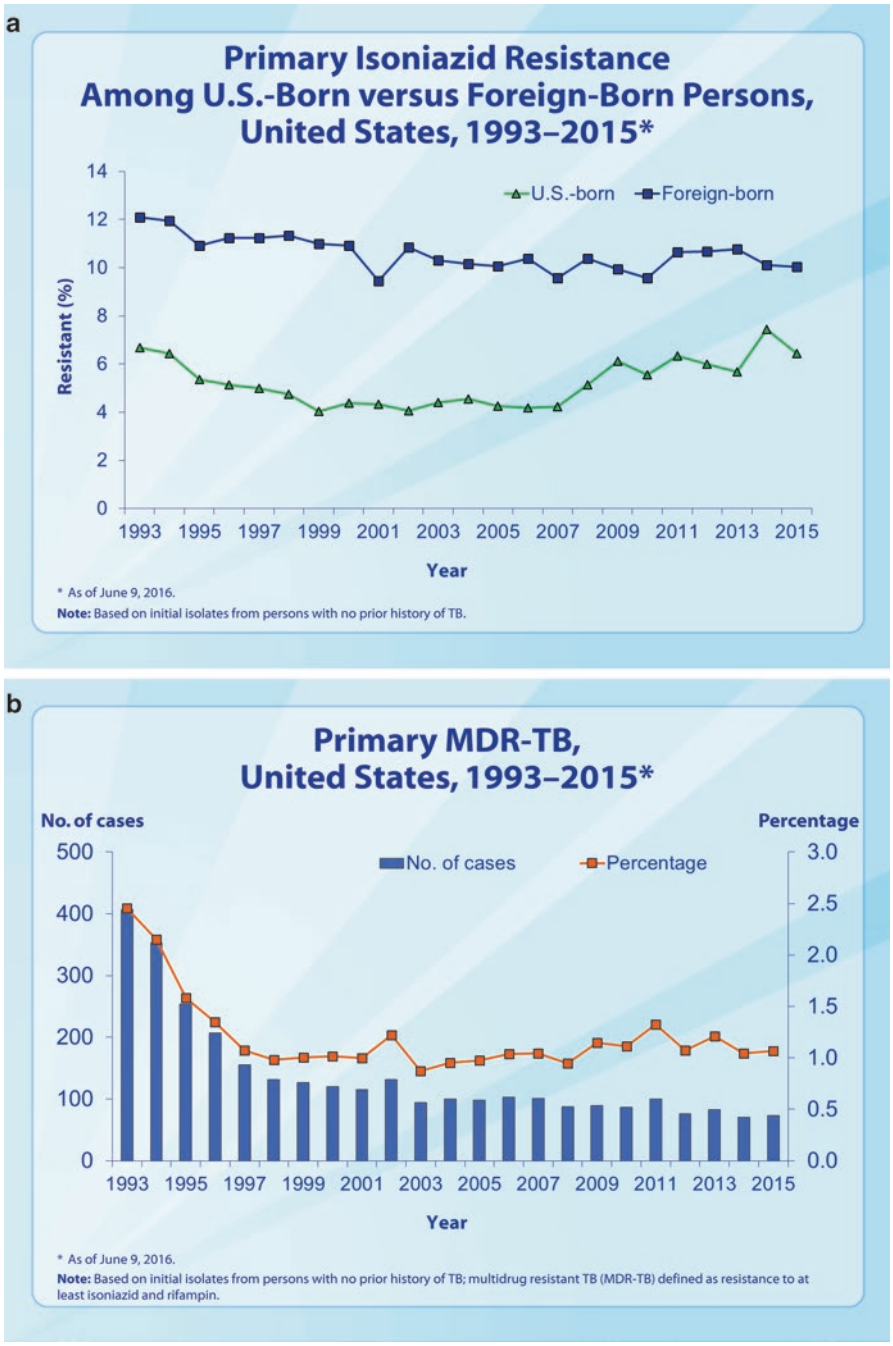

Fig. 64.3 (**a**) This graph shows primary INH resistance in US-born vs. foreign-born persons. The percentage of primary INH resistance has remained higher among foreign-born persons than among US-born persons for all years measured. In foreign-born persons, the percentage declined from 12.1 % in 1993 to 10 % in 2015. In US-born persons, the percentage decreased from 6.7 % in 1993 to 4.2 % in 2007 but has increased since then to 6.4 % in 2015. (**b**) This graph focuses on trends in primary MDR-TB in the United States from 1993 through 2015. The number of primary MDR-TB cases steadily declined from 407 in 1993 to 132 in 2002. Since then, the total number of primary MDR-TB cases has fluctuated between 87 and 103 cases, with 89 cases reported for 2015. Primary MDR-TB decreased from 2.5 % in 1993 to approximately 1.0 % in 1998 and has fluctuated around 1.0 % since then. In 2015, the percentage of primary MDR-TB was 1.1 %. (**c**) This graph highlights primary MDR-TB in US-born versus foreign-born persons. The proportion of primary MDR-TB cases in the United States that are attributed to foreign-born persons increased from approximately 25 % in 1993 to 86 % in 2015 (not shown on slide). Among the US born, the percentage with primary MDR-TB has been less than 1 % since 1997 and was 0.5 % in 2015. The percentage among foreign-born persons has fluctuated year by year, although it has remained between 1.2 and 1.8 % since 1995. In 2015, the percentage of primary MDR-TB among foreign-born persons in the United States was 1.4 %. (**d**) This graph shows the annual number of counted XDR-TB cases from 1993 to 2015. One case of XDR-TB was reported in 2015. The most XDR-TB reported in a single year was 10 in 1993, while there were no cases reported in 2003 and 2009, and no apparent trend exists in the number of cases over time

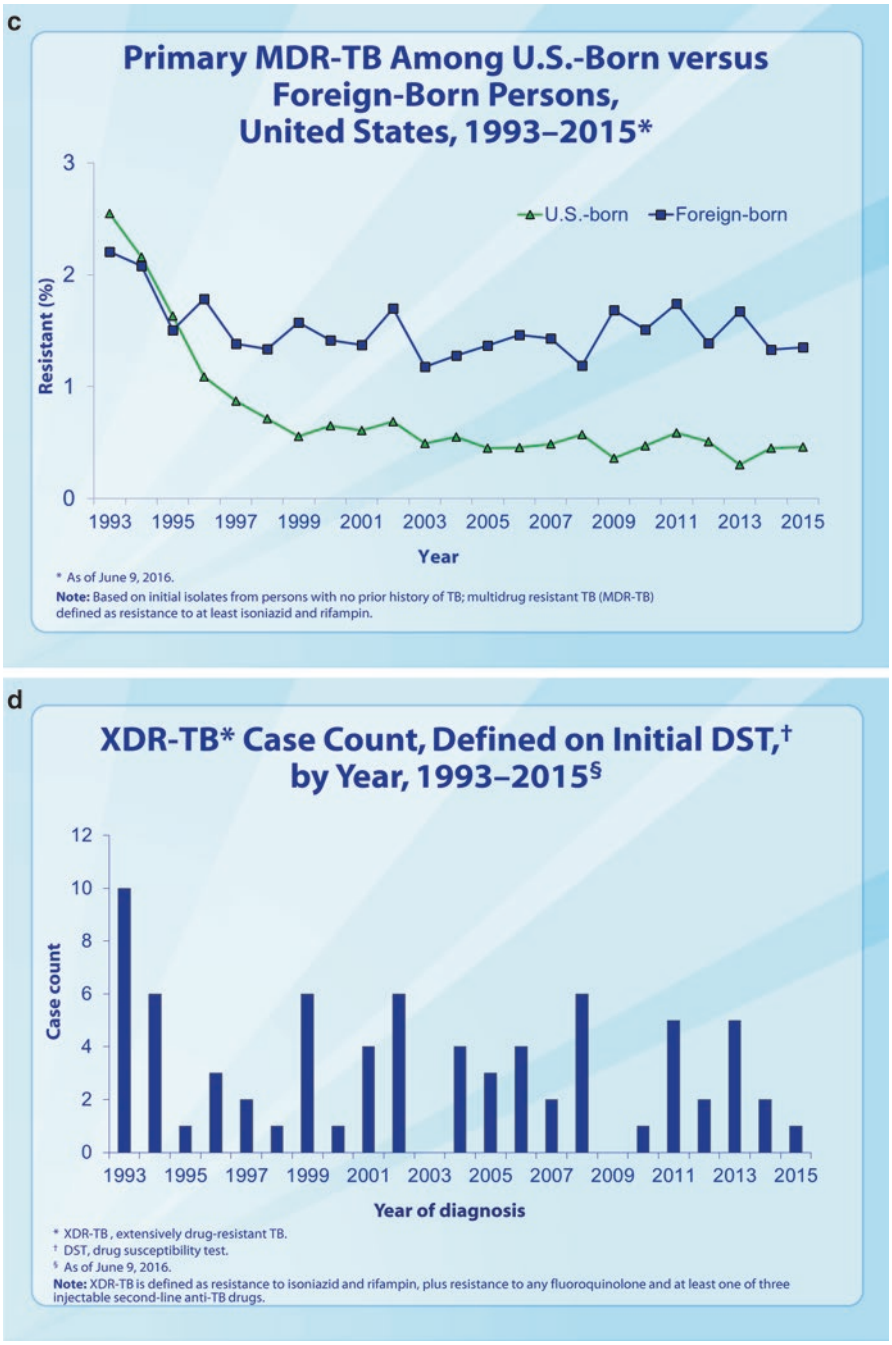

Fig. 64.3 (continued)

socioeconomic crisis and deterioration of the healthcare system. Currently, all high-burden MDR-TB countries in the WHO European Region are in the east, and 99 % of the region's MDR-TB cases occur in these countries [5].

The emergence of drug-resistant TB is a challenge to TB control in Europe. Guenther et al. evaluated second-line AST in *M. tuberculosis* isolates from patients with MDR-TB, pre-XDR-TB, and XDR-TB at 23 Tuberculosis Network European Trials (TBNET) sites. Table 64.4 shows low AST figures for test performed for later-generation FQs (levofloxacin and moxifloxacin) and linezolid at these sites in 16 European countries. Isolates from patients with pre-XDR-TB showed >30 % resistance to any FQ and almost 70 % to any second-line injectable drugs such as amikacin,

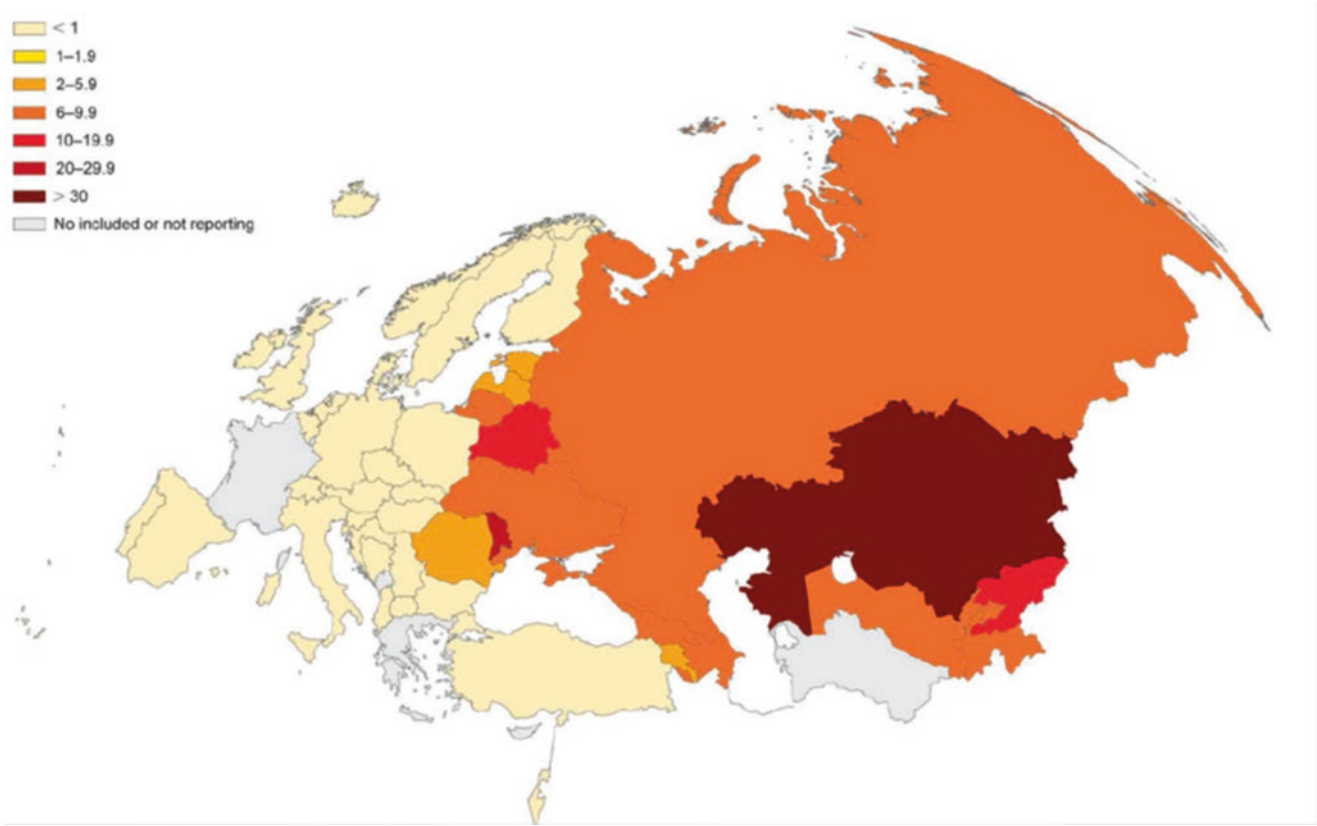

Fig. 64.4 Notification rates of MDR-TB cases/100,000 population, European Region, 2012 (reproduced with permission from Tuberculosis Surveillance and Monitoring in Europe 201411)

Table 64.4 Resistance to first- and second-line antituberculosis drugs in MDR-, pre-XDR-, and XDR-TB patients at 23 Tuberculosis Network European Trial sites in 16 countries in Europe

	MDR-TB (*n* = 258)		Pre-XDR-TB (*n* = 89)		XDR-TB (*n* = 33)	
Drug	Total tested *n* (%)	Resistant *n* (%)	Total tested *n* (%)	Resistant *n* (%)	Total tested *n* (%)	Resistant *n* (%)
Group 1						
Ethambutol	250 (96.9)	141 (56.4)	88 (98.9)	52 (59.1)	33 (100)	27 (81.8)
Pyrazinamide	98 (38.0)	44 (44.9)	56 (62.9)	44 (78.5)	18 (54.5)	17 (94.4)
Streptomycin	247 (95.7)	218 (88.3)	86 (96.6)	83 (96.5)	31 (93.9)	30 (96.8)
Group 2						
Any SLI	234 (90.7)	NA	89 (100)	60 (67.4)	33 (100)	33 (100)
Amikacin	97 (37.6)	NA	56 (62.9)	26 (46.4)	27 (81.8)	16 (59.3)
Capreomycin	171 (66.3)	NA	67 (75.3)	26 (38.8)	28 (84.8)	19 (67.9)
Kanamycin	189 (73.3)	NA	72 (80.9)	44 (61.1)	27 (81.8)	24 (88.9)
Group 3						
Any FQ	230 (89.1)	NA	89 (100)	29 (32.6)	33 (100)	33 (100)
Levofloxacin	17 (6.6)	NA	10 (11.2)	3 (30.0)	5 (15.2)	1 (20.0)
Moxifloxacin	39 (15.1)	NA	23 (25.8)	8 (34.8)	11 (33.3)	9 (81.8)
Ofloxacin	209 (81.0)	NA	82 (92.1)	23 (28.0)	33 (100)	33 (100)
Group 4						
Cycloserine/terizidone	129 (50.0)	6 (4.7)	65 (73.0)	6 (9.2)	25 (75.8)	11 (44.0)
Ethionamide/prothionamide	234 (90.7)	65 (27.8)	87 (97.8)	38 (42.7)	33 (100)	9 (31.0)
PAS	193 (74.8)	6 (3.1)	69 (77.5)	7 (10.1)	29 (87.9)	9 (31.0)
Group 5						
Amoxicillin/clavulanic acid	0	NA	0	NA	0	NA
Clarithromycin	12 (4.7)	3 (25.0)	2 (2.2)	0	3 (9.1)	0.0
Imipenem	0	NA	0	NA	0	NA
Linezolid	40 (15.5)	0	21 (23.6)	0	8 (24.2)	2 (25.0)
Meropenem	0	NA	1 (1.1)	1 (100)	0	NA
Clofazimine	0	NA	0	NA	0	NA

MDR-TB multidrug-resistant tuberculosis, *XDR-TB* extensively drug-resistant TB, *SLI* second-line injectable, *NA* not available, *FQ* fluoroquinolone, *PAS* para-aminosalicylic acid

capreomycin, and kanamycin. Of the tested XDR-TB, *M. tuberculosis* strains showed phenotypic resistance to pyrazinamide (PZA) and ethambutol (EMB), >90 % and >80 %, respectively. Additional resistance to prothionamide/ethionamide was high in isolates from both pre-XDR (43 %) and XDR-TB patients (49 %). AST against Group 5 drugs was rarely performed [6].

3 Clinical Significance and Treatment

3.1 Drug-Susceptible Tuberculosis

Treatment of drug-susceptible TB is highly effective and is based on a standardized strategy proved over several decades in international clinical trials; a treatment regimen includes a combination of drugs administered for a defined period, usually 6–9 months depending on the form of TB and history of anti-TB treatment in the past. Modern treatment regimens include four so-called first-line drugs [INH, RIF, PZA, and EMB or streptomycin (SM)], and treatment is divided into a 2-month intensive phase using three or four of the first-line drugs followed by a continuation phase usually with isoniazid and rifampin for a total of 6 months (sometimes 9 months or 12 months for TB meningitis). The goal of the intensive phase is to kill the actively metabolizing and multiplying bacteria, while the continuation phase is aiming to sterilize the infected tissue from the metabolically sporadically active semi-dormant pathogens [7–10]. Tables 64.5 and 64.6 describe the main doses, different forms of treatment of first-line drugs [7–10].

In case of a higher than 4 % rate of INH resistance in setting of the patient, treatment should be initiated with the combination of INH, RIF, PZA, and EMB or SM for 2 months (intensive phase) followed by INH and RIF for 4 months (continuation phase) [7, 9, 10]. The role of SM in first-line therapy has diminished based on the more common occurrence of resistance and the inconvenience of parenteral administration. Inclusion of EMB or SM in the regimen is not necessary if the rate of INH resistance is below 4 %, or the administration of these drugs can be discontinued if AST results are available before the end of the intensive phase and indicate the presence of a fully susceptible strain. However, if AST results are not available at the end of the intensive phase, treatment should be continued with EMB (in combination with INH and RIF); however, a dose adjustment is necessary to avoid ototoxicity [7–10]. Bacteriologic follow-up is recommended with acid-fast bacilli (AFB) smears and culture bi-weekly or at least monthly until two subsequent cultures are negative and at the end of the treatment. Time to culture negativity (culture conversion) is one of the most valuable measures of response to therapy.

The likelihood of relapse after completion of therapy shows a high correlation with the initial extent of the disease and the results of cultures at the end of the intensive phase. Therefore, in patients with advanced and cavitary disease (and AFB smear positive) whose month 2 cultures are still positive, it is recommended to extend the duration of the continuation phase with INH and RIF for an additional 3 months and prolong the total time of therapy to 9 months [7]. This approach has proven to be an effective measure to significantly decrease the chance of later relapse of the disease in these patients.

If cultures during the continuation phase are repeatedly positive or become positive again in spite of a properly administered therapy, additional molecular screening and conventional AST are highly recommended to rule out emerging drug resistance.

The completion of therapy is based both on the duration of treatment and the number of recommended doses taken (Table 64.6) [7]. However, it is not uncommon that the required number of doses is not or could not be completed during the recommended duration of the therapy. If treatment of the intensive phase is interrupted for more than 2 weeks, the treatment should be restarted from the beginning. If the interruption is less than 2 weeks, treatment should be

Table 64.5 Doses of first-line antituberculosis drugs for adults and children

	Adults/children	Daily	Twice weekly	Thrice weekly
INH	Adults (max.)	5 mg/kg (300 mg)	15 mg/kg (900 mg)	15 mg/kg (900 mg)
	Children (max.)	10–15 mg/kg (300 mg)	20–30 mg/kg (900 mg)	–
RIF	Adults (max.)	10 mg/kg (600 mg)	10 mg/kg (600 mg)	10 mg/kg (600 mg)
	Children (max.)	10–20 mg/kg (600 mg)	10–20 mg/kg (600 mg)	–
PZA	Adults	1.0 g (<55 kg)	1.5 g (<55 kg)	2.0 g (<55 kg)
		1.5 g (56–75 kg)	2.5 g (56–75 kg)	3.0 g (56–75 kg)
		2.0 g (75+ kg)	3.0 g (75+ kg)	4.0 g (75+ kg)
	Children (max.)	15–30 mg/kg (2.0 g)	50 mg/kg (4.0 g)	–
EMB	Adults (max.)	25 mg/kg first 2 months	50 mg/kg	30 mg/kg
	Children (max.)	15 mg/kg last 4 months (max. 2.5 g)	30–50 mg/kg	–

Adult doses are recommended for children older than 14 year or >40 kg
INH isoniazid, *RIF* rifampin, *PZA* pyrazinamide, *EMB* ethambutol

Table 64.6 Drug regimens and doses for patients with drug-susceptible tuberculosis

	Initial phase	Continuation phase		
Drug	Regimen	Drug	Regimen	Range of total doses
INH	7 days per week, 56 doses (8 weeks) or 5 days per week, 40 doses (8 weeks)	INH + RIF	7 days per week, 126 doses (18 weeks) or twice weekly, 36 doses (18 weeks)	182–130 doses (26 weeks)
RIF				92–76 doses (26 weeks)
PZA				
EMB				
INH	7 days per week, 14 doses (2 weeks), then twice weekly, 12 doses (6 weeks) or 5 days per week, 10 doses (2 weeks), then twice weekly, 12 doses (6 weeks)	INH + RIF	Twice weekly, 36 doses (18 weeks)	62–58 doses (26 weeks)
RIF				
PZA				
EMB				
INH	Thrice weekly, 24 doses (8 weeks)	INH + RIF	Thrice weekly, 54 doses (18 weeks)	78 doses (26 weeks)
RIF				
PZA				
EMB				
INH	7 days per week, 56 doses (8 weeks) or 5 days per week, 40 doses (8 weeks)	INH + RIF	7 days per week, 217 doses (31 weeks) or twice weekly, 62 doses (31 weeks)	273–195 doses (39 weeks)
RIF				118–102 doses (39 weeks)
EMB				

INH isoniazid, *RIF* rifampin, *PZA* pyrazinamide, *EMB* ethambutol

continued with the goal to give the total number of the doses recommended for the intensive phase within 3 months. If the total number of doses of the intensive phase could not be completed within 3 months, the treatment should be restarted. In the case treatment of an initially AFB smear-negative patient is interrupted during the continuation phase and the patient has completed at least 80 % of the recommended doses for this period, prolongation of the treatment may not be necessary. However, for an initially AFB smear-positive patient, completion of the therapy is recommended with the total number of doses [7].

Those patients that received less than 80 % of the recommended doses for the continuation phase may have two options. If the duration of interruption was less than 3 months, the continuation phase therapy should be continued. The patient should take all the initially planned doses for this period within 6 months (after the restart or within 9 months of original start date) if cultures performed after the return of the patient are negative. If these cultures are positive, restart the four-drug first-line regimen. If the lapse was 3 months or more, the entire treatment (both initial and continuation phase) should be restarted from the very beginning. However, in case of a negative follow-up culture, treatment may be stopped if the patient has received a total of 9 months therapy [7].

Conducting and enforcing directly observed therapy (DOT) in patients with any therapy interruption is indispensable. Bacteriologic follow-up of patients with treatment interruption is extremely important, and if cultures after the return of the patients are positive, additional molecular and conventional AST is recommended without delay to reveal any drug resistance.

Recently, three major phase 3 non-inferiority trials assessed the efficacy of FQs in shortening the duration of the 6-month treatment of susceptible tuberculosis to 4 months [11–13]. The concept of these trials was based on the findings of in vitro and murine model studies that indicated that moxifloxacin and gatifloxacin in combination with INH, RIF, and PZA or high-dose rifapentine resulted in a reduced time to sterilize the lung tissue from *Mycobacterium tuberculosis* and cure using a 4-month regimen. The Rapid Evaluation of Moxifloxacin in Tuberculosis (REMoxTB) trial was a randomized, double-blinded, placebo-controlled trial that evaluated two regimens in which INH or EMB was substituted to moxifloxacin in a 4-month regimen. The Ofloxacin-Containing Short-Course Regimen for the Treatment of Pulmonary Tuberculosis (OFLOTub) evaluated the standard 6-month regimen against a 4-month regimen in which gatifloxacin was substituted for EMB. The High-Dose Rifapentine with Moxifloxacin for Pulmonary Tuberculosis (RIFAQUIN) trial examined the efficacy of a 4-month and 6-month combination in which moxifloxacin replaced INH in the intensive phase. Unfortunately none of these trials were able to demonstrate that FQs could achieve the goal and shorten the duration of treatment as anticipated. However, it is important to note that in the RIFAQUIN trial, the 6-month regimen with moxifloxacin and rifapentine once weekly in the continuation phase was very effective. Using DOT, this once-weekly approach for the last 4 months of the treatment could be extremely beneficial in certain settings to assure compliance, simplify successful completion of treatment, and spare resources for follow-up. Additional studies are ongoing to examine the role of rifapentine, high-dose RIF, or clofazimine to shorten treatment of susceptible TB [14].

The early institution of appropriate therapy is essential to both prevent the emergence of MDR-TB and to treat it when it occurs [9, 10]. The WHO has adopted the directly observed therapy short course (DOTS) strategy, pioneered in studies performed in many parts of the world to treat drug-susceptible TB and to prevent MDR-TB from developing. A DOTS-Plus strategy is used to treat cases that do occur [9, 10]. Ensuring adherence and completion of therapy is the key aim, and DOTS is one effective strategy for achieving it.

3.2 Drug-Resistant Tuberculosis

3.2.1 Treatment of Different Forms of Single Drug Resistance

The standardized treatment approach is greatly jeopardized by the presence of drug resistance. Testing for molecular drug resistance mutations and complete phenotypic AST including quantitative phenotypic AST can provide valuable support to determine the best approach to successfully handle single drug resistance. This treatment may be the extension of therapy with the remaining first-line drugs, the addition of second-line drugs, or an increase of the dose of the drug involved.

INH mono-resistance can be quite high in certain high-burden settings such as India, where the rate of INH mono-resistance may reach 10 % [15]. In case of isolated phenotypic INH resistance, rapid molecular resistance screening is especially important to adequately orientate the clinician if INH could be continued or should be excluded from the regimen. In the presence of *inhA* mutations, a low level of phenotypic INH mutation can be expected which usually can be successfully controlled with high-dose INH therapy [16, 17]. In these patients, treatment could be started with an increased dose of INH in combination with RIF, EMB, and PZA for 2 months followed by high-dose INH, RIF, and EMB for 4 months. With quantitative AST, the isolates of these patients usually show resistance to 0.1 μg/mL and susceptibility at 0.4 μg/mL to INH in the liquid culture-based MGIT system [18]. However, when rapid molecular screening confirms the presence of a mutation of the *katG* gene in locus 315, the presence of a clinically meaningful and high-level phenotypic resistance (resistance at both 0.1 and 0.4 μg/mL INH in the MGIT system) is confirmed, which may clearly indicate that INH treatment is not an option for these patients and should not be continued in the treatment regimen, not even with an increased dose [19, 20]. Although molecular testing can be quite informative for the clinician, one would not need to exercise caution in the case of detection of a less common *katG* mutation (other than locus 315 mutations) which may be associated with moderate-level phenotypic resistance before making any therapeutic decisions. The level of phenotypic resistance of a less common *katG* mutation needs to be assessed with quantitative AST, i.e., minimal inhibitory concentrations (MICs). Patients with moderate, or especially with high level of INH resistance that cannot benefit from high-dose INH treatment, may be treated with RIF, EMB, and PZA for 6 months [7]. The length of therapy in patients with isolated INH resistance may be prolonged to 9 months if the patient was initially AFB smear-positive and had cavitary disease and follow-up cultures at second month were still positive. The addition of an FQ to the regimen may not be beneficial due to the unfavorable serum concentration changes due the interactions between RIF and the FQs [21, 22] but may be considered in case of an extensive disease. However, it is important to keep in mind that the RIFAQUIN trial that examined the efficacy of moxifloxacin replacing INH in the intensive phase followed by moxifloxacin and rifapentine once weekly in the continuation phase was very effective [13]. Patients who do not tolerate PZA, or in case of pregnancy, a regimen with RIF and EMB for 12 months could be an option [7]. *Ex juvantibus* high-dose INH treatment may not be recommended to all patients with isolated INH resistance since treatment in case of high-level phenotypic resistance may lead to serious polyneuropathy in these patients unnecessarily. Therefore, every effort should be taken to clarify the level of INH resistance.

Isolated rifamycin resistance is usually rare. However, a recent review of the data from various diagnostic settings worldwide supported by 14 WHO supranational laboratories revealed that RIF mono-resistance was as high as 11.6 % in certain geographic regions [23]. Mukinda et al. reported that in the Western Cape of South Africa, RIF mono-resistance tripled in 5 years, and 12 % of these patients were falsely diagnosed and treated for MDR-TB instead of a 12-month regimen with the more effective remaining first-line drugs enhanced with FQ [24]. Rufai et al. also reported a high rate of RIF mono-resistance (22.2 %) in a selection of 285 smear-positive MDR-TB suspects in India after line probe assay AST [15]. Patients with isolated RIF resistance can be treated with INH, PZA, EMB, and FQ for a minimum of 12 rather than 18 months. The preferable choice of FQ is moxifloxacin over ofloxacin due to its more favorable pharmacokinetics and MIC [7–10, 25]. This regimen can be enhanced by the addition of an injectable drug for the first 2–3 months in case of an advanced (cavitary) disease to rapidly decrease the bacterial load and/or to shorten duration to 12 months. Alternatively, an INH, PZA, and SM (or another injectable drug) combination may also be considered that could be given for 9 months [7–10, 25]. Mutations in the *rpoB* gene generally result in cross-resistance to all rifamycins. However, mutations at codon 516, Phe514PhePhe, and Ser522Leu are associated with resistance to RIF but susceptibility to rifabutin [26]. In these patients, detection of these mutations by molecular *rpoB* screening may be extremely beneficial because a standard rifabutin-based 6-month regimen can be implemented which may offer a significantly better outcome, shorter treatment period, and potentially fewer side effects.

Isolated resistance to PZA is extremely uncommon in case of *M. tuberculosis* and is usually associated with *M. bovis*, *M. bovis* BCG, or *M. canettii* infections due to their natural resistance to PZA [27]. Therefore, differentiation between the members of the *M. tuberculosis* complex is recommended by rapid identification molecular methods especially in case of phenotypic PZA mono-resistance. Treatment for a patient with mono-resistant PZA can be administered by a 9-month regimen with INH, RIF, and EMB for 2 months and with INH and RIF for 7 months thereafter [7–10, 25].

In case of isolated SM resistance, the standard first-line treatment regimen for drug-susceptible *M. tuberculosis* still applies (INH, RIF, PZA, and EMB for 2 months followed by INH and RIF for 4 months if the strain is susceptible to INH and RIF). For isolated EMB resistance (which is also uncommon), the intensive phase remains 2 months of INH, RIF, and PZA followed by 4 months of INH and RIF [7–10, 25].

To follow up the efficacy of treatment in patients with isolated antituberculosis drug resistance, a follow-up monthly AFB smear and culture with minimum bimonthly phenotypic AST and monthly molecular resistance testing could be appropriate.

3.3 Treatment of Multidrug- and Extensively Drug-Resistant Tuberculosis (MDR-TB and XDR-TB)

Treatment of MDR-TB is complex and often challenging. In order to ensure the best possible treatment outcome, the following principles should be followed regarding (1) the number of drugs necessary to treat, (2) the rational use of most effective and less effective drugs available, (3) and the duration of therapy also on emphasis on the length of administration of the injectable drug, when designing and initiating an MDR-TB regimen. Main doses and potential side effects of first- and second-line drugs are summarized in Table 64.7 [7–10, 25, 29].

According to the most recent WHO and International Union Against Tuberculosis and Lung Disease guidelines, a second-line treatment regimen should include at least four drugs certain to be effective [7–10, 25]. An injectable agent should be used for at least after 4–6 months after culture conversion [9, 10, 25]. A minimum of 18- to 24-month treatment should be given after culture conversion using DOT during the entire course of treatment with daily administration of the drugs [7–10, 25, 29]. Intermittent therapy is not recommended. Whenever it is possible based on the toxicity and tolerability of the medication, high-end doses of the available drugs should be administered [7–10, 25, 29].

Treatment can be initiated with an expanded empiric therapy based on the treatment history of the patient, potential cross-resistance mechanisms based on first-line phenotypic and molecular AST results, local antimicrobial resistance survey findings, and information on drugs commonly used in the area. Initial empiric treatment can thereafter be optimized later in line with second-line molecular and phenotypic AST results [7–9, 25, 29]. Quantitative AST results could especially be helpful to clarify the presence of cross-resistance within or among drug classes and to determine if the level of resistance, especially in case of the more potent drugs such as rifamycins, FQs, or aminoglycosides, is high or low so that their expected clinical impact can be better evaluated [30].

Antituberculosis drugs are organized into five groups presented in Table 64.8, based on the potency and efficacy (bactericidal or bacteriostatic) and route of administration [2, 7–9, 25, 29]. The selection of at least four efficient drugs should be started from Group 1 with the inclusion of all possible drugs. There is a prevailing assumption that since silent mutations in the *rpoB* gene are rare, molecular assays that screen for the presence or absence of mutations in this gene can be adequate tools for the identification of MDR-TB patients [20, 30]. Information on the type of the *rpoB* mutation can be indispensable to rule out cross-resistance to rifabutin (RFB) since mutations at codon 516, Phe514PhePhe, and Ser522Leu are usually associated with RFB susceptibility in spite of RIF resistance [26, 30]. Therefore, if this information is available and properly interpreted, these MDR-TB patients may receive a rifamycin-based therapy that may offer a much better clinical outcome [20, 30, 31]. A recent study from Bangladesh that was based on MIC testing of MDR-TB patient isolates showed that 19 % of the 62 RIF-resistant isolates in this setting showed susceptibility to RFB [32]. Molecular testing that could have provided a more rapid elucidation in this regard was not available for these patients. In addition, it is clear that some of these less common mutations (*rpoB* Leu511Pro, Asp 516Tyr, Leu533Pro, His526Leu/Ser, Ile572Phe), which may reach 22 % of all RIF-resistant cases in certain settings [33, 34], are associated with a <1.0 μg/mL phenotypic RIF resistance, which is also the definition criteria for MDR-TB. Since phenotypic tests usually only test for this single RIF concentration in the absence of routine quantitative AST (for additional lower concentrations) and molecular resistance screening, these patients are often detected as infected with fully RIF-susceptible strains. Preliminary results indicate that patients with such mutant strains may fail more often under first-line therapy with standard RIF doses [33]. Therefore, future molecular and conventional AST using a quantitative approach must be able to rapidly and adequately identify these mutations as well. However, the question has to be raised if these patients should also be considered MDR-TB in cases of associated low- or high-level INH resistance. Should one, therefore, reexamine the current critical concentration used

Table 64.7 First- and second-line antituberculosis drugs, recommended dosages, and common side effects

Drug	Route	Dose in adults	Major side effects and comments
Isoniazid	Oral, IV	5 mg/kg daily	Hepatotoxicity, peripheral neuropathy, administer with pyridoxine
Rifampin	Oral, IV	10 mg/kg daily	Hepatotoxicity, gastrointestinal upset, rash, flu-like symptom, many drug interactions (e.g., antiretroviral therapy)
Rifabutin	Oral	5 mg/kg daily (up to 450 mg daily)	Hepatotoxicity, leukopenia, thrombocytopenia, uveitis, rash arthralgia, drug interactions
Rifapentine	Oral	Not recommended in the United States for intensive phase, 600–1200 mg once weekly in continuation phase	Hepatotoxicity, drug interactions
Ethambutol	Oral, IV	15–25 mg/kg daily	Retrobulbar neuritis, visual changes, color discrimination, monitor visual acuity
Pyrazinamide	Oral	25–35 mg/kg daily	Hepatotoxicity
Levofloxacin	Oral, IV	10–15 mg/kg daily	QTc interval prolongation, Achilles tendon rupture, peripheral neuropathy
Moxifloxacin	Oral, IV	400 mg/daily	QTc interval prolongation, diarrhea, concomitant administration with bedaquiline or delamanid not recommended, peripheral neuropathy
Amikacin	IM, IV	15 mg/kg daily (max 1 g daily) 15 mg/kg daily thrice weekly after culture conversion	Nephro- and ototoxicity, monitor renal function, hearing and electrolytes
Kanamycin	IM, IV	15 mg/kg daily (max 1 g daily) 15 mg/kg daily thrice weekly after culture conversion	Nephro- and ototoxicity, monitor renal function, hearing and electrolytes
Capreomycin	IM, IV	15 mg/kg daily (max 1 g daily) 15 mg/kg daily thrice weekly after culture conversion	Nephro- and ototoxicity, monitor renal function, hearing and electrolytes
Streptomycin	IM, IV	15 mg/kg daily (max 1 g daily) 15 mg/kg daily thrice weekly after culture conversion	Nephro- and ototoxicity, monitor renal function, hearing and electrolytes
Ethionamide/ prothionamide	Oral	15–20 mg/kg daily (usually 750 mg single dose)	Hepatotoxicity, hypothyroidism, peripheral neuropathy, administer with pyridoxine,
Terizidone or cycloserine	Oral	15–20 mg/kg daily (usually 750 mg single dose)	CNS toxicity, depression, psychosis, peripheral neuropathy, administer with pyridoxine,
Para-aminosalicylic acid	Oral, IV	Oral: 4 g thrice daily: iv 12 g daily	Hypothyroidism, gastrointestinal distress
Linezolid	Oral, IV	600 mg daily	Optic and peripheral neuropathy, myelosuppression, diarrhea, and nausea
Clofazimine	Oral	100–200 mg daily	Severe skin discoloration, QTc interval prolongation, photosensitivity, gastrointestinal distress, retinopathy
Amoxicillin-clavulanate	Oral, IV	40 mg/kg daily two or three times (max. 3000 mg daily)	Diarrhea and nausea
Clarithromycin	Oral	500 mg twice daily	Diarrhea and nausea, QTc interval prolongation, *M. tuberculosis* contains *erm* [28] gene which may be associated with inducible resistance to macrolides
Imipenem-cilastatin	Oral	1000 mg two or three times daily	Diarrhea and nausea
Meropenem	IV	1000 mg two or three times daily	Diarrhea and nausea

CNS central nervous system, *GI* gastrointestinal, *IM* intramuscular, *IV* intravenous

in conventional AST assays for defining RIF resistance and, consequently, the diagnosis of MDR-TB in these patients [17]? Further clinical studies should also clarify if these patients could be better treated with increased dose of RIF.

The selection of appropriate drugs should be continued by the addition of an FQ from Group 2 and an injectable agent from Group 3 [7–10, 25, 29]. Because of the known cross-resistance mechanisms within the respective classes, only one drug is recommended to be selected from either Group 2 or Group 3. However, it is important to underline that due to their more favorable pharmacokinetics, pharmacodynamics, and low MICs, newer-generation FQs such as levofloxacin, gatifloxacin, or moxifloxacin should be given preference over ofloxacin [8–10, 25]. Based on their efficacy, tolerability, and cost, the first choice of injectable aminoglycosides should be SM, followed by kanamycin, capreomycin, and amikacin [7–10, 25, 29]. However, the rate of SM resistance may often be high in drug-resistant TB, and therefore often kanamycin or amikacin is the first choice within the class. Rapid molecular prescreening for mutations in the *rpsl* or

Table 64.8 Groups of drugs to treat MDR-TB

Group 1 (first-line oral agents)	Pyrazinamide
	Ethambutol
	Rifabutin
Group 2 (injectable agents)	Kanamycin
	Amikacin
	Capreomycin
	Streptomycin
Group 3 (fluoroquinolones)	Levofloxacin
	Moxifloxacin
	Ofloxacin
Group 4 (oral bacteriostatic second-line agents)	Para-aminosalicylic acid
	Cycloserine
	Terizidone
	Ethionamide
	Prothionamide
Group 5 (agents with unclear role)	Linezolid
	Clofazimine
	Amoxicillin/clavulanate
	Imipenem/cilastatin
	Meropenem
	Clarithromycin
	High-dose isoniazid

rrs genes from AFB smear-positive specimens directly or on culture isolates by commercially available molecular line probe assays can be a valuable tool to confirm or rule out potential SM resistance rapidly [20, 30]. Although the side effect profile of capreomycin is similar to that of the other aminoglycosides, the occurrences of these adverse effects are lower. Therefore, this drug should be considered as a first choice in patients with renal insufficiency, hearing loss, or documented peripheral neuropathy [7–10, 25, 29].

In the routine practice, inclusion of more than four (e.g., 5–7 drugs) potentially efficient drugs is often necessary if there is a suspicion that some of the drugs may be compromised (there has been a prior use in a failing regimen), or are less potent (weaker in action), or in case of an advanced cavitary and bilateral disease presentation [7–10, 25, 29]. Therefore, many MDR-TB patients' initial regimen may require the inclusions of at least one or two drugs from Group 4.

Ethionamide and prothionamide are drugs that are often used as a first choice from Group 4 based on their efficacy and low cost. However, one has to carefully consider the inclusion of these drugs since cross-resistance with INH may be relatively common in the presence of mutations in the *inhA* [20, 30]. Therefore, these drugs should not be included in the treatment regimen if rapid molecular prescreening shows mutations in this gene. However, the inclusion of high-dose INH (although not counted as one of the minimum four effective drugs) may be beneficial to enhance the standard second-line regimen of these patients (10 mg/kg/die daily or 16–20 mg/kg/die thrice weekly) [8–10, 25, 35]. Other potential drugs that can be considered from this group are cycloserine or terizidone and finally *p*-aminosalicylic acid (PAS).

Present WHO and International Union Against Tuberculosis and Lung Disease treatment guidelines suggest that since PZA was used in the failing regimen that led to the development of MDR-TB and AST to this drug may be complicated in many settings, PZA should not be counted in the total of minimum four drugs to be selected [9, 10, 25]. However, recent clinical investigations clarified that PZA resistance has become an underestimated problem since it may occur in up to 43 % of MDR-TB strains [36, 37]. Additional treatment outcome studies that aimed to address the clinical significance of in vitro PZA resistance have shown that an FQ-based MDR regimen increased early culture conversion and treatment completion by 38 % versus a similar treatment without PZA [28, 38]. Another more recent study from Bangladesh that examined the efficacy of a 9-month MDR-TB treatment regimen also confirmed that bacteriologic treatment failures and relapses were rare, in general, except among patients with high-level FQ in the presence of PZA resistance [16]. Clearly, the consequence of losing PZA for the treatment of MDR-TB and XDR-TB is highly significant. In comparison, the addition of PZA to INH and RIF for the treatment of drug-susceptible TB increased the 2-month culture conversion by 15–20 % [28]. Therefore, rapid screening of the *pncA* and *rpsA* genes for mutations known or likely to be associated with phenotypic PZA resistance is indispensable. In addition, oftentimes, empiric MDR-TB regimens that include PZA in the initial phase of the treatment suggest discontinuing that administration of the drug in the continuation phase if 3-month follow-up cultures are negative. This approach may also need reconsideration in light of the clinical data presented above.

Group 5 consists of a diverse group of drugs of which either have a low efficacy against *M. tuberculosis*, an unfavorable toxicity profile, or experience is lacking regarding their adequate long-term dosing. Therefore, their role in a combined antituberculosis drug regimen against MDR-TB remains unclear [39, 40]. These drugs should be considered when a minimum of efficient and susceptible (confirmed by phenotypic AST) four drugs cannot be selected from the first four groups for MDR-TB treatment due to confirmed resistance, suspected inefficacy, and presence of adverse effects. They become especially important in developing an adequate therapy in case of XDR-TB. The two most potent drugs in Group 5 are linezolid and clofazimine, which should always be considered in case of treatment of XDR-TB. A recent study indicated that the use of a daily 150 mg dose of clofazimine was effective in the treatment of MDR- and XDR-TB patients. However, an even more significant indicator and contributor of treatment success was the concurrent or subsequent use of linezolid [41]. Another important clinical trial that examined the role of linezolid for treatment of chronic XDR-TB revealed that 87 % of the patients had a

negative culture conversion within 6 months after the addition of linezolid to their drug regimen. Unfortunately 82 % of the patients developed clinically significant adverse effects in spite of the dose reduction of the drug to either 600 mg or 300 mg per day. The frequency of adverse effects was higher (88 %) among patients that received daily 600 mg linezolid compared to that of those (69 %) that received daily 300 mg [42]. It is important to note that three of the four patients that developed drug resistance against linezolid were from the group with the lower dose of the drug, although the frequency of drug resistance (11 %) was relatively low. In order to optimize prolonged linezolid treatment of XDR-TB patients, a more recent study reported favorable treatment outcome and adverse event frequencies with more optimized serum concentration when using a 1–4 month once-daily 800 mg linezolid treatment guided by culture status and tolerance, followed by a 1200 mg daily thrice-weekly therapy until over 1 year after culture conversion [43]. In addition, it is important to keep in mind that both aminoglycosides and linezolid inhibit the protein synthesis by targeting the mycobacterial ribosome. Therefore, theoretically linezolid may have a more significant effect in XDR-TB patients that do not receive aminoglycoside treatment, and consequently there is no target competition between the two drugs in these patients.

The recommended treatment duration for MDR-TB is 18–24 months, with a continuation phase of minimum 12–18 months after culture conversion [8–10, 25, 35]. A recent meta-analysis based on data of 9153 MDR-TB patients recommends an intensive phase of at least 8 (7–8.4) months (regardless of follow-up AFB smear and/or culture result) and a total duration of at least 20 [19–21] months in patients without previous MDR-TB history or treatment [10]. It is important to note that only 14 % of these patients had access to later-generation FQ. The transition from the initial treatment phase to the continuation phase in MDR-TB treatment is marked by the discontinuation of the injectable drug. Usually the injectable drug is not given during the entire course of the treatment based on their toxicity and adverse effects and due to their low sterilizing capacity. Therefore, with the decrease of the bacterial load which can be monitored by culture conversion, it can be discontinued. However, the ideal length of aminoglycoside therapy after culture conversion is still controversial. Previous WHO guidelines recommended discontinuing aminoglycoside therapy 4 months after culture conversion or 6 months after AFB smear negativity, while others recommend a minimum of 6-month treatment after culture conversion [9, 10]. The 2013 International Union Against Tuberculosis and Lung Disease guideline indicates that if a regimen is based on at least three effective drugs from Groups 1, 2, and 4 after discontinuation of the aminoglycoside, the injectable therapy can be stopped when AFB smear and/or cultures become negative. When there are less than three effective drugs available, or any of the three belongs to Group 5, a longer treatment is needed with the aminoglycoside [25]. However, this approach may oversimplify the problem, since one would also need to consider the grade of drug resistance (e.g., the presence of additional FQ or PZA resistance), the radiologic extent of the disease, and the resulting lung damage. The clinical status of the patient may well justify an aminoglycoside therapy even during the entire length of the treatment. Van Deun and coworkers reported notable treatment outcomes in MDR-TB patients with no prior evidence of treatment with second-line drugs using a shorter, 9-month regimen with an intensive phase of a minimum of 4 months of gatifloxacin, clofazimine, EMB, PZA, kanamycin, and high-dose INH followed by a 5-month continuation phase treatment with gatifloxacin, clofazimine, EMB, and PZA. The relapse-free cure rate was 87.9 % in these patients [44]. Based on these findings, a randomized controlled clinical trial STREAM (Standardized Treatment Regimen of Anti-TB drugs for patient with MDR-TB) was initiated to evaluate a shorter, 9-month treatment regimen.Based on the findings of this study, in May 2016 the WHO made a conditional recommendation regarding this new treatment regimen (gatifloxacin was replaced by moxifloxacin) to eligible patients (patients with non-complicated MDR-TB) [45].

A meta-analysis for response to treatment of 6724 MDR-TB patients from 26 centers revealed that treatment success of patients with only MDR-TB was 64 %, with MDR-TB plus aminoglycoside resistance 56 %, with MDR-TB plus FQ resistance 48 %, and with XDR-TB 40 % [46]. This clearly indicates the need for routine molecular screening for FQ and aminoglycoside resistance-associated molecular markers so that the different types of MDR- and XDR-TB associated with significantly different clinical outcomes can be rapidly identified and the treatment of these patients can be better optimized without delay [30]. However, since discontinuation of FQ has such a significant impact on the outcome of therapy and due to different levels of cross-resistance between aminoglycosides, detection of FQ and aminoglycoside resistance-associated mutations and phenotypic drug resistance to these drugs should not be automatically interpreted regarding resistance to the entire class of the drug. A more meaningful interpretation of these laboratory results depending on the type of the mutation and the associated different level of phenotypic resistance by conventional AST may enable the continuation of treatment with these key drugs under certain conditions. Indeed, laboratory studies indicate that mutations associated with *gyrA* and *gyrB* mutations are associated with low or moderate levels of phenotypic drug resistance to FQs [47]. It is important to note that while this level of resistance already results in MICs for ofloxacin which are already at or above the achievable drug serum concentration, later-generation FQs within the class may still be considered as a therapeutic option in the case of certain mutations [48]. The reason is that the associated elevated MIC of these newer FQs may still be below the achievable drug

serum concentration [20]. Therefore, it is important to clarify what FQ therapy the patient received. In case of a previous ofloxacin treatment (and the absence of newer-generation FQ therapy), if the mutation profile and the confirmatory quantitative AST for the level of resistance supports, and if the toxicity profile allows, increased doses of some of these newer FQs (such as levofloxacin or sparfloxacin) may further assure therapeutic efficacy in MDR-TB patients or offer more hope in case of XDR-TB. This concept was confirmed by the recent clinical trial from Bangladesh that revealed that treatment of MDR-TB patients with low levels of resistance to sparfloxacin resulted in a 90.5 % favorable outcome probability compared to that of high-level FQ-resistant MDR-TB patients with 51 % [16].

Rapid screening for mutations in the *eis*, *rrs*, and *tlyA* genes can provide similarly valuable information: first, regarding the presence of resistance to the aminoglycoside and polypeptide class of agents such as kanamycin, amikacin, and viomycin or capreomycin and, second, on the level of predictable phenotypic resistance that can be confirmed by quantitative AST [20, 30]. Mutations in the *eis* are associated with low levels of aminoglycoside resistance which is much lower than that of the drug serum concentration so that exclusion of the drug from the regimen may not be necessary, especially if a high-end dose is administered [20, 30]. In addition, the type of *rrs* mutations cannot only predict aminoglycoside and polypeptide resistance but may also provide information on the absence or presence of cross-resistance within these classes. TB strains with mutation *rrs* A1401G are usually highly resistant to kanamycin and amikacin, while they are susceptible to viomycin, or show low levels of resistance to capreomycin, which is still significantly below the achievable drug serum concentration [49, 50]. Strains with *rrs* C1402T mutations are usually associated with high levels of resistance to capreomycin, viomycin, and kanamycin but susceptibility to amikacin, while strains with *rrs* G1484T are usually highly resistant to all aminoglycosides and polypeptides. Mutations in the *tlyA* are good predictors of clinically meaningful polypeptide resistance with intact aminoglycoside susceptibility.

It is also well known that there is no cross-resistance between the SM and the other aminoglycoside or polypeptide drugs [20, 30]. The reason is that phenotypic SM resistance is usually associated with different genetic alterations, in the *Rpsl* and most commonly in the codons 513–517 of the *rrs* gene, than that of with second-line injectable drugs [20, 30]. In case of clinically significant resistance to kanamycin, amikacin, or capreomycin, the absence of mutations in these loci may suggest phenotypic susceptibility to SM of which, when confirmed by conventional AST, can be extremely valuable in the treatment of XDR-TB. This was also underlined by the observation that SM susceptibility was found to be an important predictor of long-term survival of patients with pre-XDR [51, 52]. This information, when well interpreted and completed with quantitative AST for adequate confirmation of the level of resistance in a particular patient and communicated to the healthcare provider, may allow continuation of the treatment of the patients with another aminoglycoside. This could save the patient from being labeled as XDR-TB and treated accordingly with a less potent regimen or offered potentially more effective treatment for XDR-TB. However, in these patients the clinician should clearly consider a longer than 4–6-month treatment with the aminoglycoside or continue it during the entire treatment [7–10, 25, 29].

The need to provide more comprehensive molecular and quantitative phenotypic AST information on the level of resistance of a particular class of drug is also underlined by a recent clinical finding. This finding shows that aggressive therapy (minimum of six drugs in the initial phase and four drugs in the continuation phase) [46] or treatment with at least five so-called efficacious drugs [53] provides a significantly better outcome for patients. In order to adequately determine such a powerful regimen, the laboratory must rapidly guide the healthcare provider with a comprehensive analysis and interpretation of more detailed molecular and phenotypic AST results.

The approach to design a treatment regimen for XDR-TB using drugs from the five groups recommended is the same as with MDR-TB. Regimens based on this approach have resulted in a cure rate of 56–83 % with MDR-TB [7–10, 25, 29, 35]. In case of a focal and cavitary disease, surgery can or should be considered depending on pattern and level of resistance and the efficacy of available treatment of the patient (also based on toxicity and occurrence of adverse effects) [7–10, 25, 29, 35]. The treatment of patient with XDR-TB is far more complicated in the absence of adequate number of potent drugs, and, therefore, surgery should always get an even stronger consideration in these patients [7–10, 25, 29, 35].

3.4 New Drugs

Recent advances in searching for new therapeutic options for the treatment of drug-susceptible and drug-resistant TB resulted in the development of two promising new drugs: bedaquiline and delamanid.

Bedaquiline inhibits the proton pump ATP synthase of *M. tuberculosis*, and in vitro studies indicate an increased bactericidal activity over INH or RIF [14]. The results of a recent phase 2 trial showed a significant and rapid improvement of the culture conversion rate with a 2-month bedaquiline therapy as an add-on to an MDR-TB regimen [14]. As a consequence, WHO and US Centers for Disease Control and Prevention recommended the use of bedaquiline at a dose of 400 mg per die for 2 weeks followed by 200 mg per die three times a week for 22 weeks as a therapy added to an optimized MDR-TB treatment regimen in adults when phar-

macovigilance is available and informed consent is ensured with adequate QT monitoring [54, 55].

Delamanid and pretonamid (PA-824) are first-generation nitroimidazoles that exhibit an inhibitory effect on mycolic acid synthesis and showed good effect on improving the treatment outcome of MDR-TB patients [14]. They are currently in phase 2 and 3 clinical trials. Tuberculosis-354 is a second-generation nitroimidazole that recently entered to a phase 1 trial [14]. WHO recommends the use of delamanid at the dose of 100 mg twice daily for a 6-month period in combination with an optimized MDR-TB regimen in adults when pharmacovigilance is available and informed consent is ensured [56]. Safety studies are ongoing to assess the side effects and toxicity of the combined use of bedaquiline and delamanid [14].

Recently several new or repurposed drugs (metronidazole, avermectin, disulfiram, tigecycline, inhaled colistin, benzothiazinones, and sutezolid) were reported with promising in vitro activity against TB or MDR-TB that could be potential candidates for evaluation in controlled clinical trials [14].

The availability of these new drugs for the treatment of MDR- and XDR-TB requires a careful update and reorganization of the hierarchical selection order of drugs from the five groups so that development of acquired drug resistance, unfavorable interactions, and cross-resistance for these new drugs can be minimized. In addition, the development of accurate molecular and conventional AST and their routine implementation to guard these drugs is also indispensable. The present lack of these approaches is indicated by recent reports that identified a surprising cross-resistance mechanism between bedaquiline and clofazimine and the first cases of non-trial clinical drug-resistant case with both bedaquiline and delamanid [57–60].

3.5 Special Considerations for MDR- and XDR-TB

3.5.1 Children

Sufficient evidence is missing on adequate therapeutic recommendations for management of MDR-TB and XDR-TB in children or in children exposed to infectious MDR-TB and XDR-TB cases. Diagnosis and, in turn, establishment of an appropriate and effective treatment regimen is often hampered by the fact that these patients are usually paucibacillary and a culture isolate therefore cannot be obtained. Treatment under such circumstances can be designed in line with the ADST of the isolate of the index case [7–10, 25, 29, 35].

3.5.2 Pregnancy

Pregnancies should not be terminated due to MDR-TB or XDR-TB. Although the use of aminoglycosides is not recommended, safe treatment regimens without adverse effect on the newborn can be developed using an individualized treatment approach [7–10, 25, 29, 35]. The use of PZA in pregnancy is not recommended in the United States but based on more recent evidence [61] is recommended by WHO and International Union Against Tuberculosis and Lung Disease [7–10, 25, 29, 35]. The same report also indicated that the treatment of pregnant females was also harmless to both them and their children with amikacin, ofloxacin, prothionamide, and cycloserine. Patients that are under therapy and are not AFB smear positive may continue breast-feeding.

3.5.3 HIV

The association of HIV and risk of MDR-TB and XDR-TB is low. However, HIV-coinfected individuals have a significantly higher mortality rate as that of HIV-negative MDR-TB or XDR-TB patients. Therefore, rapid diagnosis and confirmation of TB or drug-resistant TB are pivotal in these individuals. Intermittent therapy is not recommended for HIV-infected individuals even in case of treatment of drug-susceptible tuberculosis to avoid development of drug resistance especially for RIF. Antiretroviral therapy (ART) should be started without delay and regardless of CD4 cell count following the initiation of antituberculosis therapy because it reduces the risk of death and increases cure rate in patients with MDR-TB or XDR-TB [7–10, 25, 29, 35].

3.5.4 Surgery

Surgery to cure patients with MDR-TB or XDR-TB should be considered when culture conversion is not obtained in spite of 4–6-month treatment and/or the AST shows high level of drug resistance. In turn, treatment options are only with less efficient and potent drugs, and a curative therapy with chemotherapy alone seems to be questionable. In such circumstances, surgery is primarily recommended to those patients with a presentation of a focal cavitary disease. However, in dire clinical situations with limited therapeutic options, surgical resection of a primary site of a focal non-cavitary disease may also be considered. Surgery does not allow the shortening of the therapy, and patients must receive a full course of MDR-TB or XDR-TB treatment [7–10, 25, 29, 35].

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Drug Resistance of Non-tuberculous Mycobacteria

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1 Introduction

Non-tuberculous *Mycobacterium* species (NTMs) are considered opportunistic pathogens as they cause disease in animals as well as in susceptible humans. Infections can vary from asymptomatic nodules to chronic, debilitating lung and skin infections. Depending on species- and strain-specific characteristics, some NTM infections respond readily to antibiotic treatment, while others exhibit intrinsic and non-intrinsic resistance to multiple antibiotics. Intrinsic drug resistance by NTMs may have evolutionary roots in the soil.

Antiretroviral therapy has decreased AIDS-associated NTM disease, but the reported incidence of *M. avium* complex (MAC) infection of non-AIDS patients has increased in recent years, especially among women [1–6]. Exposure to NTM infection remains poorly understood, but is thought to occur mainly from diverse environmental sources. Most NTMs are slowly growing mycobacteria like their close cousin, *M. tuberculosis*, with which they share many similarities in genomic composition, cellular physiology, and mechanisms of pathogenesis. Chemotherapeutic treatments, and mechanisms of resistance to these treatments, also bear many similarities to tuberculosis. However, there are critical distinctions, especially in the case of the most common NTM pathogen of humans, MAC. This chapter addresses the spectrum of common NTM infections, the species associated with human disease, treatment issues, prophylaxis and prevention, and biological mechanisms of intrinsic and acquired drug resistance.

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2 Clinical Presentations

NTMs cause five major categories of human disease: skin, lymphadenitis, medical device and nosocomial infections, pulmonary disease, and disseminated disease. The type of infection has significant bearing on treatment decisions.

Skin and soft tissue infections. M. marinum, isolated from fresh- and saltwater, is the prototypic NTM skin infection. The bacteria gain access to the skin through minor wounds from trauma, and the first lesion is an erythematous papule which progresses to a violaceous plaque. Occasionally, the infection spreads along the lymphatic drainage of the initial inoculation site, resulting in a clinical presentation similar to sporotrichosis.

Cosmetic procedures from pedicures to tattooing to liposuction offer the opportunity for NTM to establish in the skin and cause cellulitis and abscesses. Skin and soft tissue infections are often associated with rapid-growing mycobacteria like *M. chelonae*, *M. fortuitum*, and *M. abscessus* [7].

M. ulcerans causes the Buruli ulcer, a slowly developing, ulcerating subcutaneous nodule that is common in many tropical areas. Buruli ulcer can be diagnosed with the help of PCR and newer data supports antibiotic therapy followed by surgical excision [7].

Lymphadenitis. NTM lymphadenitis occurs in children ages 1–5 years old without HIV or known immunosuppressoin. It presents with non-tender, enlarging cervicofacial adenopathy which, left untreated, will form fistulas and drain via sinus tracts. In North America, MAC is the agent most commonly implicated in NTM lymphadenitis [8].

Medical device and nosocomial NTM infections. NTMs have been recognized since 1983 as etiologic agents in peritonitis and exit-site infections in patients receiving continuous ambulatory dialysis through peritoneal catheters [9]. The most common agent in CAPD-related infections is *M. fortuitum* [10, 11],

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but other NTMs have also been documented [12–15]. Since the first description of NTM peritonitis, it has been recommended to include NTM as a possible etiologic agent if peritoneal cultures are negative at 48 h with a clinical syndrome of CAPD-related peritonitis [9, 11].

Mycobacteria have been shown to form biofilms on medical devices including central venous catheters leading to bloodstream infections [16–18]. Biofilm formation offers additional asylum and protection to the opportunistic mycobacteria; therefore, device-related NTM infection usually requires removal of the offending device.

NTMs have been implicated in other nosocomial and healthcare-related infections. Outbreaks of postinjection abscesses have been linked to injections in the Netherlands, New England, Texas, and Colombia [19]. Inadequate sterilization of equipment and contamination of the injected material was implicated in these outbreaks. Surgical operations without implanted medical devices have been complicated by NTM infections. There are case reports of postoperative NTM infections after cardiac surgery, gastric cancer surgery, and Mohs micrographic dermatologic procedures [20, 21].

Pulmonary disease. Pulmonary NTM disease is believed to be increasing in prevalence, at least in the USA [2, 4–6]. Host susceptibility factors include advanced age, certain HLA types, cystic fibrosis transmembrane receptor mutations, chronic obstructive pulmonary disease (COPD), immunomodulatory and steroid drug use, and (among women) thoracic skeletal abnormalities and rheumatoid arthritis [2, 22–24]. As with other NTMs, infection comes mainly from environmental sources, although recent reports raise the possibility of direct or indirect human-to-human transmission of *M. abscessus* among cystic fibrosis patients [25–28]. Recent genotypic studies have identified globally dispersed clinical isolates of *M. abscessus* and *M. avium*, suggesting that certain pathogen strains may be disproportionately associated with human disease [29–31].

The American Thoracic Society (ATS) and Infectious Diseases Society of America (IDSA) last released a joint statement reviewing the diagnosis and management of NTM disease in 2007 [32]. Diagnosis of NTM pulmonary disease requires respiratory symptoms associated with radiographic evidence of cavities or nodular bronchiectasis and culture of NTM from more than two sputa or a single bronchoalveolar lavage [32].

In the USA, the most commonly identified etiologic agent of NTM pulmonary infection is MAC, but a survey of cultures from Asia, Africa, Europe, South America, and Australia shows variation in the distribution of various NTM [33]. NTM can present as fibrocavitary lung disease in patients with pre-existing lung disease such a chronic obstructive pulmonary disease, or NTM can complicate established bronchiectatic pulmonary disease as in *M. abscessus* disease in patients with cystic fibrosis [34]. Slow-growing agents like MAC have also been isolated in middle and lingular lobe nodular bronchiectasis in an older, nonsmoking generally female population without prior lung disease [35]. In general, the fibrocavitary presentation has a more aggressive and predictable clinical course of decline. MAC disease presenting with nodular bronchiectasis has a less predictable course and requires clinical judgment on the institution of drug therapy, which is often poorly tolerated [36].

Disseminated disease. In patients with HIV, disseminated infections with slow-growing agents like MAC and *M. kansasii* occur when CD4 counts drop below 100 cells/μL. Up to 40 % of HIV patients with CD4 counts less than 50 cells/μL develop such infections. Disseminated MAC presents with fever, weight loss, diarrhea, adenopathy, and hepatosplenomegaly. Disseminated NTM infections are also seen in immunocompromised patients without HIV [37]. Multiple host factors have been implicated in non-AIDS disseminated NTM infection including iatrogenic immunosuppression in solid organ and hematopoietic stem cell transplants [38], T-cell deficiencies [37], IFN-gamma receptor abnormalities [39], anti-IFN-gamma autoantibodies [40], and IL-12 receptor defects [41, 42].

NTM disease and biologic therapies. Patients receiving anti-TNF alpha therapies as well as other biologic therapies like rituximab for autoimmune diseases like rheumatoid arthritis have been found to have increased risks for both pulmonary and disseminated NTM disease [43–45].

3 Therapy

Drug regimens. The ATS and ISDA have outlined guidelines for NTM therapy and susceptibility testing [32]. Recommended drug regimens and treatment strategies outlined in Table 65.1 are adapted from several sources [6, 7, 32, 36, 46, 47]. Most recommendations are based on retrospective reviews and case studies; therefore, providers should remain vigilant for new therapies as well as multimodal approaches to therapy, such as intraperitoneal streptomycin for refractory CAPD catheters or surgical debridement in conjunction with antimycobacterial therapy [13]. Treatment of NTM disease can be lengthy and expensive [48], and adherence to treatment guidelines has been reported to be problematic [49].

Susceptibility testing. Drug susceptibility testing remains controversial in NTM infection, in part due to a paucity of data related to its efficacy. Evidence supports the use of drug susceptibilities in three specific settings: macrolide sensitivity in new MAC lung disease, rifamycin sensitivity in

Table 65.1 Treatment regimens for NTM disease

Disease state	Common etiology	Therapy	Alternative therapy	Duration	Outcome notes
Pulmonary disease	*M. kansasii*	Isoniazid (300 mg)	Clarithromycin	18 months minimum with 12 months of culture negativity	Relapse rate: 0.8 %
		Rifampin (600 mg)	Moxifloxacin		
		EMB (25 mg/kg) × 2 months then 15 mg/kg)	Surgical resection		
	M. avium complex without HIV disease	C 500–1000 mg QD or AZ	C 1000mg TIW or AZ 250 mg–300 TIW*	1 year after negative culture	Best outcome with therapy following ATS or BTS guidelines
		RIF 450–600 mg QD or RFB	RIF 600 mg TIW*		
	With cavities	EMB (15 mg/kg) QD	EMB 25 mg/kg TIW*		
		Streptomycin 2–3 × week if tolerated	Surgical resection		
Disseminated disease in HIV	*M. avium* complex	C 500–1000 mg QD	AZ 500 mg QD + RFB + EMB	Lifelong if no HAART	High mortality without concomitant HAART
		± RFB 300 mg QD	(RCT with benefit to C over AZ) [57]	12 months if clinical response to HAART [60]	C + RFB + EMB improved survival [113]
		EMB 15 mg/kg QD	Fluoroquinolones		
		HAART	Amikacin		
	M. kansasii	Isoniazid 5 mg/kg (max 300 mg)	Adjust RFB on PI or	Lifelong if no HAART	High mortality without concomitant HAART
		RIF 10 mg/kg (max 600 mg)	Clarithromycin		
		EMB 15 mg/kg	Moxifloxacin		
		HAART			
Disseminated disease in non-HIV	*M. avium* complex	C 500–1000 mg QD	Azithromycin 250–500 mg QD	Consider adjunctive therapies and referral to specialty center	Not well characterized
		± Rifamycin			
		EMB 15 mg/kg QD			
Lymphadenitis	*M. avium* complex	Surgical excision ±	Clarithromycin regimen alone [114]	2–6 months	Good outcome
	M. scrofulaceum	C 500 mg PO BID if refractory or residual disease in parotid gland			
	M. malmoense				
Skin infections	*M. marinum*	C and EMB	Tetracyclines trimethoprim/sulfa	12–24 weeks	No mortality; spontaneous resolution reported
		Add RIF for deep tissue involvement	Surgery for deep involvement	Continue 8 weeks after lesion resolves	
			Amikacin		
	M. ulcerans	Surgical excision	May consider C + RIF post-excision	–	Antibiotics disappointing
					Excision can be deforming
	M. haemophilum	Combination therapy with C + amikacin, ciprofloxacin, and RIF or RFB	Consider surgical debridement	6–9 months	Not well characterized
	M. chelonae	Macrolides, amikacin, cefoxitin (except *M. chelonae*) imipenem-quinolones (*M. fortuitum*), linezolid	Consider surgical debridement	Minimum 4 months	Variable
	M. fortuitum				
	M. abscessus				

Abbreviations: *C* clarithromycin, *AZ* azithromycin, *RIF* rifampin, *RFB* rifabutin, *EMB* ethambutol, *HAART* highly active antiretroviral therapy, *ATS* American Thoracic Society, *BTS* British Thoracic Society, *PI* protease inhibitor, *PO* by mouth, *BID* twice a day, *QD* every day, *TIW* thrice weekly
Without cavities

M. kansasii, and identification of all susceptible agents in rapidly growing mycobacteria (RGM) [32].

Macrolide susceptibility is an important determinant of treatment success and mortality in MAC infections; macrolide resistance without sputum conversion is associated with increased mortality [50]. Macrolide resistance can develop on the recommended multidrug therapy including ethambutol and a rifamycin (4 %), but it is less frequent in this situation than when macrolide monotherapy is given (20 %) [50]. In vitro susceptibility predicted relapses in MAC disease when the MIC increased from ≤4.0 to ≥32 μg/mL [51]. Gardner and colleagues found that 17 % of HIV-associated MAC showed resistance to macrolides, and resistant isolates were more common in patients with prior macrolide exposure [52]. Therefore, susceptibility testing for clarithromycin is recommended in newly diagnosed and relapsed cases of MAC disease [32].

Similarly, resistance to rifamycins predicts treatment failure in *M. kansasii* disease. Resistance can develop on appropriate therapy including rifamycins; therefore, newly diagnosed and relapsed cases of *M. kansasii* should be assessed for sensitivity to rifamycins, and resistant isolates should be tested more broadly to identify other agents for therapy [32]. Drug susceptibility testing should be performed on all rapidly growing NTMs, as susceptibilities can vary intra- and interspecies [36].

Treatment outcomes and prognosis. Few specific NTM therapies have been evaluated in prospective, randomized controlled studies. Most treatment recommendations are derived from uncontrolled prospective studies or retrospective studies.

Prior to the use of rifampin, 4-month sputum conversion rates for *M. kansasii* therapy ranged from 52 to 81 %, with relapse rates of 10 % after completion of therapy [36]. With the addition of rifampin to treatment regimens for *M. kansasii*, sputum conversion rates at 4 months approached 100 % [36]. In 180 patients treated with a regimen containing rifampin, only two patients developed resistance to rifampin while on therapy and had *M. kansasii* reappear in their sputa.

Prior to the era of highly active antiretroviral therapy (HAART), disseminated *M. kansasii* disease was usually progressive and fatal. A retrospective review comparing outcome of disseminated *M. kansasii* in HIV patients between 1991 and 1996 and 1997–2002 revealed a decrease in total number of cases and 100 % survival of patients treated with HAART and a rifamycin plus INH and ethambutol [53]. Similar retrospective findings have been noted [46, 54], and new recommendations regarding length of therapy and prophylaxis have been published [55].

Macrolide therapy, which is ineffective against tuberculosis, has proven far more useful against MAC. In the pre-macrolide era, 4-month sputum conversion rates were dismally low in MAC lung disease, and relapse rates were frustratingly high. With the current recommended regimen of clarithromycin, ethambutol, and a rifamycin, sputum conversion rates of 90 % have been seen [36]. These reflect only the patients who are able to tolerate the regimen, and completion rates of NTM therapy are not well documented.

To improve regimen tolerance in patients with a lower burden of disease, thrice-weekly therapy with a macrolide, a rifamycin, and ethambutol is recommended by the ATS/IDSA for MAC patients with nodular bronchiectatic, non-cavitary disease. Thrice-weekly therapy has been shown to have an acceptable sputum conversion rate in selected patients [32]. A retrospective single-center study of thrice-weekly therapy versus daily therapy showed no difference in sputum conversion rates and no difference in response between treatment with clarithromycin and azithromycin [56] In HIV-related disseminated MAC disease, there is evidence of superiority of clarithromycin over azithromycin. In a randomized open-label study, Ward et al. [57] found that median time to sterilization of blood cultures in HIV patients with disseminated MAC was shorter in a clarithromycin/ethambutol-treated group compared to an azithromycin/ethambutol-treated group (4.38 weeks vs. >16 weeks).

In MAC pulmonary disease with macrolide resistance and disease isolated to individual lobes or subsegments of the lung, emerging data supports combination therapy with injectable aminoglycosides and surgical resection [50]. In 2008, Mitchell and colleagues shared their experience with surgical resection in 236 patients with MAC pulmonary disease in a retrospective review [58]. Surgical resection of NTM lung disease was associated with a mortality rate of 2.6 % with a morbidity rate of 11.7 %. Most notable, their patients had a relapse rate of only 5 % and cleared their sputum 93 % of the time [58].

Surgical excision of affected lymph nodes has been the gold standard in treatment of pediatric NTM lymphadenitis and a recent randomized controlled trial confirmed surgical resection as a more effective therapy than antibiotics alone [47].

Adjunctive therapies. Host defense is an important variable in NTM infections. Human environments are teeming with NTM, and a normal human host can face a daily assault of NTM by showering, inhaling dirt, and eating contaminated foods. NTMs are opportunistic pathogens that exploit known or unrecognized weaknesses in immunity. Therefore, diagnosis and therapy of a patient with NTM disease may require an examination of the host's immunity and inclusion of adjunctive therapies for patients, especially those who are known to be immunodeficient.

Highly active antiretroviral therapy (HAART) is a necessary adjunctive for patients with MAC with HIV coinfections. HAART has sharply decreased AIDS-associated disseminated MAC, which had previously been a leading cause of death in AIDS. Since the institution of HAART, revised guidelines call for the cessation of MAC therapy and prophylaxis in the HIV patient whose CD4 count rises above 100 cells/μL for 6 months or longer [55, 59, 60]. Institution of HAART therapy can uncover previously subclinical MAC in about 3.5 % of HIV+ patients with CD4 counts <100 cells/μL triggering a mycobacterial immune reconstitution syndrome [61].

Cytokines like interferon-gamma are integral in the host defense against mycobacteria. Case studies have reported clinical success using adjunctive therapy with interferon-gamma in non-HIV patients with T-cell deficiencies and disseminated mycobacterial infections, and inhaled interferon-gamma has been used in refractory *M. abscessus* pulmonary disease, resulting in a clearance of the organism from the sputum [37, 62]. Unfortunately, a randomized, placebo-controlled trial of inhaled interferon-gamma-1b failed to show efficacy in pulmonary MAC [50]. Other immunomodulatory agents which have been considered for their role in antimycobacterial defense include TNF-alpha, IL-12, and GM-CSF [59].

Emerging antimicrobials. Multidrug-resistant tuberculosis has driven research in the area of antimycobacterials. Many of the agents discussed here have been used in tuberculous therapy or were developed for antituberculosis therapy. Few have been tested clinically in the treatment of NTM diseases, but many of the agents show promising in vitro data for future therapeutic trials.

Linezolid, which has become an important tool against methicillin-resistant *Staphylococcus aureus*, has been reported to have in vitro activity (MIC ≤ 8 μg/mL) against rapidly growing mycobacteria and some slowly growing mycobacteria [63–66]. In the study reported by Brown-Elliot et al. [65], the species most likely to be susceptible to linezolid in vitro included *M. marinum*, *M. szulgai*, *M. gordonae*, and *M. kansasii*. Unfortunately, most isolates of MAC, the *M. terrae* complex, and *M. simiae* complex lacked susceptibility to linezolid with MIC ≥ 32 μg/mL. Linezolid has been reported clinically as a salvage therapy in an immunosuppressed patient with cutaneous disease due to clarithromycin-resistant *M. chelonae* [67].

Moxifloxacin and other fluoroquinolones have swept into the antimycobacterial armamentarium, and they have activity against many NTMs including MAC and *M. kansasii* (Table 65.1). Levofloxacin has been shown to exhibit synergy in vitro when combined with ethambutol and clofazimine [68], but quinolone therapy combined with a macrolide is insufficient to prevent the development of macrolide resistance in MAC pulmonary disease [50].

The discrepancy between successful in vitro killing of mycobacteria by the emerging antibiotics and the lack of clinical outcomes was further exposed in a recent retrospective review of a Japanese population of pulmonary NTM disease who had refractory disease to the ATS treatment regimen. Only a limited number (15.7 %) had a response to a treatment regimen of clofazimine, moxifloxacin, rifabutin, and linezolid [69].

Drug toxicities and intolerances. Killing NTMs with antimicrobials is only half the battle. Many of the anti-NTM drugs have undesirable side effects and drug interactions. Mild side effects can be tolerated by patients for short courses, but it is difficult to ask a septuagenarian to tolerate daily nausea and vomiting for 12–18 months of treatment for a slowly progressive case of nodular bronchiectasis. Drug toxicities and intolerances are major contributing factors to the failure to complete treatment. This in turn contributes to the development of drug resistance. Research aimed at overcoming these problems may be one way to reduce the problem of drug resistance NTM diseases. A brief summary of documented intolerances and side effects follows.

Ethambutol is recognized to cause retrobulbar optic neuritis, which presents as loss of color discrimination and visual acuity. Griffith et al. noted that 6 % of their study population receiving daily ethambutol (25 mg/kg for the first 2 months, then 15 mg/kg) developed ocular toxicity, compared to none of their patients receiving every other day ethambutol therapy (25 mg/kg) [70]. In the tuberculosis therapy literature, ethambutol ocular toxicity is dose related with an incidence of 5–6 % at dose of 25 mg/kg/day for two months and <1 % at a dose of 15 mg/kg/day [70]. The most recent guidelines have decreased the daily dosage of ethambutol in NTM disease, decreasing the likelihood of toxicity. Current recommendations call for periodic and symptomatic testing and ophthalmologic consultation for any visual complaints.

Clarithromycin and the other macrolides can cause nausea, vomiting, and diarrhea. In 1178 HIV-positive patients enrolled in a study of MAC prophylaxis, 2.5 % of patients taking clarithromycin alone couldn't eat for 3 days or experienced severe GI discomfort. When clarithromycin was combined with rifabutin, complaints of GI distress increased to 4.6 % [71]. Diarrhea occurred at similar frequencies. Clarithromycin inhibits hepatic metabolism of many drugs and may increase arrhythmias and toxicities in conjunction with Seldane™, digoxin, and other drugs [72].

The rifamycins cause orange staining of secretions and urine, which offers an excellent measure of compliance but can be upsetting to patients. Other side effects include hepatitis, nausea, vomiting, and hypersensitivity reactions. The recent ATS/ISDA NTM statement recommends liver function testing (LFT) based on clinical symptoms, but does not endorse regular LFT monitoring while on therapy [32]. The rifamycins can alter hepatic metabolism of many commonly prescribed drugs including clarithromycin and the protease inhibitors that may be part of multidrug therapy for these patients. Rifabutin, often chosen in HIV patients receiving concomitant protease inhibitors, can cause leukopenia, uveitis, arthralgias, and myalgias.

The antimycobacterial aminoglycosides, including amikacin, streptomycin, and tobramycin, are all nephrotoxic and ototoxic. Tetracyclines are not recommended in children under 8 years of age, as they are deposited in calcifying regions like the teeth and bones. They can lead to photosensitivity as well as nausea, vomiting, and diarrhea. Quinolones can also cause GI symptoms as well as the more unusual side effect of tendinopathies, neuropathy, and neuropsychiatric effects. Clofazimine should be considered carefully before use in HIV-infected patients, as increased mortality with clofazimine was seen prior to widespread HAART [73]. Clofazimine can also cause pigmentary changes to the skin which resolves with discontinuation.

4 Prophylaxis and Prevention

Despite decreased mortality with the institution of HAART, NTM infections remain an important cause of HIV-related mortality and morbidity [74]. Prophylactic macrolide therapy is recommended for patients with CD4 counts <50 cells/μL [55].

As noted earlier in this chapter, reported NTM infections appear to be increasing in non-HIV coinfected adults. A recent study on pulmonary MAC highlighted the importance of host susceptibility factors relative to known risk factors for environmental exposure [22]. NTMs have evolved to survive many environmental threats including microbicides, elevations in temperature, and alterations in pH [75–79]. Although elimination of NTM from water supplies can be difficult, viable counts of NTM in treatment effluents are typically very low. Exposure may come about as a result of colonization of downstream sites, including end-user plumbing and taps. In some cases, NTM infections have been linked to inadequate disinfection procedures. At a dialysis center in Louisiana, 25/140 patients developed *M. chelonae* infections before sampling of the water supply identified extensive contamination [80].

5 Mechanisms of Resistance

Primary and acquired resistance associated with genetic alterations of drug targets. Resistance to commonly used antimycobacterial drugs may be primary (meaning that the patient was infected with a drug-resistant strain) or acquired (meaning that resistance developed over the course of the patient's treatment). These mechanisms differ from those associated with intrinsic resistance, defined as the innate characteristics of some *Mycobacterium* species that exclude certain antibiotics from the antimycobacterial armamentarium.

In *M. tuberculosis* resistance usually results from mutations in genes coding for drug targets, or in genes required for the activation of prodrugs [81–84]. For example, resistance to isoniazid can result from mutations in *katG*, which codes for the catalase activity required for INH activation, in *inhA*, a target enzyme in the mycolic acid biosynthetic pathway, or in other genes. Rifampin binds to the β-subunit of RNA polymerase, and resistance almost always results from point mutations in a short section of that protein's structural gene, *rpoB*. Multidrug-resistance results from the accumulation of multiple individual resistance mutations.

Resistance of NTM isolates to individual drugs has been correlated with analogous altered-target phenomena. Rifampin resistance has been correlated with *rpoB* mutations in clinical isolates of *M. kansasii* [85] and, to a more limited extent, in *M. avium* [86]. Similarly, missense mutations in the petidyltransferase region of the 23S rRNA gene have been correlated with macrolide resistance in *M. kansasii* [87], *M. chelonae* [88, 89], *M. abscessus* [89], and *M. avium* [90–92]. Given the importance of macrolides for NTM treatment, it is unfortunate that most slowly growing bacteria have only one copy of the rRNA operon, a characteristic that may make them more susceptible than most bacteria to single-step mutations leading to macrolide resistance [89].

Morphotypic antibiotic resistance of MAC. Among the most clinically significant treatment challenges associated with NTM infection is the multidrug resistance of MAC. Macrolides, fluoroquinolones, rifabutin, ethambutol, amikacin, and clofazimine are effective against primary isolates, but they lose effectiveness relatively quickly unless administered in combinations that often are poorly tolerated by patients.

There is a correlation between multidrug resistance and colony type of MAC. Virtually all isolates form multiple colony morphotypes on laboratory media. Colony types vary with regard to infectivity and drug susceptibility. Reversible morphotypic switches are seen in virtually all isolates of MAC, suggesting that they confer selective advantages. One such switch is opaque-transparent, in which transparent colony-type variants are more resistant to multiple antibiotics than their opaque counterparts. Transparent variants also predominate in patient samples and grow better in animal and macrophage models of disease. Opaque variants predominate after passage in vitro. The molecular basis for the reversible opaque-transparent switch remains poorly understood.

An additional morphotypic switch, termed red-white, is visible among clinical isolates of MAC grown on media containing the lipoprotein stain Congo red (CR) [93, 94]. The red-white switch operates independently of the opaque-transparent switch, such that red-opaque (RO), red-transparent (RT), white-opaque (WO), and white-transparent (WT) morphotypes can be distinguished by CR staining. White variants are more common than red variants in patient samples that have undergone minimal transfer and storage in vitro [94]. White variants also grow better in animal and macrophage models of disease [93, 94]. The white morphotype is expressed during infection and is likely to be relevant to disease and treatment outcomes. However, the red colony type can also be recovered from patient samples [94]. White variants are more resistant than their red counterparts to multiple antibiotics in vitro [93]. The list of affected drugs includes macrolides, rifamycins, penicillins, and quinolones.

The morphotypic multidrug resistance of MAC has been ascribed to the cell wall, although additional factors may contribute. Cell wall factors have been inferred from indirect observations. For example, the genetic markers of rifampin, macrolide, and streptomycin resistance seen in other mycobacteria often are missing in resistant MAC isolates [86, 91, 95, 96]. Conditions that compromise cell wall integrity have been reported to increase the susceptibility of MAC to multiple drugs [97–99]. More recently, mutational analysis identified gene products that are required for the multidrug resistance associated with the white and transparent morphotypes [100, 101].

In order to study the genetics of morphotypic drug resistance in MAC, we have used uptake of the fluorescent nucleic acid stain SYTO16 as a surrogate marker of cell envelope permeability [100, 102, 103]. Cell populations of cultured

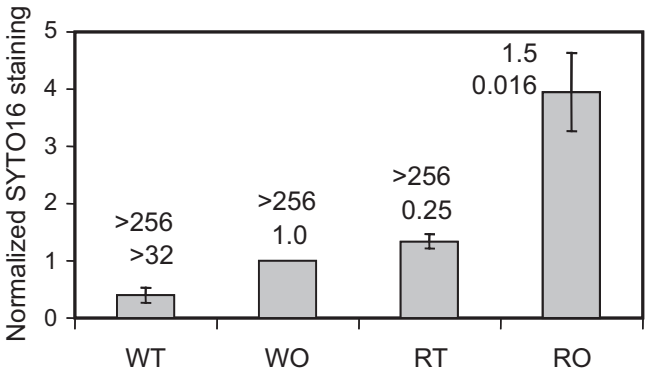

Fig. 65.1 Correlation between SYTO16 permeation and multidrug susceptibility. Naturally occurring WT, WO, RT, and RO morphotypic variants of *M. avium* clinical isolate HMC02 were assessed for SYTO16 uptake as described in the text and in reference [100]. Data were normalized to the WO strain, and means and standard deviations of three measurements are shown. Numbers above each data bar are susceptibility in μg/mL to azithromycin (*upper number*) and ciprofloxacin (*lower number*) by E-test

MAC are morphotypically heterogeneous with regard to permeability, so we quantify staining as the percentage of cells that take up the stain [100]. Mutations leading to loss of morphotypic drug resistance, including those in the genes *mtrAB*, *pks12*, and Maa2520, exhibited increased permeability to SYTO16 ([100] and unpublished results). The two-component regulatory system *mtrAB* was subsequently shown to have broad roles in growth and cell wall homeostasis in diverse *Mycobacterium* species [104, 105].

The correlation between drug resistance and cell envelope impermeability is seen in naturally occurring morphotypic variants of MAC strains, as illustrated in Fig. 65.1. Azithromycin and ciprofloxacin susceptibilities of WO, RO, RT, and WT variants of *M. avium* clinical isolate HMC02 were measured by E-test. MIC values are printed above the bar corresponding to the SYTO16 permeability of each clone in Fig. 65.1 (azithromycin above ciprofloxacin). The multidrug-resistant WT, WO, and RT forms excluded the stain, while the more drug-susceptible RO form was strongly stained. The red-white and opaque-transparent morphotypic switches are reversible and do not require drug selection, enabling these clones to toggle freely between multiresistant/impermeable and pan-sensitive/permeable forms. Morphotypic segregation into multiresistant/impermeable and pan-sensitive/permeable forms has also been observed in *M. abscessus* [106, 107].

It is difficult to conceive of a cell envelope permeability barrier that excludes structurally diverse antibiotics but not beneficial compounds. For MAC and other NTMs, the solution may be their morphotypic switches, which enable the organisms to toggle between permeable and impermeable forms. Such mechanisms may help enable these environmental pathogens to survive and flourish in diverse environments.

A hypothetical permeability barrier is unlikely to be the only mechanism of drug resistance in NTMs. Bacterial drug susceptibility can be impacted in cumulative fashion by multiple resistance mechanisms that function in a given cell. Thus, decreased permeability can function synergistically with increased expression of efflux pumps, resulting in reduced intracellular concentrations of a drug. This in turn can amplify the effects of missense mutations that reduce binding affinities of drug to target [108]. Any of these mechanisms might function in the multidrug resistance of individual NTM strains. However, a full understanding of the problem requires an understanding of how the bacteria regulate and maintain their morphotypic permeability barriers.

Intrinsic drug resistance. Mycobacterium species are innately resistant to many antibiotics that are commonly used to treat other bacterial infections. Penicillins and glycopeptides such as vancomycin are useless against most mycobacteria. NTMs may share environmental niches with related organisms, including the *Streptomyces* species that naturally produce many of the antibiotics that we use in the clinic. These evolutionary roots could have led to the selection of the intrinsic antibiotic resistance seen in many environmental mycobacteria [108].

Mechanisms of intrinsic resistance appear to differ between *Mycobacterium* species. For example, genomic comparisons in silico suggested that *M. tuberculosis* and MAC have different mechanisms of intrinsic resistance to macrolides and penicillins [101]. A 23S rRNA methyltransferase gene, *erm*, functions in the high-level resistance of *M. tuberculosis* to macrolides [109]. Expression of *erm* is controlled by WhiB7, a novel regulatory gene product that controls the expression of multiple genes, including at least some intrinsic drug resistance factors [110]. WhiB7 is found in most or all *Mycobacterium* species, including soil saprophytes, consistent with an ancestral physiological function. Resistance of *M. tuberculosis* to penicillins is thought to be mediated by at least one major β-lactamase, *blaC*, and possibly by altered expression of several penicillin-binding proteins [111, 112]. The genome sequence of MAC strain 104 has homologs to penicillin-binding proteins and putative macrolide efflux pumps found in *M. tuberculosis*, but homologs to *ermMT* and *blaC* were not found in its genome [101]. Conversely, mutational analysis showed that *pks12*, a gene required for morphotypic multidrug resistance in MAC, is not required for intrinsic resistance of *M. tuberculosis* to macrolides and penicillins [101].

6 Concluding Remarks

The prevalence of NTM disease is elevated by its persistent nature and by the challenges associated with treatment in patient populations that often have impaired immune

function. NTM infections are chronic, stubborn, and debilitating. As our population ages, the number of people susceptible to such infections may continue to rise. In order to meet this challenge, new drugs are needed and existing drugs must be preserved. It is also imperative that we improve our understanding of drug resistance in these pathogens.

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Part IX

Fungal Drug Resistance: Clinical

The Role of Resistance in *Candida* Infections: Epidemiology and Treatment

66

Jack D. Sobel and R.A. Akins

1 Introduction

During the last three decades, there was a marked increase in the population of immunocompromised and severely ill individuals at risk of developing opportunistic fungal infections [1]. In particular, the increased use of immunosuppressive agents particularly in organ transplant patients, chemotherapy, and lifesaving medical technology resulted in this increase of both superficial and serious invasive fungal infections [2–4]. The initial increase in fungal infections occurred at a time when there were few available, effective, systemic antifungal agents. Parenteral and systemically active oral azoles only became available in the 1980s. Accompanying the introduction of these newer azoles, an explosion in numbers of patients with AIDS at high risk of developing oropharyngeal and esophageal candidiasis was encountered.

It was during the 1990s that drug resistance became an important problem in virtually all populations of patients at risk, but predominantly in patients with AIDS [5, 6]. Reports of resistance to antifungal drugs have appeared with increased frequency. Confusion abounds as to how common *Candida* resistance is and whether fungal isolates should routinely be sent for susceptibility testing. Simultaneously, both clinical resistance and the increased incidence of fungal infections drove the development of new generations and classes of antifungal agents. Although extremely rare prior to the 1990s, antifungal drug resistance has now rapidly become a major problem in certain populations. The highest-risk population has been the most vulnerable, viz., patients with HIV infection. Thus, in the decade of the 1990s, up to a third of advanced-stage AIDS patients had drug-resistant strains of *Candida albicans* isolated from the oral cavities. However, it is no longer HIV-infected patients that demonstrate major clinical problems with antifungal resistance [7]. Nevertheless, occasional cases of clinical and in vitro resistant mucosal candidiasis due to *C. albicans* continue to be reported; however, the availability of new azoles, e.g., posaconazole and parenteral echinocandins, usually resolves the therapeutic challenge. Unfortunately, highly immunocompromised patients following both bone marrow and solid organ transplants have become a focus of rising antifungal resistance to both azole and echinocandin antifungal drugs. The purpose of this chapter is to review the epidemiology, pathogenesis, risk factors, and treatment of resistant candidiasis. Understanding cellular and molecular mechanisms of antifungal drug resistance and associated risk factors is crucial to developing successful prophylactic and treatment strategies to prevent emergence of resistant fungi and is discussed in the subsequent chapters. Management of refractory fungal disease caused by resistant *Candida* species will be reviewed together with methods available to prevent further development of antifungal drug resistance in candidiasis.

2 Epidemiology of Candidiasis

Oropharyngeal candidiasis (OPC) is most prevalent in infants, the elderly, and compromised hosts and also associated with serious underlying conditions including diabetes, leukemia, neoplasia, steroid use, antimicrobial therapy, radiation therapy, and chemotherapy. At least a quarter of cancer patients not receiving antifungal prophylaxis develop OPC, whereas other investigators have observed OPC in more than half of all immunocompromised patients. Prolonged neutropenia appears to be the single most important risk factor for both oropharyngeal colonization with a *Candida* species and subsequent symptomatic disease [8]. Approximately 80–90 % of patients with HIV infection will develop OPC at some stage of the disease [6], and 60 % of untreated patients

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develop AIDS-related infection within 2 years of appearance of OPC [9]. *Candida albicans* remains the most common species responsible for OPC [10]. A small unique population at high risk for developing azole antifungal resistance are individuals with immunodeficiency-related chronic mucocutaneous candidiasis [11, 12].

Vulvovaginal candidiasis (VVC) is considered to be the second most common form of vaginitis worldwide affecting millions of immunocompetent women. More than 90 % of infections are caused by *Candida albicans* [13]. The high prevalence of this infection in otherwise healthy females is responsible for significant morbidity and use of antifungal therapy.

During the 1980s, data from the National Center for Health Statistics (NCHS) reported that bloodstream infections (BSIs) were the 13th leading cause of death in the USA. *Candida* bloodstream infection has an attributable mortality of approximately 35 % [1]. Fungal infections, particularly due to *Candida* species, increased dramatically and accounted for 8–15 % of all nosocomial bloodstream infections [14–17]. The National Hospital Discharge Survey (NHDS) reported rates of oropharyngeal and disseminated candidiasis to have increased fourfold and 11-fold, respectively, between 1980 and 1989, a trend that continued over the next two decades [18]. Bloodstream *Candida* infections previously predominantly seen in cancer patients became common in ICUs and pediatric wards [19]. The SCOPE study reported that for the 3-year period ending in 1998, *Candida* species remained the fourth most common cause of nosocomial bloodstream infection [16, 20]. Risk factors for the increased incidence of candidemia have been reviewed [21, 22]. Moreover, candidemia has the highest crude mortality (40–50 %) of all nosocomial bloodstream infections [20, 23, 24]. Autopsy studies have also confirmed the increase in the incidence of disseminated candidiasis. Candidemia is associated with prolongation of hospital stay 70 vs. 40 days compared to matched nonfungemic patients as well as considerable increase in costs of therapy [25].

At present, *C. albicans* accounts for ~40–60 % of all nosocomial invasive *Candida* infections, reflecting a continued shift toward *Candida* species other than *C. albicans* has occurred, and of relevance because of intrinsic or acquired antifungal resistance in several of these species [2, 15, 23, 26–28]. Within the hospital setting, areas with the highest rates of candidemia include intensive care units, surgical units, trauma units, and neonatal ICUs. In fact, 25–50 % of all nosocomial candidemia occurs in critical care units. Neutropenic patients, formerly the highest-risk group, are no longer the most vulnerable subpopulation, likely as a result of the use of fluconazole prophylaxis during neutropenia [29]. In some tertiary care centers, *C. albicans* is no longer the most frequent bloodstream isolate, having been replaced by *C. glabrata*, which in turn replaced *C. tropicalis* as the most prevalent non-*albicans* species, causing 3–50 % of all candidemias. The increased frequency of *C. glabrata* in ICUs is also attributed to fluconazole exposure in ICU patients [30, 31]. There is a wide global variation in the predominance of particular species with *C. tropicalis* common in South America and *C. parapsilosis* common in Europe [32].

3 Mechanism of Action of Antifungal Drugs [33]

3.1 Polyenes [34]

The most important polyenes include amphotericin B and nystatin. Amphotericin B binds to sterol, the primary fungal cell membrane altering membrane permeability and ultimately cell death. Amphotericin B also causes oxidative damage to fungal cells (Vol. 1, Chapter 26).

3.2 Fluoropyrimidines

Flucytosine, or 5-fluorocytosine (5-FC), is a synthetic fluorinated pyrimidine. It is transported into susceptible fungal cells by the action of an enzyme cytosine permease and then converted by cytosine deaminase to fluorouracil. The latter molecule is incorporated into RNA in place of uracil. In addition, flucytosine blocks thymidylate synthetase, an essential enzyme for DNA synthesis (Vol. 1, Chapter 27).

3.3 Azoles

The azole antifungal agents in clinical use contain either two or three nitrogens in the azole rank and are therefore classified as imidazoles (ketoconazole, miconazole, clotrimazole, econazole, and butoconazole) or triazoles (itraconazole, fluconazole, terconazole). The newer azole agents include voriconazole, posaconazole, ravuconazole, and albaconazole. The azoles inhibit ergosterol synthesis in the fungal cell membrane through their action on the cytochrome P450-dependent enzyme lanosterol 14α-demethylase. Differences among various azoles relate primarily to their pharmacokinetics as well as their affinity for the target enzymes. There are also some differences in antifungal spectrum. Voriconazole and posaconazole have activity against many yeasts and filamentous fungi as well (Vol. 1, Chapter 27).

3.4 Echinocandins

This new class consists of parenteral caspofungin, micafungin, and anidulafungin. These agents inhibit fungal cell wall synthesis of an enzyme 1,3-β-D-glucan synthase, preventing the

formation of 1,3-β-D-glucan, an essential component of the fungal cell wall. These agents result in a weakened cell wall resulting in fungal cell lysis and are considered candidacidal [35] (Vol. 1, Chapter 29).

4 Definition of Resistance

4.1 Refractory Candidiasis

This by no means uncommon condition refers to treatment failure of symptomatic patients with antifungal agents. Only one of the many causes of therapeutic failure is due to the presence of in vitro confirmed resistant *Candida* spp. (Box 66.1) (Fig. 66.1). Treatment failure can also be the result of failure of the antifungal agent to reach the target site of infection in sufficient concentrations due to inadequate dosing, impaired absorption (food, gastric pH), poor compliance, and drug interactions. Other causes of treatment failure include local factors that either interfere with drug action, e.g., purulent material in an undrained abscess, or prevent access to organisms seeking refuge in a biofilm, e.g., prosthesis both intravascular and intra-articular [32]. A profoundly depressed immune system may also be responsible for failure. Both adequate numbers of functioning polymorphonuclear leukocytes and cell-mediated immunity are also essential in eradicating *Candida* infection. Clinical resistance refers to treatment failure despite microbial susceptibility in vitro.

Box 66.1. Causes of Treatment Failure Resulting in Refractory Candidiasis

1. In vitro antifungal resistance
   (a) Primary (intrinsic)
   (b) Secondary
2. Failure of drug to reach the site of infection in effective concentration
   (a) Poor adherence
   (b) Inadequate dosing
   (c) Impaired oral absorption
   (d) Drug interactions
3. Failure to drain abscess
4. Local protective mechanisms, e.g., biofilm (catheter, prosthetic valve, device, foreign body)
5. Impaired host immune/defense mechanism
   (a) PMNs
   (b) CMI

*Mechanisms 2–5 result in clinical resistance with failure associated with susceptible microorganisms.

4.2 Primary or Secondary Resistance

An organism that is resistant to a drug prior to exposure is defined as having intrinsic or primary resistance. Examples of primary resistance include *C. krusei* to fluconazole and *C. krusei* and *C. lusitaniae* to flucytosine. Acquired or

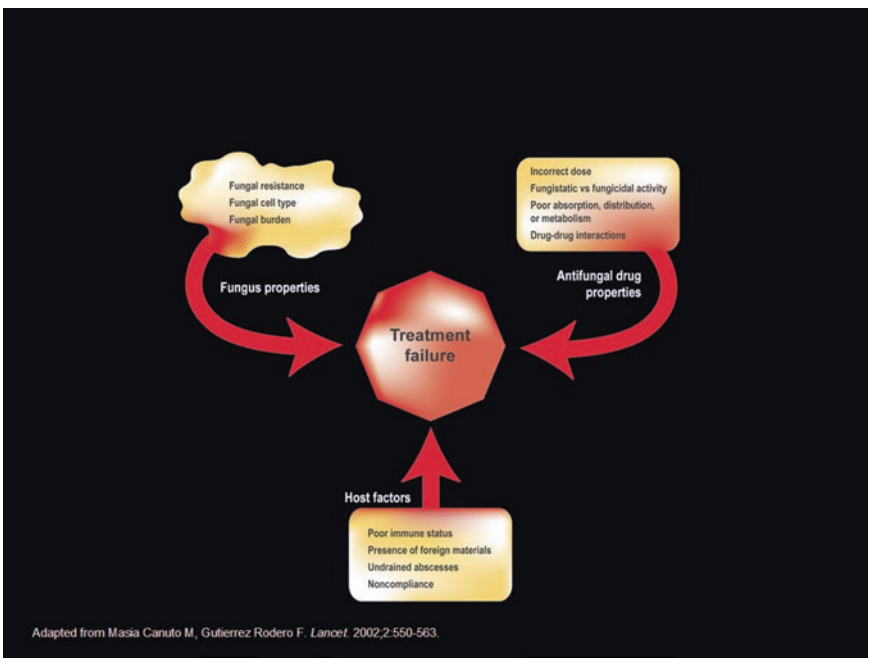

Fig. 66.1 Principal causes of failure of antifungal therapy

secondary resistance develops during or after exposure to an antifungal agent, e.g., HIV-infected patients with fluconazole-resistant OPC and esophageal candidiasis due to *C. albicans*. Cross-resistance refers to multidrug resistance either within the same class or multiple classes. Heteroresistance refers to variable in vitro susceptibility of different colonies of the same isolate obtained from the same agar plate. All forms of in vitro resistance may be temporary, transient, or irreversible.

5 Antifungal Susceptibility Tests

5.1 Methods

Testing methods and breakpoints for antifungal drugs were first suggested by Rex [36–39]. However, considerable change in methods followed to produce standardized, reproducible susceptibility methods for fungi resulting in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and the EUCAST methodology [40–47]. Accordingly, interpretive breakpoints determined by these methods are available for testing *Candida* species to fluconazole, itraconazole, voriconazole, flucytosine, amphotericin B, caspofungin, anidulafungin, and micafungin [42, 48–53] (Table 66.1). Recently, new interpretative standards have been introduced which profoundly impact upon determination and definition of susceptible and resistance isolates, potentially causing confusion for uninformed clinicians. Firstly, a new epidemiologic cutoff value (ECV) is now available and represents a more sensitive measure of change in susceptibility breakpoints [17, 43, 44]. The ECV method statistically determines the distribution of MICs within a given microbial species and is defined as the MIC value that excludes non-wild-type strains, specifically an isolate likely to contain a resistant mutation. Reliance upon the ECV results in variable breakpoints for different *Candida* species and in many cases a severalfold lowering of the susceptibility breakpoint, e.g., the previous *C. albicans* breakpoint for susceptibility to fluconazole was ≤8 mg/L, but with the new interpretation, this value is reduced to ≤2 mg/L and elevated to ≤16 mg/L for *C. glabrata*. The ECV method is valuable for detecting emergence of resistance in a *Candida* species in an institution. Using this method, most breakpoints have declined, and results of M27-A3 (CLSI) and EUCAST match more frequently. Moreover, as breakpoints decrease more isolates are deemed resistant, but no increased risk of treatment failure has been reported. This conclusion applies to both the triazoles and echinocandins with the new CLSI guidelines (Table 66.1). In the final analysis, therapeutic decisions are always individualized based upon the patient's response to therapy at the time susceptibility results become available.

In general, the susceptibility of the *Candida* isolate to the currently available antifungal agents is generally predictable if the species of the infecting isolate is known. However, individual isolates may not follow this general pattern [17].

In the past susceptibility testing of *Candida* isolates, even blood isolates, was not recommended on a routine basis. Testing was recommended only for persistent disease and failure of organism eradication in symptomatic patients with appropriate antifungal therapy. This principle was based upon the cost and lack of testing facilities available, but also driven by the rarity of in vitro resistance. However, recent surveillance suggests the emergence of reduced susceptibility of some *Candida* species in relation to azoles and echinocandins. Triazole resistance among *C. glabrata* isolates has increased to an extent that it is difficult to rely upon triazoles for therapy without performing susceptibility testing [32]. Unfortunately more recently, a similar trend has begun to emerge for a smaller proportion of *C. glabrata* isolates and the echinocandins [1, 13]. Accordingly, susceptibility testing is now required and recommended to guide the management of candidiasis. It is now recommended the laboratories perform routine antifungal susceptibility testing against both the triazole and echinocandins for *C. glabrata* isolates from blood and sterile sites and for other *Candida* species that have failed to respond to antifungal therapy. Although controversial, based upon the overall infrequency of antifungal resistance in *C. albicans*, routine testing for this species is not indicated in the absence of treatment failure. The value of testing for other *Candida* species is less clear, although occasional resistance among *C. tropicalis* and *C. parapsilosis* has been reported in certain hospitals with high use of antifungals. Hence, some authorities recommend triazole susceptibility testing for all bloodstream and clinically relevant *Candida* isolates, whereas testing for echinocandin susceptibility should be considered in patients who have had prior treatment with an echinocandin.

The objective of susceptibility testing is to differentiate infecting strains that are susceptible and hence likely to respond to a given antifungal drug from those strains resistant and hence more likely to fail therapy. With regard to echinocandins, it is essential that susceptibility tests capture high-MIC strains containing *FKS* mutations. To date the CLSI has used limited clinical data but also microbiologic data to define clinical breakpoint for all three echinocandins against *Candida* spp. [54]. Unfortunately, some resistant *Candida* strains were often misclassified by this breakpoint [55, 56]. As a result, new breakpoints were determined by CLSI that better accounted for *FKS* mutations [32, 47] (see Table 66.1). EUCAST established *Candida* species-specific and echinocandin-specific clinical breakpoints (Vol. 2, Chapter 18).

NCCLS M27-A methodology has only a limited ability to measure MICs of *Candida* isolates to amphotericin B. Rex et al. recommended the use of antibiotic medium 3 broth to measure

Table 66.1 In vitro susceptibility of *Candida albicans* and interpretative breakpoints

	Clinical breakpoints (in μg/mL)			
Organism	Susceptible	Susceptible dose dependent	Intermediate	Resistant
C. albicans				
Caspofungin	≤0.25	–	0.5	≥1
Anidulafungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole	≤2.0	4.0	–	≥8
Itraconazole	≤0.12	0.25–0.5	–	≥1
Voriconazole	≤0.12	–	0.25–0.5	≥1
C. parapsilosis				
Caspofungin	≤2	–	4	≥8
Anidulafungin	≤2	–	4	≥8
Micafungin	≤2	–	4	≥8
Fluconazole	≤2	4.0	–	≥8
Voriconazole	≤0.12	–	0.25–0.5	≥1
C. tropicalis				
Caspofungin	≤0.25	–	0.5	≥1
Anidulafungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole	≤2	4.0	–	≥8
Voriconazole	≤0.12	–	0.25 – 0.5	≥1
C. glabrata				
Caspofungin	≤0.12	–	0.25	≥0.5
Anidulafungin	≤0.12	–	0.25	≥0.5
Micafungin	≤0.06	–	0.12	≥0.25
Fluconazole		≤32	–	≥64
C. krusei				
Caspofungin	≤0.25	–	0.5	≥1
Anidulafungin	≤0.25	–	0.5	≥1
Micafungin	≤0.25	–	0.5	≥1
Fluconazole[a]	–	–	–	–
Voriconazole	≤0.5	–	1	≥2
C. guilliermondii				
Caspofungin	≤2	–	4	≥8
Anidulafungin	≤2	–	4	≥8
Micafungin	≤2	–	4	≥8

24 h 100 %, MIC end points read as 100 % inhibition at 24 h incubation; 24 h 50 %, MIC end points read as 50 % inhibition at 24 h incubation
[a]Fluconazole breakpoints are not available for *C. krusei* since this species is considered intrinsically resistant to this compound. All strains should be reported as resistant

resistance [48, 57]. In general, current methods are limited to identifying *Candida* isolates associated with clinical failure, although breakpoint minimal lethal concentrations (MLCs) and MICs of ≥1 μg/mL at 48 h have been recommended to more accurately predict mycologic *Candida* spp. failure with amphotericin B [58]. In a multicenter study of candidemia in non-neutropenic patients, all blood isolates demonstrated amphotericin B MICs less than 1.0 μg/mL. As with fluconazole, clinical failures (10–15 %) were all associated with in vitro susceptible isolates with low amphotericin B MICs [50].

The Etest is often used as an alternate to broth dilution methodology and certainly is useful in the setting of refractory clinical disease, and there is no other testing method available. The Etest is considered suitable for testing *Candida* spp. against amphotericin B or flucytosine, but less reliable for azole susceptibility [49, 51–53, 57].

5.2 In Vitro Susceptibility and Resistance of *Candida* Species (Table 66.2)

5.2.1 Azoles

The triazole, voriconazole, posaconazole, and ravuconazole exhibit greater potency and spectrum than either fluconazole or itraconazole but are still essentially fungistatic.

Table 66.2 Susceptibility of *Candida* spp. to antifungal agents

MIC_{50}	Amphotericin B	Fluconazole	Itraconazole	Voriconazole	Flucytosine	Caspofungin
C. albicans	0.5	0.5	0.12	0.03	≤0.25	0.12
C. tropicalis	0.25	1	0.06	0.06	≤0.25	0.25
C. glabrata	0.5	16	0.25	0.25	≤0.25	0.12
C. parapsilosis	0.25	1	0.12	0.03	≤0.25	1.0
C. krusei	0.25	64	0.5	0.5	16	0.5
C. lusitaniae	≥1	2	0.25	0.03	≤0.25	1.0

Shown are typical species-specific MIC_{50}s (μg/mL) adapted from reports describing collections of clinical isolates [50, 59–61]. MICs were obtained by the NCCLS M27 methodology (National Committee for Clinical Laboratory Standards, 1995) for all drugs but amphotericin B. If this method fails to detect amphotericin B-resistant *Candida* [50], then reported amphotericin B MICs were obtained by a more sensitive method based on the use of antibiotic medium 3 in an agar-based testing format

The activity of this broad-spectrum triazole extends to some fluconazole-resistant strains of *Candida*.

Primary and secondary azole resistance is species dependent and also shows marked geographic variation [59, 62]. There is no clear evidence for a correlation between the agricultural use of azoles and an increase in antimycotic resistance in *Candida* species. Primary resistance to azoles remains uncommon in candidiasis, with the exception of *Candida glabrata* and *Candida krusei*. Most acquired azole resistance emerged in AIDS patients with OPC and EC following prolonged azole therapy in the presence of advanced immunodeficiency. Azole resistance in other settings is uncommon [32, 63].

1. *C. albicans*. Primary resistance to fluconazole and itraconazole is extremely rare. Moreover, outside the realm of AIDS, acquired or secondary resistance has likewise remained uncommon especially with regard to bloodstream isolates. Each year, thousands of randomly obtained BSIs isolated from all over the world are tested in a single site (SENTRY), and over several years fluconazole-resistant *C. albicans* remains <5 % and shows no evidence of changing [60, 63, 64]. In contrast, Antoniadou et al. reported that 9 % of bloodstream isolates of *C. albicans* were resistant to fluconazole (MIC > 64 μg/mL) [65]. Spontaneous fluconazole resistance in the absence of prior azole therapy is rare but has been reported in otherwise healthy adults [66]. Based upon molecular modeling studies, it has been reported that certain mutations in *ERG* II result in significant levels of resistance to fluconazole and voriconazole but have less effect on the susceptibility of the organisms to itraconazole and posaconazole, possibly due to the more extensive binding of the latter agents to the target enzymes [67].
2. *C. tropicalis*. Occasional strains of *C. tropicalis* demonstrate azole resistance although MIC_{90} values indicate continued susceptibility. This species has a proclivity to produce trailing grown in vitro often misinterpreted as resistance.
3. *C. parapsilosis* strains are usually highly susceptible to all azoles [67].
4. *C. krusei*. This species is intrinsically resistant to fluconazole and has higher MICs to itraconazole in the S-DD range. Voriconazole is, however, very active against *C. krusei* [60, 68]. *C. krusei* incidence has remained stable over the last decade.
5. *C. dubliniensis*. This species has been increasingly identified and implicated in OPC in HIV-infected agents and is usually identified as *C. albicans*. Most *C. dubliniensis* strains are susceptible to fluconazole although in vitro resistance can be induced. Acquired resistance develops much more rapidly than in *C. albicans*.
6. *C. glabrata*. Among pathogenic yeast species, *Candida glabrata*, which accounts for 5–40 % of all yeast isolates, ranks second in all clinical forms of candidiasis today and in some studies of nosocomial candidemia is more common than *C. albicans* [32, 69]. This opportunistic pathogen is particularly relevant in immunocompromised patients including those receiving cytotoxic chemotherapy, undergoing transplantation, and infected with HIV. This critical *Candida* species represents the Achilles heel of the azole class [70, 71]. *C. glabrata* isolates exhibit bimodal susceptibility to azoles with 10–15 % of bloodstream isolates demonstrating fluconazole resistance (≥64 μg/mL) [60, 64]. Patterns of fluconazole susceptibility vary by geographic area, patient population, risk factors, and azole exposure [72]. In particular, clinical isolates obtained from patients with AIDS and OPC/EC and those with underlying malignancy show reduced susceptibility to fluconazole and itraconazole. Fluconazole resistance is lowest in Asia-Pacific and Latin-American regions (3–4 %) and highest in North America (10–15 %). Both the frequency of *C. glabrata* occurrence and azole susceptibility are profoundly affected by azole exposure, with 30–40 % of isolates being S-DD. International surveillance reveals that recently submitted bloodstream isolates (2001–2005) of *C. parapsilosis* and *C. tropicalis* in contrast to *C. albicans* did reveal a slight increase in fluconazole resistance. A similar increase in resistance was

observed for *C. glabrata* with sustained high rates of fluconazole resistance (14.3–18.3 %) over this period [63]. In general, whereas most *C. glabrata* isolates are still susceptible to voriconazole, most fluconazole-resistant *C. glabrata* isolates are resistant to itraconazole, and half are also resistant to voriconazole and posaconazole [60, 69, 73]. Not surprisingly, several reports of voriconazole-resistant *C. glabrata* breakthrough fungemia in bone marrow transplant recipients receiving long-term voriconazole prophylaxis have been reported [74].

5.2.2 Flucytosine

Intrinsic resistance among *C. albicans* has been described in 6.5–33 % of isolates and is invariably associated with serotype B isolates [75]. More recent studies have shown lower resistance frequency possibly due to infrequent use. Pfaller et al. studying 8803 clinical isolates of *Candida* spp. reported susceptibility as follows: *C. albicans* (97 %), *C. tropicalis* (92 %), *C. guilliermondii* (100 %), *C. dubliniensis* (100 %), *C. parapsilosis* (99 %), and *C. glabrata* (99 %) [64]. The least susceptible species was *C. krusei* (5 % susceptible, 67 % intermediate, and 28 % resistant). A smaller study reported that 82 % of *C. glabrata* were susceptible to flucytosine. The pharmacokinetics and in vitro activity of flucytosine make the agent particularly useful for azole-resistant *Candida* infections in relatively inaccessible sites such as CSF and the genitourinary tract.

Unfortunately, secondary acquired resistance is common (30 %) and acquired rapidly to flucytosine when used as monotherapy. Accordingly, flucytosine is almost always used in combination with other antifungals.

5.2.3 Polyenes

Resistance to amphotericin B may be intrinsic or acquired [76]. *C. albicans* resistance is extremely rare, although the NCCLS M27-A methodology may be underestimating its occurrence. For amphotericin B, NCCLS methodology generates a narrow MIC range limiting its ability to identify isolates likely to cause therapeutic failure [58]. Moreover, more important than resistance is the phenomenon of reduced susceptibility without frank resistance. Powderly et al. reported reduced amphotericin B sensitivity of blood isolates of *C. albicans* in neutropenic patients and correlating higher MICs with poor outcome [77]. Fortunately, such strains are rare and secondary resistance is uncommon [78]. Resistance in *C. parapsilosis* and *C. dubliniensis* but not *C. tropicalis* is rare [79]. Although *C. glabrata* and *C. krusei* isolates are usually considered susceptible to amphotericin B, they tend to have higher MICs, justifying initial empiric use of amphotericin B at a higher dose of 1.0 mg/kg/day. Sterling reported the emergence of resistance to amphotericin B during therapy for *C. glabrata* infection in an immunocompetent host [80]. Many but not all *C. lusitaniae* and some *C. guilliermondii* isolates demonstrate intrinsic resistance to amphotericin B [81]. Acquisition of secondary polyene resistance in species, in addition to *C. albicans*, includes *C. lusitaniae* and *C. guilliermondii* during amphotericin B therapy especially in myelosuppressed patients [78, 82–84]. Rare cases of fatal septicemia reported of amphotericin B-resistant *C. lusitaniae* [85]. Resistance to amphotericin B desoxycholate implies that the organism will be resistant to the various lipid formulations of amphotericin B.

5.2.4 Echinocandins

Early reports of clinical and/or in vitro resistance to any of the echinocandin agents were rare. In 2003, the in vitro activities of caspofungin against 3959 isolates of *Candida* spp. from 95 different medical centers were determined and compared with fluconazole and itraconazole [61]. No resistant strains of *C. albicans* were detected. Against all *Candida* species, 96 % of MICs were ≤2 μg/mL. *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. glabrata* were the most susceptible species, and *C. guilliermondii* was the least susceptible (MIC_{90} >80 μg/mL). *C. parapsilosis* MIC_{90} 2–4 μg/mL was significantly increased versus *C. albicans* 0.25 μg/mL [61]. Echinocandins remain very active against azole-resistant isolates of *C. albicans* and *C. glabrata* (99 % of MICs were ≤1 μg/mL). There is no evidence of a significant impact of azole resistance mediated by CDR pumps on echinocandin resistance in clinical *Candida* isolates.

Similarly, large multinational *Candida* isolate collections have been used to evaluate in vitro resistance to micafungin and anidulafungin, and identical almost universal susceptibility has been reported and once more higher MICs of *C. parapsilosis* emerged [63]. Interestingly, caspofungin is not fungicidal for isolates of *C. parapsilosis* or *C. guilliermondii* [86].

Breakpoints for the echinocandin class of agents were delayed in appearance since in vitro and in vivo analyses were hampered by a dearth of resistant isolates. As a result Kartsonis et al. failed to establish any relationship between baseline caspofungin MICs and clinical outcome with isolates from both mucosal and invasive *Candida* infections [87]. An echinocandin MIC of ≤2.0 μg/mL, a blood concentration easily achievable in vivo under normal dosing, would encompass 99.7 % of all clinical isolates of *Candida* species [63].

While clinical failure due to echinocandin-resistant *Candida* isolates has been rare, acquired in vivo resistance following echinocandin exposure undoubtedly occurs, and resistant isolates have increasingly been reported. All the resistant isolates were shown to have homozygous mutations in the *FKS*1 gene. Clinical failure with all *Candida* species has also increasingly been reported [88–90].

Hernandez et al. in 2004 reported a patient with azole-refractory OPC/EC which in spite of initial improvement

eventually failed on caspofungin [91]. Initial isolates exhibited low caspofungin MICs, whereas a late isolate had higher MIC. The clinical response was reproduced in a murine model correlating MIC with the clinical response to caspofungin. Similarly, a case of progressive loss of echinocandin activity following prolonged use for treatment of *C. albicans* esophagitis was reported [92].

Moudgal et al. in 2005 described a patient with aortic valve endocarditis due to *C. parapsilosis* [93]. After initially responding to combination therapy with caspofungin (MIC 2 μg/mL) and fluconazole, he cleared his fungemia and was discharged on fluconazole only. He returned three months later with recurrent *C. parapsilosis*, now resistant to both fluconazole and caspofungin (MIC > 16 μg/mL) and also voriconazole and micafungin but not anidulafungin. Similar case reports regarding acquired echinocandin resistance in *C. glabrata* are reported more than a decade ago predicting a future likelihood of increased resistance in this species (see Chapter. 29, Volume 1) [94].

5.3 Correlation of In Vitro Susceptibility Testing and Clinical Outcome of Treatment with Antifungal Agents

In vitro susceptibility is only one of the many factors that influence the outcome of therapy of fungal infections [37]. A variety of pharmacokinetic and pharmacodynamic drug factors as well as a multitude of host factors (neutropenia, compliance, catheter presence, APACHE scores, abscess drainage) all interact to impact upon clinical outcome(s). Even the definition of clinical outcome is controversial, ranging from clinical improvement to mycologic evaluation (short or long term) on patient survival (days or weeks). Nevertheless, in vitro susceptibility determination may serve as an objective, reproducible measure that can profoundly influence drug selection with physicians recognizing the limitations of in vitro susceptibility testing.

Establishing that an isolate is resistant to an antifungal agent in vitro is an immensely useful step in selecting therapy. Determining that the isolate is susceptible to antifungal agents in no way predicts survival or fungal eradication. Clinicians should recall the old 90-60 rule in which a clinical response of 90 % or more can be expected when an in vitro sensitive strain is treated with an appropriate antibiotic in comparison to a 60 % response when a resistant strain is treated with drugs showing reduced or no activity in vitro.

With regard to candidiasis, in vitro and clinical outcome correlations have mainly been applied to OPC/EC and candidemia, where the 90-60 rule appears to have been met, recognizing this is merely a minimal standard. The most important principle applied is that organisms deemed resistant in vitro are much less likely to respond in vivo. Yet within the candidemia RCTs involving hundreds of patients, almost all patients failing did so with highly susceptible strains. This emphasizes the principle that susceptibility in vitro does not guarantee successful therapy. Most studies evaluating the 90-60 rule have applied to azoles, specifically fluconazole, and the best correlation was in OPC/EC in AIDS patients. The clinical predictability of amphotericin B susceptibility is less well established. Moreover, Sobel et al. found poor correlation between in vitro MICs and response to fluconazole therapy for VVC [95]. Finally, any discussion of clinical correlation must distinguish resistance developing in a given strain of the same species from the problem of acquiring less susceptible strains from the same or different species.

5.4 Indications for Antifungal Susceptibility Testing in *Candida* Infections

Apart for reasons of periodic epidemiologic surveillance and resistance monitoring, routine susceptibility testing by any of the aforementioned methods is not indicated. Testing is justified for all bloodstream *Candida* isolates, especially if associated with persistent, breakthrough, and recurrent candidemia and also refractory mucosal candidiasis, anticipated prolonged or critical therapy, e.g., endocarditis, osteomyelitis especially with non-*albicans Candida* invasive infections. Also, testing is essential with selected non-*albicans Candida* species, e.g., *C. glabrata*, initially treated by non-azole regimens anticipating a switch to oral therapy with either fluconazole or voriconazole to complete therapy. Given the increase of parenteral echinocandins as first-line therapy for candidemia only to have the remainder of the therapeutic course completed by oral triazoles, the Infectious Society of America now recommends that all first bloodstream isolates should be tested for antifungal susceptibility [96].

6 Epidemiology and Risk Factors for Resistant Candidiasis

Does azole use select for antifungal drug resistance? In this context, clinical resistance is encountered with (a) the presence of organisms with intrinsic, de novo resistance to antifungals usually seen with non-*albicans Candida* and rarely *C. albicans*, (b) alternately, evolution may occur of the initially sensitive strain to an identical strain that has undergone genetic and molecular changes, or (c) there is replacement of the strain with a new resistant strain of the same species or finally replacement with a new strain of a different species.

Evidence links empirical, prophylactic, and therapeutic use of azoles and selection for yeasts other than *C. albicans* that exhibit decreased susceptibility to azoles, e.g., *C. glabrata* and *C. krusei* infections in patients receiving fluconazole prophylaxis [97–99]. Most of the early data came from AIDS patients. The emergence of antifungal-resistant *C. albicans* fungemia has increasingly been reported in bone marrow transplant recipients being administered with long-term fluconazole prophylaxis [100]. Similarly, isolated reports of fluconazole-resistant fungi in surgical ICUs are emerging [101].

While molecular changes in a single strain invariably reflect a single or more usually multiple genetic mutations, the dynamics of acquisition of a new strain or species is less well understood. New more resistant *Candida* strains or species may be acquired during hospitalization from medical staff carriers. This process has been well documented with *C. albicans* and *C. parapsilosis*, but *C. glabrata* is rarely identified on the hands of carriers or in hospital environment. It is hypothesized that patients may be colonized in the gastrointestinal tract simultaneously by multiple strains of *Candida*, including the possibility of multiple species. Routine culture only captures the dominant strain or species. After antifungal drug ingestion or pressure, more susceptible strains are eliminated or so reduced in number so as to allow growth and emergence and recognition of more resistant strains or species that have coexisted long term but were previously not recognized.

6.1 HIV/AIDS

AIDS patients have been the focal point of much of the scientific inquiry into fluconazole resistance. On the one hand, oral and esophageal candidiasis became extremely common as a clinical manifestation of AIDS in the 1980s. The availability of fluconazole as both treatment and subsequently prophylaxis in patients with recurrent disease was an enormous boon to care. Within a few short years, clinical and in vitro fluconazole resistance was widespread and caused major alarm among AIDS practitioners [6, 32, 102]. Several studies, mainly retrospective, identified risk factors for acquisition of fluconazole resistance (Box 66.2). In addition to the status of the immune system (CMI), i.e., CD4 lymphocyte count, most studies concluded that patterns of fluconazole use particularly drug dose were the dominant factors associated with resistance acquisition [103–106]. In the majority of patients, mutation of a previously susceptible strain of *C. albicans* to a resistant strain is likely to have occurred, together with coinfection with *Candida* species resistant to fluconazole, e.g., *C. glabrata* [107].

Box 66.2. Risk Factors for Azole Resistance in Candidiasis

1. HIV/AIDS
   (a) Advanced immunosuppression (low CD4 cells)
   (b) High viral load
   (c) Fluconazole administration
      - Poor compliance
      - Past fluconazole exposure
        – Total dose
        – Intermittent therapy
        – Prophylaxis versus therapeutic
        – Low dose
2. Hematologic malignancy/BM transplantation
   (a) Azole exposure (prophylactic)
3. Prosthetic devices—foreign bodies
   (a) Biofilm

In a prospective, randomized, controlled trial conducted by the Mycoses Study Group, episodic treatment versus continuous prophylaxis with fluconazole was studied. The first conclusion was that overall resistance acquisition was uncommon in this HAART-compliant study population. Secondly, the use of episodic compared to continuous fluconazole prophylaxis was not shown to be protective in preventing emergence of resistance [108]. In general, no pattern of fluconazole prescription or ingestion has been consistently identified as contributing to azole resistance selection, although both dosing and duration have been widely implicated in emergence of resistance. Most importantly, it has not been established whether lower doses used for longer periods of time lead to antifungal resistance and whether intermittent therapy, especially using higher doses for shorter periods, prevents resistance [109]. In contrast to the above, occasionally resistant species were isolated in patients with HIV infection and no prior exposure to fluconazole [110].

It is noteworthy that in the last decade, because of the availability of potent and better tolerated ART, the occurrence of fluconazole-resistant OPC and *Candida* esophagitis has become infrequent.

6.2 Hematologic Malignancies and Transplant Patients

This growing population is the second focus of resistant candidiasis. Empiric systemic antifungals are widely used as empiric therapy for antibiotic-resistant fever in addition to azole prophylaxis both in neutropenic patients (usually fairly short term) and non-neutropenic high-risk posttransplant patients (often long term). Once more, azole exposure both

oral and systemic is recognized as (a) infrequent cause of azole-resistant *C. albicans* and (b) a more frequent and important cause of selection of non-*albicans Candida* species, both colonizing the gastrointestinal tract and as a cause of the ensuing infrequent invasive candidiasis [99, 111]. Primary fluconazole resistance has been reported in patients with severe neutropenia [112, 113]. Candidemia due to *C. krusei* has been associated with prior exposure to fluconazole [94, 114, 115].

6.3 Prosthetic Devices/In Vivo Biofilm

Evidence has been presented based upon in vitro, animal models and clinical studies that *Candida* organisms found in biofilm may show significant reduced susceptibility to azole drugs [116]. The implications are self-evident, since infections involving intravascular catheters and prosthetic valves and devices invariably fail intensive antifungal therapy and require surgical removal for cure. Clinical failure may also be due to failure of the antifungal drug to penetrate the biofilm access of yeast cells found within the biofilm [117]. The most important explanation for biofilm-related resistance appears to be the phenotypic and genotypic changes that are reported in biofilm containing yeast cells demonstrating in vitro antifungal resistance when compared to planktonic isotype cells. Nett et al. reported increased β-1,3-glucan content in *C. albicans* cell walls from biofilm compared to planktonic organisms thought to be responsible for polyene resistance and fluconazole resulting in limited intracellular penetration [118]. Biofilm-associated yeast cells are more susceptible to β-glucan inhibitors, i.e., echinocandins [119].

6.4 Antifungal Drugs

While most of the information available on drug-induced resistance followed the use of fluconazole and ketoconazole, usually as oral agents, little is known about the potential for broader-spectrum (itraconazole, voriconazole, posaconazole, caspofungin, micafungin, anidulafungin) or more active/potent in vitro drugs (voriconazole, posaconazole, echinocandins) or Candidacidal drugs (echinocandin) to select for less susceptible *C. albicans* or non-*albicans Candida* isolates.

Invasive infections due to amphotericin B-resistant *Candida* isolates have infrequently been reported in association with the use of this agent [58, 77, 120]. Many *C. lusitaniae* and some *C. guilliermondii* isolates demonstrated primary resistance to amphotericin B, but secondary resistance to amphotericin B appears to be uncommon. Acquired resistance associated with disseminated infections due to *C. glabrata*, *C. krusei*, and *C. albicans* that developed during therapy is described but is uncommon [121]. Resistance appears to be due to alteration or a decrease in the amount of ergosterol in the cell membrane. Yoon demonstrated in vitro reversible switching of *C. lusitaniae* with acquired amphotericin B resistance [122]. Nystatin-resistant *C. rugosa* was reported a burn unit following extended use of prophylactic topical nystatin [123].

A growing mass of data indicates that frequent and prolonged exposure to azole may influence the emergence of non-*albicans Candida* species especially *C. glabrata* but may also select for acquired resistance in *C. albicans* strains particularly following prolonged exposure to subinhibitory azole concentrations [32, 100, 111, 115]. However, the overall effect of azoles on *Candida* species distribution and resistance development is incompletely understood [124, 125]. Blott et al. reported that over an 11-year period in a single institution, the volume of fluconazole consumption did not correlate with *Candida* sp. distribution [124].

6.5 *Candida* Vaginitis

In spite of widespread use and abuse of over-the-counter (OTC) imidazole antifungals, little evidence has emerged of azole resistance in *C. albicans* or selection of non-*albicans Candida* spp. [126, 127]. However, prolonged use of long-term, low-dose (150 mg/week) fluconazole maintenance prophylaxis, in women with recurrent vulvovaginal candidiasis (RVVC), has recently been reported to contribute to both fluconazole and azole class resistance resulting in refractory vaginitis caused by in vitro resistant *C. albicans* [128, 129]. Moreover, in a study of HIV-positive women with RVVC receiving fluconazole, some evidence did surface of emergence of *C. glabrata* as a more frequent pathogen [130, 131].

6.6 Azole Cross-Resistance

Given that the azole class of antifungal agents share a common mechanism of action and in most cases of resistance, development of cross-resistance is common.

When selecting antifungal treatment, it is essential to establish whether the patient has received previous antifungal therapy because patients may harbor *Candida* species resistant to multiple azole agents [132–134]. Both in vitro and clinical studies have clearly demonstrated high frequency of azole cross-resistance [135]. Several studies indicated cross-resistance to itraconazole, ketoconazole, and other imidazoles in isolates resistant to fluconazole [32, 136]. Most of the strains concerned were fluconazole-resistant isolates of *C. albicans* obtained from patients with advanced AIDS and refractory OPC [137, 138], but others have reported cross-resistance in virtually all species of *Candida* exposed to non-fluconazole azoles, e.g., itraconazole and ketoconazole [132, 133, 139, 140]. Moreover, resistance found to first- and second-generation azoles may

extend, even in the absence of exposure, to newer triazoles, voriconazole and posaconazole, either as absolute resistance or more frequently as higher MIC values [136, 141–144]. In general, fluconazole-resistant strains had higher MICs to voriconazole and posaconazole. Nevertheless, cross-resistance varies considerably among species; hence, some but not all *C. parapsilosis* and *C. albicans* isolates maintain susceptibility to itraconazole, posaconazole, and voriconazole despite fluconazole resistance. Cross-resistance is often more predictable for *C. tropicalis* isolates, and lack of cross-resistance is seen with *C. krusei*. The development of resistance to azoles invariably requires more than one mutation; hence, isolates with resistance to both fluconazole and itraconazole exhibit multiple mechanisms or types of resistance and therefore are more likely to demonstrate resistance or reduced susceptibility to newer azole agents. Cross-resistance is a very common if not universal feature in azole-resistant *C. glabrata* isolates, especially in those that are capable of expressing multiple mechanisms of resistance [145, 146].

Susceptibility testing of 6970 *Candida* isolates from 200 centers worldwide by Pfaller et al. revealed that *C. albicans* and *C. glabrata* strains resistant to both fluconazole and itraconazole were less susceptible to posaconazole, ravuconazole, and voriconazole [60]. Slightly less than 50 % of *Candida* species isolates resistant to fluconazole maintained susceptibility to newer triazole agents [147]. In a study of azole cross-resistance, fluconazole MICs of ≤32 μg/mL predicted susceptibility, and MICs of ≥64 μg/mL predicted resistance of *Candida* spp. to voriconazole and posaconazole [147]. Voriconazole was active against *C. krusei* regardless of azole susceptibility. While much has been written of fluconazole prophylaxis leading to widespread azole resistance, similarly itraconazole prophylaxis was shown to be associated with cross-resistance to fluconazole [133, 148]. While much of the literature on azole cross-resistance has focused on mucosal candidiasis, similarly, large surveillance surveys of *Candida* spp. causing invasive infection including candidemia have shown evidence of cross-resistance.

6.7 Drug Pharmacokinetics, Pharmacodynamics, and Resistance in Candidiasis

Andes et al. reported the impact of fluconazole dosing regimens and pharmacodynamics on resistance development in *C. albicans* [149, 150]. Fluconazole regimens that produced prolonged sub-MIC concentrations were associated with resistance development. The emergence of the resistant phenotype was associated with increased expression of *CDR1*- and *CDR2*-encoded efflux pumps but not *MDR1*-encoded pumps or *ERG II* [149, 150]. In a murine systemic candidiasis model, the more frequently administered dosing regimens prevented the emergence of a resistant cell phenotype.

A correlation between in vitro susceptibility and response to therapy of non-mucosal candidiasis has been demonstrated in some studies [151, 152] but not others. Clancy et al. in 2003 evaluated 32 bloodstream *Candida* isolates and concluded that geometric mean MIC and fluconazole dose/MIC ratio predicted clinical failure [153]. Inadequate dosing of fluconazole (≤200 mg/day) and ratio <50 correlated with therapeutic failure, but not necessarily with resistance development.

6.8 Echinocandin Resistance

Candida sp. isolates resistant to echinocandins were first reported in 2005 [88], but reports of resistance were rare, at <2–3 % with *C. albicans* and most *Candida* species [43, 62, 154, 155]. However with time, reports of clinical failure with isolates demonstrating high MIC were increasingly but still not frequently seen [92, 156–165]. Overall, echinocandin resistance among most *Candida* species has been largely unchanged in the past few years [32]. However, this does not apply to *C. glabrata*, where echinocandin resistance is increasing and there is justifiable concern especially since many isolates also demonstrate azole resistance [166–168]. The SENTRY Antimicrobial Surveillance Program revealed that 8.0–9.3 % of blood isolates of *C. glabrata* from 2006 to 2010 were echinocandin drug resistant [154]. Of concern, Alexander et al. reported an increase in echinocandin-resistant *C. glabrata* bloodstream isolates, in Duke Hospital from 2–3 % in 2001–2006 to more than 13 % in 2009–2010 [166]. This is not widespread throughout the USA in that one recent study showed 3.1–5.7 % resistance in *Candida* isolates [62, 168]. Nevertheless, echinocandin resistance was similarly linked to azole resistance in *C. glabrata*. In this large Pham study, nearly all isolates containing an *FKS* mutation were resistant to at least one echinocandin, and 36 % were also resistant to fluconazole [168].

6.8.1 Mechanism of Acquired Echinocandin Resistance

Echinocandin resistance results from modification of glucan synthase, which is encoded by genes *FKS1* and *FKS2*. Unlike azole drugs, echinocandins are not substitutes for multidrug transporters [169]. Echinocandin resistance is nevertheless well characterized, conferred by restricted mutations in two highly conserved "hot spot" regions of the *FKS* genes [167]. The *FKS* mutations result in amino acid mutations that induce MIC values from 20- to 100-fold and reduced sensitivity of glucan synthase to drug by 50–30,000-fold [170]. These less susceptible fks mutant strains respond poorly to echinocandin drugs in pharmacodynamic models of infection [171, 172] and are associated with reduced clinical response [173, 174]. The *FKS* resistance mechanisms have been observed in many *Candida* species [175]. In all *Candida* species, except *C. glabrata*, mutations occur within two "hot spot" regions of *FKS1* [55] (see Chapter

29, Volume 1). In *C. glabrata* mutations occur in the homologous hot spot regions of *FKS1* and *FKS2* [55, 155, 170].

The echinocandin drugs are highly serum protein bound potentially reducing susceptibility testing. Serum is considered to reduce the fungicidal characters of the echinocandins, resulting in fungistatic activity against certain *Candida* species [175].

Biofilms also play a role in antifungal resistance [176]. Decreasing glucan production, accompanying echinocandin use increases susceptibility of yeast organisms contained within the biofilm to the effects of these drugs [177].

7 Refractory Candidiasis: Clinical Resistance Syndromes and Their Management

7.1 Oropharyngeal and Esophageal Candidiasis

Refractory OPC and EC represent the commonest manifestation of clinical azole resistance and failure that is supported by concomitant in vitro azole resistance. Most patients present with highly symptomatic episodes with oropharyngeal pain and debilitating dysphagia and odynophagia requiring hospitalization. The majority of patients with refractory upper gastrointestinal candidiasis have AIDS and advanced immunodeficiency. In the 1990s, the annual incidence of clinical failure of fluconazole in OPC was approximately 5 % [104–107]. Accordingly, refractory superficial candidiasis peaked and became a major clinical problem during the decades of the 1990s prior to the availability of highly active antiretroviral therapy (HAART) [110, 178, 179]. The majority of these patients have refractory disease caused by *C. albicans* [180]. Only a minority have non-*albicans Candida* spp. usually *C. glabrata*, strains of which are usually resistant in vitro to fluconazole. Resistant strains of *C. albicans* and *C. glabrata* are frequently, but not invariably, cross-resistant to itraconazole and ketoconazole [181]. Refractory mucosal candidiasis has also been reportedly associated with *C. tropicalis* and *C. krusei* [5]. In the absence of coinfection with non-*albicans Candida* species, refractory candidiasis is seen with both in vitro resistant and sensitive *C. albicans*. The reason for treatment failure caused by azole-sensitive *C. albicans* is usually the result of noncompliance with ART therapy, drug underdosing, or drug interactions. Another major factor is simply advanced immunodeficiency. With refractory esophagitis, it is important to exclude concomitant pathology such as CMV or HSV esophagitis. Other explanations for the in vitro-in vivo discrepancy in compliant patients relate to heteroresistance in individual colonies of *Candida*, with chance selection of a "susceptible" colony. Most patients with refractory OPC and EC almost always have usual *Candida* spp. isolates with in vitro resistance.

Finally, some experts have questioned the virulence capacity of non-*albicans Candida* species to induce OPC and EC, let alone refractory disease [5, 182]. It is true that refractory candidiasis in patients with AIDS, from whom NAC strains are isolated, usually represents mixed infections with coexistent *C. albicans*; however, resistant disease due to *C. glabrata* in the absence of *C. albicans* is now widely accepted.

The availability of HAART was rapidly followed by a marked decline in the frequency of refractory OPC and EC [183]. It was assumed that enhanced mucosal immune function was responsible for this phenomenon. However, this issue is more complex in that refractory disease resolved within days and weeks of initiation of HAART, preceding demonstrable improvement or change in CD4 lymphocyte cell count or any other marker of CMI, suggesting that some other beneficial effects might be responsible [184]. Another observation included the disappearance of azole-resistant strains of *C. albicans* and *C. glabrata* with the reappearance of azole-sensitive strains. How was improved mucosal CMI selecting susceptible strains of *Candida*? Another more recent hypothesis relates to a direct effect of HIV structural components in directly influencing genes carried by *Candida* responsible for virulence expression including development of azole resistance. Accordingly, HIV gp 160 and gp 41 may influence *Candida* in vitro, selecting for azole resistance [185]. According to this hypothesis, the mucosal viral load (HIV RNA) would enhance *Candida* virulence in situ and finally induce or select for azole resistance. Introduction of HAART and rapid decrease in viral load, before immune recovery, would explain early resolution of refractory mucosal candidiasis and reemergence of azole-susceptible strains. Therapeutic protease inhibitors may further reduce *Candida* virulence by inhibiting fungal secretory aspartyl proteinases [186].

It follows that in the post-HAART era, the frequency of refractory disease as well as in vitro azole resistance declined substantially. The majority of patients with chronic and refractory disease are usually noncompliant AIDS patients infected with susceptible *C. albicans*. In a study of in vitro susceptibility of oral isolates in the HAART era, Tacconelli showed a reduction in azole resistance from 37 to 7 % [187]. The explanation for the reduced or diminished at-risk population is thought to relate to reduced fluconazole exposure, i.e., fewer low-dose regimens and less continuous long-term therapy; however, this hypothesis is unproven. Barchiesi et al. reported that most patients on HAART are colonized by strains of *C. albicans* susceptible to fluconazole (93 % sensitive) [188]. Most cases of OPC in the HAART era are caused by fluconazole-sensitive *C. albicans*.

A high prevalence of non-*albicans Candida* species (*C. albicans* 49 %, *C. glabrata* 24 %) with frequent resistance to fluconazole and itraconazole has also been reported in patients with advanced cancer, especially head and neck malignancy [189, 190]. Another small but critically important patient population includes patients with the various genetic

forms of chronic mucocutaneous candidiasis such as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) patients [191]. Frequent decreased susceptibility of *C. albicans* to fluconazole is a common complication of prolonged fluconazole use in this population.

Clinical management of refractory OPC requires evaluation and determination of etiological mechanisms responsible for clinical resistance, including CD4 count, compliance with HAART therapy, previous OPC, and exposure to azoles, usually fluconazole [192]. Finally, clinical resistance implies failure to respond despite adequate delivery of a tolerable therapeutic concentration of the drug. Once in vitro resistance is suspected, cultures are obtained and susceptibility determined of the responsible organisms. Most commonly, *C. albicans* is present, sometimes together with a second species usually *C. glabrata*. While awaiting microbiology and susceptibility results, treatment is initiated. Therapeutic strategies are listed in Box 66.3. Initial options include progressive increasing doses of oral fluconazole from 100 to 400 mg/day, including fluconazole suspension [193] or swish-and-swallow amphotericin B suspension (100 mg/mL, taken as 1 mL qid) [194]. Although cross-resistance with other triazoles is common, in the event of retained itraconazole sensitivity, itraconazole suspension (10 mg/mL, taken as 10 mL bid) is often effective, although usually on a temporary basis only [195]. However, the most important advance in therapy of fluconazole-refractory OPC is oral posaconazole. Although initially available only as an oral suspension, it is now prescribed as posaconazole tablet 400 mg bid for 14 days. Given its safety profile, posaconazole is used preferentially to oral voriconazole.

Box 66.3. Therapy of Fluconazole-Refractory Oropharyngeal (OPC) and Esophageal Candidiasis (EC)

OPC

- High doses of fluconazole tablets
- Fluconazole suspension
- Itraconazole capsules/suspension
- Amphotericin B oral suspension
- IV amphotericin B/lipid formulation
- Posaconazole oral/IV
- Voriconazole oral/IV
- IV echinocandin
- Immunomodulation
  - G-CSF
  - GM-CSF
  - α-Interferon

EC

- IV echinocandin
- Fluconazole
- IV lipid formulation of amphotericin B
- IV voriconazole*

*If susceptible in vitro

Parenteral antifungals have become the last resort employing intravenous amphotericin B, echinocandin, or voriconazole [196]. All these options may successfully control and eradicate acute symptomatic infection; however, unless immune reconstitution follows, relapse is inevitable. Potentially, the aforementioned parenteral antifungals could be given on an intermittent maintenance basis; however, maintenance suppressive therapy with oral posaconazole 400 mg per day is effective [197].

While HAART therapy offers a definite solution in AIDS patients, the same cannot be said from CMC patients with progressive azole resistance starting with fluconazole and extending sequentially to itraconazole and then voriconazole with either *C. albicans* or *C. glabrata*. Intermittent parenteral echinocandins or lipid formulation of amphotericin B will be necessary, although the use of oral posaconazole is preferred [198].

7.2 Refractory Esophageal Candidiasis (Box 66.3)

As for refractory OPC, clinically resistant EC is mainly seen in untreated AIDS patients with advanced immunodeficiency, with a history of sporadic previous treatment with fluconazole. Refractory, especially chronic, EC is associated with a profound impact on general health leading to weight loss, malnutrition, and overall reduced general health status. Oral cultures usually reveal the *Candida* species responsible for esophageal disease, recognizing that more than one resistant species may coexist. Most cases of fluconazole-resistant EC are similarly resistant to itraconazole [199]. In a minority of patients still capable of swallowing, oral posaconazole is still a therapeutic possibility. If swallowing is not possible, therapeutic options now include amphotericin B deoxycholate or lipid formulations used parenterally in hospitalized patients, and while widely recognized as efficacious, there are little published data documenting efficacy. Cost with the use of lipid formulations and toxicity associated with conventional AmB remain issues. Regardless of which formulation is chosen, low-dose regimens frequently fail in patients with azole-resistant *C. albicans* and/or *C. glabrata*. Response to IV therapy is frequently slow, and >0.8 mg/kg AmB or 5 mg/kg of lipid AmB should be used.

Fortunately, the drugs of choice are IV echinocandins. Studies confirm similar efficacy with daily IV caspofungins, anidulafungin, and micafungin. Accordingly, caspofungin was found to have ~70 % efficacy rate in treating patients with fluconazole-refractory EC [6, 198, 200–202]. No cross-resistance exists between azoles and echinocandins. Similar efficacy for EC has been observed with parenteral voriconazole, also achieving ~70 % response rates but with little experience published with fluconazole-resistant species [203]. Table 66.1 shows the impact of fluconazole-resistant

C. albicans on susceptibility to voriconazole; hence, higher doses of voriconazole may well be indicated [87, 144]. The recent availability of parenteral posaconazole increases therapeutic options, and oral posaconazole is recommended as de-escalation therapy to complete parenteral echinocandin treatment.

Regardless of the parenteral regimen selected, the dominant issue remains the maintenance antifungal prophylaxis in these severely immunocompromised individuals. It cannot be emphasized sufficiently that the key to preventing further recurrences or inevitable relapses of refractory EC lies with successful initiation of HAART therapy. Noteworthy several studies indicated that relapse rates of EC are higher following initially successful echinocandin treatment [204]. Until HAART therapy reverses susceptibility, maintenance prophylaxis is best afforded with oral posaconazole.

7.3 Refractory *Candida* Vaginitis (VVC)

Two forms of vulvovaginal candidiasis (VVC) exist. In the first place, an individual episode of symptomatic vaginitis may not respond to conventional topical or oral antifungal therapy. The other form of refractory disease is found in a larger population of women with frequently recurring episodes of relapsing symptomatic vaginitis although each individual episode of VVC responds to conventional therapy (RVVC).

Failure to achieve clinical improvement and symptom resolution, i.e., azole-resistant vaginal *C. albicans*, is still uncommon but has increasingly been reported in both HIV-positive and HIV-negative women [205]. It is actually remarkable that resistance is not more frequent given the widespread use of low-dosage fluconazole as single-dose therapy or once-weekly maintenance prophylaxis for RVVC. Nevertheless, any patient with acute *Candida* vaginitis, failing to improve with a standard regimen of oral or topical azoles, with persistent symptoms, positive microscopy, and culture, should be treated with topical vaginal boric acid 600 mg daily for 14 days. At the same time, the *C. albicans* isolate should be sent for azole susceptibility testing. The same cannot be said for acute *C. glabrata* vaginitis which responds to azole agents with a 50 % rate only [206]. Acute *C. glabrata* vaginitis should be treated with topical boric acid 600 mg suppositories daily for 11–21 days with an anticipated clinical and mycological response rate of ~70 % [179]. Higher cure rates (>90 %) can be obtained with topical 17 % flucytosine intravaginal cream, 5 g nightly for 14 days, although the cream must be compounded and is not widely available and hence is expensive [206, 207]. High cure rates also follow daily intravaginal amphotericin B 50 mg suppository for 14 days or in combination with topical flucytosine [208].

Acute vaginitis due to *C. krusei*, although rare, will not surprisingly fail to respond to oral fluconazole, due to innate or primary resistance [209]. Occasionally, patients may respond to oral itraconazole or topical miconazole or clotrimazole prescribed for 14 days. *C. krusei* is also resistant to flucytosine, and hence vaginitis due to this species is often extremely difficult to control.

It should be emphasized that refractory acute vaginitis is extremely rare, although busy practitioners might not agree. This is because of incorrect diagnosis on the part of practitioners who treat vaginitis on an empiric basis, invariably failing to measure vaginal pH, perform microscopy, and obtain a vaginal culture. Several studies have confirmed the poor diagnostic acumen of practitioners. Self-diagnosis by women is no better. Other species of *Candida* can cause vaginitis, but tend to rapidly respond to azole therapy.

Much more common and affecting millions of women, in their childbearing decades worldwide, is recurrent vulvovaginal candidiasis (RVVC) thought to affect 6–8 million women in the USA. Under these circumstances recurring episodes of vaginitis respond appropriately to antifungal therapy regardless of route, only for symptoms and signs to recur within a month or two but rarely monthly [210]. RVVC is mostly caused by azole-sensitive *C. albicans* (>90 %) and less commonly by *C. glabrata* (5 %). RVVC is rarely a manifestation of drug resistance but of host factors that predispose to genital tract yeast colonization and host immune response hyperreactivity to *Candida* antigens [210]. RVVC is best controlled by once-weekly fluconazole maintenance prophylaxis administered for 6 or more months [128], although other forms of suppressive azole therapy are effective but less convenient [211, 212]. Boric acid has also been used effectively [213].

The management of azole-refractory vaginitis due to in vitro confirmed fluconazole-resistant *C. albicans* is initially managed with daily vaginal boric acid for 2 weeks, while in vitro susceptibility tests become available. Acute, nonrecurrent vaginitis may require no additional therapy; however, women suffering from RVVC will of necessity require a maintenance antifungal regimen. Possible alternatives to weekly fluconazole are daily ketoconazole or itraconazole 100–200 mg, provided that susceptibility is confirmed in vitro. As per standard protocols, the maintenance daily regimens are continued for at least 6 months. In the event of frequently reported azole cross-resistance, no oral azoles are likely reasonable safe alternative agents. In this scenario, long-term maintenance therapy can be achieved with topical boric acid or nystatin for the same long-term duration, but little published data are available. Similarly, daily combination therapy with boric acid and nystatin is effective for symptomatic recurrent VVC due to *C. glabrata* although such cases are rare.

Box 66.4. First-Line Antifungal Drug Therapy of Candidemia (Parenteral)

1. Amphotericin B (conventional deoxycholate)
2. Lipid formulation AmB
3. Fluconazole (400 mg/day)
4. Fluconazole (800 mg/day)
5. Itraconazole
6. Voriconazole
7. Caspofungin
8. Amphotericin B + flucytosine
9. Amphotericin B + fluconazole

7.4 Refractory Candidemia and Disseminated Candidiasis

The incidence of bloodstream infections (BSIs) due to *Candida* spp. has increased worldwide, with accompanying significant mortality. Fortunately, in parallel with this increase has been an increase in the therapeutic armamentarium for candidemia (Box 66.4). The purpose of this chapter is not to review management of candidemia (see reviews [96, 114, 214]). Drug resistance is monitored by a variety of study organizations in multiple countries. Perhaps the most comprehensive antifungal susceptibility monitoring organization is the SENTRY system receiving in excess of 2000 bloodstream *Candida* isolates annually from all over the world [63]. Compiled data are shown in Table 66.3. Nevertheless, given the proportional and occasionally found absolute increase in cases of invasive candidiasis and candidemia due to non-*albicans Candida* species especially *C. glabrata*, together with the availability of safe and in the past predictable effective echinocandins, guidelines from national and international infectious disease societies have recently been issued which acknowledge the reduced azole susceptibility of non-*albicans Candida* species. Hence, until information of the identity of the *Candida* species responsible for the bloodstream infection is available, echinocandins are considered drugs of first choice to be prescribed [96].

Table 66.3 Species distribution of *Candida* from cases of invasive candidiasis[a]

	% of total no. of cases[b]					
Species	1997–1998	1999	2000	2001	2002	2003
C. albicans	73.3	69.8	68.1	65.4	61.4	62.3
C. glabrata	11.0	9.7	9.5	11.1	10.7	12.0
C. tropicalis	4.6	5.3	7.2	7.5	7.4	7.5
C. parapsilosis	4.2	4.9	5.6	6.9	6.6	7.3
C. krusei	1.7	2.2	3.2	2.5	2.6	2.7
C. guilliermondii	0.5	0.8	0.8	0.7	1.0	0.8
C. lusitaniae	0.5	0.5	0.5	0.6	0.5	0.6
C. kefyr	0.2	0.4	0.5	0.4	0.4	0.5
C. rugosa	0.03	0.03	0.2	0.7	0.6	0.4
C. famata	0.08	0.2	0.5	0.2	0.4	0.3
C. inconspicua			0.08	0.1	0.2	0.3
C. norvegensis			0.08	0.1	0.07	0.1
C. dubliniensis			0.001	0.08	0.1	0.05
C. lipolytica			0.06	0.06	0.06	0.08
C. zeylanoides			0.03	0.08	0.02	0.04
C. pelliculosa				0.06	0.05	0.04
Candida spp.[c]	3.9	6.0	3.7	3.3	7.9	4.9
Total no. of cases	22,533	20,998	11,698	21,804	24,680	33,002

[a]Data compiled from the ARTEMIS DISK Surveillance Program, 1997–2003

[b]Includes all specimen types and all hospitals from a total of 127 different institutions in 39 countries

[c]*Candida* species not otherwise identified

7.4.1 *C. albicans*

Despite the widespread use of fluconazole over the last 15 years, fluconazole resistance in *C. albicans* blood isolates remains below 5 %, with no evidence of a progressive increased resistance with time or associated with a specific geographic area [60]. It is not fear of an azole-resistant strain of *C. albicans* that drives principles of antifungal drug selection. Candidemia due to drug-resistant *C. albicans* is rare, but has been rarely reported in patients with hematologic malignancy [100]. However, *C. albicans* is no longer the most prevalent *Candida* species responsible for BSI, and rarely is drug resistance a management issue. Should an azole-resistant *C. albicans* isolate be responsible for the candidemia, the clinical manifestations include persistent candidemia on fluconazole therapy, relapsing candidemia or possibly increased mortality, and finally breakthrough candidemia. In the last decade, results of at least five randomized prospective controlled studies have been published involving fluconazole and other antifungal drugs [215–220]. Attempts have been made to correlate clinical outcome with in vitro MICs. In none of these studies has *C. albicans* antifungal resistance, specifically fluconazole resistance emerged as a cause of drug failure [216, 217]. The lack of fluconazole resistance in *C. albicans* BSI isolates after all these years remains reassuring, but the altered epidemiology is less so. In contrast to other studies, correlation between in vitro susceptibility and response to fluconazole therapy has been demonstrated, but rarely is persistent fungemia due to azole-resistant *C. albicans* but rather non-*albicans Candida* species [221].

7.4.2 *C. glabrata*

As evident in Table 66.2, candidemia due to *C. glabrata* has increased especially in North America and Europe. Fluconazole resistance in bloodstream *C. glabrata* isolates is evident in 7–10 % of strains, with an addition of 27–30 % of isolates considered S-DD indicating reduced fluconazole susceptibility of *C. glabrata* isolates. Accordingly, only 50–70 % of *C. glabrata* bloodstream isolates are highly susceptible to fluconazole. Several studies involving *C. glabrata* have shown a similar susceptibility pattern [63, 64].

Documented failure or suboptimal response to fluconazole and other antifungals has been forthcoming in some studies and is impressively present in others [221]. When failure was always apparent, this may simply reflect small numbers of patients with *C. glabrata* fungemia, i.e., some published studies have lacked the power to show any differences in outcome by *Candida* species.

Supporting the in vitro data are numerous case reports of fluconazole failure to eradicate *C. glabrata* fungemia subsequently responsive to parenteral polyene or echinocandin therapy as well as retrospective analysis of patients with persistent candidemia [151, 221]. Accordingly, most experts would recommend avoiding any azoles, including voriconazole, initially in patients with candidemia caused by *C. glabrata* and initiate therapy with an echinocandin. Until the *Candida* isolate (species) is identified and species identity is becoming more and more rapidly established, then given the increased likelihood of *C. glabrata* and other reduced fluconazole susceptibilities, selection should include the possibility and commence with an echinocandin. In candidemia patients doing well on azoles, continued therapy with the azole would be perfectly reasonable.

8 Adjuvant Therapy for Resistant Candidiasis

The use of immune and nonimmune adjuvants to treat refractory candidiasis is almost exclusively seen in patients with AIDS or chronic mucocutaneous candidiasis (CMC). Even with the latest generation of azoles (voriconazole, posaconazole) and polyene and echinocandin use, refractory mucosal disease is still reported due to resistant *C. albicans*, *C. glabrata*, and rarely other *Candida* species. There have been anecdotal successes reported with immunostimulators mainly recombinant human granulocyte-macrophage colony-stimulating factor (rhu GM-CSF) [222, 223]. Also, interferon gamma has occasionally been given [196]. Unfortunately, investigators tend to publish only successful therapeutic endeavors and failures are more frequent [224, 225]. Even so, long-term use and success of these growth factors have not been forthcoming especially associated with CMC. The use of these agents, given these expenses, requires the performance of randomized controlled studies which are unlikely given the current infrequency of these refractory cases. The value of GM-CSF in invasive candidiasis has not been demonstrated but may have a role in persistently neutropenic patients. Monoclonal antibodies were shown to prevent disseminated candidiasis in a mouse model and have been the bases for vaccine development. Likewise, the administration of anti-*Candida* heat shock antibodies may have an adjuvant role together with antifungals for resistant or refractory candidemia.

9 Prevention of Antifungal Resistance in *Candida* Species

In general, standard principles of infection control that apply to all microorganisms and particularly nosocomial infections should be applied to prevent antifungal resistance.

Avoidance of prophylactic or suppressive therapy and a preference for repeated short course of azoles for OPC in the late stages of AIDS are an attractive but unproved measure for delaying the appearance of azole resistance. In a study conducted in patients with recurrent OPC and AIDS, episodic fluconazole therapy was compared to continuous fluconazole therapy aimed at evaluating likelihood of inducing fluconazole resistance and refractory oropharyngeal candidiasis [98]. The study failed to show a difference in the two arms with regard to selection or induction of azole resistance. This somewhat disappointing result may reflect the fact that the study was conducted during the HAART era with relatively few individuals presenting with refractory mucosal disease, with advanced immunodeficiency and unavailability of HAART therapy. The study outcome is in sharp contrast to clinical experience obtained in the pre-HAART era.

It goes without saying that all unnecessary use of azoles should be avoided, whether as prophylaxis or therapy. Many clinicians prescribe a lower than recommended prophylactic dose of oral fluconazole in neutropenic patients, i.e., 100 mg vs. 400 mg daily. To date, no evidence has emerged of increased fluconazole resistance as a specific consequence of this reduced daily dose. Nevertheless, many experts advise against the use of azole prophylaxis in neutropenia of short duration. Paterson suggested that combining oral amphotericin B with azoles may prevent the emergence of resistant *Candida* species in neutropenic patients; however, oral amphotericin B is poorly tolerated and noncompliance is common [226].

Studies have indicated that most *Candida* species are carried and readily transferred manually by nursing physicians and other medical personnel [227]. Accordingly, adherence to strict handwashing principles applies equally to *Candida* and specifically the transfer of resistant strains of *C. albicans* and other *Candida* species [228, 229]. In particular, *C. parapsilosis* is frequently isolated from the hands in contrast to *C. glabrata* which appears to be endogenously acquired from GIT carriage only. Isolation of patients with resistant strains of *Candida* is not indicated in this era of universal precautions. Perhaps, the most controversial is the use of antifungal prophylaxis in selected high-risk patients in intensive care units. Several studies suggest limited benefit and only in selected high-risk ICU patients [230, 231, 232].

10 Conclusion and Perspective

During the last two decades, enormous strides have been made in understanding the subcellular, molecular, and genetic basis of antifungal resistance. All in all, clinically refractory candidiasis is uncommon. The explosion in clinically resistant cases of OPC and EC early in the AIDS epidemic has not stood the test of time with the arrival of antiretroviral therapy. Of course, clinically resistant cases still occur and remain a therapeutic challenge, but the majority of cases of mucosal disease are caused by azole-sensitive *Candida albicans*. There has been an increase in non-*albicans Candida* species causing invasive candidiasis. Much, but not all, evidence points to widespread prophylactic, empirical, and therapeutic use of fluconazole. Nevertheless, blood isolates of *C. albicans* remain remarkably and predictably susceptible to fluconazole and other azoles, and this is a worldwide experience. There is no doubt that certain *Candida* species are less susceptible and/or resistant to fluconazole and show cross-resistance to all azoles. This species-specific (*C. krusei*, *C. glabrata*) azole resistance has a major influence in antifungal drug selection. These two species not only expose vulnerability of the azole class but require higher doses of polyenes. As such, fungal susceptibility tests in the past were rarely available and infrequently and selectively used. This however has changed with increased availability. The newer generations of azoles are often active against non-*albicans Candida* species (*C. glabrata* and *C. krusei*) and as such offer early confident broad-spectrum therapy. Moreover, they are frequently active against fluconazole-resistant *C. albicans*. The echinocandins have further eased the concern of azole resistance in candidiasis, but time has yet to determine the potential for echinocandin-acquired resistance in candidiasis.

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Antifungal Drug Resistance in *Aspergillus*

67

P.H. Chandrasekar and Elias K. Manavathu

1 Introduction

A recent database search revealed a steady increase in the number of publications (almost 1000 papers) on antifungal drug resistance/lack of drug susceptibility in aspergillus in peer-reviewed scientific journals from 2000 to 2014 (Fig. 67.1). In contrast, about only 57 publications appeared in scientific journals directly dealing with antimicrobial drug resistance in aspergillus prior to 2000. Until recently, unlike in Candida, drug resistance in aspergillus was poorly appreciated. This rapid rise in publications signifies a revitalized interest in aspergillus drug resistance in clinical settings that is augmented by the upward spike in aspergillus infections in humans, primarily in the immunocompromised patient population. Although more than 200 *Aspergillus* species are described in the literature (less than 10 % cause disease in man), more than half (50.63 %) of the published reports deal with drug resistance/lack of drug susceptibility in clinically most common *A. fumigatus* followed by *A. niger* (11.8 %), *A. flavus* (11.3 %), *A. terreus* (7.7 %), and *A. nidulans* (7.0 %) (Fig. 67.1). Approximately 12 % of the publications describe drug resistance/lack of drug susceptibility in other disease-causing *Aspergillus* species. This somewhat skewed distribution of publications on antimicrobial drug resistance in aspergillus is not surprising considering that about 60–70 % of the isolates obtained from clinical specimens are *A. fumigatus*.

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Several reasons could be attributed to the increased interest in the area of antimicrobial drug resistance in *Aspergillus* species, namely, (1) effective prophylaxis against *Candida* infections in highly vulnerable immunocompromised patient population (e.g., organ transplant recipients and cancer patients undergoing aggressive chemotherapy) has made the opportunistic *Aspergillus* species a major clinical problem, (2) effective and safer anti-aspergillus drugs have become available, (3) improved modern diagnostic techniques facilitate better identification of aspergillus infection, (4) introduction of standardized susceptibility testing has led to the identification of more drug-resistant clinical strains, (5) introduction of culture-independent molecular techniques to identify potential drug resistance causing gene mutation has enhanced the level of detection of drug resistance, and (6) increased use of antimycotic drugs (e.g., azole derivatives) as agrochemical pesticides has increased the frequency of selection of drug-resistant environmental isolates.

Despite the availability of potent antifungal agents, systemic fungal infections continue to cause significant morbidity and mortality. While *Candida*-related deaths have declined since the late 1980s, those due to aspergillosis remain high. A high percentage of patients continue to die with invasive aspergillosis (IA) despite treatment [1–3]. Scientific discussions regarding unsuccessful treatments reason that susceptible hosts, particularly cancer patients and transplant recipients, are profoundly immunocompromised with neutropenia and/or impaired monocyte/macrophage dysfunction. There is universal agreement that the outcome of IA is largely dictated by the host immune status [4–6]. Regardless of the antifungal drug(s) employed, the poor outcome or failure of antifungal therapy is generally attributed to persistently, compromised host defenses and, in most cases, not considered to be due to drug-resistant fungi. Also, failure of antifungal drugs may be due to inappropriate dose, fungistatic activity, high protein binding, poor absorption/distribution, and metabolism or drug interactions. Until recently, drug resistance in aspergillus was not adequately examined.

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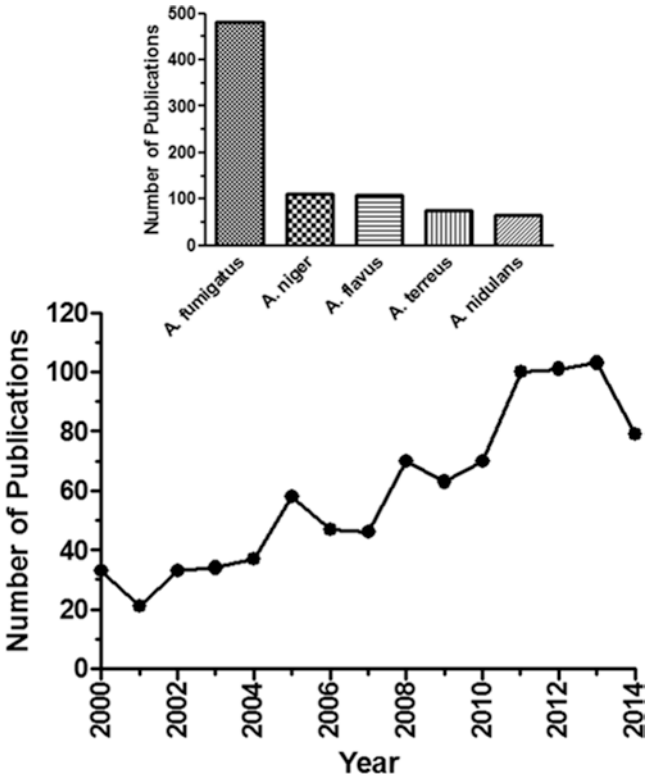

Fig. 67.1 Number of peer-reviewed yearly publications on antimicrobial drug resistance in *Aspergillus* species from 2000 to 2014

In a contrasting argument, azole resistance takes center stage (Fig. 67.2). Availability of better-tolerated effective azoles has led to their widespread and prolonged use for prophylactic or therapeutic purposes, particularly in compromised hosts; as a result, clinically significant azole resistance is increasingly encountered. Failure to available drug therapy can no longer be entirely attributed to the immunocompromised status of the host. Additionally, common use of azoles in agricultural industry in many countries has resulted in the recognition of multi-azole resistance in environmental isolates of aspergillus. Drug resistance in aspergillus is becoming a global phenomenon; strategies and guidelines are urgently needed for the management of suspected or proven drug-resistant aspergillosis.

The cornerstone for the successful management of IA includes decrease in immunosuppression, immune restoration, surgical debridement, and optimal antifungal drug therapy. The antifungal drugs available for therapy of IA are listed in Table 67.1. The most recent addition is isavuconazole, a water-soluble, anti-mold azole available in oral and IV forms. Data on aspergillus exhibiting resistance to drugs listed are limited. This limitation has largely been due to lack of interest in the past as the incidence of infection was low; amphotericin B being the only effective drug, the pathogen not readily recovering from most infected patients; lack of information on resistance to newer drugs; and more importantly, nonavailability of a reliable susceptibility test method to correlate in vitro findings to clinical outcome. The rising incidence of aspergillosis, the recent availability of a standardized in vitro method to test susceptibility of filamentous fungi, and the entry of new drugs have kindled the interest and made it feasible to study drug resistance in aspergillus [7].

2 In Vitro Resistance

2.1 Mechanisms of Antifungal Resistance

Resistance can be described as primary (innate) when a fungal pathogen is intrinsically resistant to the antifungal drug or secondary (acquired) when an organism develops resistance during drug exposure either due to spontaneous mutation or the acquisition of the resistance trait from an external source by genetic transfer. The known cellular and molecular mechanisms responsible for reduced in vitro and in vivo susceptibility to antifungal drugs fall into two broad categories, namely, reduced intracellular accumulation of the antifungal drug compared to that in the susceptible cells and quantitative or structural alteration of the fungal drug target.

The reduced intracellular drug accumulation occurs either due to efflux of the drug from the cell mediated by efflux proteins or due to reduced penetration of the drug into the cell because of selective drug-permeability barrier(s). The efflux proteins belong to two groups, ATP binding cassette (ABC) transporters and major facilitators. The efflux proteins pump out drugs accumulated in the cell at the expense of energy and maintain the concentration of the drug inside the cell below the level normally required for the inhibition of growth. Thus, even in the presence of high concentration of the drug outside the cell, the organism is able to grow and function physiologically more or less normally. The energy required for the expulsion of the drug is generally derived from hydrolysis of ATP. When an organism develops resistance to a certain drug due to efflux, the pump proteins are overproduced compared to the amount present in drug-susceptible cells. In general, the efflux proteins are native to the cell carrying out essential nutrient transport but fortuitously adapted to perform transport of substances toxic to the cell, including antimicrobial drugs.

A second, less well-known mechanism for the reduced accumulation of antifungal drugs inside the fungal cell is diminished penetration of the drug because of selective permeability barrier(s). This type of mechanism is known to be responsible for resistance to antibacterial drugs in Gram-negative bacteria such as *Pseudomonas* species where the outer cell membrane or biofilm acts as a selective permeability barrier [8]. In the case of fungi, the reduced penetration is often associated with other factors such as the chemical

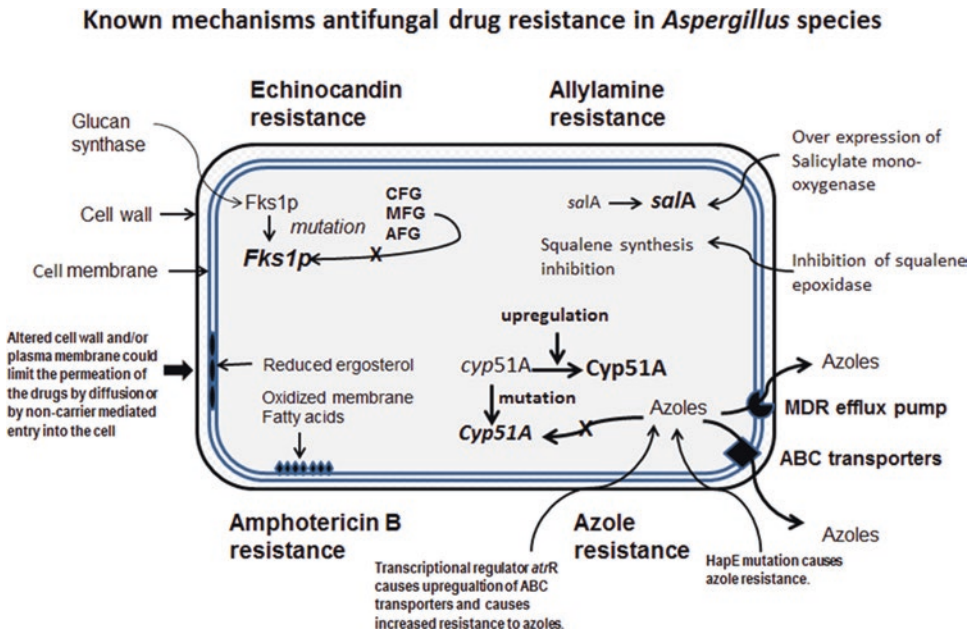

Fig. 67.2 A diagrammatic illustration of the known mechanisms of antimicrobial drug resistance in *Aspergillus* species. See the text for details

Table 67.1 Drugs for invasive aspergillosis

A. Polyenes
Amphotericin B deoxycholate (AmBD)
Amphotericin B lipid complex (ABLC), Abelcet®
Liposomal amphotericin B (LamB), Ambisome®
Amphotericin B colloidal dispersion (ABCD), Amphocil®
B. Azoles
Voriconazole, V-fend®
Itraconazole, Sporanox®
Posaconazole, Noxafil®
Isavuconazonium sulfate, Cresemba®
C. Echinocandins
Caspofungin, Cancidas®
Micafungin, Mycamine®
Anidulafungin, Eraxis®

changes in the cell wall and production of hydrophobic compounds such as pigments. Excessive production and incorporation of pigment(s) in the cell wall often act as a barrier for the penetration of toxic substances, including antifungal agents [9]. Since the presence of cell wall pigment provides an added advantage to drug-resistant cells for survival in the presence of antifungal drugs compared to the susceptible ones, the synthesis of cell wall pigment(s) is often considered as a virulence factor [10].

Modification of the fungal drug target (with which the drug molecules interact to bring about their antifungal activity) is a well-known mechanism responsible for the emergence of antifungal drug resistance in medically important fungi. The modification of the drug target is achieved at two levels: quantitative and structural (qualitative). Quantitative drug target modification is obtained by the enhanced production of the drug target by upregulation of its synthesis or by the increased dosage of the gene(s) responsible for the synthesis of the drug target. In either case, the increased amount of the fungal drug target requires higher concentration of the drug to elicit an inhibitory effect. Thus, fungal cells with increased amount of the drug target will survive in the presence of increased amount of the drug compared to a susceptible cell that possesses the base level of the drug target. The structural modification of drug target occurs by the mutational acquisition of genetic variation affecting its synthesis or primary structure (protein). Variation of the primary structure of protein often leads to secondary and tertiary structural changes that affect the binding and processing of drug molecules that mimic the natural substrate (in the case of enzyme target) or ligand (in the case of receptor molecules). Usually, drug target modification-dependent mechanism alone or in combination with other resistance mechanism leads to high-level cellular resistance to the antifungal drug.

2.2 Resistance to Polyenes

Amphotericin B is a typical polyene antifungal drug approved for primary therapy against a wide variety of fungal infections since 1953 [11] and remained as the unchallenged gold standard until recently. It is an amphoteric molecule composed of a hydrophilic polyhydroxyl chain along one side and a lipophilic polyene hydrocarbon chain on the other. It interacts with the fungal membrane-associated ergosterol forming channels or pores spanning across the plasma membrane disrupting the osmotic integrity and the selective permeability of the fungal

plasma membrane. The loss of osmotic integrity and the selective permeability of the membrane result in leakage of essential intracellular cations such as calcium, potassium, and magnesium as well as various metabolites [12]. This indiscriminate massive loss of essential nutrients and ions is believed to be primarily responsible for the death of fungal cells when treated with amphotericin B, although other biochemical reactions such as oxidation of plasma membrane-associated phospholipids and their derivatives affecting the proper functioning of the fungal plasma membrane may also play a major role for the fungicidal activity of amphotericin B [13].

In spite of the extensive use of amphotericin B as the primary antifungal drug against fungal infections over a period of nearly five decades, the emergence of high-level resistance to this compound in clinical isolates of fungi, including *Aspergillus* species, is very rare. The reason(s) for the lack of emergence of resistance to amphotericin B among clinical isolates of fungi is not understood. However, the occurrence of the so-called conventional drug target modification-dependent acquired resistance to amphotericin B requires the synthesis of a modified ergosterol that is biologically functional, but unaffected by the inhibitory action of amphotericin B. The possibility of spontaneous emergence of such a sterol synthetic pathway capable of synthesizing an altered amphotericin B-resistant biologically functional ergosterol in fungi, including *Aspergillus* species, by genetic variation is remote. Hence, it is not surprising that high-level amphotericin B resistance in fungi, including *Aspergillus* species, due to drug target modification is comparatively rare, although other mechanisms of antifungal resistance may occasionally confer reduced susceptibility to this antifungal drug. The clinical and laboratory isolates of *Aspergillus* species showing reduced in vitro or in vivo susceptibility to amphotericin B reported in the literature may belong to this group.

Few reports of low-level amphotericin B resistance among clinical isolates of *Aspergillus* species are available in the literature [14–18]. When attempts were made to evaluate the in vitro resistance (defined as elevated MICs compared to that obtained for the susceptible isolates) to in vivo resistance using animal models, the correlation was poor [15, 16]. On the other hand, when clinical outcome of amphotericin B treatment was retrospectively compared with the in vitro resistance, there was good correlation between amphotericin B failure and elevated MIC of the drug. Because of the paucity of clinical isolates of *Aspergillus* species resistant to amphotericin B, Manavathu et al. [14] have selected *Aspergillus fumigatus* isolates showing low-to-medium-level in vitro resistance to amphotericin B in the laboratory by UV irradiation followed by selection on Sabouraud dextrose agar containing amphotericin B. Using a murine pulmonary aspergillosis model, these investigators have demonstrated correlation between in vitro and in vivo resistance to amphotericin B [19].

Although high-level amphotericin B resistance among clinical isolates of *Aspergillus fumigatus* is rare, *Aspergillus terreus* is inherently less susceptible to amphotericin B, perhaps due to innate resistance to this drug. Exact reason for its reduced susceptibility to amphotericin B is not known. Walsh et al. [18] have investigated the in vitro susceptibility of several clinical isolates of *A. terreus* by the CLSI broth microdilution method M-38A. The MIC of amphotericin B for these isolates ranged from 2 to 4 μg/mL, considerably higher than that of other susceptible *Aspergillus* species such as *A. fumigatus*. Moreover, when tested in an animal model [18], a representative of this group of organisms showed reduced susceptibility to amphotericin B therapy. Therefore, amphotericin B is not the preferred drug for the treatment of aspergillus infection caused by *A. terreus*.

In addition, Seo et al. [17] have selected an *A. flavus* isolate highly resistant to amphotericin B (MIC 100 μg/mL) in the laboratory by sequential transfer of a susceptible strain (MIC ≤ 1 μg/mL) to agar plates containing increasing concentrations of amphotericin B. Further investigation by these authors revealed that the resistant isolate had significant chemical modification to its cell wall which presumably results in poor penetration of the drug to the cell. Balajee et al. [20, 21] have shown that *A. lentulus* and *A. udagawae* previously erroneously identified as *A. fumigatus* are resistant to multiple antifungal drugs, including amphotericin B.

In spite of its high efficacy, a wide spectrum of activity and relatively low cost, conventional amphotericin B deoxycholate is rarely used as the frontline antifungal drug now because of its high potential for toxicity. Liposomal amphotericin B with considerably less toxicity is used as an alternative to treat aspergillus infection. Drugs of the azole and echinocandin classes have considerably diminished the clinical role of amphotericin B.

2.3 Resistance to Triazoles

The triazoles are second-generation members of the azole family of antifungal drugs characterized by the presence of heterocyclic head region carrying three nitrogen atoms instead of two found in imidazole molecule. The addition of an extra nitrogen atom to the imidazole ring moiety not only improved the spectrum of activity but also the potency of the molecule [22–24]. This is not surprising since the heterocyclic ring moiety carrying the nitrogen atoms is the active functional group of the molecule, while the hydrophobic aliphatic chain contributes significantly to the specificity and the pharmacologic properties of the molecule [25]. Itraconazole and newer triazoles such as voriconazole (Pfizer Pharmaceuticals), posaconazole (Schering-Plough Pharmaceuticals, now part of Merck Pharmaceuticals), and isavuconazole (Astellas Pharmaceuticals) possess excellent in vitro and in vivo (clinical and/or animal models) activity against various *Aspergillus* species.

All triazoles are believed to have the same mode of action at clinically relevant concentrations. The primary molecular target of this group of compounds is the cytochrome P450-dependent

14α-sterol demethylase ($P450_{14DM}$), an enzyme responsible for the removal of the methyl group on carbon 14 of 14α-sterol compounds. Although $P450_{14DM}$ is the primary molecular target of triazoles, at high concentrations these drugs may have rather nonspecific effect by directly interfering with the membrane function for which the mechanism is not understood. For instance, it is possible that these molecules, having the capacity to mimic certain sterols, could be randomly inserted into the membranes and as a result affects the function of the plasma membrane. Recently, it was noted that voriconazole has a second target in the sterol synthetic pathway, namely, 24-methylene dihydrolanosterol demethylation [26].

The *cyp*51 gene coding for $P450_{14DM}$ has been characterized from a wide variety of saprophytic and pathogenic fungi, including human pathogens [27–29]. A comparison of the primary structure of $P450_{14DM}$ from various fungal species representing major groups of pathogenic fungi showed six highly conserved regions (CR) arbitrarily called CR1 to CR6 (Fig. 67.3). These conserved regions are known to make important contribution to either the enzyme activity or susceptibility of $P450_{14DM}$ to azole antifungal drugs. In the case of *A. fumigatus* P450 14α-sterol demethylase A protein, these conserved regions are located all throughout the protein sequence (CR1: P38-G89, CR2: V101-S140, CR3: I183-P227, CR4: L267-V315, CR5: V354-V371, CR6: D430-V472). Not

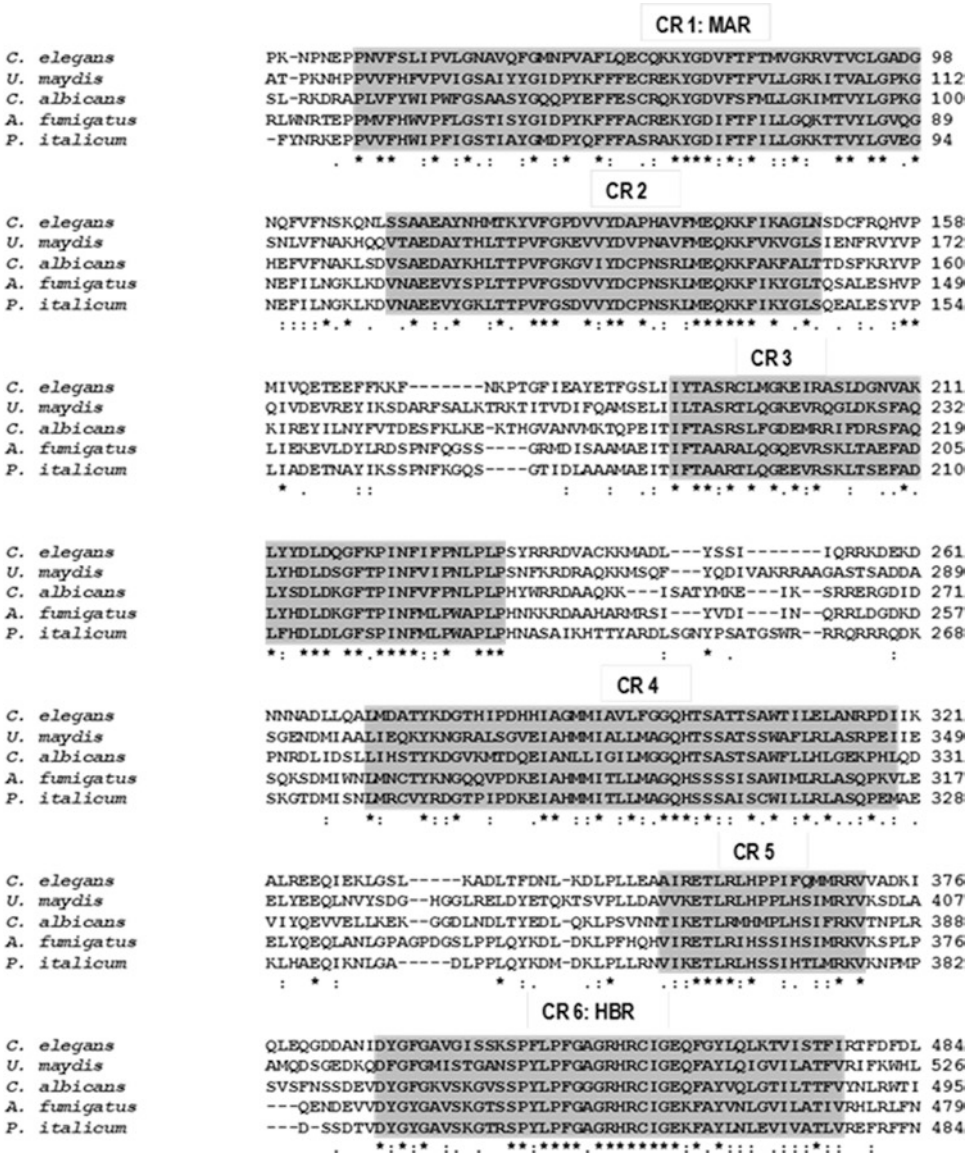

Fig. 67.3 A comparison of the primary structures of P450 lanosterol demethylases ($P450_{LDMs}$) from various fungi. The deduced amino acid sequence of $P450_{LDMs}$ from *Cunninghamella elegans* (Accession AAF20263), *Ustilago maydis* (Accession XM011391846), *Candida albicans* (Accession XP_716761), *Aspergillus fumigatus* (Accession AAK73659), and *Penicillium italicum* (Accession Q12664) was compared by multiple sequence alignment using Clustal Omega as described in Materials and Methods. The highly conserved regions are shaded in *gray*. *CR* conserved region, *MAR* membrane-anchoring region, *HBR* heme-binding region. Asterisks (*) represent identity, colon (:) indicates conserved substitution, whereas period (.) denotes semiconservative substitution

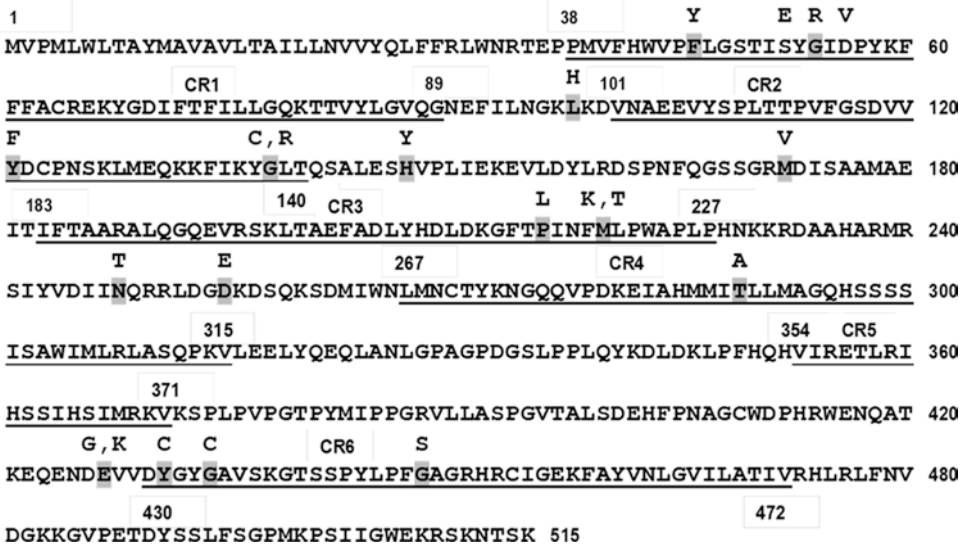

Fig.67.4 Primary structure of *A. fumigatus* Cyp51A protein (Accession AAK73659) showing amino acid substitutions known to affect azole susceptibility in *A. fumigatus*. The highly conserved regions are underlined, and 12 of 16 known substitutions are located in or in close proximity to the conserved regions. Only CR5 is without a known amino acid change affecting azole susceptibility. Among the six conserved domains, the membrane-anchoring (CR1) and the heme-binding (CR6) regions have the most amino acid substitutions and appeared to be the "hot spots" for mutational changes affecting azole susceptibility. The substituted amino acids are shown in *gray*, and the corresponding replacement(s) is shown above. *CR* conserved region

surprisingly, 12 of the 16 loci of *A. fumigatus* P450 14α-sterol demethylase A known to have amino acid substitutions that resulted in azole resistance are located in or in close proximity of these highly conserved regions (Fig. 67.4).

The role of two such highly conserved regions to triazole resistance in *A. fumigatus* has been examined. Proximal to the N-terminus of the protein lies a region starting from amino acid P38 to G89 (CR 1) commonly known as the membrane-anchoring region (MAR) mainly consisting of hydrophobic amino acid residues. It is generally believed that this region of the polypeptide is responsible for anchoring the enzyme to the plasma membrane of the cell. The hydrophobic amino acid residues dominating this region facilitate the insertion of the polypeptide into the lipid bilayer of the membrane. Membrane anchoring places the enzyme molecule in the most favorable position to interact with the incoming substrate (14α-sterol) for binding to the active site for subsequent processing. Thus, plasma membrane-anchored $P450_{14DM}$ will be more efficient for rapid catalysis of the demethylation of 14α-sterol.

The most highly conserved region of the $P450_{14DM}$ is the heme-binding region located at the carboxyl-terminal region from D430-V472 (CR 6) of the protein. Since heme is an essential prosthetic group of all cytochrome P450-dependent enzymes, this region of the polypeptide is highly conserved in all $P450_{14DMS}$. Alignment of 25 $P450_{14DMS}$ from various sources ranging from *Homo sapiens* to the fungus *Cunninghamella elegans* showed that F447, G448, G450, R451, H452, and C454 are perfectly conserved at the core region of the HBR of $P450_{14DMS}$ (Fig. 67.5). Genetic and biochemical studies have shown that C470 in *S. cerevisiae* (C454 in *A. fumigatus*) is involved in substrate binding possibly by providing a sixth coordinate and presumed to be involved in the correct alignment of the incoming substrate molecule for maximum catalytic efficiency [23, 24]. Mutant enzymes carrying variants of this residue lack enzyme activity. Conservation of the critical amino acid residues at the heme-binding region is not only essential for the enzyme function but also necessary for the maintenance of azole susceptibility of the protein. The exact role of each of the highly conserved amino acid residues for the binding and the processing of the substrate is not known at present due to the paucity of adequate X-ray crystallographic data.

In contrast to pathogenic yeasts such as *Candida* species in which a single gene codes for $P450_{14DM}$, in *A. fumigatus*, there are two highly homologous genes *cyp*51A and *cyp*51B coding for P450 14α-sterol demethylases A (Cyp51A) and B (Cyp51B). Several reports of clinical and laboratory isolates of *Aspergillus* species, primarily *A. fumigatus*, showing reduced susceptibility to triazoles have been published recently [21, 30–42]. In several cases, resistance to one member of the triazole group failed to show cross-resistance to other triazole(s) [31, 37, 39, 41]. Amino acid alteration of Cyp51A appears to be the most commonly found mechanism of resistance to triazoles in *A. fumigatus*. Alteration of G54 of Cyp51A to K, E, or R confers resistance to itraconazole and posaconazole [31–33, 36, 41, 42] but not to voriconazole [31, 41]. In contrast, alteration of G448S primarily confers resistance to voriconazole but only a modest reduction of susceptibility to posaconazole and itraconazole

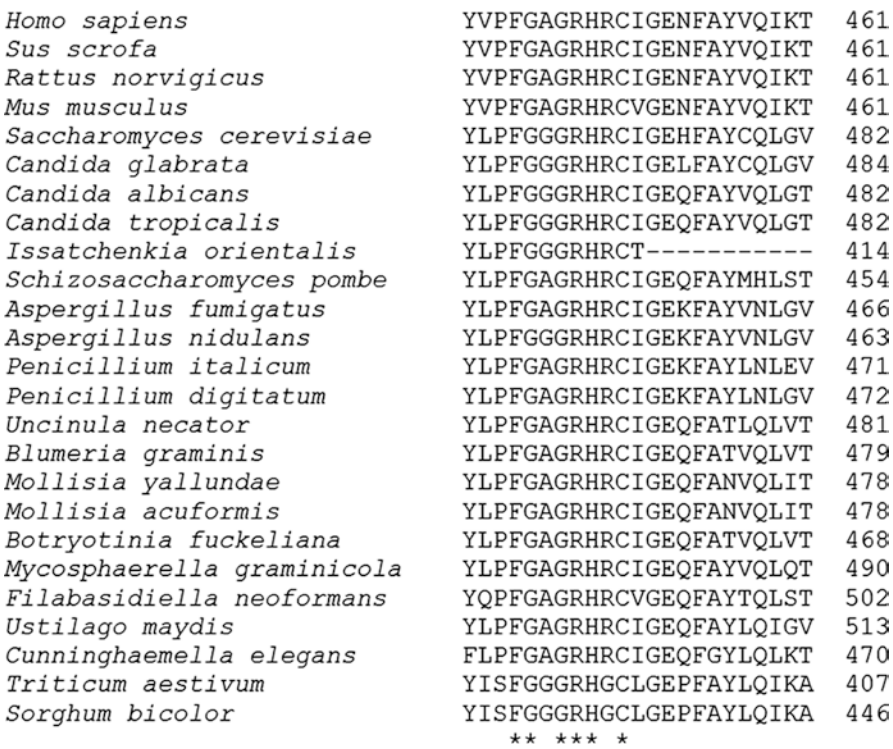

Fig. 67.5 Amino acid alignment of the heme-binding region of 25 $P45014_{DMS}$ from *Homo sapiens* to *C. elegans*. The highly conserved amino acid residues are marked by *asterisks*. The numbers on the right indicate the amino acid residue number. The alignment was done by DNA and protein sequence analysis Program Omiga. The amino acid sequences were obtained from the NCBI protein data bank

[31, 41]. Modeling experiments [43–45] suggest that the heme-binding region is part of the active site of $P450_{14DM}$, and any amino acid change at the active site makes the organism resistant to all triazoles to a lesser or greater degree. In contrast, amino acid variation at the putative membrane-anchoring region confers resistance to triazoles with long aliphatic tail region. These results thus suggest that cross-resistance to triazoles in *A. fumigatus* is at least partly dependent on region-specific amino acid variation of $P450_{14DM}$. On the other hand, alteration of G138 to either C [38] or R [31] confers resistance to multiple triazoles. Initially Mellado et al. [34] and subsequently other investigators [35, 36] have noted that alteration of M220 in Cyp51A to V, K, T [34], or I [35, 36] makes the organism harboring the mutant enzyme resistant to itraconazole. Interestingly, both G138 and M220 are located in CR 2 and CR 3 (Fig. 67.4) of Cyp51A, respectively.

The most widely distributed and frequently reported mechanism of azole resistance in *A. fumigatus* is a combination of *cyp*51A promoter modification coupled with one or more amino acid substitution in Cyp51A protein. The first reported promoter modification involves tandem repeat of a 34 bp (5'GAATCACGCGGTCCGGATGTGTGCTGAGC CGAAT3') nucleic acid sequence (TR34) of the promoter region 288 bp upstream to the ATG codon that specifies the initiation of translation. This promoter modification is often associated with L98H amino acid substitution [40, 46–49], and the TR34/L98H change gives rise to a Cyp51A protein whose activity is relatively unaffected by clinically relevant concentrations of voriconazole, itraconazole, and posaconazole. Hence, the organism that harbors such a variant Cyp51A protein develops pan azole resistance, and the clinician is left with very few options for effectively treating invasive aspergillosis.

A second more recently detected mechanism of azole resistance in *A. fumigatus* involving simultaneous promoter modification and amino acid substitution involves a 46 bp (5'-TCTAGAATCACGCGGTCCGGATGTGTGCTGAGC CGAATGAAAGTTG-3') tandem repeat (TR46) in association with Y121H/T289A amino acid substitutions [50–55]. TR46/Y121H/T289A changes in *cyp*51A/Cyp51A develop high-level resistance to voriconazole but not to posaconazole, although the MIC of posaconazole is slightly elevated [56]. However, there are conflicting reports of the need for the combined presence of TR46/Y121H/T289A simultaneously for the development of azole resistance. For instance, Y121F change alone is able to confer resistance to voriconazole in *A. fumigatus* [57]. Molecular modeling experiments revealed that Y121 is part of a molecular pocket involved in the initial docking of the incoming triazole molecule prior to binding to the active site [58]. Thus, any significant changes in the docking site would have serious implications in the binding of the drug to the target, thus affecting its bioactivity. A comparison of the *cyp*51A promoter regions carrying TR34 and TR46 revealed that the latter is a derivative of TR34 and the two promoters differed by the 12 nucleotide sequence GAAAGTTGTCTA missing from EU626235. If the missing sequence is inserted at the junction of TR34 repeat together with the adjacent flanking sequences, it would generate a perfect 46 bp repeat producing TR46

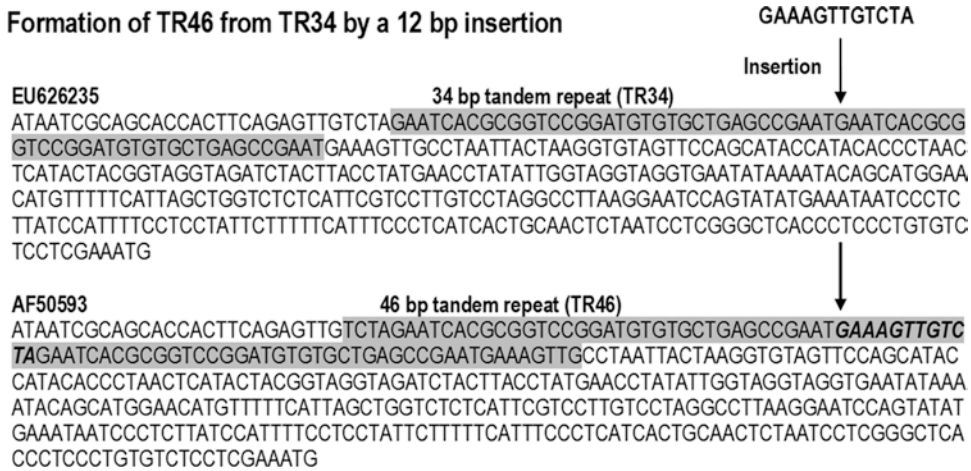

Fig. 67.6 Schematic illustration of the possible formation TR46 from TR34 or vice versa. The TR34/TR46 sequences are highlighted in *gray*. The suspected 12 nucleotide insertion sequence (GAAAGTTGTCTA) is shown in *bold italics*. EU626235 is the GenBank accession number of *cyp*51A gene sequence carrying TR34. AF50593 is an *A. fumigatus* clinical isolate harboring *cyp*51A gene carrying TR46

(Fig. 67.6). Conversely, a deletion of the 12 nucleotide stretch from TR46 would give rise to TR34. Which one of these two possibilities occurred first in nature is unclear.

The TR34/L98H- and TR46/Y121H/T289A-dependent mechanisms of azole resistance have been reported in many regions of Europe and Asia but not in North America. Mutational alteration of critical amino acid residues involved directly or indirectly in the binding of the drug to the target together with alteration of the noncoding regulatory sequences that has the potential to regulate the synthesis of the drug target 14α-sterol demethylase is a powerful mechanism for conferring high-level drug resistance. It is intriguing that the TR46/Y121F/T289A changes on the *cyp*51A gene confer resistance to voriconazole (MIC > 16 μg/mL), yet the organism remains relatively susceptible to itraconazole (MIC 2 μg/mL) and posaconazole (MIC 0.5 μg/mL).

Epidemiological studies have shown that the TR34/L98H and TR46/Y121H/T289A mechanisms of resistance initially emerged in environmental aspergillus isolates probably due to a widespread use of azole-related demethylase inhibitors (DMIs) as pesticides for crop protection in agriculture [59, 60]. The fact that these isolates are widespread and show cross-resistance to currently used azoles jeopardizes their efficacy as antifungal drugs for the treatment of invasive aspergillosis. In the future, patients who demonstrate clinical failure to voriconazole will have to be investigated for the presence of voriconazole resistance in their post-therapy isolates.

Although the efflux-mediated drug resistance is well documented in pathogenic yeasts [61–66], such mechanism of drug resistance in pathogenic filamentous fungi, including *Aspergillus* species, is not well investigated. In *A. fumigatus*, itraconazole is able to induce the expression of an ABC transporter gene called *atr*F, but the role of this gene conferring resistance in clinical isolates to itraconazole is not established. Multiple drug resistance (MDR) membrane proteins called *afu*MDR1 and *afu*MDR2 were previously identified and characterized from *A. fumigatus* [67]. But their actual function or contribution to antifungal drug resistance was not investigated. Nascimento et al. [33] have showed that *Afu*MDR1 and *Afu*MDR2 may not be involved in efflux-mediated triazole resistance in this organism. On the other hand, *Afu*MDR3 and *Afu*MDR4 were overexpressed in *A. fumigatus* resistant to itraconazole, but not *Afu*MDR1 and *Afu*MDR2 suggesting that *Afu*MDR3 and *Afu*MDR4 may play a role in efflux-mediated triazole resistance in *A. fumigatus*. However, these researchers were unable to document the accumulation of itraconazole in the resistant and susceptible isolates of *A. fumigatus*. By itraconazole uptake study in mycelia, Manavathu et al. [68] have previously demonstrated that the intracellular accumulation of radioactive itraconazole was significantly lower in laboratory-selected *A. fumigatus* isolates showing reduced in vitro susceptibility to itraconazole compared to that in the susceptible parent. The efflux of drug in combination with drug target modification has made laboratory-selected *A. fumigatus* isolates highly resistant (MIC ≥ 100 μg/mL) to itraconazole suggesting that multiple mechanisms of drug resistance may coexist in the same cells to make them highly resistant to drug [33]. Through expression of *cyp*51A in an autonomously replicating multicopy plasmid, Osherov et al. [29] transformed an itraconazole-susceptible *A. fumigatus* to a drug-resistant strain.

2.3.1 HapE-Mediated Azole Resistance

Recently, Camps et al. [69] have described a novel mechanism for itraconazole resistance in *A. fumigatus* mediated by the transcription factor HapE by comparative genome analyses of drug-resistant and drug-susceptible *A. fumigatus* serial isolates obtained from a patient suffering from chronic granulomatous disease (CGD). These investigators analyzed the genomes of two drug-susceptible and two drug-resistant isogenic isolates collected serially from the CGD patient in a relatively short period of time (17 weeks apart) by whole genome analysis. By whole genome analysis coupled with sexual crossing and RT-PCR analysis, they identified P88L mutation in the transcription factor HapE subunit. HapE is nucleotide-binding protein which binds to CCAAT sequence

and modulates the promoter activity of a specific gene. Although the exact mechanism by which HapE mutation brings about itraconazole resistance in *A. fumigatus* is unclear at present, two distinct possibilities exit. One, HapE could be a transcription enhancer and by binding to the CCAAT region upregulate *cyp*51A. The increased synthesis of Cyp51A will result in the need of an increased amount of the drug to inhibit the enzyme and sterol synthesis. Two, alternatively, binding of the mutant HapE (or lack of binding) will result in the downregulation of *cyp*51A resulting in the synthesis of less enzyme. Less Cyp51A results in slow rate of sterol synthesis and poor growth. Since most antibiotics are optimally effective against actively growing cells, low growth rate will result in poor killing by the drug, and the organism shows resistance/tolerance to the drug. However, additional investigation is required to pinpoint the exact mechanism of HapE-mediated azole resistance in *A. fumigatus*.

2.3.2 Transcriptional Regulator atrR-Mediated Azole Resistance

Hagiwara et al. [70] have recently identified a new mechanism of azole resistance in *A. fumigatus* which these investigators initially identified and characterized in *Aspergillus oryzae*. This new mechanism of resistance is dependent on the expression level of a crucial transcriptional regulator called *atrR* in aspergillus. *atr*R regulates the level of expression of multiple genes, including *cyp*51A, *cdr*B1, and those genes responsible for *A. fumigatus* to grow under hypoxic conditions. When *atr*R is expressed at normal or elevated level, the expression of azole target gene *cyp*51A and the multidrug efflux pump *cdr*B1 is upregulated producing increased amounts of Cyp51A and CdrB1 proteins. These increases result in elevated MICs for miconazole, itraconazole, and ketoconazole [70], but not for unrelated antifungal drugs such as micafungin and amphotericin B. The reverse situation is also true. In the absence of *atr*R expression, the organism becomes more susceptible to azole antifungal drugs. However, these investigators in their initial investigation did not use newer generation of azole such as voriconazole and posaconazole. So cross-resistance to this newer generation of azoles in the presence of elevated level of *atr*R is not known. In addition, *atr*R regulates the levels of expression of a cluster of genes that enable *A. fumigatus* to grow under hypoxic condition (1 % O_2 level). In contrast, deletion of *atr*R gene results in the loss of hypoxic growth characteristics.

2.4 Resistance to Echinocandins

The echinocandins such as caspofungin, micafungin, and anidulafungin are semisynthetic cyclic lipohexapeptide antifungal drugs designed to render specific interaction with fungal cells with a minimum level of toxicity to host cells at therapeutic doses. The molecular target of echinocandins is believed to be 1,3-β-D-glucan synthase (GS; E. C. 2.4.1.34; UDP glucose: 1,3-β-D-glucan 3-β-D-glucosyltransferase), a multimeric membrane-bound enzyme that catalyzes the synthesis of $\beta(1 \rightarrow 3)$ glucan, even though there is no direct molecular evidence to support this view [71]. However, it is safe to say that these compounds inhibit cell wall synthesis in a wide variety of fungi, and cell wall synthesis is the target of the echinocandin compounds. What distinguishes the members of the echinocandin family of antifungal drugs from the polyenes and the azoles is their specificity against fungi with relatively little mechanism-based toxicity against the host.

Recently, a pair of reports of echinocandin resistance in aspergillus has been published. Gardiner et al. [72] have isolated two classes of *A. fumigatus* mutants in the laboratory showing reduced susceptibility to caspofungin. Site-directed mutation of the target gene coding for glucan synthase Fks1p, including the catalytic subunit, produced mutants showing low-level in vitro resistance (≈16-fold increase of MIC) to caspofungin. Subsequent characterization of one such mutant showed S678P alteration of Fks1p [73].

These investigators also isolated a number of spontaneous mutants of *A. fumigatus* in the laboratory showing a biphasic susceptibility pattern. At low concentrations of the drug (<0.5 μg/mL), these isolates were highly susceptible to the growth inhibitory effect of caspofungin. However, at drug concentrations >0.5 μg/mL but <16 μg/mL, these spontaneous mutant isolates showed near normal growth pattern, whereas at drug concentrations >16 μg/mL, they showed partial susceptibility to growth inhibition. No target gene mutation or upregulation of Fks1p expression was noted in these isolates. These authors speculate that the biphasic pattern of susceptibility to caspofungin is caused by a novel mechanism of echinocandin resistance in *A. fumigatus* perhaps due to the remodeling of cell wall components [72]. In addition to the reports of the acquired resistance to caspofungin in *A. fumigatus* laboratory isolates, Balajee et al. [21] have shown that *A. lentulus* is intrinsically resistant to caspofungin.

2.5 Resistance to Allylamines

Terbinafine (Novartis Pharmaceuticals) is an allylamine antifungal drug that possesses excellent in vitro activity against *Aspergillus* species, including *A. fumigatus*. The MICs of this compound against *Aspergillus* species are in the submicrogram range, usually two- to fourfold lower than that of amphotericin B. Preliminary experiments indicate that terbinafine is a fungicidal agent against *Aspergillus* species [74–78]. Only limited amount of in vivo data (animal models and case reports) is available at present, and the indication is that it is less impres-

sive in vivo than it is in vitro, perhaps because of poor availability of the drug due to the excessive protein binding.

The molecular target of terbinafine is squalene epoxidase (also known as squalene monooxygenase), an enzyme involved in the oxidation of squalene to 2,3-oxidosqualene (also called squalene 2,3-epoxide). The squalene epoxidase, in the presence of NADPH and oxygen, catalyzes the addition of an oxygen atom from molecular oxygen to the end of the squalene chain, forming an epoxide. The cofactor NADPH reduces the other oxygen atom to molecular oxygen and water. The inhibition of 2,3-oxidosqualene synthesis leads to the inhibition of ergosterol synthesis that eventually results in plasma membrane malfunction and fungal cell death.

The investigation of the effect of terbinafine on *Aspergillus* species is at its infancy, and an understanding of the spectrum of terbinafine resistance in *Aspergillus* species will be few years away. To date, there are only three reports of terbinafine resistance in *Aspergillus* species. In all three cases the resistant isolates were either genetically engineered or induced by UV irradiation followed by selection in the presence of terbinafine. Liu et al. [78] have expressed multiple copies of the gene coding for squalene epoxidase in *A. fumigatus* using a multicopy plasmid. Transformants harboring multiple copies of squalene epoxidase gene showed decreased in vitro susceptibility to terbinafine.

Graminha et al. [79] have investigated terbinafine resistance in a laboratory isolate of *A. nidulans*. These investigators generated a number of terbinafine-resistant mutant isolates of *A. nidulans* by UV irradiation followed by selection in the presence of the drug. One such terbinafine-resistant isolate was characterized by molecular genetic techniques. The resistant isolate had multiple copies of *salA*, a gene coding for salicylate 1-monooxygenase, an enzyme known to be responsible for the degradation of naphthalene ring structures in other microorganisms. So it is quite possible that the expression of multiple copies of *salA* may be responsible for the reduced susceptibility of *A. nidulans* to terbinafine.

In contrast to the gene dosage-dependent terbinafine resistance in *A. nidulans* and *A. fumigatus*, Rocha et al. [80] have recently reported terbinafine resistance in a genetically engineered laboratory isolate of *A. fumigatus* harboring an altered squalene epoxidase gene. These investigators genetically engineered replacement of phenylalanine 389 of squalene epoxidase with leucine (F389L). This single amino acid change was sufficient to confer resistance to terbinafine in an isolate carrying the mutant enzyme. Since the terbinafine target is squalene epoxidase, an enzyme involved in the initial stage of ergosterol synthesis, spontaneous mutants resistant to terbinafine will ultimately emerge in nature with the increased use of this drug against aspergillus infection.

3 Stress Response and Antimicrobial Drug Resistance

Aspergillus species are ubiquitous, and as such they are the most widely distributed filamentous fungi pathogenic to man. They are commonly found in the environment and constantly exposed to a wide variety of environmental stress factors. As a group, members of the genus *Aspergillus* successfully adapt to environmental changes such as temperature variations, pH changes, change in osmolarity in the ecological niche, exposure to toxic pollutants, xenobiotics, and antimicrobial drugs. Exposure of aspergillus cells to antimicrobial drugs or other types of xenobiotics induces a tremendous amount of physiological stress, and these organisms are equipped with the necessary tools to successfully cope with such environmental stressors, including exposure to antimicrobial drugs. To effectively prevent from the inhibitory effect(s) of the antibiotics, the organism must develop drug resistance/tolerance. The exposure of an organism to such environmental stressors is often sensed by membrane receptors, and the information is relayed to the intracellular environmental stress response pathway to take appropriate corrective action against the threat. To cope with the life-threatening exposure to antimicrobial drug(s), the organism either develops resistance/tolerance to the antifungal drug utilizing the stress response pathway or adopts other means to nullify the inhibitory effect of the drug [81–89].

The role of stress response pathway on the development of antimicrobial drug resistance has been studied extensively in several saprophytic [90–97] and pathogenic fungi [98–102]. Central to the stress response pathway is a series of mitogen-activated protein kinase cascades commonly known as the MAP kinase (MAPK) pathway. The MAPK cascade is a three-kinase module which receives (sense), transmits (transduces), and regulates the cellular developmental or physiological processes in response to the external signal to counter or nullify the adverse (often deleterious) effect(s) of the external stress [103]. The classical MAPK pathway contains a highly conserved three-tiered module consisting of a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK), and the final MAP kinase (MAPK). In their inactive form the MAPKs are non-phosphorylated. When sensing extracellular stress signals, the MAPK cascades are activated by sequential dual phosphorylation of highly conserved threonine and tyrosine residues [104]. It is the MAPKKK that usually sense the extracellular stress signal at cell surface level (either cell wall or cell membrane), and signal is relayed (transduced) intracellularly by MAPKK to the nucleus, and activation of MAPK results in activation of transcription factors that regulate synthesis of cellular molecules required to correct or negate the harmful (deleterious) effect of the external stress.

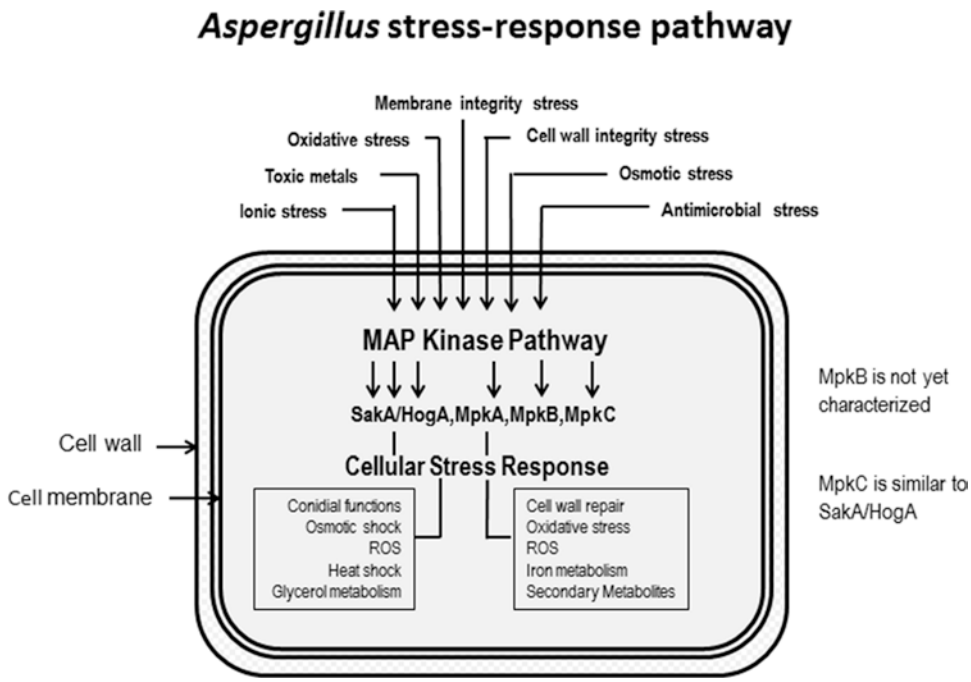

Fig. 67.7 Diagrammatic illustration of the stress response pathway in *Aspergillus fumigatus*. See text for details

The genome of *A. fumigatus* encodes four MAP kinase genes, namely, *sak*A/*hog*A, *mpk*A, *mpk*B, and *mpk*C [105], and their corresponding gene products are designated SakA/HogA, MpkA, MpkB, and MpkC. Of the four MAPKs in *A. fumigatus*, only SakA/HogA and MpkA have been investigated in detail so far. MpkC appears to be very similar to SakA/HogA module, and MpkB has not been characterized yet. A simplified diagrammatic illustration of common external stressors and the known components of the MAPK cascade that respond to stress signals are shown in Fig. 67.7. At times more than one MAPK module is activated in response to the same extracellular stress signal, and this redundancy is to secure added protection to the cell from deleterious stress factors. For example, both the SakA/HogA and MpkA modules in *A. fumigatus* appear to respond when the cell experiences deleterious oxidative stress such as ROS.

SakA/Hog (high-osmolarity glycerol) A module is one of the best characterized well-studied MAPK cascades in fungi, including *A. fumigatus*. It is the primary module of the MAPK pathway that controls transcription, translation, transport, and cell cycle adaptations in response to extracellular stress. Once the osmotic stress signal is received, HogA is activated (phosphorylated) and elicits specific responses for glycerol synthesis and cell cycle arrest [106–109]. In addition to responding to osmotic stress, in *S. cerevisiae* HogA has a broad range of involvement in the responses to UV, heavy metals, heat, citric acid, hypoxia, and oxidative stresses as well as in the response to cell wall-interfering agents. Gene characterization reveals that *A. fumigatus* *sak*A/*hog*A codes for a 366-amino acid protein with a molecular mass of 42 kDa showing high similarity to Hog1p of *S. cerevisiae* (82 % identity), SakA of *A. nidulans* (84 % identity), and Hog1p of *C. albicans* (79 % identity).

A. fumigatus *sak*A/*hog*A regulates the transcription of DprA and DprB involved in eliciting response to osmotic and pH stresses. The response to oxidative stress is a major function of the HOG pathway and is considered playing a key role in resistance to phagocytic killing [110]. Interestingly, amphotericin B and itraconazole are considered to be oxidative stress drugs because of their modes of action mediated by the involvement of the cellular oxidative stress response. The *sak*A/*hog*A deletion mutant of *A. fumigatus* is significantly more sensitive to the cytotoxic effect of these antifungal drugs indicating that it plays a role in antifungal tolerance [111].

The role of MpkA MAPK module in stress response pathway of *A. fumigatus* has been studied by a number of investigators [112–115]. Their results show that MpkA plays a critical role in maintaining the integrity of the cell wall, oxidative stress response, pigment formation by tyrosine degradation pathway, secondary metabolite synthesis (e.g., gliotoxin), iron metabolism by regulating the synthesis of iron-carrying molecule such as siderophore, oxidative stress response (ROS), conidial germination and growth under nutrient deficient/starvation conditions, osmotic shock (hypertonic or hypotonic conditions) response, and in response to temperature variations (heat shock). Preliminary studies have shown that MpkC is able to sense alternate carbon source(s) when the primary carbon source is limited or depleted [116].

The MAPK pathway-mediated antimicrobial drug resistance in *A. fumigatus* appears to be multifactorial and not dependent on any specific resistance mechanism(s) [83, 85,

86, 117–124]. Majority of the reported cases of MAPK-dependent drug resistance in *A. fumigatus* is associated with the cell wall integrity and repair pathway. When the integrity of the cell wall is breached by chemical or mechanical insults, the MpkA module will be activated and repair the damaged cell wall by a rebuilding and restructuring process. The chemical composition and the architecture of the rebuilt wall could be different enough that the cell wall-acting antimicrobial drugs will be rather ineffective.

The cell wall integrity restoration pathway-dependent antimicrobial drug resistance in *A. fumigatus* is often mediated by the molecular chaperone heat shock protein 90 (HSP90). Repression of HSP90 synthesis in *A. fumigatus* not only affects critical cellular process such as conidia formation and cell wall integrity but also potentiates the effects of azoles and cell wall inhibitors such as the echinocandins and Congo red [83, 85, 117, 118]. Similarly, when HSP90 was inhibited chemically, similar results were obtained. Also, HSP90 inhibition either genetically or by pharmacological interaction markedly reduced the high-level resistance of *A. fumigatus* biofilm to azoles and echinocandins [125]. Blum et al. [81, 86, 126, 127] have recently studied the role of HSP90 on *Aspergillus terreus* resistance to amphotericin B (AMB). When HSP90 function was inhibited, both *A. terreus* and *A. fumigatus* became more susceptible to AMB, and the MIC of AMB for *A. terreus* was reduced by 100-fold (32–0.38 μg/mL). Recently, Dirr et al. [128] studied the effect of voriconazole and posaconazole on an *A. fumigatus* mutant lacking *mkk2* (MAPKK) of the cell wall integrity pathway. The mutant strain was significantly more susceptible to voriconazole and posaconazole.

Although research on MAPK pathway-mediated antimicrobial drug resistance in *A. fumigatus* is in its early stages, these results clearly show that MAPK pathway plays a key role in drug resistance/tolerance in fungi, including *A. fumigatus*. Perhaps, this will give an opportunity to explain at least in part the clinical failure of antifungal therapy in the absence of any known mechanism-based resistance to the drug in question in the infecting clinical strain.

4 Animal Models

Animal models have been used extensively to study pathogenesis, host responses, disease transmission, and therapy of aspergillus infection [129]. Animal (mouse, rat, guinea pig, and rabbit) models have been used to evaluate the in vivo efficacy of antimycotic drugs against pulmonary and disseminated aspergillosis caused by drug-susceptible and drug-resistant *Aspergillus* species [130–133]. The selection of a particular animal model suitable for drug therapy study is dependent on the pharmacodynamics of the drug, ideally one that mimics the parameters in human. Most data are from rodent, particularly mouse models of aspergillosis. These models have been critical to the advancement of therapy. Using one or more of these animal models, several investigators examined the efficacy of polyenes, triazoles, echinocandins, and allylamines against aspergillus isolates showing elevated MICs of the drug in vitro.

Using transiently immunocompromised murine [15, 16, 19] and guinea pig [16] models for disseminated [15, 16] and pulmonary [19] aspergillosis, the in vivo efficacy of amphotericin B against *Aspergillus fumigatus* isolates with either elevated amphotericin B MIC or isolate obtained from a patient who failed amphotericin B therapy was examined. No consensus for the correlation of in vitro resistance to in vivo failure was obtained in these studies. For instance, Odds et al. [16] obtained no interpretable relationship either in mouse or guinea pig model when the MIC of amphotericin B was ≥2 μg/mL. In other words, no correlation of in vitro resistance to in vivo resistance was found. On the other hand, Verweij et al. [15] have investigated the efficacy of amphotericin B and caspofungin against two *A. fumigatus* clinical isolates (AF210 and AF65) with more or less similar amphotericin B MICs, although AF65 was obtained from a patient failing amphotericin B therapy. Amphotericin B treatment at doses 0.5, 2, and 5 mg/kg/day failed to improve the survival and reduction of fungal burden in the animals infected with *A. fumigatus* AF65, but not with AF210. On the other hand, anidulafungin (LY303366) treatment at doses of 10 and 25 mg/kg/day was highly effective against AF210 and AF65 infections. These data suggest that even in the absence of elevated MIC, drug failure in the clinical situation is correlated with reduced therapeutic efficacy in the animal model. In contrast, Manavathu et al. [19] have investigated the in vivo efficacy of amphotericin B against a laboratory-selected *A. fumigatus* isolates with elevated amphotericin B MICs in a pulmonary aspergillosis model. Animals infected with the mutant isolate showing in vitro resistance also showed poor survival and increased fungal burden compared to those infected with the drug-susceptible parent. These conflicting limited data suggest the need for additional experiments to establish in vitro-in vivo correlation for amphotericin B resistance in *A. fumigatus* using animal models.

Walsh et al. [18] have examined the effect of conventional and liposomal amphotericin B on *Aspergillus terreus* infection utilizing an experimental invasive pulmonary aspergillosis model in transiently neutropenic rabbit. As mentioned previously, greater than 90 % of the *A. terreus* isolates show high-level in vitro resistance to amphotericin B [134]. Treatment of rabbits infected with *A. terreus* with conventional and liposomal amphotericin B failed to improve survival and reduce fungal burden compared to those obtained for control untreated animals, whereas treatment with posaconazole and itraconazole improved survival with significant reduction of fungal

burden. Thus, for *A. terreus* isolates, their innate in vitro resistance to amphotericin B is well correlated with lack of amphotericin B treatment efficacy in the rabbit model.

Among various triazoles that are effective against *Aspergillus* species, itraconazole was examined to assess the correlation of either in vitro or clinical resistance by an animal model. Denning et al. [30] investigated the susceptibility of a clinical isolate of *A. fumigatus* obtained from an immunocompromised patient with IA who failed itraconazole therapy. The clinical failure of this isolate was associated with elevated MIC for itraconazole with modest rise of MICs for other triazoles. Itraconazole treatment of neutropenic mice infected with this isolate failed to improve survival and reduce fungal burden, whereas the echinocandin anidulafungin was highly effective in prolonging the survival of the animal and significantly decreased the fungal burden. Likewise, micafungin was effective in neutropenic mice infected with an itraconazole-resistant strain of *A. fumigatus* and a strain of *A. terreus* demonstrating in vivo resistance to amphotericin B [135]. In contrast, Odds et al. [16] have obtained no clearly interpretable results showing a correlation between in vitro resistance and failure of drug treatment when the itraconazole MIC of the isolate was greater than 1 μg/mL. Once again, the paucity of sufficient data makes it difficult to draw any conclusion regarding the in vitro-in vivo correlation of triazole resistance in the animal model. In a non-neutropenic murine model of IA using voriconazole-susceptible and voriconazole-resistant *A. fumigatus*, the investigators found the combination of voriconazole and anidulafungin to be synergistic in voriconazole-susceptible IA, but only additive in voriconazole-resistant IA. Survival of 100 % was observed only in groups receiving the highest doses of both drugs [136]. This study suggests the possible utility of combination therapy in azole-resistant IA.

5 Clinical Data: Resistance

A. fumigatus is by far the most common *Aspergillus* species causing human infection. Little is known about the true prevalence of resistant aspergillus infections because there are no national surveillance programs, and most laboratories do not perform susceptibility testing. Primary resistance to antifungal drugs among the isolates of *A. fumigatus*, *A. flavus*, and *A. niger* is infrequent. Among the uncommon species, *A. ustus* is poorly susceptible to all antifungals, and there are case reports of *A. ustus* causing invasive aspergillosis with poor outcome, mostly in allogeneic stem cell recipients [137]. *A. ustus* infection and zygomycosis have emerged as breakthrough infections in stem cell recipients receiving voriconazole and caspofungin [137–139]. *A. ustus* isolates were found resistant to amphotericin B, triazoles, and echinocandins.

5.1 Resistance to Polyenes

A. terreus appears innately resistant to amphotericin B, both in vitro and in animal models [18, 134]. Available clinical data support such observations; in a 12-year retrospective analysis of IA caused by *A. terreus*, infection progressed rapidly resulting in a 91 % mortality despite amphotericin B therapy [140]. Steinbach and colleagues reported a mortality rate of 73 % in patients with *A. terreus* infection and treated mostly with a polyene (amphotericin B or amphotericin B lipid formulation) [141]. The same investigators reported a significantly better survival (56 %) in similar patients treated with voriconazole. Based on in vitro, animal, and limited clinical observations, it appears polyenes are best avoided and azoles are preferred agents in the therapy of *A. terreus* infection. Extremely limited clinical data exist for polyene resistance in non-Aspergillus terreus species. In a retrospective study of 29 immunocompromised patients with IA and treated with amphotericin B, in vitro susceptibility to the drug predicted clinical outcome [142]. Remarkably, 22 of 23 patients infected with aspergillus resistant to amphotericin B (MIC > 2 μg/mL) (both *A. terreus* and non-terreus aspergillus) died, while none of the remaining six infected with susceptible aspergillus died. This study however used "older," nonstandardized methods of susceptibility testing; furthermore, no details of clinical features were provided. Clinical failure with liposomal amphotericin B was noted during the treatment of severe cutaneous aspergillosis in two premature infants with extremely low birth weight; both infants were successfully treated with voriconazole [143].

Verweij et al. [15] described the recovery of amphotericin B-resistant *A. fumigatus* isolates from the lung of a patient with refractory infection treated with the same drug. Introducing the resistant isolate in the animal model, the investigators observed similar poor outcomes in those treated with amphotericin B and untreated controls. Also, a higher inoculum was required to produce disease in the animal model. Of interest, Lionakis et al. [144] found that preexposure of cancer patients to amphotericin B or triazoles was associated with increased frequency of recovery of non-Aspergillus fumigatus species. Moreover, such postexposure isolates were amphotericin B-resistant but not azole-resistant. Since clinical failures are common, emergence of aspergillus resistance to polyenes during therapy of invasive aspergillosis is of interest. Whether resistance to amphotericin B emerges is not clear since most clinical failures have been attributed to poor host factors and perhaps infarcted tissue with poor drug penetration. Unlike with bacterial infections, difficulty in obtaining sequential isolates of the fungus during an episode of infection makes it hard to evaluate emergence of resistance. Available limited data suggest that the emergence of resistance to polyenes during therapy is uncommon [144–146].

5.2 Resistance to Azoles

Azole resistance in aspergillus is increasingly reported and may emerge as a significant problem in the management of aspergillosis. Resistance may be acquired during azole therapy or through environmental exposure to fungicide-exposed strains. Prospective evaluations in several countries suggest prevalence of azole resistance, particularly in *A. fumigatus*, to be around 1–2 % [53, 147–149]. More recent international surveillance data suggest 3–8 % azole resistance among aspergillus isolates [150, 151]. Centers in the UK and the Netherlands have described particularly high frequencies of azole resistance (15 % and 10 %, respectively). Attributable reasons include long-term azole therapy in patients with chronic aspergillosis in Manchester (UK) and high use of azoles in the agrochemical industry in the Netherlands.

Routine susceptibility testing of aspergillus isolates is to be strongly considered in patients with relapsed infection on long-term or subtherapeutic triazole therapy and in other relevant cases. In a recent epidemiological study in lung transplant recipients on long-term azole prophylaxis, Mayo Clinic investigators identified 16.5 % patients with invasive

Table 67.2 Azole resistance in *Aspergillus*—human data

Reference/first author	Clinical data	Comments
154/Chryssanthou	Three of 80 patients: initial ITZ-susceptible, then ITZ-resistant *A. fumigatus* [ITZ use: 5 months–3 years]	No genotyping done (possibly different strains)
30/Denning 155/Oakley	*Patient 1*: Hodgkin's disease—ITZ-susceptible *A. fumigatus* pleuropericarditis. Oral ITZ × 9 months, then sputum: *A. fumigatus* (ITZ MIC > 16 μg/mL)	*A. fumigatus* strains with in vitro ITZ—resistance: good correlation in animal model Mechanism of resistance: primary or secondary
	Patient 2: AIDS—invasive aspergillosis due to *A. fumigatus*; AmB for 3 months; relapsed infection (*A. fumigatus*[1]—recovered from sputum); improved with ITZ. Sputum culture—*A. fumigatus*.[2] Both *A. fumigatus*[1] and *A. fumigatus*[2] had ITZ MIC > 16 μg/mL	
	Animal model: no decreased mortality in animals receiving ITZ for infection with ITZ-resistant aspergillus	
156/Dannaoui	*Patient*: bronchiectasis; sputum, ITZ-susceptible *A. fumigatus*	Pre- and post-therapy Isolates had similar RAPD
	Rx: ITZ × 5 months	Patterns (i.e., same strain)
	Relapse of infection; subsequent *A. fumigatus* ITZ MIC > 16 μg/mL	
	Animal model: poor efficacy of ITZ in animals infected with ITZ-resistant aspergillus	
157/Verweij	1945–1998: collection of clinical isolates of aspergillus in the Netherlands from 114 patients (170 isolates)	Three ITZ-resistant *A. fumigatus* isolates recovered from a lung transplant recipient receiving ITZ
158/Balajee	1991–2000 (Seattle, USA)	Ten patients with ITZ-resistant *A. fumigatus* (no previous exposure to ITZ)
	10 of 128 *A. fumigatus* isolates: ITZ-resistant (MIC ≥ 1 μg/mL)	
	Also, cross resistant with VCZ/caspofungin/amphotericin B	Exact mechanisms of cross-resistance unknown
159/Warris	*Patient*: chronic granulomatous disease	*A. fumigatus* resistant to ITZ/RCZ and reduced susceptibility to VCZ/PCZ
	A. nidulans infection → successful therapy with VCZ; maintenance (6 years) on ITZ; subsequent aspergillosis with *A. fumigatus* (ITZ-resistant)	
	Successful therapy with high-dose VCZ	
37/Howard	*Patient*: sarcoidosis with chronic cavitary aspergilloma (*A. fumigatus*); therapy with ITZ, then VCZ; some response to IV caspofungin	*A. fumigatus* resistant to ITZ/VCZ/PCZ and RCZ Mutation (G138C) in the target gene (CYP51A) encoding 14α-sterol demethylase
38/Dannaoui	*Patient*: sarcoidosis complicated by aspergilloma; ITZ for 3 years; recovery of ITZ-resistant *A. fumigatus*, treated with VCZ (MIC 1 μg/mL) and obtained good response	Mutation (M220L) in CYP51A plus increased expression of multidrug transporters
160/Verweij	Nine patients (13 isolates): four with primary IA and five with breakthrough IA (prior therapy with ITZ or VCZ); two died	Mutation (L98H) in CYP51A with a tandem repeat in the same promoter region. (No clonal spread)
	A. fumigatus resistant to ITZ/VCZ/PCZ/RCZ	
161/van Leer-Buter	*Patient*: oropharyngeal carcinoma with pulmonary cavities	Autopsy: *A. fumigatus* and *A. niger*
	Bronchoalveolar lavage: *A. fumigatus* and *A. niger*. Therapy with VCZ for about 10 days	*A. fumigatus*: azole-susceptible and azole-resistant (L98H in CYP51A and a tandem repeat in the promoter region) phenotypes

VCZ voriconazole, *ITZ* itraconazole, *PCZ* posaconazole, *RCZ* ravuconazole, *AmB* amphotericin B, *MIC* minimum inhibitory concentration

fungal infection. *A fumigatus* was the most common species; of interest, azole resistance was uncommon in these breakthrough cases [152]. Itraconazole, the first triazole effective against aspergillus, became available for clinical use in the mid-1990s. Data on resistance to itraconazole in the clinical context are limited, as shown in Table 67.2 [30, 37, 38, 153–160]. In a nationwide survey of 21 Dutch hospitals, no patients with multiple triazole-resistant *A. fumigatus* were found (0 of 114 patients) during 1945–1998 as compared with 10 of 81 patients with such isolates since 2002 ($P < .001$). Noteworthy is a case report—a patient with chronic granulomatous disease, who, while receiving long-term prophylaxis with itraconazole, developed itraconazole-resistant invasive aspergillosis and required high-dose voriconazole for successful outcome [158]. The *A. fumigatus* isolate had reduced susceptibility to other azoles (voriconazole and posaconazole) as well. High-level panazole-resistant *A. fumigatus* was recovered from four patients with chronic lung disease, one of them developing progressive resistance following long-term azole therapy and switching between antifungal azoles. The phenotypes were not associated with a specific CYP51A gene mutation [161]. Overall, azole-resistant aspergillosis is likely to become a significant clinical issue. No management guidelines exist to guide clinicians encountering or suspecting azole-resistant aspergillosis.

In theory, "clinical resistance" may be anticipated during therapy with a polyene following exposure to azole, because the azole may have depleted the common target (i.e., ergosterol). No clinical study, however, has implicated prior azole exposure (as prophylaxis or therapy) as a cause for subsequent failure with polyene therapy, but such patients may need close observation. This situation may occur with increasing frequency with the standard use of voriconazole as primary therapy of or prophylaxis against aspergillosis. Voriconazole has been used with satisfactory results (~50 % response) as salvage therapy in most patients initially treated with amphotericin B [162].

Given the suboptimal response with single drug use for invasive aspergillosis, particularly in profoundly compromised hosts (e.g., with persistent neutropenia), the strategy of drug combinations is increasingly employed. From previous in vitro observations, clinicians have been concerned about antagonism with the use of drug combinations, leading to clinical failure. However, data from in vitro studies and animal models suggest no antagonistic interactions when azoles are combined with polyenes or echinocandins [163–166]. Experimental and retrospective data suggest synergistic efficacy with combination of azole (voriconazole) plus caspofungin [167, 168]. A prospective clinical study of combination antifungal therapy of voriconazole and anidulafungin for invasive aspergillosis concluded that, in the subgroup of patients with probable aspergillosis (diagnosed by suggestive radiography plus galactomannan positivity), higher survival rate was noted. However, superiority of the combination over monotherapy was not conclusively demonstrated [169]. Azole resistance was not addressed in this paper.

5.3 Resistance to Echinocandins

No clinical data on aspergillus resistance to echinocandins are reported. Susceptibility testing is not routinely performed, and the methods for testing are not standardized. Clinical breakthrough cases have been reported, and resistance has been confirmed in vivo [170]. In high-risk patients treated with caspofungin, 13 cases of breakthrough aspergillosis were noted in a retrospective study. Rate of breakthrough aspergillosis was estimated to be 4.2 % in onco-hematology patients [171]. Emergence of resistance is to be anticipated due to the lack of cytocidal activity of echinocandins against aspergillus and the potential for point mutation resulting in altered glucan synthase. With the increased use of echinocandins for prophylaxis, empiric or definitive therapy, resistance to these drugs in aspergillus isolates needs to be closely monitored.

6 Conclusion

Unlike the situation with candida, data on drug resistance in aspergillus are slow to accumulate. Recent standardization of susceptibility of testing for filamentous fungi has made it possible to study the phenomenon of resistance. Of the four drug classes, aspergillus resistance in azoles is most commonly described; resistance to polyene class has remained remarkably low. Mechanisms of azole resistance in aspergillus are better understood. The widespread nonclinical use of azoles in the agricultural industry appears to be a significant contributor to resistance among environmental isolates. With the increasing incidence of aspergillosis and the widespread use of orally administered anti-aspergillus azoles for prolonged periods in different settings (prophylaxis or therapy), particularly in compromised hosts or in those with a heavy burden of the organism, the emergence of drug resistance in aspergillus is likely to escalate.

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Drug Resistance in Cryptococcosis

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1 Introduction

There are over 30 different *Cryptococcus* species (spp.), with *C. neoformans* and *C. gattii* being the two primary human pathogens responsible for the fungal disease, cryptococcosis. These encapsulated basidiomycetous yeasts have important differences in their geographic distribution, ecological niches, and predilection to cause life-threatening disease in immunocompromised as compared to seemingly immunocompetent hosts.

C. neoformans is found throughout the world and primarily causes infection in immunosuppressed patients. Its primary habitat is around pigeon roosts and in soil containing avian droppings. *C. neoformans* strains consist of two varieties and three serotypes that are based on capsular epitopes and five genotypes. Nearly all *C. neoformans* infections in patients with the *human immunodeficiency virus* (HIV) involve *C. neoformans* variety (var.) *grubii* (serotype A), except in Europe, where *Cryptococcus neoformans* var. *neoformans* (serotype D) and some A/D hybrid strains are responsible for clinical disease. *C. gattii* (serotypes B and C with four genotypes) is found in the soil around certain types of trees and has historically been linked to outbreaks in healthy hosts living in tropical and subtropical regions. More recently, *C. gattii* has emerged in temperate portions of the Pacific Northwest (USA and Vancouver, Canada) and has been linked to opportunistic infections in HIV–AIDS patients from southern California.

The two pathogenic cryptococcal species can further be subdivided into nine major molecular types: VNI to VNIV and VNB for *C. neoformans* and VGI to VGIV for *C. gattii*. There is evidence that the various molecular types may actually represent cryptic species, with important differences in virulence and antifungal susceptibility. VNI and VGI molecular types are widespread and cause the majority of disease attributed to *C. neoformans* and *C. gattii*, respectively. In contrast, VGIII, VGIV, and VNIV appear to be geographically restricted, with VGII strains responsible for the Pacific Northwest outbreak.

Cryptococcal infection typically begins after inhalation of the yeast or basidiospores into the lung, which may be followed by hematogenous spread to extrapulmonary tissue, and is usually considered to represent reactivation of a dormant infection [1]. Most patients present for medical attention with subacute signs and symptoms such as fever, headache, lethargy, and/or mental status changes. The five most common anatomic sites of cryptococcal involvement are the lungs, central nervous system (CNS), skin, prostate, and eye. In HIV-infected patients, the two species share similar clinical aspects, but disease manifestations may differ in patients without HIV. *C. neoformans* has a unique predilection for neural tissue and is an important cause of meningoencephalitis. Alternatively, some reports have suggested that *C. gattii* more frequently produces pulmonary infections, but may also involve the CNS [2]. In otherwise healthy hosts, intracranial *C. gattii* infection has a higher propensity to cause focal brain lesions on radiographs than *C. neoformans* and has been associated with a delayed response to therapy as well as more frequent requirements for neurosurgical intervention [3].

Despite serological evidence for widespread infection in select human populations, cryptococcosis is a relatively uncommon disease in individuals with a healthy immune system. Patients with T-cell deficiencies are at the highest risk of developing invasive cryptococcal infection, with a prevalence of up to 30 % in AIDS patients in sub-Saharan Africa. Life-threatening infections caused by this pathogen have been increasingly recognized worldwide, largely due to the AIDS epidemic and the expanded use of immunosuppressive drugs and chemotherapeutic agents. Progress in the

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fields of organ transplantation and management strategies in hematology–oncology have led to an increased number of immunosuppressed survivors at risk for invasive fungal infections. Precise estimates of the incidence of cryptococcal disease are not available in the era of antiretroviral therapy (ART). The disease was thought to affect between 6 and 10 % of AIDS patients in the USA, Australia, and Western Europe prior to the advent of ART [4], but its frequency has decreased in the current clinical climate [4].

Cryptococcal disease in both HIV-positive and HIV-negative hosts continues to be associated with significant morbidity and mortality. Successful treatment has historically relied on the use of amphotericin B (AmB), flucytosine (5-FC), and fluconazole. To date, in vitro antifungal drug resistance to amphotericin B is uncommon, and there are geographical differences in rates of fluconazole resistance. Elevated fluconazole MICs have been reported in settings where prophylactic fluconazole use is routine and in cases of relapsed cryptococcal disease after initial fluconazole monotherapy. Despite the availability of antifungal agents with activity against *Cryptococcus* species in vitro and the use of ART to treat HIV, treatment failures continue to occur for a variety of reasons including direct antifungal drug resistance. Recent improvements in antifungal susceptibility testing have allowed for the identification of resistant strains in vitro. However, substantial discrepancies across different susceptibility test methods exist, and the correlation between in vitro drug activity and clinical outcome has not been fully elucidated. AIDS patients with cryptococcal meningitis who survive beyond initial induction therapy may require prolonged maintenance therapy to prevent disease relapse. Secondary prophylaxis or maintenance therapy in conjunction with the prolonged use of antifungal agents for the treatment or prevention of other fungal infections has generated concern that less susceptible cryptococcal strains may emerge.

The potentially devastating clinical ramifications of antifungal drug resistance have led to intensified efforts to better define the scope of the resistance problem. To this end, a significant amount of work has gone toward improving and standardizing systems capable of identifying fungal resistance when it occurs, delineating the molecular mechanisms responsible for the development of drug resistance, and designing new and improved strategies to treat patients with resistant cryptococcosis. The aim of this review is to summarize the current understanding of clinical resistance in *Cryptococcus neoformans* and to discuss future directions for the prevention and management of antifungal drug resistance when it occurs.

2 Definitions

Drug resistance is an important clinical problem in a variety of infectious diseases. Classically, the term resistance is used to describe an in vitro phenomenon in which a microorganism displays relative insensitivity to a specified antimicrobial agent as compared with other isolates of the same species. Resistance can either be primary or secondary. Primary resistance occurs in microorganisms never exposed to the drug of interest. Primary resistance in *C. neoformans* and *C. gattii* is relatively uncommon but has been reported to occur with 5-FC [5] and fluconazole [6]. Secondary resistance, also known as acquired resistance, results from previous drug exposure. This form of drug resistance has been increasingly observed in *C. neoformans* with the azole class of antifungals. Secondary resistance to 5-FC was primarily a concern in the 1970s when this agent was used as monotherapy for the treatment of cryptococcal meningitis [7]; but with the use of the combination of flucytosine and AmB, the development of flucytosine resistance is less common. Intrinsic resistance has been defined as an inherent resistance of all isolates of one species to a certain drug. This type of resistance is observed for the echinocandin class of drugs against both *C. neoformans* and *C. gattii*, and thus, at present, this class of agents has not been useful in treating cryptococcosis.

Lastly, clinical resistance has also been termed clinical failure. Clinical resistance describes an in vivo phenomenon in which a microorganism continues to cause evidence of disease despite therapeutic concentrations of an appropriate antimicrobial at the site of infection. For example, progressive neurologic dysfunction after the initiation of antifungal therapy for cryptococcal meningoencephalitis may result from failure to control intracranial pressure or rapid immune reconstitution and does not necessarily imply underlying antifungal drug resistance.

2.1 Clinical Treatment Failure

Potential reasons for clinical treatment failure are many and include (1) host factors such as immune status and treatment compliance, (2) site of infection, (3) drug characteristics including bioavailability and toxicity profile, and (4) fungal factors such as the virulence of the infecting strain as well as the direct minimum inhibitory concentration (MIC). Arguably, the most important long-term prognostic factor for the successful treatment of cryptococcosis is the ability to treat the patient's underlying disease process as opposed to the organism's MIC.

2.1.1 Clinical Resistance Patterns in Patients Without HIV Infection

Treatment failure as a result of impaired host defenses has been clearly described in the setting of neoplastic disease. In the classic prognostic analysis conducted by Diamond and Bennett in 1974, patients that died on amphotericin B therapy were more likely to have an underlying lymphoreticular malignancy and/or to have received corticosteroid therapy

[8]. Furthermore, patients with cryptococcal meningitis who relapsed after antifungal therapy were more likely to have received 20 mg or more of prednisone a day. Improved clinical outcomes were noted if the corticosteroids were reduced to below 20 mg of prednisone daily.

Outcomes related to invasive cryptococcal disease in HIV-negative patients have been reevaluated in the era of effective azole therapy. Overall mortality was 30 %, and mortality attributable to cryptococcal meningitis was 12 % in a study of 306 HIV-negative patients conducted at 15 US medical centers from 1990 through 1996 [9]. Cause-specific mortality was highest for patients with organ failure syndromes (34 %) and second highest for patients with hematologic malignancies (21 %). Solid organ transplant (SOT) recipients are also at risk for developing cryptococcal disease. Cryptococcosis has been reported to occur in 0.3–5 % SOT recipients, with dissemination beyond the lungs and/or CNS documented in 52–61 % of these patients [10, 11]. Mortality rates have ranged from 15 to 20 % to as high as 40 % in those with CNS infection [10, 11]. Recently, it was shown in one large medical center that mortality was higher in the non-HIV, non-transplant group (31 %) versus the HIV and transplant recipient groups (16 %). These findings likely reflect heterogeneous comorbidities and the fact that the non-HIV/non-transplant groups had substantially longer duration of symptoms prior to diagnosis [12].

2.1.2 Clinical Resistance Patterns in Patients with HIV Infection

Currently recommended antifungal treatment regimens in conjunction with ART have improved the prognosis for patients with HIV-associated cryptococcosis; however, acute mortality remains unacceptably high. Robinson and colleagues [13] reported that 37 % of 204 evaluable AIDS patients with cryptococcal meningitis enrolled between 1986 and 1993 failed to have a negative CSF culture after 10 weeks of combination therapy with AmB and 5-FC. Twenty-nine deaths were reported within the first 2 weeks of the study, and a total of 62 deaths occurred prior to the 10-week assessment. Multivariate analysis identified the CD4 cell count as one of the characteristics associated with treatment outcome at 10 weeks. Furthermore, investigators using quantitative cultures have also shown that an initial high burden of yeasts in CSF during cryptococcal meningitis is associated with a worse prognosis [14]. Both host and yeast factors contribute to final outcome of this disease.

2.1.3 Clinical Resistance and Pharmacologic Limitations

The location of cryptococcal infection in combination with the pharmacologic properties of currently available antifungal drugs also plays a role in clinical outcome. A vivid example of this was the observation that antifungal therapy incompletely eradicated *Cryptococcus* from the genitourinary tract of patients with prostatic involvement and HIV infection [15]. Similarly, ketoconazole with its inconsistent oral absorption and limited penetration into the central nervous system has been shown to be ineffective for treating cryptococcal meningitis in spite of in vitro activity [16]. Drug side-effect profiles and patient adherence are also important considerations in treatment failure. Nephrotoxicity and infusion-related side effects, for example, can limit the clinical effectiveness of amphotericin formulations and have frequently shifted the polyene of primary use from amphotericin B deoxycholate to a lipid formulation of amphotericin B. The development of bone marrow and gastrointestinal side effects has been a problem with flucytosine therapy, and frequently, blood levels of flucytosine are not readily available for adjusting doses.

2.1.4 Cryptococcal Virulence Factors and Clinical Resistance

Cryptococcal pathogenicity also influences clinical resistance patterns. Intrinsic virulence differences among *Cryptococcus* strains have been shown to exist under controlled conditions in animal models, and cryptococcal infection in humans may be linked to the infecting strain's inherent virulence characteristics. It has recently been shown that certain cryptococcal genotypes (i.e., burst groups) were associated with a poorer prognosis in HIV-infected patients [17]. Additionally, Mitchell and colleagues [18] performed a retrospective review of patients with cerebral cryptococcosis in Australia between 1985 and 1992. Infection with *C. gattii* was associated with a poorer prognosis despite prolonged AmB administration and careful management of increased intracranial pressure. Furthermore, the outbreak VGIIa strains from Vancouver Island also appear more virulent than non-outbreak VGIIb strains in macrophage and murine models [19]. In addition to the cryptococcal variety, the yeast's ability to produce a melanin-like pigment in vitro has also been linked to pathogenesis [20]. Melanin may protect the yeast from UV damage, extremes in temperature, oxidative stresses, and host macrophages. Van Duin et al. [21] demonstrated that melanization reduced the susceptibility of *C. neoformans* to AmB and caspofungin using in vitro killing assays. The work of Odom and colleagues [22] has also shown that the calcineurin pathway is required for *C. neoformans* virulence in warm temperatures that mimic the host environment, but not lower environmental temperatures. Lastly, the cryptococcal capsule has also been shown to play a key role in virulence. Acapsular mutants are typically avirulent, whereas encapsulated organisms display varying degrees of pathogenicity. Capsule size has been associated with intracranial pressure and host immune responses [23].

We have highlighted several of the host characteristics, pharmacologic limitations, and fungal virulence factors

thought to be an integral part of cryptococcal clinical resistance. In addition to these variables, the in vitro antimicrobial susceptibility has been shown in animal models and some clinical reports to be an important predictor of outcome in cryptococcal infection. Primary and secondary antifungal drug resistance has become clinically important as the number of immunocompromised patients requiring long-term antifungal therapy has grown. The remainder of this review will focus on the identification of resistant cryptococcal isolates in the microbiology laboratory in addition to a review of the epidemiology and molecular mechanisms of antifungal drug resistance in *C. neoformans*.

3 Susceptibility Testing

Antifungal susceptibility testing has accrued substantial interest in recent years as the incidence of invasive fungal infections and the number of available antifungal agents have increased. A great deal of effort has gone into the development of reproducible and clinically relevant reference methods for yeast susceptibility testing. This collaborative work has promoted standardization across laboratories, and, although imperfect, it has given clinicians an in vitro benchmark to assist in the selection of antifungal therapy.

3.1 Broth Dilution Methods

The internationally recognized reference method for yeast susceptibility testing is broth dilution, a technique that involves serial twofold dilutions of an antifungal drug in a liquid medium that is inoculated with a standardized number of yeast cells and incubated for a prescribed period of time. A substantial body of work has shown excellent correlation between broth macrodilution, which utilizes larger volume individual tubes as compared to microdilution comprised of trays with wells that hold a much smaller volume. The broth microdilution (BMD) adaptation is less labor-intensive and is the broth dilution technique of choice in most microbiology laboratories.

Currently there are two standardized methods for BMD antifungal susceptibility testing of yeast: the Clinical and Laboratory Standards Institute (CLSI documents M27-A3 and M27-S4) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods. The two approaches are similar in that they both use RPMI-1640 broth as the base medium and have the same incubation durations and a prominent inhibition endpoint (i.e., 50 % for fluconazole and flucytosine, 100 % for AmB) for MIC determination relative to the growth control. Differences between the methods include the inoculum density, glucose content of the medium, the shape of the microdilution wells, and a visual (CLSI) versus spectrophotometric (EUCAST) endpoint reading. Both methods include guidance for the testing of isolates of *Cryptococcus*, specifically.

3.2 Modifications to the CLSI Broth Microdilution Method

Some cryptococcal isolates grow slowly or suboptimally in the CLSI-recommended RPMI-1640 medium. Therefore, the recommended incubation time for *Cryptococcus* is 70–74 h as opposed to 24–48 h for *Candida*. In addition, the use of Yeast Nitrogen Base (YNB) in place of standard RPMI-1640 media may facilitate the growth of *C. neoformans* and improve the clinical relevance of the MIC, an alteration that was first suggested by Ghannoum et al. [24]. Subsequent multicenter studies have confirmed strong interlaboratory agreement using the CLSI microdilution method combined with YNB [25]. Cryptococcal meningitis, especially in patients with uncontrolled HIV, is a high fungal burden disease; therefore, it has also been suggested that a larger inoculum size improves the predictive value of fluconazole MIC testing. Variation of inocula from 10^3 to 10^5 cells per mL has shown small but significant inoculum effects in determining MICs for fluconazole, AmB, and flucytosine for *C. neoformans* [24]. Therefore, many in vitro studies have used 10^4 colony-forming units/mL (CFU/mL) as the final inoculum for susceptibility testing.

3.3 Disk Diffusion Susceptibility Testing

Both CLSI (Document M44) and EUCAST provide standardized methodologies for disk diffusion testing of *Candida* spp. These methods have also been extended to *Cryptococcus*. Pfaller et al. [26] compared fluconazole disk diffusion zone diameters to MICs determined by M27-A2 using a total of 276 clinical *C. neoformans* isolates. Method comparisons yielded an overall categorical agreement of 86 %, with 0 % very major errors (i.e., the disk diffusion method never indicated that an organism was susceptible when the reference method indicated resistant), 2 % major errors (i.e., disk diffusion suggested that an organism was resistant, but the reference method reported susceptible), and 12 % minor errors.

3.4 The E-test for Antifungal Susceptibility Testing

The E-test method consists of a plastic strip impregnated with a predefined gradient of antifungal drug concentrations that is used to determine an organism's MIC. Several investigators have compared the E-test and CLSI microdilution

methods for determining susceptibility of *C. neoformans* isolates and have observed mixed results. Using RPMI-1640 medium with 2 % glucose (RPG agar) for the E-test, Aller et al. [27] reported that fluconazole and flucytosine MICs measured by the E-test showed good agreement with BMD methods (81.1 and 89.2 % ± one twofold dilutions agreement, respectively). However, only fluconazole showed a statistically significant agreement between methods. Itraconazole and AmB MICs showed poor correlation (54 % and 13.5 % ± 2 dilutions agreement, respectively). No itraconazole- or AmB-resistant isolates were included for analysis. Using the same medium, Maxwell and colleagues [28] showed good agreement between the E-test and BMD for voriconazole (94 %) and AmB (99 %). Similarly, 98 % agreement was observed for isavuconazole, without any significant discrepancies (i.e., >2-well dilution differences) [29]. Lozano-Chiu et al. [30] reported that antibiotic 3 medium was superior to both the YNB and the RPMI-1640 media for consistently identifying AmB-resistant cryptococcal isolates in broth by the M27-A2 method. When these investigators used an E-test agar diffusion method, both the RPMI-1640 and the antibiotic 3 medium allowed ready detection of the amphotericin B-resistant isolates. In addition, the investigators reported a high level of agreement between the broth and E-test methods.

The etiology of discrepancy in AmB results across these studies is unclear. Based on available data, the E-test is likely to be a useful alternative to the M27 microdilution techniques for determining the susceptibility of *C. neoformans* to flucytosine, fluconazole, voriconazole, isavuconazole, and possibly AmB. The E-test may be especially helpful for detecting AmB-resistant isolates.

3.5 Interpretive Breakpoints

Clinical interpretive breakpoints (CBPs) for in vitro antimicrobial susceptibility may be used to identify clinical yeast isolates that are likely to respond to treatment with a given antifungal drug administered using the approved dosing regimen for that agent [31]. Yeast CBPs have only been established for *Candida* and were determined based on extensive clinical experience with mucosal and invasive candidiasis.

Even though recent refinements in susceptibility testing have provided improved standardization, continued technical variability and the lack of data on antifungal PK/PD as well as clinical outcomes relative to MIC have prevented the establishment of CBPs for *C. neoformans* and *C. gattii* by either the CLSI or EUCAST. At this time, there is no exact MIC or zone size endpoint to identify resistant cryptococcal phenotypes. Anecdotally, resistance breakpoints (μg/mL) for *C. neoformans* and *C. gattii* have been proposed and applied in surveys of large collections of isolates. These cutoffs include a MIC (μg/mL) of either ≥16 or ≥64 for fluconazole, ≥1 for itraconazole, ≥2 voriconazole, ≥32 for flucytosine, and ≥1 for AmB. Others have used zone diameters of ≤14 mm as a threshold for non-susceptibility. It is important to note that these breakpoints must be interpreted cautiously in the context of the clinical scenario. Prospective studies are ultimately required to identify accurate CBP determinations for antifungal drug resistance. Furthermore, routine in vitro susceptibility testing is not currently recommended for treatment of cryptococcosis [32].

3.6 Epidemiologic Cutoff Values

Epidemiologic cutoff values (ECVs) potentially represent the most sensitive benchmark for differentiating organisms with decreased antimicrobial susceptibility [33]. ECVs may be useful for identifying isolates that are less likely to respond to treatment due to acquired resistance mechanisms when CBPs have not been established as well as for following in vitro susceptibility trends over time. The ECV is defined as an MIC cutoff value that discriminates wild-type (WT) isolates from non-WT strains (i.e., organisms harboring mutational or acquired resistance mechanisms) [34]. The ECV takes into account the WT MIC distribution, modal MIC, and the inherent variability of the susceptibility test method (±1 doubling dilution). The MIC distribution for WT organisms typically covers 3–5 doubling dilutions surrounding the modal MIC [31, 35, 36]. For most MIC distributions, the ECV occurs at a threshold of approximately two dilutions above the modal MIC and encompasses ≥95 % of the WT MIC distribution [31].

Multiple international studies have determined cryptococcal ECVs using large global collections of isolates tested according to the CLSI M27-A3 method (RPMI-1640 medium and 72 h incubation). The first report focused on the azole class of drugs [37] and included 285 invasive *C. neoformans* clinical isolates. The ECVs (μg/mL), along with the percentage (%) of isolates that had an MIC ≤ ECV for fluconazole, posaconazole, and voriconazole, were 8 (96.9 %), 0.25 (96.5 %), and 0.12 (95.1 %), respectively. Interestingly, temporal trends in fluconazole ECVs between 1996 and 2008 showed a decreasing frequency of non-WT strains. This observation is in agreement with previous surveys from developed countries, which suggest that *C. neoformans* susceptibility to fluconazole has improved with the introduction of antiretroviral therapy.

The second two studies included large numbers of *C. gattii* as well as *C. neoformans* strains and reported ECVs according to molecular type; but not all isolates had genotype determination available [38, 39]. Fluconazole ECVs (μg/mL) for different *C. neoformans* genotypes were 8 (VNI) or 16 (non-typed and VNIII). Fluconazole ECVs for

C. gattii ranged from 8 (non-typed, VGI, VGIIa, VGIII) to 32 (VGII). ECVs for the other triazole drugs also showed genotype-specific differences. Posaconazole ECVs ranged from 0.25 (*C. neoformans* non-typed and VNI) to 0.5 (*C. gattii* non-typed and VGI). Similarly, voriconazole ECVs spanned 0.12 (VNIV), 0.25 (non-typed isolates of both species, VNI, VNIII, VGII, and VGIIa), and 0.5 (VGI). MIC distributions also differed by species and molecular genotypes for AmB and 5-FC. For AmB, the ECVs (with the percentage of isolates for which MICs were less than or equal to the ECV listed in parentheses) were 0.5 μg/mL for *C. neoformans* VNI (97.2 %), *C. gattii* VGI (99.2 %), and VGIIa (97.5 %) and 1 μg/mL for *C. neoformans* (98.5 %), *C. gattii* non-typed (100 %), and VGII (99.2 %) isolates. ECVs for 5-FC were 4 μg/mL for *C. gattii* non-typed (96.4 %) and VGI (95.7 %), 8 μg/mL for VNI (96.6 %), and 16 μg/mL for *C. neoformans* non-typed (98.6 %) and VGII (97.1 %).

These observations suggest that ECVs for the *Cryptococcus neoformans–gattii* species complex should be both species specific and potentially molecular-type specific. Limited sample sizes precluded assessments of ECV differences for molecular types other than those listed here. In general, the triazole ECVs for *C. neoformans* molecular types were lower than those for *C. gattii*, and the frequency of MICs above the ECV (non-WT strains) was higher for fluconazole (1.7–9.5 %) than for the other triazoles (0–5.7 %).

4 Epidemiology of Reduced Cryptococcal Antifungal Susceptibility

Several large studies have examined the prevalence of cryptococcal strains with reduced antifungal susceptibility in vitro.

4.1 Global Surveillance Studies

Using standardized disk diffusion testing, 2230 *C. neoformans* isolates collected from 134 study sites in 40 countries were tested against fluconazole and voriconazole over a 10.5-year period [40]. For study purposes, interpretive breakpoints (zone diameters) for fluconazole were susceptible ≥19 mm and resistant ≤14 mm. For voriconazole, ≥17 mm was considered susceptible and ≤13 mm resistant. Overall, 10.4 % of isolates were resistant to fluconazole while only 1.7 % was resistant to voriconazole. Importantly, a significant proportion of fluconazole-resistant isolates (13.6 %) showed cross-resistance to voriconazole.

4.2 Population-Based Surveys

Brandt and colleagues [41] reported on an active surveillance program conducted by the Centers for Disease Control and Prevention (CDC) in four metropolitan areas of the USA between 1992 and 1994 and 1996 and 1998. A total of 732 isolates from 522 patients were evaluated as part of this surveillance. In vitro susceptibilities for AmB were measured using the E-test and MICs for flucytosine, fluconazole, and itraconazole and were measured by the CLSI broth microdilution method. A broad range of MICs were observed over the study period. Interestingly, the MIC_{50} and MIC_{90} for the four drugs did not change by more than a one log dilution between the first 3 years of surveillance as compared to the follow-up 3-year period. No geographical differences were noted and the AmB MIC was ≥2 μg/mL for just two isolates in the entire study. Both isolates were identified in the 1996–1998 surveillance period. Individual histories of AmB exposure were not described for these isolates. Six isolates (0.6 %) collected between the years of 1992 and 1994, and four isolates (1.6 %) collected between 1996 and 1998, had flucytosine MICs ≥32 μg/mL. The incident isolate MIC for fluconazole was ≥64 μg/mL for 6 of 253 patients (2.4 %) between 1992 and 1994 and ≥64 μg/mL for 2 of 269 patients (0.7 %) between 1996 and 1998. The investigators also compared fluconazole susceptibilities for 172 serial isolates of *C. neoformans* collected at least 1 month apart from 71 patients. Thirteen of the 71 (18 %) patients with follow-up isolates had a fourfold or greater increase in fluconazole MIC as compared with the initial isolate. The remaining 58 patients (82 % of serial isolates) showed either no change in MIC (33 patients) or up to a one log dilution change (25 patients). Clinically, this is an interesting observation given that the group of patients with serial isolates available for comparison had presumably been receiving fluconazole maintenance therapy.

A population-based surveillance program was also conducted in South Africa [42]. In many parts of the developing world, the fungicidal combination of AmB and flucytosine for cryptococcal meningitis is precluded by cost, availability, and difficulties with drug administration and/or monitoring. In these settings fluconazole monotherapy, often administered at a relatively low dose (≤400 mg daily), has been the standard initial treatment for CNS disease. Additionally, long-term fluconazole prophylaxis to prevent and treat HIV-associated mucocutaneous candidiasis is common. To determine whether this practice may contribute to the emergence of cryptococcal isolates with reduced fluconazole susceptibility, cases of laboratory-confirmed cryptococcosis were reported to the Mycology Reference Unit, National Institute for Communicable Diseases in Johannesburg, with

testing performed for isolates from 2002 to 2003 and 2007 to 2008. The MICs for six antifungal drugs (AmB, fluconazole, flucytosine, voriconazole, posaconazole, and itraconazole) were determined for incident isolates using the standard M27-A3 method. An incident case was defined as the first episode of laboratory-confirmed disease in a patient. In addition, serially collected isolate pairs were also tested for susceptibility to fluconazole. Of the 487 incident isolates tested, only 3 (0.6 %) demonstrated a fluconazole MIC of ≥16 μg/mL; all of these were from 2002 to 2003. Three additional isolates from the earlier surveillance period had elevated itraconazole MIC values (MIC ≥ 1 μg/mL). All incident isolates were inhibited by low concentrations of AmB (MIC_{90} = 0.19 μg/mL). The MICs for voriconazole and posaconazole were also low (≤0.25 μg/mL and ≤0.5 μg/mL for voriconazole and posaconazole, respectively). Despite no flucytosine use in South Africa during the surveillance period, 17 of 237 (7 %) isolates had MIC values of 8 μg/mL or 16 μg/mL. There were no differences in MIC_{50} and MIC_{90} between the two surveillance periods for any of the antifungal drugs tested. Lastly, of the 67 cases with serially collected isolate pairs, only one case had a follow-up isolate (collected more than 30 days after the incident culture) with a fluconazole MIC significantly higher than the corresponding incident isolate.

4.3 Studies Involving Patients with Relapsed Meningitis

Bicanic et al. [43] described 32 episodes of relapsed cryptococcal meningitis in 27 HIV-positive subjects after initial treatment with fluconazole at a dose of 400 mg daily. Seventy-six percent of culture-positive relapses (n = 21) were associated with isolates that had reduced susceptibility to fluconazole, and these cases carried a high associated mortality regardless of whether or not the patient was on ART. Interestingly, 44 % of patients infected with fluconazole-resistant isolates had been receiving rifampicin without adjustment of fluconazole dose. Rifampicin is known to induce fluconazole metabolism.

Yildiran et al. [44] also investigated the in vitro susceptibilities of 213 CSF isolates from 192 patients against fluconazole, voriconazole, and posaconazole using the M27-A macrodilution method. This *C. neoformans* collection was comprised of isolates previously submitted to the University of Texas Health Science Center in San Antonio between 1990 and 1999. The MIC_{50} and MIC_{90} for each of the triazoles studied remained essentially unchanged over the 10-year observation period. Overall, posaconazole was the most active triazole (MIC_{90}, 0.06 μg/mL) followed by voriconazole (MIC_{90}, ≤0.125 μg/mL) and then fluconazole (MIC_{90}, 8 μg/mL). Twenty patients with relapsing meningitis who had serial isolates submitted at least 1 month apart were reviewed. Nine patients (45 %) had the same fluconazole MICs (±1 dilution) for the initial as compared to the final isolates; six patients (30 %) had a 4- to 16-fold rise in the fluconazole MIC; and the remaining five patients (25 %) had a 4- to 16-fold decrease in MICs. The voriconazole MICs remained unchanged over time (±1 dilution). Sixteen patients (80 %) had equivalent (±1 dilution) posaconazole MICs for the original and final isolate; two (10 %) patients had a four-fold rise in MICs, and the final two (10 %) had a 4- to 16-fold decrease. The observed changes in fluconazole MICs over time did not necessarily predict the directional changes observed in the posaconazole MIC. Proposed explanations for changes in the posaconazole MICs seen over time in some isolates include speculation that a different cryptococcal strain could be causing relapse. Previous studies, however, have shown that relapses are most often caused by the initial infecting strain.

Taken together, these relatively large studies provide us with some insight into the prevalence of reduced susceptibility to antifungal drugs, over diverse geographic regions and prolonged periods of time. Although the majority of isolates appeared to be susceptible to a variety of antifungal agents in vitro, acquired azole resistance has clearly been demonstrated following fluconazole exposure. Posaconazole appears to be the most active drug in vitro, and some, but not all, studies have observed that elevated fluconazole MICs predict elevated voriconazole MICs. Continued surveillance with documentation of clinical outcomes in relation to MIC is warranted. This is especially important in instances of relapsed cryptococcosis. The IDSA Cryptococcosis Treatment Guidelines have supported the importance of saving all cryptococcal isolates so they can be tested concurrently if there is an apparent relapse. Although empirical as a recommendation, these guidelines suggest a three-tube dilution rise in MIC as a marker for development of direct drug resistance [32].

4.4 Susceptibility Differences Across Cryptococcal Species

There has been significant interest in whether antifungal susceptibility differences exist between *C. neoformans* and *C. gattii* species. *C. gattii* infections are often associated with a delayed response to antifungal therapy [3], but this observation is not necessarily linked to higher MICs. There are conflicting reports comparing MICs between *C. neoformans* and *C. gattii* strains, with some studies reporting similar antifungal MICs among the two species [45], while others show higher azole and flucytosine MICs for *C. gattii* than for *C. neoformans* [46]. It is not clear whether the differences in MICs are truly between the two species complexes or potentially related

to the molecular genotype, as the ECV reports have suggested.

5 Clinical Relevance of In Vitro Fungal Susceptibility Results

Multiple studies have assessed the correlation between susceptibility test results and clinical response in cryptococcal disease. The majority of these reports have focused on the clinical predictive value of fluconazole and/or AmB MICs.

5.1 Correlation Between Fluconazole Susceptibility and Clinical Outcomes

Aller et al. [47] reviewed 25 episodes of predominantly AIDS-related cryptococcal infection in 25 patients from 1994 to 1996 from the USA and Seville, Spain. Therapeutic failure was observed in 5 of 24 patients with AIDS. There was a statistically significant association between elevated fluconazole MICs (≥16 μg/mL) and mortality rate as well as treatment failure. Susceptibility testing in this study was performed following the CLSI guidelines described in document M27-A. Similarly, Menichetti et al. [48] conducted a study of high-dose fluconazole therapy in 14 consecutive AIDS patients with cryptococcal meningitis. The reported median time to first negative CSF culture was 56 days for patients who had an isolate with a fluconazole MIC of 4 μg/mL and 16 days for patients with an isolate MIC of <4 μg/mL. Although the difference in median time to CSF sterilization did not reach statistical significance, 40 days difference may have clinical relevance. An analysis correlating clinical outcome with fluconazole MIC was not conducted in this study.

Witt and colleagues [49], using both BMD with the YNB modification as well as the CLSI macrodilution method, attempted to determine whether in vitro fluconazole susceptibility in conjunction with clinical variables might predict treatment outcome for patients with acute AIDS-associated cryptococcal meningitis. The study population consisted of patients who had enrolled in one of two clinical trials evaluating varying doses of fluconazole with or without flucytosine. Treatment was considered successful if the patient was alive with a sterile CSF culture at the end of 10 weeks of therapy. The mean log MIC for fluconazole was significantly higher for the isolates from patients who failed therapy as compared to those that had treatment success. This was only true, however, when the MIC was measured by the modified BMD method. There was no statistically significant difference in the mean log MIC distribution when the MIC was measured by the standard M27 macrodilution technique. The authors suggested this discrepancy could be due in part to enhanced growth in the YNB medium as compared to the RPMI used for the macrodilution technique.

Although these reports suggest that elevated fluconazole MICs correlate with poorer prognosis, the data are derived from single-center studies with small numbers of patients. It is important to note that fluconazole failures have also been reported with MICs as low as 2–4 μg/mL [50].

5.2 Correlation Between Amphotericin B Susceptibility and Clinical Outcomes

Elevated AmB MICs in *C. neoformans* and *C. gattii* are relatively rare in clinical practice. However, several reports correlating AmB MIC with clinical outcome have been published. Powderly et al. [51] evaluated four serial isolates from a single patient with AIDS-associated cryptococcal meningitis. They reported a rise in AmB MICs from 0.4 to 1.6 μg/mL, which correlated with clinical relapse. Alternatively, others have described susceptibilities of serial *Cryptococcus neoformans* isolates from patients with relapsed meningitis whose isolates showed no decrease in AmB susceptibility relative to the initial isolate [52] and no trend toward higher MICs for strains isolated from patients who failed to respond to antifungal therapy regardless of the drug and susceptibility test method used (i.e., CLSI–RPMI, CLSI–YNB modification, or E-test) [53].

Given the lack of robust correlation between in vitro susceptibility as determined by standard methods and early clinical outcomes, there has been substantial interest in alternative approaches. Larsen and colleagues [54] tested pre- and posttreatment *C. neoformans* CSF isolates from 13 patients using CLSI–RPMI to measure MIC as well as a modified broth macrodilution. In this study, duplicate testing was performed using a standardized inoculum and an inoculum that corresponded to the pretreatment "patient-specific inoculum" as defined by quantitative colony counts per milliliter of CSF. AmB MICs ranged from 0.125 to 0.25 mg/L, a spread too narrow to predict fungal response in culture at day 14 of therapy. Alternatively, a statistically significant association between drug concentration and day 14 quantitative culture was observed when the patient-specific inoculum was used. The authors concluded that assessments of pretreatment fungal burden are required to reliably predict the microbiologic treatment response. These observations were subsequently replicated in an additional independent cohort of AIDS patients [55].

In addition to the patient-specific inoculum approach, there is evidence that minimum fungicidal concentrations (MFCs) may be better predictors of clinical outcome than MICs. In one study, 16 isolates of *C. neoformans* obtained from AIDS patients with meningitis had AmB MIC and MFC testing performed [56]. The MFC was defined as the

concentration of an antifungal agent at which the number of colony-forming units upon subculture in the presence of the drug is essentially zero. An AmB concentration of 1 μg/mL had fungicidal activity against most of the isolates, but four isolates from patients who did not respond to therapy showed a persistent or tolerant antifungal effect. In contrast, the MIC values obtained from all isolates suggested that they were susceptible.

Testing conditions for the reproducible determination of cryptococcal MFCs have not been established. Similarly, a standardized procedure for the quantification of yeast in the initial CSF culture does not currently exist. Both approaches are more labor-intense and technically challenging than the standard BMD methods because they involve meticulous colony counts and therefore may not be practical for most clinical laboratories. However, the current evidence suggests that alternative in vitro approaches are required to optimally predict microbiologic outcomes.

5.3 In Vitro–In Vivo Susceptibility Correlations

There are multiple potential explanations for the lack of reproducible correlations between cryptococcal MICs and clinical outcome. First, it is difficult to directly compare drug susceptibility reports given the heterogeneity of the patient populations studied, differences in testing methodologies, and the variable clinical endpoints used to define treatment success. Another important confounder is the pathobiology of the organism itself. In the environment, cryptococcal strains with different genotypes, serotypes, or mating types are frequently isolated from the same geographic site [57]. Thus, human infection with multiple strains acquired from the environment is plausible. Mixed infections involving different serotypes or genotypes have been reported when single colonies were purified and analyzed due to variable colony morphologies observed in the same culture [58, 59], and molecular analyses of unpurified isolates demonstrated that close to 20 % of cryptococcal infections are actually mixed infections [60]. Microevolution in the host during infection has also been postulated [61–63]. The ability of the organism to undergo recombination with the production of haploid or diploid progeny [64] and the accumulation of translocations, duplications, and even formation of aneuploidy strains is assumed to be a stress response that contributes to genetic diversity and potentially drug resistance. Antifungal therapy may select for the strains that have acquired chromosomal duplications and aneuploidies conferring reduced antifungal susceptibility through duplication of chromosomes or part of chromosomes containing ErgII or drug pumps such as AFR1; but ex vivo, these isolates may then lose their chromosomal abnormalities and thus resistant phenotype when grown on nutrient agar (i.e., non-stress conditions). Going forward, new approaches to susceptibility testing that incorporate direct analysis of CSF (i.e., unpurified isolates) in the presence of drug- or patient-specific inoculum with measurement of antifungal drug effects on fungal viability will likely be required to better replicate in vivo conditions in the laboratory.

6 Molecular Mechanisms of Antifungal Resistance

There are a limited number of antifungal agents available for the treatment of cryptococcosis. The major classes of drugs in use today are the polyenes, azoles, and fluoropyrimidines. Studies have also evaluated the activity of echinocandin analogs alone and in combination with other antifungal agents against *C. neoformans*. The mode of action of the antifungal agents used to treat invasive fungal infections can be divided into three broad categories which include (1) fungal plasma membrane disruption (polyenes and azoles), (2) DNA and RNA synthesis inhibition (fluoropyrimidines), and (3) 1,3-β-D-glucan synthase inhibitors (echinocandins). The development of drug resistance can occur at several sites along the fungal metabolic pathway. Research on the mechanisms of antifungal drug resistance has focused on several areas such as alterations of the drug target, impairment of drug entry into the cell, drug efflux out of the cell, and inactivation of drug within the target cell.

6.1 Polyenes

Amphotericin B deoxycholate (AmB) was first discovered in 1956 in the aerobic actinomycete *Streptomyces nodosus*. AmB was licensed for use in 1959 and is active against a variety of fungi including *Cryptococcus neoformans*. The polyene antifungals, including AmB and the newer less toxic lipid formulations, are fungicidal agents. These drugs work by targeting ergosterol, the principal sterol in most fungal plasma membranes. Ergosterol is important for maintaining structural integrity. It has been hypothesized that 8–10 molecules of drug bind to form a pore within the fungal lipid bilayer, thus promoting spillage of potassium ions and disruption of the cellular proton gradient. In addition to the cell membrane effects, polyenes are also thought to induce oxidative damage in fungal cells [65]. Several investigators have described potential mechanisms for AmB resistance. Kelly et al. [66] described two *C. neoformans* isolates collected from a patient with AIDS-associated cryptococcal meningitis who had failed therapy with AmB and fluconazole. When the pre- and posttreatment isolates were compared, the investigators found the posttreatment isolate to have depleted cell

membrane ergosterol concentrations as a result of a newly acquired defect in sterol delta$^{8\rightarrow7}$ isomerase. This target defect conferred AmB resistance but did not affect the post-treatment isolates' susceptibility to fluconazole.

Ghannoum et al. [67] also described characteristics of cryptococcal sterol composition in relation to AmB and fluconazole susceptibilities. They evaluated 13 isolates from five patients with recurrent cryptococcal meningitis. Strain typing with DNA probes showed that the initial and relapse isolates were identical. All five patients had received fluconazole, and three of the five had also received AmB in the interval between initial diagnosis and relapse of infection. Relapse isolates differed from the initial isolates in sterol composition. None of the relapse isolates had a change in AmB susceptibility, but several relapse isolates did differ in their susceptibility to fluconazole. The investigators concluded that the sterol changes could have been a result of the selective pressure of the antifungal regimen or potentially a result of unidentified in vivo host selection pressures.

The use of azole antifungals, which also inhibit fungal ergosterol synthesis, may theoretically result in a lack of a binding site for AmB. Joseph-Horne and colleagues [68] identified *C. neoformans* mutants that were cross-resistant to azoles and AmB but found that this cross-resistance was not related to sterol biosynthesis. The frequency with which the cross-resistant phenotype was detected in their study was 10^{-8}. The authors suggest that a single mutation may be responsible for the cross-resistance and hypothesized that reduced cellular content of drug could account for the observed multidrug resistance. Unfortunately, no direct measure of AmB drug accumulation could be performed in this investigation. In another study, the same investigators [69] were able to isolate a series of *C. neoformans* mutants resistant to AmB that retained the ability to accumulate ergosterol. They postulated that there are at least several categories of AmB-resistant mutants found among *C. neoformans* isolates. These categories include (1) sterol mutants and (2) mutants with normal sterol biosynthesis with or without cross-resistance to fluconazole.

An animal study conducted by Currie et al. [70] suggested that host factors may also play a role in the development of antifungal drug resistance. In this study, serial passage of five environmental *C. neoformans* isolates in a mouse resulted in statistically significant increases in AmB MIC_{50s} for all isolates, but no significant differences in the fluconazole MICs were noted. Mouse passage was associated with changes in cell membrane sterol content and composition for all five of the passed cryptococcal isolates. Paradoxically, ergosterol content increased in four of the five isolates, all of which were more resistant to AmB after serial passage. This finding highlights the complexity of AmB resistance mechanisms and suggests, at least in the murine model, that drug-resistant variants may arise in vivo without prior drug exposure. As of yet, there has not been a report of primary amphotericin B resistance in a *C. neoformans* strain isolated from a human.

6.2 Fluoropyrimidines

Flucytosine (5-FC) is a fluorinated pyrimidine that was discovered in 1957 as part of a search for novel chemotherapeutics, with subsequent FDA approval in 1971 for the treatment of invasive mycoses. Flucytosine is structurally similar to both fluorouracil (5-FU) and floxuridine and has minimal protein binding and excellent penetration into body fluids. The drug is taken up into fungal cells by a cytosine permease and then deaminated to 5-FU by cytosine deaminase, an enzyme not present in human tissues. The deaminated compound is converted intracellularly to a nucleoside triphosphate termed fluorouridine triphosphate (FUTP) that becomes incorporated into fungal RNA where it causes miscoding and ultimately abnormal protein synthesis. Fluorouracil may also be converted to a deoxynucleoside capable of disrupting DNA synthesis [49, 53].

Inherent resistance to flucytosine has been demonstrated in *C. neoformans* [5] and is thought to result from one of several mechanisms. First, a loss of cytosine permease or deaminase activity may lead to decreased uptake or deamination of the drug. These enzymatic defects confer intrinsic resistance to flucytosine. The next mechanisms of resistance are defects in the activity of uracil phosphoribosyltransferase or uridine-5-monophosphate pyrophosphorylase, enzymes integral to the pyrimidine salvage pathway. Block et al. [7] found that cryptococcal isolates resistant to flucytosine also acquired significant resistance to fluorouracil. This cross-resistance suggested an abnormality in the protein or genes associated with uracil phosphoribosyltransferase or uridine-5-monophosphate pyrophosphorylase.

De novo flucytosine resistance may arise in *C. neoformans* as a result of mutations in either of two non-linked genes. The genes named FCY1 and FCY2 act as simple Mendelian determinants that recombine freely, but have not yet been specifically isolated or sequenced. Studies have examined the frequency of the appearance of flucytosine-resistant mutants within susceptible clinical isolates [71]. In an in vitro experiment, resistant mutants appeared in <0.001 % of randomly selected colonies. The average mutation rate was 70 ± 17.9 mutants per 10^7 cryptococcal cells, suggesting that flucytosine resistance is possibly a single mutational event. These data also suggest that the mutation rate is such that flucytosine resistance could easily be selected for at infection sites such as the CSF, where the burden of yeast can reach 10^7 CFU/mL or greater [71].

Hespenthal and Bennett [72] published their early experience with flucytosine as monotherapy for cryptococcal

meningitis. Their data, collected before the first AmB/flucytosine trials, showed that secondary resistance occurred in 6 of 13 patients who did not respond to therapy or relapsed. In the isolates that developed secondary resistance, flucytosine MICs rose from ≤2.5 to >320 μg/mL and remained at this level for all subsequent testing. The overall treatment failure rate for flucytosine monotherapy in this study was 57 % (13 out of 23 patients).

In a seminal article, the combination of AmB and flucytosine for the treatment of cryptococcal meningitis diminished the frequency of flucytosine resistance in relapse strains [73]. Subsequent clinical experience has shown that flucytosine should always be used in combination with other antifungal drugs such as AmB or fluconazole for the treatment of life-threatening cryptococcosis because of the high rate of secondary drug resistance [73, 74].

6.3 Azoles

Discovery of the azole derivatives in the late 1960s marked a major therapeutic advance for the treatment of invasive mycoses. This class of antifungal agents is totally synthetic and consists of two groups, the imidazoles and the triazoles. The triazoles have three nitrogen molecules within the azole ring, while the imidazoles have two nitrogen atoms. The azoles are fungistatic drugs. The newer azole compounds (voriconazole, posaconazole, and isavuconazole) have a broadened spectrum of antifungal activity including activity against most yeasts as well as some filamentous fungi. Itraconazole, fluconazole, ketoconazole, voriconazole, posaconazole, and isavuconazole have all been shown to have in vitro activity against environmental isolates of *C. neoformans*.

6.3.1 Sterol Biosynthesis

Like the polyenes, the azole class of antifungal drugs acts by interrupting sterol biosynthesis, a multistep process involved in the conversion of lanosterol to ergosterol. Specifically, azoles inhibit lanosterol 14α-demethylase ($P450_{14dm}$), a cytochrome P450-dependent enzyme containing a heme moiety in its active site. Azole compounds function by binding to the iron atom within the $P450_{14dm}$ heme group through an unhindered nitrogen in the azole ring. The azole–heme complex prevents the demethylation of lanosterol required for ergosterol formation. Resultant ergosterol depletion in conjunction with the accumulation of lanosterol and other methylated sterol precursors interferes with fungal membrane structure and function.

Several investigators have attempted to better delineate the mechanisms responsible for azole drug resistance in *C. neoformans*. These appear to be multiple processes that play a role in azole resistance which include changes in the affinity of the target enzyme (sterol 14α-demethylase), inhibition of 3-ketosteroid reductase, drug uptake defects, overexpression of the target enzyme, and genetic mutations encoding for multidrug efflux pumps. Each will be reviewed here.

Venkateswarlu and colleagues [75] evaluated 11 *Cryptococcus neoformans* isolates in an attempt to determine the biochemical basis of tolerance to fluconazole. The investigators focused on variability in sterol composition, inhibition of $P450_{14dm}$ by fluconazole, and the cellular concentration of fluconazole. Sterol analysis was conducted in the presence and absence of fluconazole. Exposure to fluconazole produced a decrease in ergosterol levels to below 20 % of normal in all isolates. All treated isolates accumulated obtusifolione and eburicol, indicative of the inhibition of 3-ketosteroid reductase (a NADPH-dependent enzyme catalyzing C-4 demethylation required for ergosterol biosynthesis) and $P450_{14dm}$, respectively. Eburicol and obtusifolione cannot support cell growth because they are methylated at the C-4 position, and it has been postulated that optimal membrane function requires C-4-demethylation. The investigators suggest that the inhibition of 3-ketosteroid reductase and $P450_{14dm}$ may result from direct azole effects or possibly from the retention of a C14α-methyl group in the substrate. Inhibition of $P450_{14dm}$ was tested by measuring the incorporation of [2-^{14} C] mevalonate into C-14 demethylated sterols in cell extracts. It was noted that only the isolates with low-level fluconazole resistance displayed decreased $P450_{14dm}$ sensitivity to fluconazole. Finally, cellular concentrations of fluconazole were measured using radiolabled drug. The most resistant strains were observed to have a 10- to 20-fold reduction in drug accumulation. The authors hypothesize this could have resulted from the presence of multidrug resistance transporters similar to those found in azole-resistant strains of *C. albicans*. In summary, these data suggest low-level fluconazole resistance may be related to changes in the affinity of the $P450_{14dm}$ target enzyme for fluconazole, while high-level fluconazole resistance may result from decreases in the cellular concentration of fluconazole.

Lamb et al. [76] also studied the P450 system of *C. neoformans* in relation to azole tolerance. In their analysis, sterol composition did not change in the azole-tolerant clinical isolates. All strains accumulated approximately 70 % egosterol, similar to previous sterol analyses of wild-type *C. neoformans*. The investigators also evaluated P450 using microsomal fractions. The specific P450 content was observed to be higher in the azole-tolerant isolates, with approximately twice the P450 content of the susceptible strains. They also noticed that the intracellular concentration of fluconazole was reduced in all of the tolerant isolates, but the drug concentration remained in excess of the microsomal P450 content per cell, suggesting ample drug was available to exert antifungal effect. Lamb's group concluded that alterations in drug target cytochrome P450 may be responsible for azole

tolerance and that this alteration could result in diminished affinity for drug at the enzyme's active site.

6.3.2 14 α-Demethylase (ERG11)

Complimentary to the body of work contributing to an improved understanding of the biochemical basis for azole drug resistance in *C. neoformans*, recent attention has also turned to the potential genetic mechanisms of azole drug resistance in *C. neoformans*. The gene encoding 14α-demythelase (*ERG11*) has been evaluated to determine whether molecular modifications such as mutation or overexpression may lead to antifungal drug resistance in yeasts. The majority of this work has been done with *C. albicans*. The role of *ERG11* alteration in the development of fluconazole resistance in *C. neoformans* was evaluated by examining five isolates from one AIDS patient with recurrent cryptococcal meningitis exposed to fluconazole over a 14-month period [77]. DNA fingerprinting showed that all five isolates were the same strain. Isolates 1–4 were considered susceptible to fluconazole (MIC 1–2 μg/mL), while the fifth isolate showed an MIC of 16 μg/mL and was considered resistant. PCR amplification and gene sequencing of ERG11 for the first four isolates did not show any base changes. The fifth strain displayed a point mutation (g1855t) in a highly conserved region of the *ERG11* protein. An equivalent substitution has been described at the G464S position in *C. albicans* and has been linked previously to fluconazole resistance in this organism. This analysis is one of the first studies to link a point mutation to drug resistance in *C. neoformans*.

6.3.3 Multidrug Efflux Pumps

Posteraro et al. [78] designed a cDNA subtraction library technique to compare gene expression between a fluconazole-resistant mutant and its original azole-susceptible clinical isolate. The azole-resistant mutant was generated by in vitro exposure to fluconazole. The resistant phenotype, with a fluconazole MIC of 64 μg/mL, was stable after 20 consecutive subcultures on drug-free medium. DNA fingerprinting was performed on the two strains, yielding identical RFLP patterns. The investigators then identified cDNA expressed in the resistant mutant but not the fluconazole-susceptible parental strain. Sequence analysis revealed that a portion of cDNA expressed only in the resistant mutant was homologous to known members of the ATP-binding cassette (ABC) transporter superfamily. ABC transporters are groups of genes known to code for multidrug efflux pumps. The unique mutant cDNA was then used as a probe to isolate the entire gene from the *C. neoformans* genomic library. Subsequent sequencing identified an ABC transporter gene that encodes a protein with a significant degree of similarity to other ABC transporters. The researchers named the gene *C. neoformans* Antifungal Resistance 1 (*CnAFR1*, GenBank accession number AJ428201). The *CnAFR1* locus in the resistant isolate was disrupted by homologous recombination to determine whether *CnAFR1* is involved in fluconazole resistance. Disruption of the gene resulted in improved susceptibility to fluconazole in the null mutant. Furthermore, reintroduction of *CnAFR1* led to restoration of the resistance phenotype.

Thornewell et al. [79] also identified a *C. neoformans* gene encoding a protein related to the ABC transporter multidrug resistance proteins. However, the cellular function of this CneMDR1 protein has not been clearly established, and the investigators concluded further experiments are required to determine whether CneMDR1 is actively involved in antifungal drug resistance.

6.3.4 Heteroresistance

Mondonet al. [80] were the first to describe heterogeneity in fluconazole and voriconazole MICs among the clonal subpopulations of a single isolate derived from a HIV-negative man, who had never been treated with antifungal drugs. In addition, these investigators outlined steadily increasing fluconazole MICs among six sequential isolates from an AIDS patient with recurrent meningitis. When single colonies obtained from the isolates of both patients were grown on medium containing 64 μg/mL of fluconazole, a homogeneous population of resistant cells was observed. Upon return to drug-free medium, the majority of these subclones lost their resistance and reverted to the initial phenotype. This pattern of intrinsic azole resistance has been termed heteroresistance and is defined as the emergence of a minor subpopulation of resistant cells, within a single colony of a susceptible strain. The resistant subpopulations can adapt to increasing concentrations of the drug in a stepwise manner. However, repeated transfer on drug-free media causes the highly resistant subpopulation to revert to the original level of heteroresistance. The level of heteroresistance has been defined as the lowest concentration of azole drug at which resistant subpopulations emerge.

Both *C. neoformans* [81] and *C. gattii* [82] strains have been shown to harbor heteroresistance to the triazoles. In one study of clinical and environmental isolates, a considerably higher proportion of *C. gattii* strains (86 %) than *C. neoformans* strains (46 %) exhibited heteroresistance at fluconazole concentrations of ≥16 μg/mL, but there was no apparent correlation between serotype or molecular type with heteroresistance [82]. Yamazumi et al. [83] also investigated the prevalence of heteroresistance in clinical cryptococcal isolates obtained over a broad geographic distribution. In their report, 4.7 % of strains (5 of 107) exhibited heteroresistance to fluconazole.

Comparative genome hybridization and quantitative real-time PCR studies have shown that *C. neoformans* adapts to high concentrations of fluconazole through the duplication of multiple chromosomes [84]. Strains that adapted to fluconazole concentrations higher than their MICs contained

disomies in chromosome 1, with accumulation of additional duplications in several other chromosomes in the presence of increasing drug pressure. Two important determinants of fluconazole susceptibility reside on chromosome 1: *ERG11*, the target of fluconazole, and *AFR1*, the major transporter of azoles in *C. neoformans*. Upon removal of drug exposure, strains returned to their initial level of fluconazole susceptibility and lost the extra chromosomes. Importantly, as proof of principle in subsequent animal model experiments, clones with chromosome 1 disomy emerged in the brain of mice with prolonged fluconazole exposure and biological stress in a strain-dependent manner [85]. Further work is warranted to help determine the clinical significance of fluconazole heteroresistance in human infections, the role it might play in treatment failure, and in ability to develop accurate in vitro susceptibility breakpoints.

6.4 Glucan Synthesis Inhibitors

The fungal cell wall has also been an attractive focus of antifungal drug research and development. Although the composition of the cell wall varies among fungal species, there are common pathways not found in mammalian cells that have been evaluated as potential antifungal drug targets. The general components of these synthesis pathways include chitin, mannoproteins, and 1,3-β-D-glucan. The echinocandins are cyclic hexapeptides that inhibit the biosynthesis of 1,3-β-D-glucan. Specifically, these compounds function as noncompetitive inhibitors of 1,3-β-D-glucan synthase, an enzyme involved in the production of glucan polymers in the fungal cell wall. The current generation of echinocandins includes caspofungin, anidulafungin, and micafungin. These agents have potent activity against a variety of fungi including *Candida* species, *Aspergillus* species, and *Pneumocystis carinii*, but limited activity against *C. neoformans* [86]. It has been hypothesized that the lack of anti-cryptococcal activity displayed by the echinocandins may result from few 1,3-β-D-glucan linkages in the cryptococcal cell wall, absent or low levels of the target enzyme, or limited binding of the synthase inhibitors to the target enzyme. However, the exact etiology of intrinsic resistance has not been definitively established.

Feldmesser et al. [87] undertook an ultrastructural analysis of the cryptococcal cell wall in an attempt to better define glucan linkages and thereby investigate one of the proposed mechanisms of echinocandin drug resistance in *C. neoformans. C. neoformans* cells were grown with and without caspofungin in cell culture. Affinity-purified rabbit antiserum against 1,3-β- and 1,6-β-D-glucan was used to determine whether these epitopes were present in the cell wall of *C. neoformans* cells. Using immunoelectron microscopy and gold particle quantitation, the investigators were able to show that both 1,3-β- and 1,6-β-D-glucan linkages were present in *C. neoformans* cells grown in vitro as well as in infected murine pulmonary tissue. The researchers detected fewer glucan epitopes when the *C. neoformans* cells were grown in caspofungin concentrations typically fungicidal for other fungal species. The group concluded that the absence of 1,3-β-D-glucan linkages does not explain the relative lack of efficacy of caspofungin. They also found that caspofungin partially inhibited the formation of 1,3-β-D-glucan linkages as measured by epitope detection. The authors suggest that *C. neoformans* 1,3-β-D-glucan synthase may be relatively resistant to inhibition by caspofungin and offer this as an explanation for the drug's lack of efficacy against *C. neoformans*.

Previous studies of *C. albicans* and *S. cerevisiae* mutants have identified the transmembrane subunit of the 1,3-β-D-glucan synthase as the target for the echinocandins. The enzyme is a heteromeric complex consisting of two subunits, a large 215-kDa catalytic subunit in the plasma membrane and a small GTP-binding subunit which activates the catalytic portion of the enzyme. Fks1p is the proposed catalytic subunit and is encoded by two homologous genes FKS1 and FKS2. Single disruptions of either gene in *S. cerevisiae* have not been shown to affect fungal viability, however, a double disruption is lethal [88]. Similar FKS genes have been identified in *C. albicans* and *Aspergillus* species [89]. Thompson et al. [90] cloned and sequenced the FKS1 homolog from a *C. neoformans* strain by cross hybridization to *S. cerevisiae*. Sequence analysis of the cryptococcal Fks1p protein was 58% identical to both *C. albicans* and *S. cerevisiae* FKS1 and 62% homologous to *A. fumigatus* FKS1. Only one copy of FKS was found in the *C. neoformans* genome. Amino acid sequences known to be essential for echinocandin susceptibility in *S. cerevisiae* were conserved in the cryptococcal analysis. Thompson's group then disrupted the FKS1 gene in order to evaluate its role in cryptococcal viability. Homologous integrative transformation with a plasmid equally capable of integrating into one of two unique positions within the FKS1 gene was employed to statistically show the essentiality of the gene products for viability. Only one of the two possible integration orientations was capable of disrupting gene function. The demonstration of essentiality derives from exclusive recovery of integrations in the non-disrupting orientation. The investigators observed 23 homologous recombination events in the non-disrupting orientations and no integrations in the disrupting orientation. The probability of this result, assuming an equal chance of recombination in either orientation, is 1.19×10^{-7}. The authors felt this was a strong statistical argument for the essentiality of the FKS1 gene in *C. neoformans*. They also concluded that the gene encoding 1,3-β-D-glucan synthase is present in *C. neoformans* and that glucan synthesis is required for fungal viability.

The relatively low efficacy of echinocandins against *Cryptococcus* also does not appear to result from limited drug activity against the cryptococcal 1,3-beta-glucan synthase [91]. As for the notion that limited drug access to the target enzyme may play a role in echinocandin resistance, it has been demonstrated that acapsular *C. neoformans* strains have caspofungin MICs similar to the capsular isolates described in previous studies [90]. Therefore, the cryptococcal polysaccharide capsule does not appear to play a significant role in the relative lack of efficacy of caspofungin against *C. neoformans*. It is possible that an as of yet undiscovered mechanism of action is operative in other fungal pathogens, but not in *C. neoformans*/*C. gattii*.

7 Strategies to Overcome Drug Resistance in *Cryptococcus neoformans*

Effective strategies to prevent antifungal drug resistance are needed. Plans for the management of existing drug resistance, especially fluconazole resistance, are paramount. This section focuses on six strategies that should be considered in the clinical approach to the prevention and/or management of antifungal drug resistance in *C. neoformans*.

7.1 Primary Prophylaxis

The simplest and most cost-effective strategy to manage cryptococcal drug resistance is to prevent infection entirely. *Cryptococcus gattii* has been found in association with several species of *Eucalyptus* and other trees, while varieties *neoformans* and *grubii* have been isolated from fruit, trees, and bird excreta. Patients at high risk for cryptococcal infection should avoid these environments when possible. Complete elimination of all yeast exposure, however, is highly unlikely. Prevention of the development of cryptococcosis could also involve either chemoprophylaxis or immunization, targeting individuals at the highest risk for disease. Unfortunately, adoption of a prophylactic strategy in high-risk patients has the potential to increase the incidence of drug resistance as a result of prolonged exposure to antifungal drugs, while cryptococcal vaccines await the results of clinical trials in humans.

Several studies have assessed the efficacy of azole prophylactic therapy for the prevention of cryptococcal disease in high-risk AIDS patients. Both fluconazole and itraconazole are effective for preventing cryptococcosis [92, 93]. None of the prevention trials, however, have shown a survival benefit. In addition, the expense, potential for selection of resistant fungi (in both *Candida* and *Cryptococcus* species), and possible drug–drug interactions make most physicians reluctant to use azoles for primary prophylaxis. Also, current use of ART and its associated immune reconstitution have significantly reduced the risk for cryptococcosis in patients with HIV infection. Currently, the recommendations from the Infectious Diseases Society of America (IDSA) and US Public Health Service (USPHS) do not endorse primary prophylaxis for fungal disease in patients with AIDS [94].

A polysaccharide-protein conjugate vaccine composed of cryptococcal capsular glucuronoxylomannan covalently coupled to tetanus toxoid has been developed. Subsequently, the vaccine has been shown to produce a protective antibody response in mice with high levels of capsular antibodies identified after active and passive immunization [95]. The finding that antibodies to the capsular polysaccharide glucuronoxylomannan could mediate protection against infection has led to substantial excitement in the cryptococcal vaccination field. A phase I clinical trial evaluating the safety, pharmacokinetics, and effectiveness on antigen elimination of a protective antibody in humans has been completed [96]. However, currently, there are no fungal vaccines or serotherapeutics available for routine clinical use.

7.2 Host Immune Function Modulation

A significant proportion of the drug resistance problems associated with cryptococcosis are related to clinical resistance. Enhancing the overall immune function of the host with ART or the reduction of immunosuppressive agents when possible for transplant and autoimmune disease patients is also likely the most effective means of preventing cryptococcosis. Effective augmentation of the host immune response along with appropriate fungicidal therapy capable of promoting rapid tissue sterilization is an ideal strategy for preventing antifungal drug resistance.

7.2.1 Cytokine Therapy

A significant amount of work in the last decade has gone toward defining host cell signaling through cytokines in addition to the potential of antibody-based therapies. Commercially available cytokines include granulocyte, granulocyte–macrophage, and macrophage colony-stimulating factors (G-CSF, GM-CSF, and M-CSF), as well as γ-interferon, interleukin 12 (IL-12), IL-18, and IL-2. These agents have produced remarkable results against *Cryptococcus* in vitro, particularly when used in combination with antifungal agents [97–101]. The best studied of the cytokines has been γ-inferferon. A clinical study comparing two different doses of γ-interferon three times per week versus no cytokine treatment as adjunctive therapy in patients receiving standard drugs for cryptococcal meningitis was conducted [102]; a second follow-up study also confirmed the improved killing of *Cryptococcus* with addition of γ-interferon treatment to antifungal drugs [103], but more

studies need to be done using cytokines in the treatment of human cryptococcosis before the clinical utility of these agents can be fully realized.

7.2.2 Antibody Therapy

Casadevall [104] has written a cogent review of antibody-based therapies for emerging infectious diseases. Theoretical benefits of antibody-based therapy include pathogen-specific targeting of therapy, toxin neutralization, the enhancement of host effector cell function, and exploitation of favorable pharmacokinetic profiles as has been seen with human IgG (i.e., long half-life, good tissue penetration, and positive safety and tolerability record). Potential problems with antibody-derived therapy include the emergence of antibody-resistant variants, triggering neutralizing antibody production and/or allergic response, limited CNS penetration, and cost. Several experimental studies have shown that monoclonal antibodies to *C. neoformans* capsular glucuronoxylomannan can enhance the therapeutic efficacy of flucytosine [105], AmB [106], and fluconazole in mouse models. Passive immunization with melanin-binding monoclonal antibodies has also been shown to improve survival and reduce fungal burden in *C. neoformans*-infected mice [107]. Although studies evaluating the safety and kinetics of adjunctive serotherapy with monoclonal antibodies for treatment of human cryptococcosis have been performed [96], human efficacy data are lacking at the present time.

7.3 Pharmacotherapeutic Strategies

Optimal pharmacologic therapy should be individualized, and several variables need to be considered when attempting to curtail the emergence of antifungal drug resistance. These variables include drug selection and dose, drug administration schedule, duration of therapy, site of infection, and host immune status. Although none of these factors have been evaluated specifically for their contribution to antifungal drug resistance in cryptococcosis, we can make some inferences based on our experience with pharmacotherapeutic efficacy and antimicrobial drug resistance in other disease states. The continued use of higher fluconazole doses for cryptococcal meningitis illustrates an attempt at therapeutic drug optimization for dosing.

7.3.1 Drug Dosing

One might hypothesize the use of less toxic antifungal drugs at high doses for as brief a time as possible would optimally reduce the emergence of resistance. AmB has transformed cryptococcal meningitis from a uniformly fatal infection to one that is potentially curable. Recent studies have suggested that treatment regimens containing a higher daily dose of AmB (0.7 mg/kg/day) are associated with more rapid CSF sterilization [13] and may decrease short-term mortality in AIDS patients with meningitis as compared with regimens employing lower doses of the polyene [108].

7.3.2 Drug Selection

One limitation to high-dose AmB has been the increased incidence of toxic side effects. Lipid preparations can be given at higher doses with fewer adverse side effects. Currently, the most clinical experience exists with liposomal amphotericin B (AmBisome) at doses of 3–6 mg/kg/day for the treatment of AIDS-associated cryptococcal meningitis. Liposomal amphotericin B appears at least clinically equivalent to conventional AmB [109].

The favorable therapeutic index of the azoles makes dose escalation an attractive option to promote cure and prevent the emergence of antifungal drug resistance. Although optimal dosing for the acute treatment of cryptococcosis is not precise, fluconazole doses of 800 mg/day for meningitis and 400 mg/day for pulmonary disease are likely to give improved results, and even higher doses of fluconazole (i.e., 1200–2000 mg/day) are being tested. A review by Duswald et al. [110] illustrates that higher daily doses of fluconazole than are currently approved may be well tolerated and improve clinical outcomes in selected patient populations for a variety of indications. Furthermore, as we better understand the relationship between MIC and clinical outcomes, the use of newer azole preparations may become important additions to the armamentarium of anti-cryptococcal agents.

In vitro studies comparing the newer triazoles to fluconazole and itraconazole against clinical isolates of *C. neoformans* have been very encouraging. Independent studies have found that the newer triazoles appear to be highly active in vitro against *C. neoformans*. Pfaller et al. [111] evaluated 566 clinical isolates from the USA and Africa and found voriconazole to be more active against *Cryptococcus* isolates than either fluconazole or itraconazole. As the fluconazole MICs increased in this study, so did the MICs of itraconazole and voriconazole. Despite this finding, 65% of the isolates with fluconazole MICs in the range of 16–32 μg/mL remained highly susceptible to voriconazole (MIC ≤ 0.12 μg/mL), and 99% of isolates with fluconazole MICs ≥ 16 μg/mL were inhibited by ≤1 μg/mL of voriconazole. These results suggest there is no automatic cross-resistance among the azoles for *C. neoformans*. Unfortunately, studies with voriconazole in patients with refractory cryptococcosis have a reported success rate of only 39% [112]. This represents a very select group of patients meeting strict criteria for treatment failure, and it is possible that certain patients are refractory to all azole therapy. Further clinical studies need to be done to confirm the promising in vitro results described with the newer triazoles for cryptococcosis. We need to better define which patients may benefit most from treatment with these agents. Future clinical research efforts should not only evaluate specific

drugs and dosing regimens but also explore the role the host immune status may have in the development of antifungal resistance.

7.4 Combination Therapy

Therapeutic regimens utilizing antifungal drug combinations offer multiple potential advantages: (1) a more rapid fungicidal response, (2) reduced resistance development, (3) enhanced spectrum of activity prior to identification of drug susceptibilities, and (4) reduced relapse rates. Several antifungal combinations have been critically evaluated for the treatment of cryptococcal disease, and the IDSA has published comprehensive practice guidelines which include the currently recommended drug combinations [32].

7.4.1 Amphotericin B Plus Flucytosine

Amphotericin B combined with 5-FC for 2 weeks, followed by 8 weeks of treatment with either itraconazole or fluconazole for the initial treatment of AIDS-associated cryptococcal meningitis, has been evaluated in a randomized double-blind multicenter trial [108]. The addition of flucytosine to induction therapy with AmB followed by fluconazole consolidation was independently associated with CSF sterilization and reduced relapse rates. Another study using quantitative yeast counts in the CSF showed that AmB plus flucytosine more rapidly sterilized the CSF of patients compared with patients receiving AmB alone, AmB plus fluconazole, or all three drugs together [113]. Additionally, a recent study showed the combination not only killed yeast faster than single-drug therapy but also had a survival benefit at 70 days [114].

7.4.2 Fluconazole Plus Flucytosine

Flucytosine plus fluconazole has also been evaluated in human studies. A prospective randomized open-label trial of 58 Ugandan patients with AIDS showed that the combination of fluconazole, 200 mg once a day for 2 months in combination with flucytosine at a dose of 150 mg/kg/day for the first 2 weeks, improved survival at 180 days as compared to fluconazole monotherapy [115]. In a noncomparative prospective open-label pilot study of 32 subjects with AIDS, the clinical success rate of fluconazole combined with flucytosine at 10 weeks was reported to be greater than previous reports of either drug alone [74].

7.4.3 Other Combinations

Amphotericin B and fluconazole given in combination is an alternative recommendation for use set forth in the IDSA treatment guidelines [32]. Recent positive studies with this combination [116] and our personal experience with polyenes and azoles administered concomitantly for the treatment of cryptococcosis have not shown antagonism nor have we noticed an increase in the incidence of antifungal drug resistance. Rex and colleagues have also shown in a well-designed, randomized and blinded multicenter trial that the combination of AmB and fluconazole was not antagonistic for the treatment of candidemia in non-neutropenic adult patients as compared to fluconazole alone [117]. Therefore, the combination of AmB plus fluconazole should be considered in locales without access to flucytosine or when toxicity develops with flucytosine.

Several other interesting drug combinations have been evaluated in vitro. Fugita and Edwards [118] have shown the combination of AmB and rifampin to be synergistic in vitro. The echinocandins have not proven to be effective against *C. neoformans* when used alone, but Franzot and Casadevall [119] showed the combination of caspofungin and AmB in vitro can be strongly synergistic. When fluconazole was combined with caspofungin in this analysis, the effects were less impressive. Barchiesi et al. [120] used checkerboard methodology to evaluate the in vitro interactions of flucytosine and posaconazole in addition to a murine model for in vivo efficacy. In this study, combination therapy with posaconazole and flucytosine was more active in vitro than either agent alone. Although a survival benefit was not demonstrated in vivo, tissue burden experiments showed a reduction in number of cryptococcal cells for those mice receiving combination therapy.

7.5 Surgical Intervention

Another strategy that may be useful, in the appropriate clinical setting, to treat drug-resistant infections is surgical excision or debulking. Surgical intervention has been described for the management of large intracerebral mass lesions >3 cm [121] and large pulmonary cryptococcomas [122], particularly with *C. gattii infections* unresponsive to conventional pharmacotherapy. Continued systemic antifungal therapy is required since surgery alone is unlikely to completely eradicate infection.

7.6 New Drug Targets and Drug Development

The development of new antifungal drugs is likely the most important long-term strategy to manage the problem of antifungal drug resistance. In addition to the agents already mentioned, there are other classes of compounds with demonstrated anti-cryptococcal activity in vitro. These novel agents diversify the range of drug targets and thus broaden therapeutic options. Several of these investigational agents will be discussed here.

7.6.1 Benzimidazole Compounds

In vitro studies have shown *C. neoformans* to be quite susceptible to selected antihelmintic benzimidazole compounds. Benzimidazoles work by binding free β-tubulin, thereby inhibiting polymerization required for the microtubule-dependent uptake of glucose. Cruz and Edlin [123] characterized β-tubulin genes and their expression in *C. neoformans*. They also identified a likely benzimidazole target in this fungal pathogen. Del Poeta et al. [124] have described two bis-benzimidazole compounds with potent in vitro activity against yeast.

7.6.2 Immunophilins and the Inhibition of Signal Transduction Pathways

The immunosuppressants cyclosporin A (CsA), FK506 (tacrolimus), and rapamycin (sirolimus) are natural products that have revolutionized the field of transplantation. These compounds are known to have antimicrobial properties and have been shown to possess activity against *C. neoformans*. Husain et al. [11] have shown that SOT recipients who developed cryptococcosis while receiving tacrolimus were statistically less likely to have CNS involvement as compared to all other transplant recipients not receiving this drug.

The immunosuppressive properties of these agents result from inhibition of cellular signal transduction pathways required for T-cell activation. All three agents diffuse into cells and bind to intracellular immunophilins present in human lymphocytes as well as yeast. CsA binds to cyclophilin A while FK506 and rapamycin bind to FKBP12. The drug-immunophilin complex targets various proteins required for signal transduction and cell proliferation. In humans as well as *C. neoformans*, the cyclophilin A-CsA and FKBP12–FK506 complexes target calcineurin, a calcium-regulated protein phosphatase [22]. Calcineurin has been shown to be essential for the virulence of *C. neoformans* and is required for its growth at 37° [22]. FKBP12–rapamycin does not affect calcineurin. Instead, the FKBP12–rapamycin complex inhibits TOR kinases integral in cell-cycle regulation [125].

Recent work has focused on identifying non-immunosuppressive analogs of these drugs and testing them in vitro against *C. neoformans* [126–128]. The results of these studies have been promising. Novel non-immunosuppressive analogs have been found and appear to retain some anti-cryptococcal activity in vitro. Further examinations of CsA, FK506, and rapamycin analogs are needed. These compounds may have promise for development as antifungal drugs for use either alone or in combination with other agents.

7.6.3 ATPase Activity and H+ Transport

The bafilomycins are a group of macrolide antibiotics that inhibit vacuolar-type proton-translocating ATPases (V-ATPases) with high affinity [129]. Bafilomycin A_1 has also been shown to inhibit plasma membrane ATPase (P-ATPase) as well as the ATP-binding cassette (ABC) transporters [130]. ATPase inhibition reduces cellular ability to withstand cation stress and has been identified as a potential new antifungal target. Manavathu and colleagues [131] examined the in vitro susceptibility of *C. neoformans* to NC1175, a novel conjugated styryl ketone with ATPase inhibitory properties in *Candida* and *Aspergillus* species. The MIC values for NC1175 were threefold to fourfold higher than those of AmB and various azoles (NC1175 $MIC_{90} = 1$ mg/L). The authors state that this compound displays fungicidal activity against *C. neoformans* in vitro, although these data were not shown, and suggest the mechanism of action is at least partly due to inhibition of P-ATPase-mediated extrusion of intracellular protons.

Studies with *Saccharomyces cerevisiae* have shown that mutants with impaired structure or function of V-ATPase were nonviable if the yeast also had cellular defects in calcineurin [132]. Del Poeta et al. [124] have taken this observation a step further and explored the in vitro effects of combining the calcineurin inhibitor FK506 or its non-immunosuppressive analog with bafilomycin A_1 against *C. neoformans*. They found that FK506 in combination with bafilomycin displayed dramatic synergistic antifungal activity. In combination, the dose of both agents could be reduced and still retain an inhibitory endpoint. This is potentially important given the immunosuppressive effects of FK506. The non-immunosuppressive analog combined with bafilomycin was not synergistic against the wild-type *C. neoformans* strains tested. Interestingly, the combination of FK506 plus caspofungin was synergistic in vitro. Again, additional studies in animal models are needed to better define the clinical potential for these novel drugs and drug combinations.

7.6.4 Sordarins

The sordarins are another class of antifungal drugs that selectively inhibit protein synthesis in a variety of yeast. Sordarin derivatives have been reported to show antifungal activity against *C. neoformans* [133]. The mechanism of action is thought to be inhibition of fungal elongation factor 2, an essential step in protein synthesis.

7.6.5 Novel Drug Combinations

Chloroquine at low concentrations has been shown to enhance the activity of human mononuclear phagocytes against *C. neoformans* [134]. The antifungal activity of chloroquine is enhanced at higher concentrations likely to be found within the acidic environment of cryptococcal phagosomes [135]. A related compound, quinacrine, was found to be between 10- and 100-fold more active against *Cryptococcus* than chloroquine on a molar basis [135]. These findings have potential clinical applicability as both drugs have proven to be safe and tolerable when administered orally, and they are available in the developing world.

Furthermore, the benefits of chloroquine in murine models of cryptococcosis have been demonstrated [136]. Experiments examining whether chloroquine and quinacrine have additive or synergistic activity when combined with other agents will bolster our understanding of the utility of these drugs, and possibly other related compounds, for the treatment of cryptococcosis.

8 Conclusions

The last 10 years have seen an explosion in laboratory, and clinical work focused on the medically important fungi, as these organisms have recently emerged as a significant group of opportunistic pathogens. With more widespread use of antifungal therapy for maintenance and/or prophylactic purposes in immunosuppressed patient populations, the problem of antifungal drug resistance is likely to continue to be an important issue. The future of cryptococcal therapy will almost certainly include novel and existing drugs used in combination to maximize fungal killing and minimize the ramifications of antifungal drug resistance. As our understanding of the molecular mechanisms of drug resistance improves, new drug targets will be identified and therapeutic strategies individualized. Conventional antifungal drugs may also eventually be combined with immunoactive cytokines or antibodies to help enhance the host's immune response to cryptococcal disease. Finally, continued clinical laboratory experience, improved microbiologic techniques, and laboratory standardization will enhance our ability to predict clinical outcome based on culture data. The foundations for a productive future in cryptococcal research and clinical care have been firmly established, and we expect the field to continue to flourish in the next few decades.

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69 Antifungal Therapy for Histoplasmosis: Focus on Susceptibility, Resistance, and Effectiveness in Humans and Experimental Infection

L. Joseph Wheat

1 Introduction

While effective therapies exist for treatment of histoplasmosis, treatment failure occurs in up to 20 % of cases with disseminated disease. The most common causes for treatment failure include inadequate drug exposure and the presence of far-advanced disease at diagnosis. Antifungal susceptibility testing is rarely performed, and consequently the role of drug resistance as a cause for failure is unknown. Resistance may cause treatment failure with fluconazole and the echinocandins. Resistance should be assessed in evaluation of new agents for treatment of histoplasmosis.

Histoplasma capsulatum var. *capsulatum* is an ascomycete from the *Arthrodermataceae* family and the causative agent of histoplasmosis. The mold form of *H. capsulatum* is found in "microfoci" containing bat or bird guano, in the soil or areas where birds or bats have inhabited. The mold consists of hyphae bearing both macroconidia and the infectious microconidia. The organism is highly endemic to certain parts of North America [1], Central America, and South America [2] and much less so in parts of Asia, Southeast Asia, China, India, Australia, Africa, and Europe.

Infection with *H. capsulatum* is initiated upon inhalation of microconidia, which germinate into yeasts upon engulfment by macrophages. The organism survives within macrophages, which transmit the yeast throughout the body. T-cell immunity is critical in recovery from histoplasmosis.

In the absence of immunocompromising conditions, most infections are asymptomatic and primarily involve the lungs. Symptoms often develop within a few weeks following heavy exposure and involve the lungs diffusely, often causing respiratory difficulty [3, 4]. This form of histoplasmosis is referred to as acute pulmonary histoplasmosis (APH). Chronic pulmonary histoplasmosis (CPH) is progressive and occurs in patients with underlying chronic obstructive pulmonary disease. Progressive disseminated histoplasmosis (PDH) usually occurs in patients with underlying immunosuppressive disorders, some of which are yet to be discovered [5].

2 Treatment

Lipid formulations of amphotericin B are the treatment of choice for more severe cases of histoplasmosis [6–8].

Amphotericin B. The mortality was 83 % in untreated patients with PDH contrasted to 23 % in those receiving amphotericin B [9]. The mortality approached 50 % in patients with the acquired immunodeficiency syndrome (AIDS) that were critically ill who were treated with the deoxycholate formulation amphotericin B [10]. Liposomal amphotericin B was more effective than deoxycholate amphotericin B in a randomized double-blind clinical trial in patients with AIDS [11]. Additionally amphotericin B induces a clinical response more rapidly than itraconazole [12–14]. Treatment usually can be changed to itraconazole within 1–2 weeks [11].

Itraconazole. Itraconazole is the preferred oral agent in patients with milder manifestations who are not felt to require hospitalization and "step-down" therapy following response to amphotericin B. In noncomparative trials itraconazole was successful in 85 to 100 % of cases CPH and APH [8]. Causes for failure of itraconazole therapy include nonadherence to therapy [15], gastrointestinal conditions that impair absorption, interactions with drugs that accelerate its metabolism or interfere with its absorption [16], and

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severe disease, due to the delay in achieving therapeutic blood levels of itraconazole [13].

Fluconazole. Fluconazole is less effective than itraconazole in histoplasmosis, based upon data from an experimental model of histoplasmosis [17] and experience in humans [8, 12]. In a study in patients with AIDS, 74 % responded to 800 mg daily, but one-third relapsed over the next 6 months after the dose was reduced to 400 mg daily [18]. Fourfold or greater increases in the minimum inhibitory concentration (MIC) to fluconazole occurred in the isolates from over half of failing patients [18]. Also, the clearance of fungemia occurred more slowly with fluconazole than with itraconazole treatment [12]. Fluconazole may be used in patients who cannot be treated with itraconazole, in whom immunity is not severely reduced.

Posaconazole. H. capsulatum is susceptible to posaconazole [19–21], and posaconazole was as effective as itraconazole and experimental infection [20, 21]. Limited information based on case reports [22, 23] and a small study [24] support these findings. Until recently, the only formulation of posaconazole that was commercially available was the oral suspension, which failed to achieve adequate serum concentrations in many patients [25]. Intravenous [26] and tablet [27] formulations are now available, which achieve therapeutic serum concentrations, overcoming this limitation.

Voriconazole. Voriconazole is active against *H. capsulatum*, but animal studies and clinical trials for treatment of histoplasmosis have not been conducted. Of note is that cross-resistance to voriconazole occurs in fluconazole-resistant strains [28]. Voriconazole has been used successfully in a small number of patients with histoplasmosis [29–32], but prior therapy with amphotericin B or itraconazole compromises assessment of its role. Voriconazole also has been used successfully in a few patients with histoplasmosis of the central nervous system [33–35]. Although voriconazole achieves higher concentrations in the CSF than itraconazole or posaconazole, the role of CSF concentration for treatment of *Histoplasma* meningitis is controversial. For example, fluconazole achieves excellent concentrations in CSF but was inferior to itraconazole and amphotericin in an experimental model of *H capsulatum* meningitis [36].

Ravuconazole. Ravuconazole is highly active against *H. capsulatum* with MICs ranging from less than 0.007 to 0.015 μg/mL [28]. Ravuconazole improved survival and reduced fungal burden to a comparable degree to itraconazole in a murine model of histoplasmosis [37]. Both were more effective than fluconazole. Clinical trials using ravuconazole for treatment of histoplasmosis have not been reported.

Isavuconazole. Isavuconazole also is active against *H. capsulatum* with MICs ranged from less than 0.0004 to 0.0063 μg/mL [38, 39]. Isavuconazole has not been evaluated in experimental models or in humans with histoplasmosis.

Echinocandins. Caspofungin [40], micafungin [41], and anidulafungin (Wheat, unpublished observation, 2004) were not active in vitro or effective treatment for experimental infection in several studies. Others reported greater in vitro susceptibility [19, 42, 43] and a more favorable outcome of experimental infection [42]. Clinical trials evaluating the echinocandins for the treatment of histoplasmosis have not been conducted.

Nikkomycin Z. Variable susceptibility was observed with nikkomycin Z, and its effectiveness in the mouse model correlated with MIC [44]. Other reported greater in vitro activity and in vivo efficacy [45, 46]. Nikkomycin Z has not been studied for treatment of histoplasmosis in humans.

Terbinafine. In vitro activity has been demonstrated with MICs below 0.39 μg/mL in 90 % of strains [47]. In vivo efficacy has been reported in animal models [48] and in patients with African histoplasmosis [49].

Combination therapy. In two studies, antagonism was noted using the combination of amphotericin B and fluconazole [17, 36]. While itraconazole was not antagonistic to amphotericin B, outcome was no better in animals receiving the combination than amphotericin B alone. Nikkomycin Z and fluconazole, used at relatively low doses, exhibited additive activity in an experimental model of histoplasmosis [45]. Micafungin and amphotericin B were evaluated in vitro and in vivo and exhibited no interaction [41]. There is no evidence that combination therapy is more effective than therapy with amphotericin B or itraconazole alone. One reason for administering itraconazole with amphotericin B for a few days before stopping amphotericin B would be to achieve therapeutic itraconazole concentrations before the antifungal effect of amphotericin B dissipated.

3 Resistance

The yeast should be tested as it represents form of the organism found in the tissues, and susceptibility to the mold does not predict susceptibility to the yeast. Susceptibility testing, however, is not available for routine testing. Modifications of the NCCLS method for yeast may be used for susceptibility testing of *H. capsulatum* [50]. Modifications include standardized of the inoculum by comparison to McFarland standard of 5 at 530 nm, which was diluted 1:100, while the *Candida parapsilosis* ATCC 90018 control was prepared according to the NCCLS method, by comparison to a 0.5 McFarland standard, and then diluted 1:2000. A second modification was prolongation of the incubation time from 96 to 120 h at 37 °C. These modifications were required because of the slow growth rate of *H. capsulatum*. Growth of *H. capsulatum* was scored by comparison to controls grown

in the absence of drug. Inhibition of at least 80 % as compared to the no drug control was defined as the MIC for the azoles only.

H. capsulatum yeast is susceptible to amphotericin B and most triazoles [20, 21, 44, 51]. Resistance has been noted with the fluconazole, voriconazole, echinocandins, nikkomycin Z, and 5-fluorocytosine. Susceptibility testing using the yeast correlated with clinical outcome in experimental models of histoplasmosis, while susceptibility using the mold did not [41, 44]. Others, however, using mold have noted greater susceptibility to the echinocandins [19, 42] and nikkomycin Z [45].

Fluconazole. Resistance as a cause for treatment failure with fluconazole has been reported [51, 52]. MICs were significantly lower in isolates from patients who responded to therapy compared to those who did not (Fig. 69.1). The median MICs were 1.0 mg/L for fluconazole, 0.015 mg/L for voriconazole, and ≤0.007 mg/L for posaconazole and ravuconazole. While the MICs to voriconazole were significantly lower than to fluconazole, they were higher than to posaconazole or ravuconazole. Of the 17 posttreatment isolates, 12 (70 %) exhibited a fourfold or greater increase in MIC to fluconazole and 7 (41 %) to voriconazole. MICs did not increase to posaconazole or ravuconazole in these isolates (Fig. 69.2).

The biochemical basis of acquired resistance to fluconazole has been examined in isolates from an AIDS patient who failed fluconazole therapy [52]. The concentration to inhibit 50 % of strains (IC_{50}) for fluconazole was threefold greater for the relapse isolate than the parent isolate. Likewise, with regard to ergosterol content, the IC_{50} for fluconazole was fivefold greater for the relapse isolate than the parent isolate. Differences in inhibition of sterol biosynthesis using [^{14}C] were also observed between the parent and relapse isolate (Fig. 69.3). Ergosterol and erosta-5, 22-diene-3-β-ol remained the predominant sterols formed in the parent and relapse isolates in the absence of drug. Inhibition of ergosterol biosynthesis by both azoles resulted in accumulation of eburicol and obtusifolione in the parent isolate, which were reduced in the relapse isolate. This suggests that the cytochrome P-450-dependent enzymes 14α-demethylase and 3-ketosteroid reductase became less sensitive to fluconazole and more sensitive to itraconazole in the relapse isolate. Comparison of the CYP51Ap amino acid sequences from a fluconazole susceptible pretreatment (MIC 1 mg/L) and a posttreatment isolate exhibiting reduced susceptibility to fluconazole (MIC 16 mg/L) identified a single substitution in the posttreatment isolate; tyrosine at position 136 was replaced by phenylalanine (Y136F) [28].

Nikkomycin Z. Nikkomycin Z was active against some strains of *H. capsulatum* and effective against one susceptible strain in experimental infection [44]. While nikkomycin Z did not prolong survival or reduce fungal burden in experimental infection with an isolate with an MIC of ≥64 μg/mL,

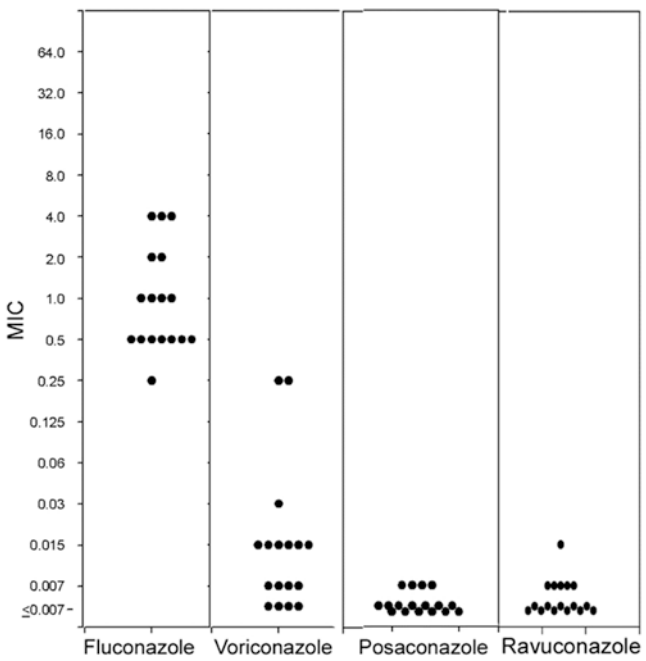

Fig. 69.1 MICs of isolates obtained at baseline before initiating treatment with fluconazole in patients with AIDS and disseminated histoplasmosis. Each *point* represents a single pretreatment isolate from individual cases. Obtained with permission of the publisher [28]

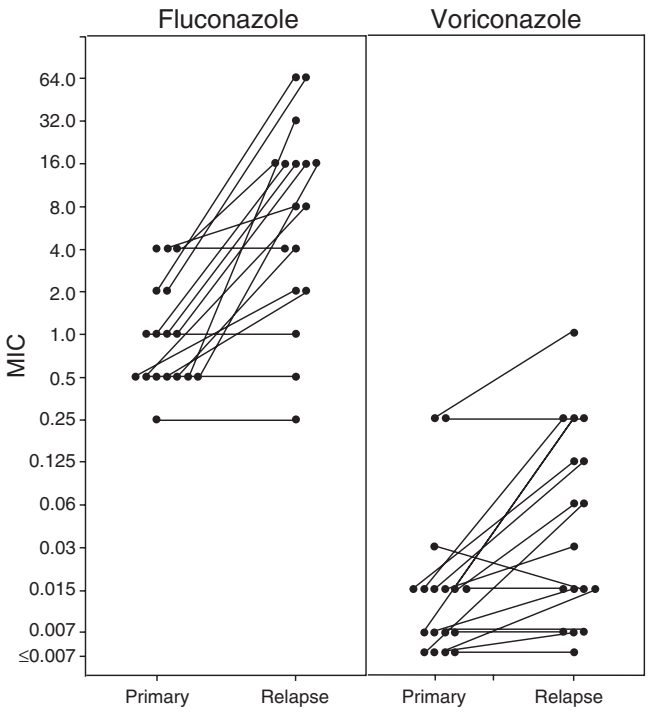

Fig. 69.2 The MIC of the pretreatment and failure isolates are connected by a *line* for each patient that failed fluconazole therapy. Pretreatment and fluconazole failure isolates remain susceptible to posaconazole and ravuconazole, median MICs of 0.007 mg/L or less (obtained with permission of the publisher [28])

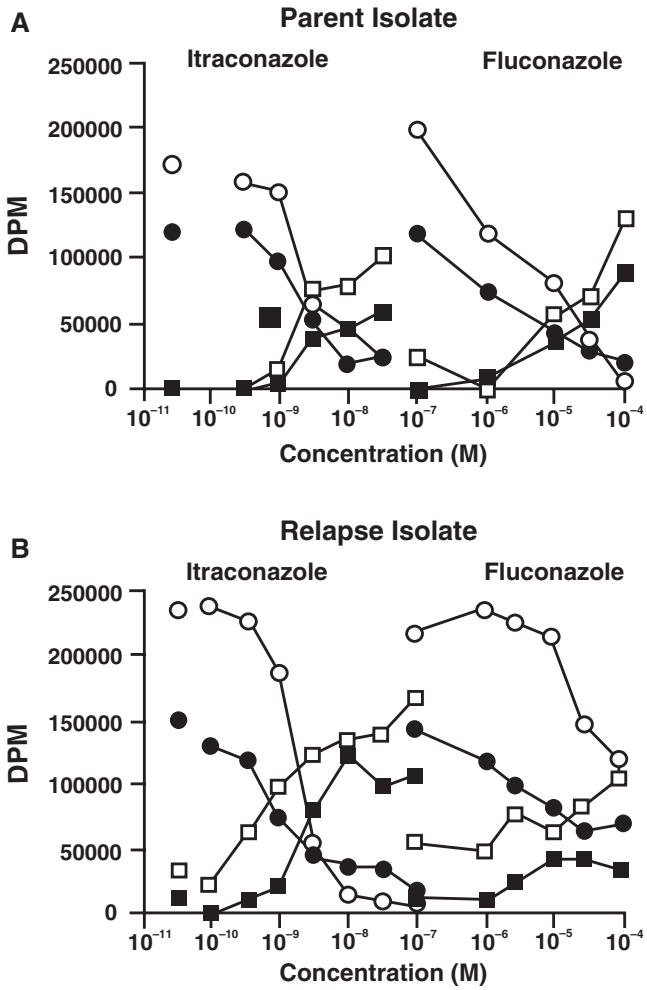

Fig. 69.3 Effects of itraconazole (ITZ) and fluconazole (FCZ) on ergosterol synthesis from [14C] acetate by the parent and relapse isolates. The sterols formed are ergosterol (*circle*), ergosta-5, 22-diene-3-ol (*filled circle*), obtusifolione (*square*), and eburicol (24-methylene-dihydrolanosterol) (*filled square*). Results for controls (ergosterol synthesis in the presence of solvent DMSO) are depicted inside the left-hand margin by the *datum points* that are not connected by *lines*. Results are mean values from four experiments (obtained with permission of the publisher [52])

nikkomycin Z was as amphotericin B against using an isolate with an MIC of 4 μg/mL. In another study using a mold with an MIC of 0.5 μg/mL, nikkomycin Z improved survival and reduced fungal burden [45].

Echinocandins. The activity of the echinocandins against *H. capsulatum* has been inconsistent. In vitro susceptibility testing using the yeast revealed MICs of 16 μg/mL or greater in over 90 % of isolates [40], and therapy was ineffective in experimental infection [40, 53]. Others reported greater in vitro activity [19, 42, 43] and effectiveness in experimental infection [42, 54]. Data on use of the echinocandin for treatment of histoplasmosis in humans is scarce. A patient receiving TNF blocker therapy treated with micafungin for presumed *Candida* sepsis died 4 days later of undiagnosed disseminated histoplasmosis [55].

5-Fluorocytosine. Resistance to 5-fluorocytosine with MICs >64 μg/mL was observed in all 20 strains tested (Wheat, unpublished observation). 5-Fluorocytosine has not been investigated in experimental histoplasmosis or in humans.

Role of melanin in response to antifungal therapy. Melanin plays a role in killing of *H. capsulatum*. Although melanized and non-melanized isolates were susceptible to amphotericin B and caspofungin, melanized strains exhibited reduced killing by these agents [56]. Melanin was postulated to reduce binding of amphotericin B and caspofungin to their targets. One possible role for combination therapy with drugs that inhibit fungal melanization and amphotericin B or echinocandin was proposed.

4 Other Agents with Antifungal Activity

Antibacterial agents. Mycelial and yeast forms were highly susceptible to trimethoprim sulfamethoxazole [57]. Isoniazid derivatives also have shown activity in vitro, including synergy with amphotericin B, which was mediated through inhibition of ergosterol synthesis [58]. Ciprofloxacin exhibited synergy with several antifungal agents for the yeast and mold form of *H. capsulatum* [59]. In vivo studies were not performed, however.

Non-antimicrobial agents. Miltefosine, developed for use in chemotherapy for malignancy, exhibited in vitro activity against the mold and the yeast form of *H. capsulatum* [60]. Maytenin and pristimerin, secondary metabolites of *Maytenus ilicifolia*, a fungus obtained from the roots of *M. ilicifolia* plants, showed antifungal activity for one reference strain and one clinical strain of *H. capsulatum* [61]. Ajoene, isolated from garlic [62], was active against mycelial form of some strains of *H. capsulatum* [63].

5 Conclusion

The liposomal formulation of amphotericin B and itraconazole are the drugs of choice for treatment of histoplasmosis. Considering in vitro susceptibility and potential for development of resistance and the availability of the tablet and intravenous formulation, posaconazole is the most promising alternative to itraconazole. Clinical trials using posaconazole and several newer triazoles for treatment of histoplasmosis are needed. Antifungal resistance limits the effectiveness of fluconazole, and potentially voriconazole, for treatment of histoplasmosis. Echinocandins have no role in treatment for histoplasmosis. Susceptibility testing and evaluation of efficacy in experimental infection are helpful in preclinical evaluation of antifungal agents and investigation of resistance as a cause for treatment failure.

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Drug Resistance in *Pneumocystis jirovecii*

70

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1 Introduction

Pneumocystis jirovecii (previously known as *Pneumocystis carinii*) is an opportunistic fungus that causes pneumonia, *Pneumocystis carinii* pneumonia (PCP), in immunocompromised individuals. Before 1982 PCP was relatively rare and primarily diagnosed among patients with congenital immunodeficiencies and patients receiving potent immunosuppressive therapy as part of an antineoplastic regimen. However with the AIDS pandemic, PCP emerged as the most common AIDS-defining diagnosis in industrialized countries. The peak incidence of PCP was observed in the late 1980s and early 1990s. Subsequently there has been a decline in the incidence of PCP because of the widespread introduction of PCP chemoprophylaxis and the introduction of increasingly potent HIV-1 antiretroviral regimens. However, PCP remains a serious opportunistic infection among heavily immunosuppressed patients who are not receiving appropriate chemoprophylaxis.

2 The Organism

Pneumocystis were identified early in the last century in guinea pigs by Chagas and in rat lungs by Carini [1, 2]. These investigators mistakenly considered the organisms as a new form of *Trypanosoma cruzi*. In 1912, *Pneumocystis* was recognized as a new species and named in honor of Carini [3]. *Pneumocystis* was first described in humans in 1942 by two Dutch investigators, van der Meer and Brug, who described it in three cases: a 3-month-old infant with congenital heart disease and in 2 of 104 autopsy cases—a 4-month-old infant and a 21-year-old adult [4]. However, *Pneumocystis* was first established as a human pathogen when Vaněk and Jirovec in 1952 identified the organism as the cause of interstitial plasma cell pneumonia among premature or malnourished infants in orphanages [5].

For most of the twentieth century, *Pneumocystis* was considered as a protozoon and single species based on its morphologic features, its resistance to classical antifungal agents, and the effectiveness of certain drugs used to treat protozoan infections. However, in 1988, based on the work by Edman and colleagues [6], phylogenetic analysis of ribosomal RNA (rRNA) sequences and observations of genome size placed *P. carinii* in the fungal kingdom. Subsequent studies including genome sequencing have confirmed that *Pneumocystis* is an *Ascomycete* fungus closely related to *Taphrina deformans* and *Schizosaccharomyces pombe* [7–12].

In contrast to most other fungi, *Pneumocystis* possesses only one copy of the nuclear ribosomal RNA locus, has a fragile cell wall, and contains no ergosterol [13]. Genomic studies suggest that *Pneumocystis* species are obligate parasites, which retrieve energy and compounds from host cells, without free-living forms [14]. During infection, two major stages have been identified morphologically: cysts (asci) and trophic forms (previously called trophozoites). The cyst has a cell wall which primarily consists of β-1,3 glucan, while the trophic form has no detectable β-1,3 glucan [15].

Pneumocystis organisms have been identified in most mammalian species in which it has been searched for. Genetic and antigenic analyses have shown that *Pneumocystis* includes a broad family of organisms, with species specificity among its mammalian hosts [13, 16, 17]. Remarkably, the level of genetic divergence between *Pneumocystis* organisms infecting different mammals is greater than the degree

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of divergence observed between certain fungi classified as distinct species [18, 19]. Phylogenetic comparisons of DNA sequences in organisms from 18 different nonhuman primate species have demonstrated that sequence divergence correlates with the phylogenetic difference between the host species, which suggests that *Pneumocystis* species have evolved together with their hosts [20].

Thus unique species of *Pneumocystis* appear to infect each unique mammalian host species. To date, rats are the only species that have been demonstrated to be infected by two unique *Pneumocystis* species. The organism infecting humans has been renamed *Pneumocystis jirovecii*, in honor of Otto Jirovec, who was among the first to describe the microbe in humans [5, 21, 22].

3 Transmission and Infection

Since *P. jirovecii* cannot be cultured in vitro, knowledge about its biology has been difficult to obtain. However, the development of molecular and immunologic techniques, including sequencing the genome from one *P. jirovecii* isolate, has permitted considerable insight into this organism and how it interacts with its host. Based on antibody and PCR findings, primary infection with *P. jirovecii* happens in very early childhood (<1 year of age) with a uniformly high incidence in all geographic areas, which suggests that *P. jirovecii* organisms are ubiquitous [23].

It was previously thought that the infection was carried lifelong and that clinical infection was a result of reactivation in immunocompromised hosts. However, molecular typing studies have questioned this view and support a more complex picture of transmission and infection.

The acquisition of infection in humans is most likely the result of person-to-person spread [24, 25]. When the organism is obtained initially as a primary infection, it is not clear whether an immunocompetent host develops a transient disease. Various investigators have proposed that primary infection might correlate with the development of upper or lower respiratory manifestations, or with the development of sudden infant death syndrome [26–28]. Following primary infection, the presumption, based on murine models, has been that the organism becomes latent, later manifesting clinically if the patient becomes profoundly immunosuppressed.

More recent data, however, suggests that human hosts can be infected with more than one strain of *Pneumocystis jirovecii*, raising the possibility that infection can be acquired on multiple occasions, leading to latency with a variety of distinct organisms [29–35]. Further, typing studies of outbreaks of PCP, especially in renal transplant patients, have provided compelling evidence that a single strain spreading among susceptible hosts can be responsible for such outbreaks. The clinical disease PCP may, therefore, occur as a reactivation of a prior latent organism, or as a result of recent acquisition of an airborne pathogen [36, 37].

Since most infants acquire antibody against *Pneumocystis* during the first year of life, the organism must be ubiquitous. Nonhuman animals are not the source, however, because, as mentioned above, each animal species is infected with a different species of *Pneumocystis*, and there is no cross species infection that has been identified. From PCR-based studies, it is now clear that infants and immunocompetent adults frequently experience colonization and likely constitute the major reservoirs for *P. jirovecii* [38]. Recently, several renal transplant centers have reported rising incidence of PCP with genotype studies showing evidence of patient-to-patient transmission, which suggest that iatrogenic exposure of immunocompromised patients is an increasing problem [39, 40].

Pneumocystis has specific tropism for the lung, where it exists in the alveoli. In rare cases organism has been detected in other organs, but it seldom causes disease at extrapulmonary sites. After inhalation, the organism attaches tightly to the surface of type I alveolar cells [41]. Adherence is potentially mediated by the major surface glycoprotein (MSG) [42, 43]. This protein is the most abundant antigen on the surface of *Pneumocystis* and is encoded by a multicopy gene family. MSG represents a family of proteins that are highly polymorphic, repeated, and distributed among most chromosomes of *Pneumocystis*. MSG provides *Pneumocystis* with a mechanism for antigenic variation by switching the expression of multiple MSG genes, with a system that resembles one used for antigenic variation in *Trypanosoma cruzi* [44, 45]. It is likely that this antigenic variation in MSG serves for avoiding the host immune response [46]. There is no detailed knowledge of the life cycle and the mode of replication has not been definitely established, but both asexual and sexual life cycles have been proposed [47, 48]. Recently, several genes, which in other fungi are involved in mating, pheromone responsiveness, and responses to environmental changes, have been demonstrated in *Pneumocystis*, suggesting that the organism has a sexual replication cycle that responds to environmental changes in the lung [49–51].

4 Drug Treatment

The major drug classes used for treatment and prophylaxis of PCP include antifolate drugs, diamines, atovaquone, and macrolides (Tables 70.1 and 70.2). Most traditional antifungal agents have no activity against *Pneumocystis*, likely due to the absence of ergosterol, which is the target of amphotericin B as well as azoles. As *Pneumocystis* was originally believed to be a protozoon, initial drug testing focused on drugs with activity against protozoan infections.

Table 70.1 Regimens for prophylaxis against *Pneumocystis* pneumonia

Drug	Oral or aerosol dose
First choice	
Trimethoprim-sulfamethoxazole	1 DS or SS daily
Alternatives	
Trimethoprim-sulfamethoxazole	1 DS three times per week
Dapsone	50 mg twice daily or 100 mg daily
Dapsone with pyrimethamine plus leucovorin	50 mg daily 50 mg weekly 25 mg weekly
Dapsone with pyrimethamine plus leucovorin	200 mg weekly 75 mg weekly 25 mg weekly
Pentamidine aerosolized	300 mg monthly via Respirgard II nebulizer system
Atovaquone	1500 mg daily
[a]Pyrimethamine plus	25–75 mg qd
Sulfadiazine	0.5–2.0 g q6h

DS double strength = 800 mg sulfamethoxazole, 160 mg trimethoprim, *SS* single strength = 400 mg sulfamethoxazole, 80 mg trimethoprim
[a]This regimen only for use in case of concurrent toxoplasmosis

In 1958, pentamidine isethionate was the first drug used to successfully treat PCP [52]. In the 1960s, the combination of sulfadoxine and pyrimethamine was used for the prevention of epidemic infantile pneumocystosis in Iran [53]. In 1966, Rifkind treated two patients with sulfadiazine and pyrimethamine: both patients died, but two patients were successfully treated 4 years later [54]. Between 1974 and 1977 studies led by Hughes et al. established that the combination of trimethoprim-sulfamethoxazole (TMP-SMX) is effective for both treatment and prophylaxis of PCP [55–57]. TMP-SMX is as effective as intravenous pentamidine for therapy and is still the treatment of choice. Additionally, TMP-SMX is the most effective chemoprophylaxis for PCP and therefore the standard for prevention.

Other drugs have proven activity for therapy, including sulfadiazine plus pyrimethamine, dapsone plus trimethoprim, atovaquone, clindamycin plus primaquine, and trimetrexate. Dapsone, dapsone-trimethoprim, atovaquone, and aerosolized pentamidine have documented efficacy in prophylaxis in patients at high risk for developing PCP. Clindamycin-primaquine has not been shown to be effective for chemoprophylaxis. Anecdotal reports and case series in pediatric populations suggest that intravenous pentamidine may be effective [58, 59]. There are other drugs that have in vitro activity or anecdotal anti-PCP activity in humans and could have a role in managing human disease if all other alternatives were not feasible. These include azithromycin, doxycycline, and echinocandins; the latter class of drugs, which target beta 1,3-glucan synthesis, have activity against the cyst but not the trophic form in animal models.

5 Prophylaxis

Among HIV-infected patients, the occurrence of PCP is closely related to the CD4 count: the lower the CD4 count, the more likely PCP is to develop. While a count of 200 cells/mm^3 is often used as an indicator or susceptibility, HIV-infected patients do in fact develop PCP at counts higher than 200 cells/mm^3, although at a lower frequency than at 200, 100, or 50 cells/mm^3.

Patients with congenital immunodeficiencies, particularly X-linked immunodeficiency with hyper-immunoglobulin M and SCID, patients receiving long-term and high-dose corticosteroid therapy, and patients receiving certain chemotherapeutic regimens for cancer therapy or transplantation are at risk of developing PCP. Among HIV-negative patients, risk factors for PCP include coexisting pulmonary disease with CMV infection, pre-existing lung disease, the use of certain anti-cytokine antibodies (e.g., adalimumab), and in particular lymphocyte-depleting agents such as alemtuzumab, fludarabine, or rituximab [60–66]. In addition, genetic factors may also contribute to risk. In patients without HIV, CD4 counts are not a reliable marker of susceptibility. Several studies have shown that the occurrence of PCP is not as predictable with these markers in diseases unrelated to HIV [62].

Systemic chemoprophylaxis against PCP was introduced by Dutz in Iran in the early 1960s. He showed that outbreaks of PCP could be aborted with the use of sulfadoxine plus pyrimethamine [67]. Hughes et al. followed this observation with a classic study of children with acute lymphocytic leukemia (ALL); they showed that PCP could be virtually eliminated by TMP-SMX prophylaxis [68]. Subsequently this prophylaxis was used for other populations of cancer and transplant recipients with a very high success rate. With the advent of the AIDS epidemic, PCP prophylaxis was used sporadically in the 1980s. After publication of a convincing study by Fischl et al., PCP prophylaxis became a standard of care for HIV-infected patients with CD4 counts less than 200 cells/mm^3 in 1989 [68]. The identification of additional risk factors for the development of PCP has led to expanded recommendations for the use of PCP chemoprophylaxis—details are provided in Table 70.3. HIV-1-infected patients with oral candidiasis or a CD4 count less than 200 cells/μL should be offered primary prophylaxis. Secondary prophylaxis should be offered to all patients following an episode of PCP. In HIV patients receiving prophylaxis, prophylaxis can safely be discontinued if immune function is improved above a CD4 count of 200 cells/μL for at least 3 months following antiretroviral therapy. If the patient subsequently fails antiretroviral therapy and the CD4 declines to below 200 cells/μL, prophylaxis should be restarted.

In non-HIV-infected individuals, conditions such as organ transplantation, high-dose corticosteroid treatment,

Table 70.2 Drug regimens for the treatment of PCP

Drug	Route	Dose	Toxicity	Advantages	Disadvantages
First choice					
Trimethoprim-sulfamethoxazole	By mouth	2 DS every 8 h	Rash and fever	Superior efficacy	Rash common
			Anemia and neutropenia	Inexpensive	
	Intravenous	Trimethoprim 5 mg/kg with sulfamethoxazole 25 mg/kg every 8 h	Hyperkalemia	Oral and iv	
			Hepatitis	Bacterial and anti-toxoplasmosis activity	
			Nephritis		
			Anaphylactoid reaction		
Alternatives					
Dapsone plus trimethoprim	By mouth	100 mg daily	Rash, nausea and vomiting, fever	Inexpensive	No i.v. formulation
	By mouth	320 mg every 8 h	Methemoglobinemia, leukopenia, and hemolytic anemia		
			Liver function abnormalities, headache		
			Dapsone may cause hemolysis in patients with G-6PD		
Clindamycin plus primaquine	By mouth, intravenous	450–600 mg every 6 h	*Clostridium difficile* diarrhea, nausea and, vomiting. Primaquine may cause hemolysis in patients with G-6PD deficiency		No i.v. formulation for primaquine
	By mouth	30 mg daily			
Pentamidine	Intravenous	4 mg/kg day	High incidence of adverse effects, particularly hypoglycemia and nephrotoxicity	Highly effective	Toxicity common. Only i.v. formulation
			Pancreatitis and dysglycemias		
			Hypotension with short infusion time		
			Pancytopenia, QT prolongation		
Atovaquone	By mouth	750 mg twice daily	Rash, nausea, diarrhea, and headache (20 %)	Well tolerated	Expensive
			Fever, increased transaminases and neutropenia		Useful for mild disease
Adjunctive therapy					
Prednisone in patients with room air $pAO_2 < 70$ mmHg (9.3 kPa)	By mouth, intravenous	40 mg twice daily for 5 days		Standard of care for moderate or severe disease	Metabolic problems especially glucose and electrolyte changes
		40 mg daily days 6–11			
		20 mg daily days 12–21 while on anti-PCP therapy			

and/or high-dose chemotherapy may confer a high risk of PCP. Prophylaxis should be offered as shown in Table 70.3. Several prophylactic regimens are available. The most efficient, cheap, and widely used regimen is daily TMP-SMX. TMP-SMX prophylaxis is relatively well tolerated by most non-HIV patients; in contrast, HIV patients have a high frequency of adverse effects, in particular rash and myelosuppression. Before the advent of antiretroviral therapy, 50 % of patients experienced an adverse effect after 12 months of prophylaxis with double-strength TMP-SMX (160/800 mg), and half would have switched to other types of prophylaxis within 3 years [69]. Fortunately, one single-strength (80/400 mg) TMP-SMX daily appears to be equally effective and is associated with fewer side effects than one double-strength tablet daily [70]. Because of its efficacy, ease of administration, and cost, every effort should be tried to maintain patients at risk of PCP on TMP-SMX. Tolerability may improve with lower-dose or intermittent regimens. For patients who have had nonlife-threatening reactions to TMP-SMX (e.g., not Stevens-Johnson syndrome), it can be safely reintroduced in many patients by dose escalation [71, 72].

Table 70.3 Recommendations for PCP prophylaxis and risk identification in selected diseases

Disease	Risk identification	Duration of prophylaxis	Comment
HIV-1 infection	Prior PCP		Prophylaxis improves survival
	CD4 cell count <200	Lifelong unless CD4 count >200 × >*3 months due to ART*	Restart prophylaxis if CD4 count falls to <200
	Oropharyngeal candidiasis		
	CD4 cell count <14 %		
	Prior AIDS-defining illness		
Organ transplantation		*General: Minimum 6 months after transplantation*	
Kidney	Depends on intensity of immunosuppression and occurrence of graft versus host disease or rejection	At least 6 months	Need for PCP prophylaxis determined by clinical experience. CD4 count is *not* a reliable predictor
Lung		Indefinitely	
Heart/liver		6–12 months	
Autologous BMT		6–12 months	
Allogeneic BMT		Minimum 1 year	
Rejection		Reinstate	
Graft versus host disease		Reinstate	
Malignancy			
Acute lymphoblastic leukemia (ALL)	During and subsequent to combination chemotherapy	During severe immunosuppression	Need for PCP prophylaxis determined by clinical experience with each chemotherapeutic regimen. CD4 count is not a reliable predictor
		Continue during maintenance therapy for childhood ALL	
Chronic lymphatic leukemia (CLL)	Treatment with fludarabine or alemtuzumab (Campath, anti-CD52)	3–6 months post-chemotherapy	
Lymphoma	Certain chemotherapeutic regimens, e.g., R-CHOP14, escalated BEACOPP, nucleoside analogs	Minimum 2 months after discontinuation or until CD4 > 200	

BMT bone marrow transplantation, *ART* antiretroviral therapy

6 Treatment of PCP

Untreated PCP is invariably fatal. In the beginning of the HIV epidemic, the mortality rate of PCP was reported to be 30–40 % [73, 74], increasing to 70–90 % among patients who progressed to respiratory failure [75]. During the 1990s, mortality rates dropped to 5–15 % [76–81]. This appears to be a consequence of earlier recognition of the infection, the introduction of adjuvant corticosteroids to patients with moderate-to-severe PCP as defined by a PaO_2 of less than 70 mmHg, better diagnostic and therapeutic abilities related to concomitant processes, and improved ICU supportive measures.

The importance of educating patients to seek medical attention early, when symptoms are still mild, must be an emphasis of patient management programs. Both patients and health-care professionals must recognize that mild symptoms such as dyspnea, cough, or low-grade fever can be the initial manifestation of PCP, especially in patients with CD4+ T lymphocyte counts below 200 cells/mm^3. Thus, clinicians should not wait for all features of PCP to be present, or for the chest radiograph to be abnormal, before initiating a workup for PCP. Moreover, once there is a high suspicion, therapy should be instituted promptly if the diagnostic procedures will be delayed.

The choice of specific chemotherapy is also important. The most potent drugs for PCP treatment are antifolate drugs, which act by blocking de novo synthesis of folates through inhibition of dihydropteroate synthase (DHPS) or dihydrofolate reductase (DHFR) (Fig. 70.1).

DHPS catalyzes the condensation of p-aminobenzoic acid (PABA) and hydroxymethyl dihydropterin pyrophosphate to produce dihydropteroate, which is later converted to dihydrofolate by dihydrofolate synthase. Subsequently, dihydrofolate is reduced by dihydrofolate reductase (DHFR) into tetrahydrofolate. Sulfa drugs are structural analogs of PABA and inhibit DHPS.

The earliest clinical trials to treat PCP were performed with sulfadiazine plus pyrimethamine on the assumption that

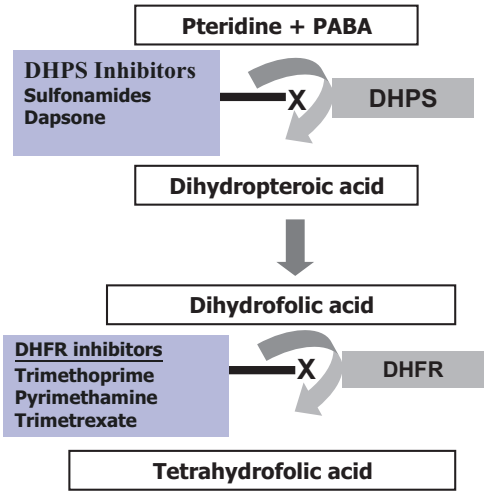

Fig. 70.1 Inhibition of folate synthesis by sulfonamides and DHFR inhibitors. *PABA* paraaminobenzoic acid, *DHPS* dihydropteroate synthase, *DHFR* dihydrofolate reductase

these drugs would have synergistic action against pneumocystis, as against plasmodia. When the commercial combination of sulfamethoxazole plus trimethoprim was developed to treat bacterial infections, this preparation was assessed for PCP therapy and prophylaxis since commercial sponsorship of studies could be obtained. At that time there was no knowledge about the relative potency of various sulfonamide preparations against pneumocystis, nor was there information about the relative potency of various DHFR inhibitors. Subsequently, it was found that sulfamethoxazole is probably as potent as any of the other commercially available sulfonamide preparations as discussed below [82, 83]. However, trimethoprim is not as potent as other available DHFR inhibitors, as also described below.

In Table 70.2, drug treatment options for PCP are listed together with the most important advantages and toxicities of each drug regimen. During the 1980s several trials investigated the efficacy of TMP-SMX compared to pentamidine [84–87]. In the only noncrossover trial ($n=70$) [86], TMP-SMX was associated with a better survival than pentamidine. However, when all trials are considered, TMP-SMX and pentamidine appear to have roughly comparable efficacy [84]. Drug toxicity occurs in 24–57 % of HIV-infected patients treated with TMP-SMX [88].

Adverse effects generally occur after 7 days of therapy and most commonly include rash, fever, and leukopenia. Hepatotoxicity characterized by elevated transaminases also occurs. There are cases of sulfamethoxazole-induced interstitial nephritis, renal calculus formation, anaphylactoid reactions, and pancreatitis reported. Trimethoprim can be associated with hyperkalemia. These toxicities are usually not life threatening, although fatal cases of Stevens-Johnson syndrome have occurred.

Pentamidine is associated with a high frequency of toxicities, some of which are treatment limiting. Early experiences with rapid infusions of pentamidine were associated with hypotension and death, so this route of administration was abandoned. Intramuscular injections were better tolerated in terms of blood pressure, but they caused a high frequency of sterile abscesses. Therapy was then administered by slow intravenous infusion, which is the best tolerated route. Inhaled pentamidine has been used for therapy and is well tolerated, but efficacy is poor. Pentamidine is nephrotoxic and causes predictable glomerular and tubular damage to the kidney. Pentamidine is toxic to the pancreas: its initial effects cause a surge of insulin release that often manifests as hypoglycemia, followed by the development of hyperglycemia. Hypoglycemia can occur days or weeks after starting therapy and may occur many days after stopping therapy. Leukopenia can also occur. Pentamidine prolongs the QT interval, and cases of torsades de pointes have been reported. Treatment-limiting toxicities with pentamidine treatment occur in 13–80 % of patients.

Alternatives for therapy to TMP-SMX and pentamidine include dapsone-trimethoprim, clindamycin-primaquine, and atovaquone (Table 70.2). Trimetrexate has activity but is no longer commercially available. Dapsone has not been studied as a single drug and thus should not be use alone for treatment. Dapsone-trimethoprim is effective, however, and probably has potency that is comparable to TMP-SMX. However, since this combination does not come as a fixed-dose combination, is only available orally, and cross-reacts with sulfa in 50 % of allergic patients, this regimen does not offer many advantages over TMP-SMX.

Clindamycin-primaquine appears to work on a different metabolic pathway than TMP-SMX. Two comparative trials of clindamycin/primaquine with TMP-SMX in moderate-to-severe PCP demonstrated apparent equivalence for clindamycin-primaquine, but both trials were underpowered [89, 90]. A retrospective observational study suggests that this regimen should be preferred for treatment of PCP, if TMP-SMX is not tolerated [91]. Clindamycin causes a relatively high incidence of hepatitis, rash, and diarrhea in HIV-infected patients. Primaquine can only be given orally.

Atovaquone is well tolerated and acts on a different metabolic pathway from TMP-SMX. However, this drug is also only available orally and does not appear to be as effective as TMP-SMX [92]. This is a good alternative for patients with mild disease who cannot tolerate TMP-SMX.

Efficacy of dapsone-trimethoprim has only been demonstrated for mild-to-moderate PCP and for atovaquone only for mild PCP [89, 92–94]. Both must be administered orally.

The optimal duration of therapy for PCP has never been properly studied. Usual recommendations are that HIV-negative patients should receive 2 weeks and HIV-positive patients should receive 3 weeks of drug treatment.

Many patients experience progressive oxygen desaturation during the first 4–5 days of therapy. This deterioration appears to be caused by the drug-induced death of *Pneumocystis* organisms with exacerbation of alveolar inflammation. This inflammation can be reduced by corticosteroids. Four randomized controlled trials demonstrated that corticosteroids could reduce mortality in HIV-infected patients with moderate or severe disease [95–98]. Based upon these results, adjunctive steroids are now recommended for all HIV patients with severe disease ($PaO_s < 70$ mmHg). In non-AIDS patients, the situation is often complicated by prior use of corticosteroids, which are themselves a risk factor for development of PCP. In this setting steroid use must be individualized to balance the beneficial anti-inflammatory effects with the potentially harmful immunosuppressive effects.

7 Sulfonamide Resistance

The widespread use of TMP-SMX and dapsone for therapy and prophylaxis of PCP among HIV patients has led to the concern that sulfa (sulfonamide or sulfone) resistance could develop in *P. jirovecii*.

In many pathogenic bacteria and parasites, resistance to sulfonamides has increased as a consequence of selective pressure and has limited the efficacy of sulfonamides [99]. Widespread use of sulfa drugs for malaria and bacterial infection in Africa has produced high rates of resistance in *P. falciparum* and many bacterial species [100]. In San Francisco, the increasing use of PCP prophylaxis among HIV patients led to a marked increase in trimethoprim-sulfamethoxazole resistance among isolates of *Staphylococcus aureus* and seven genera of *Enterobacteriaceae* [101]. In a retrospective study, trimethoprim-sulfamethoxazole resistance was more than twice as likely in blood culture isolates from HIV patients receiving trimethoprim-sulfamethoxazole compared to patients not receiving this prophylaxis [102].

In pathogens, such as *Escherichia coli*, *Neisseria meningitidis*, *Mycobacterium leprae*, and *Plasmodium falciparum*, sulfonamide resistance is caused by mutations in the primary sequence of the DHPS gene [103–105]. The mutations that confer resistance are localized within a highly conserved active site of the DHPS protein. In *Pneumocystis*, the DHPS protein is part of a trifunctional protein along with dihydroneopterin aldolase and hydroxymethyl dihydropterin pyrophosphokinase, which together are encoded by the multidomain *FAS* gene [106].

In 1997, Lane and co-workers were the first to identify non-synonymous (resulting in changes in the encoded amino acid) DHPS mutations in *Pneumocystis jirovecii* [107]. The most frequent DHPS mutations occur at nucleotide positions 165 and 171, which lead to an amino acid change at positions 55 (Thr to Ala) and 57 (Pro to Ser). The homologous Thr and Pro are highly conserved across species, including *Pneumocystis* infecting other hosts. Thus these variants appear to represent true mutations rather than allelic polymorphisms. The Th55 is homologous to Thr62 of *E. coli* DHPS, which, based on its crystal structure, binds the pterin substrate. It is hypothesized that the Thr55Ala and Pro57Ser affect the position of Arg56 (whose homologue in *E. coli* is involved in binding pterin as well as sulfa drugs), decreasing its ability to bind sulfa drugs and resulting in a consequent reduction in sulfa drug sensitivity [108, 109].

Either mutation can occur alone. However frequently, both mutations are seen in the same isolate. While the association with sulfa exposure is consistent with the concept that these mutations represent resistance that developed under drug pressure, documenting resistance is very difficult, in part because *Pneumocystis* cannot be cultured and in part because functional enzymes (recombinant or native) are not readily available.

Saccharomyces cerevisiae has been used as a model to study *P. jirovecii* DHPS resistance. The DHPS enzyme of *S. cerevisiae* has high functional and genetic similarity to the DHPS of *P. jirovecii*. This enzyme from *Saccharomyces* is also trifunctional. By site-directed mutagenesis, the in vitro effects of mutations identical to the DHPS mutations in *P. jirovecii* can be investigated. Using this model two studies reported that the double DHPS mutations Thr55Ala and Pro57Ser result in an absolute requirement for PABA, consistent with resistance being associated with altered substrate binding [110, 111]. Interestingly, the single mutation Pro57Ser conferred resistance to sulfadoxine, which is supported by clinical observations suggesting a specific association of this mutation with sulfadoxine resistance in PCP [110]. However, one study showed an increase in sensitivity of the double mutations to sulfamethoxazole, suggesting that this approach may not accurately reflect the effect of these mutations in *P. jirovecii*.

Several clinical studies have investigated the frequency and significance of DHPS mutations in *P. jirovecii*. Table 70.3 provides a summary of studies reporting frequencies of mutations in sulfa-exposed and sulfa-unexposed patients. Although the studies vary considerably in size (13–158 patients) and in definitions of sulfa exposure, a clear association between previous exposure to sulfa drugs (primarily for prophylaxis rather than therapy) and DHPS mutations has been shown in most studies. Large geographical variation in the prevalence of DHPS mutations has been reported, ranging from 0 to 100 % of isolates. In the USA, the incidence of mutations was lower in Indianapolis and Denver compared to San Francisco, where one study reported that more than 80 % of patients were infected with mutant strains [112]. Wide variations have also been observed in studies from Europe with a particularly low incidence in Italy: in one study an 8 % frequency of mutations was found among 107

HIV patients with isolates obtained between 1994 and 2001 [113]. Mutations have rarely been found in clinical isolates obtained prior to the early 1990s but seem to have increased in frequency recently presumably as a consequence of increasing selective pressure caused by the widespread use of sulfa drugs for prophylaxis (they were widely used for treatment in the 1980s) of PCP [114–116]. Importantly, DHPS mutations have also been increasingly found in patients without any previous exposure to sulfa drugs, suggesting person-to-person spread of mutant strains.

Based on a genetic analysis of multiple loci, it appears that the mutations arose independently in multiple strains of *Pneumocystis* [117]. In a genotype study of 13 European HIV patients with recurrent episodes of PCP, a switch from wild-type to mutant DHPS occurred in five of seven patients who had a recurrence of the otherwise same molecular type of *P. jirovecii* [118]. All patients had received treatment or secondary prophylaxis with trimethoprim-sulfamethoxazole or dapsone. These findings suggest that DHPS mutants may be selected in vivo (within a given patient) under the pressure of trimethoprim-sulfamethoxazole or dapsone. In addition, recent genotype studies also provide evidence that antibiotic pressure causes changes in DHPS mutant frequency [119]. The emergence of DHPS mutations appears to be specific for *P. jirovecii*, because only wild-type *Pneumocystis* DHPS has been found in other primate species [120].

The clinical significance of DHPS mutations, specifically with regard to response to prophylaxis and therapy using a sulfa-based regimen (primarily trimethoprim-sulfamethoxazole or dapsone), has been controversial. Several studies have reported a significant association of DHPS mutations with failure of low-dose sulfa prophylaxis (Table 70.4). However, the extent to which this association reflects actual drug resistance or failure to comply with prescribed prophylaxis is unknown. Hence, in spite of the emergence of mutant DHPS strains, current clinical experience supports the efficacy of trimethoprim-

Table 70.4 Prevalence of DHPS mutations and association with sulfa exposure

Study	Country (year)	No. of DHPS mutations/no. of PCP episodes	DHPS mutations/ sulfa exposed	DHPS mutations/ no sulfa exposure	Risk ratio (95 %CI)
Kazanjian et al. [115]	USA (1983–2001)	58/145 (40 %)	38/56	20/89	3.0 (2.0–4.6)
Ma et al. [121]	USA (1985–1998)	16/37 (43 %)	11/16 (69 %)	3/15 (20 %)	3.4 (1.2–9.9)
Helweg-Larsen et al. [116]	Denmark (1989–1999)	31/152 (20 %)	18/29 (62 %)	13/123 (11 %)	5.9 (3.3–10.6)
Alvarez-Martinez et al. [122]	Spain (1989–2004)	17/98 (17 %)	15/44	2/54	9.2 (2.2–38.1)
Hauser et al. [123]	Switzerland/France (1990–2000)	69/305 (20 %)	24/34	45/271	4.3 (3.0–6.0)
Visconti et al. [33]	Italy (1992–1997)	7/20 (35 %)	3/4	3/14	3.5 (1.1–11.1)
Nahimana et al. [124]	France (1993–1996)	57/158 (36 %)	25/29	32/129	3.5 (2.5–4.9)
Santos et al. [83]	France (1993–1998)	11/20 (55 %)	5/5 (100 %)	3/12 (25 %)	4. 0 (1.5–10.7)
Takahashi et al. [31]	Japan (1994–1999)	6/24 (25 %)	2/3 (33 %)	4/24 (19 %)	4.0 (1.2–13.3)
Ma et al. [113]	Italy (1994–2001)	9/107 (8 %)	6/31	3/76	4.9 (1.3–18.3)
Costa et al. [32]	Portugal (1994–2001)	24/89 (27 %)	5/16 (31 %)	19/73	1.2 (0.5–2.7)
Valerio et al. [37]	Italy (1994–2004)	14/154 (9 %)	4/38	10/116	1.2 (0.4–3.7)
Beard et al.[a] [35]	USA (1995–1998)	152/220 (69 %)	Np	Np	Na
Huang et al.[a] [30]	USA (1996–1999)	76/111 (69 %)	57/71 (80 %)	19/40 (48 %)	1.7 (1.2–2.4)
Totet et al. [125]	France (1996–2001)	0/13	0/0	2/13	Na
Zingale et al. [34]	Italy (1996–2002)	25/64 (39 %)	21/29	4/35	6.3 (2.5–16.4)
Wissmann et al. [126]	Brazil (1997–2004)	0/57	0/5	0/52	Na
Yoon et al.[a] [127]	USA (1997–2008)	232/301 (77 %)	Np	Np	2.87 (1.33–6.19)[b]
Kazanjian et al. [115]	China (1998–2001)	0/15	0/0	1/15	Na
Van Hal et al. [128]	Australia (2001–2007)	8/60 (13 %)	2/8	6/52	2.2 (0.5–8.9)
Dini et al. [129]	S. Africa (2006–2007)	85/151 (56 %)	Np	Np	Na
Taylor et al. [130]	Uganda (2007–2009)	13/13 (100 %)	Np	Np	Na
Long et al. [131]	China (2008–2011)	0/20	Np	Np	Na
Deng et al. [132]	China (2009–2013)	3/25 (12 %)	0/0	3/25	Na
Sheikholeslami et al. [133]	Iran (2010–2011)	5/34 (15 %)	Np	Np	Na
Monroy-Vaca et al. [134]	Brazil (2010–2013)	3/16 (18 %)[c]	0/0	3/16	Na

Only studies with more than ten patients included. Sulfa exposure: Current or previous exposure to sulfone drugs (trimethoprim-sulfamethoxazole, dapsone, or sulfadiazine) at diagnosis of PCP. *Np* not provided, *Na* not applicable

[a]Overlap of patients

[b]Adjusted odds ratio

[c]Only colonization

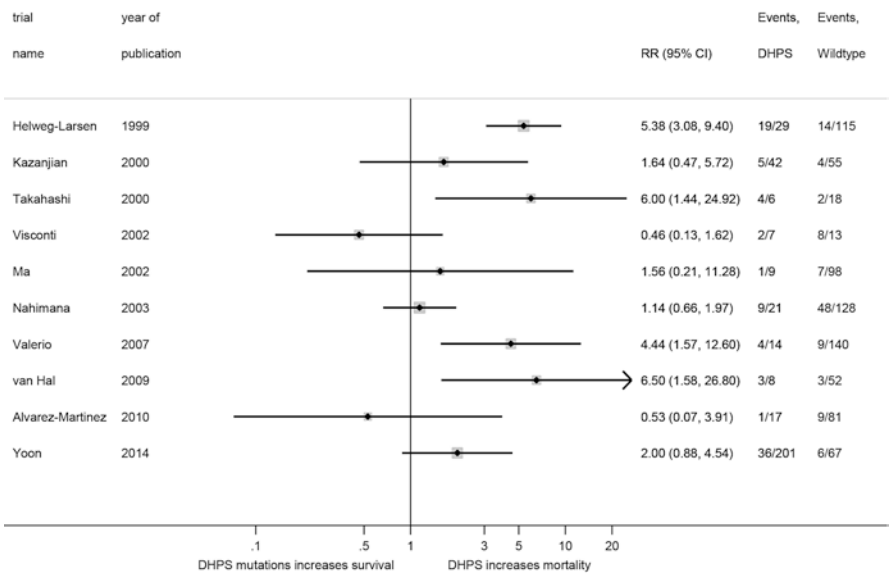

Fig. 70.2 Risk of death following PCP, comparing DHPS mutation to wild type in published observation studies. Forest plot of DHPS mutations and survival showing relative risk of deaths with 95 %CI. DerSimonian random-effect analysis

sulfamethoxazole prophylaxis when taken regularly. However, there is evidence to suggest a contributory role for DHPS mutations in breakthrough PCP in patients using alternative sulfa prophylaxis. Hauser et al. found a significant association with failure of pyrimethamine-sulfadoxine prophylaxis and the Pro57Ser mutation: all 14 patients failing this type of prophylaxis harbored this mutation [124]. Further, relatively high numbers of prophylaxis failures associated with DHPS mutations have been described in patients receiving dapsone prophylaxis. Thus, available data currently suggest that DHPS mutations contribute to low-level sulfa resistance and may be most important in failure of second-line sulfa prophylaxis. However, the major reason for PCP breakthrough continues to be poor adherence to chemoprophylaxis [135].

Studies assessing the impact of DHPS mutations on response to therapeutic, high-dose trimethoprim-sulfamethoxazole have been conflicting, as shown in Fig. 70.2. While initial case reports suggested that patients with mutant DHPS strains had increased risk of failing sulfa therapy or prophylaxis [136], subsequent studies have been more conflicting. A Danish study of 152 patients with AIDS-related PCP found that the presence of DHPS mutations was an independent predictor of decreased 3-month survival, when compared to patients harboring wild-type DHPS [116]. However, several subsequent studies have found no certain evidence for increased mortality when comparing patients with DHPS mutation to wild type [112, 124, 127]. There are several possible reasons for the discrepancy between the studies, including methodological differences in the definitions of survival endpoints or prophylaxis and treatment failures, or other confounding factors related to the difficulties in assessing clinical resistance (see Box 1). Moreover, even in studies reporting an association of DHPS mutations with failure of sulfa therapy, the majority of patients with mutant DHPS strains have been successfully treated with trimethoprim-sulfamethoxazole or dapsone/trimethoprim. These observations suggest that the currently identified DHPS mutations may confer only low-level sulfa resistance, allowing PCP to occur in the setting of prophylactic doses of sulfa drugs, that is overcome by the higher doses used for therapy. Given that *Pneumocystis* has already demonstrated an ability to mutate under antibiotic pressure, a major concern is that additional mutations may develop that produce high-level resistance.

Box 70.1. Limitations to the Study of Drug Resistance in *Pneumocystis*

Compared to other pathogenic fungi, the study of drug resistance in *P. jirovecii* has been and continues to be difficult. In spite of many attempts, there exists no in vitro culture system for propagation of *Pneumocystis*. The absence of a culture system precludes standard susceptibility testing and has greatly limited the understanding of many fundamental aspects of the organism and impeded investigations into mechanisms of drug resistance. Because knowledge of the metabolic pathways is limited, most drug development has been empiric, and the currently available treatment options for PCP have been unchanged during the last 15 years. Experimental systems have mainly relied on immunosuppressed animal, in particular the rat model of *Pneumocystis*.

Another problem is that no consistent definition of clinical failure exists. In other fungal infections, clinical resistance is classically defined as persistence or progression despite the administration of appropriate

(continued)

antimicrobial treatment. However, this definition is problematic when applied to PCP. First, persistence of *Pneumocystis* organisms may happen in spite of a successful treatment response. Studies using repeat bronchoscopy during and immediately after successful treatment of PCP have shown that clearance of organisms is slow, with approximately half of patients still harboring *Pneumocystis* at the end of 3 weeks of treatment in spite of a successful treatment response [137–140]. Although infection is eventually cleared and the viability of organisms detected at end of treatment is uncertain, it is clear that detection of organisms during or at the end of treatment cannot be interpreted as a proxy for resistance. Second, host inflammatory response rather than resistance to antimicrobial drug treatment may cause an apparent absence of response to treatment. PCP is characterized by marked pulmonary inflammation that in severe cases results in alveolar damage and respiratory failure. Although an efficient immune response is required to control the infection, it has also been demonstrated that an excessive inflammatory response, rather than direct effects of *Pneumocystis* organisms, is crucial for the pulmonary injury [141, 142]. Therefore, a severe inflammatory response with respiratory distress, rather than drug resistance, may cause treatment failure. Third, treatment of PCP is associated with a high incidence of adverse effects including fever. In clinical practice it may be difficult to know whether a slow treatment response with continuing fever is caused by the infection or by the treatment. Given the difficulties in defining clinical failure, reported failure rates for primary trimethoprim-sulfamethoxazole treatment in AIDS patients have varied considerably, ranging from 10 to 40 % of cases [53, 76, 78].

In addition, the contribution of non-adherence in presumed failure of prophylaxis may be difficult to assess. The most important reason for prophylaxis failure continues to be non-adherence to prescribed prophylaxis [134, 143, 144]. Clinical resistance has been investigated by genotyping of *P. jirovecii* isolates from patients who develop PCP in spite of prescribed chemoprophylaxis. However, in most studies assessment of adherence to prophylaxis has been based on chart reviews, which may fail to disclose non-adherence to a drug regimen. The likelihood of developing *P. jirovecii* resistance within a patient is likely to be higher with inadequate or interrupted dosing. Hence, in theory resistance mutations could be markers of poor adherence, rather than the direct cause of treatment failure.

8 DHFR Resistance

The diaminopyrimidines, trimethoprim and pyrimethamine, are competitive inhibitors of dihydrofolate reductase (DHFR), which catalyzes the reduction of the biologically inactive7,8-dihydrofolatetotheactive5,6,7,8-tetrahydrofolate in the presence of NADPH and is essential for biosynthesis of purine/pyrimidine nucleotides, thymidylate, and certain amino acids. They are used in combination with sulfonamides.

Interestingly, in animal models trimethoprim does not add any potency to sulfonamides and thus may not be contributing at all to the anti-PCP efficacy of TMP-SMX [145]. The amino acid sequence of DHFR from *P. jirovecii* differs from rat-derived *P. carinii* by 38 %. Ma and Kovacs evaluated the activity of DHFR inhibitors by using a yeast assay expressing *P. jirovecii* DHFR and observed that the human *Pneumocystis*-derived DHFR had a ~tenfold increase in sensitivity to trimetrexate and trimethoprim compared to rat *Pneumocystis*-derived DHFR. For the human *Pneumocystis*-derived DHFR yeast strain, trimethoprim and pyrimethamine were both weak inhibitors, with IC_{50}s in the micromolar range; trimetrexate was about tenfold and 40-fold more potent than trimethoprim and pyrimethamine, respectively (Table 70.5). Given that trimetrexate is much more potent against PCP than trimethoprim in vitro, the combination of trimetrexate and sulfamethoxazole may be a more potent combination than trimethoprim plus sulfamethoxazole. However, there are currently no clinical data to support this, and as noted above, trimetrexate is no longer commercially available.

In several bacterial and parasitic species, resistance to DHFR inhibitors has emerged as a consequence of selective pressure by DHFR inhibitors. In this way, resistance of *P. falciparum* and *P. vivax* to pyrimethamine has emerged and is now widespread [147]. However, despite the widespread use of trimethoprim in combination with sulfamethoxazole for prevention and treatment of PCP, only relatively few DHFR mutations have been identified in *Pneumocystis* DHFR [121, 148–150]. Ma et al. detected only a single synonymous DHFR mutation in specimens obtained from 32 patients, of whom 22 had previous exposure to TMP-SMX therapy or prophylaxis [121]. Takahashi et al. found four mutations in *P. jirovecii* DHFR from 27 patients,

Table 70.5 50 % inhibitory concentrations (IC_{50}) of DHFR inhibitors from a yeast complementation assay [146]

	IC_{50} (nM)	
DHFR inhibitor	Human-derived *P. jirovecii* DHFR	Rat-derived *P. carinii* DHFR
Trimethoprim	5700	81,000
Pyrimethamine	20,500	33,200
Trimetrexate	490	4200

of whom only three had previous exposure to TMP-SMX [148]. Two of these mutations were non-synonymous and were not associated with prior exposure to TMP-SMX. In both studies patients were successfully treated with TMP-SMZ. Nahimana et al. documented non-synonymous substitutions in 9 of 15 patients receiving a DHFR inhibitor as part of their prophylactic regimen compared to 2 of 18 not receiving a DHFR inhibitor [149]. Interestingly, 5 of 7 patients receiving pyrimethamine had non-synonymous substitutions, suggesting a greater selective pressure of this drug. A South African study found non-synonymous DHFR mutations in samples obtained between 2001 and 2003 in 3 of 27 patients. None had long-term exposure to TMP-SMX before developing PCP [130]. Finally, Matos and co-workers from Portugal reported a 27 % rate of DHFR mutations in 128 PCP episodes, without associated failure of PCP prophylaxis [151].

In conclusion, although several studies have reported DHFR mutations, there is so far no evidence that the widespread use of trimethoprim or pyrimethamine has caused emergence of clinical significant resistance to DHFR inhibitors.

8.1 Atovaquone

Atovaquone (2-[*trans*-4-(4′-chlorphenyl)cyclohexyl]-3-hydroxy-1,4-hydroxynaphthoquinone) is used to prevent and treat disease caused by *P. jirovecii*, *Plasmodium* spp., *Toxoplasma gondii*, and *Babesia* spp. [152]. Atovaquone is structurally similar to the mitochondrial protein ubiquinone (coenzyme Q) and competitively binds to the cytochrome bc_1 complex. The bc_1 complex catalyzes electron transfer from ubiquinone to cytochrome c and thereby proton translocation across the mitochondrial membrane resulting in the generation of ATP. Binding of atovaquone to the ubiquinol oxidation pocket of the bc_1 complex and the Rieske iron-sulfur protein disrupts electron transport and leads to collapse of the mitochondrial membrane potential [153]. Eventually, this presumably results in depletion of ATP within *Pneumocystis* and leads to killing of the organism [154]. Mutations of the cytochrome b gene have been identified in *Plasmodium* spp., *Toxoplasma gondii*, and *Pneumocystis*. In vitro studies of *Plasmodium* and *Toxoplasma* show that these mutations confer resistance to atovaquone. Because *Pneumocystis* cannot be propagated in vitro, similar susceptibility testing cannot be done. In vitro studies of the *Saccharomyces cerevisiae* cytochrome bc_1 complex and atovaquone have demonstrated binding to the ubiquinol pocket. Introduction of mutations near the binding pocket led to decreased activity of atovaquone [153]. Introduction of seven mutations observed in isolates of *Pneumocystis* from atovaquone-experienced patients into *S. cerevisiae* cytochrome b increased the inhibitory concentration from 25 to >500 nM [155, 156].

Results from two clinical studies have been published. In the first, sequencing of the cytochrome b gene of *Pneumocystis* from ten patients showed sequence variations in four patients [157]. Three of four patients receiving atovaquone as prophylaxis demonstrated such variations. Notably, two of them had non-synonymous changes leading to amino acid substitutions within the ubiquinol pocket. Similar mutations in other microorganisms are associated with resistance to atovaquone. One patient who had not received atovaquone prophylaxis had a synonymous change that did not confer any change in amino acid sequence. In the second study, a nested case-control study, significantly more patients who previously had been exposed to atovaquone (5 of 15 patients) had mutations than unexposed patients (3 of 45) [158]. Five different mutations near the ubiquinol pocket were described bringing the total number to seven. The high number of mutations is unusual but may be explained by a higher mutational rate and impaired proof-reading of mitochondrial genes. Survival from PCP did not differ between patients with or without mutations. Overall, these findings are consistent with the development of atovaquone resistance after selective pressure is exerted.

8.2 Pentamidine and Clindamycin-Primaquine

Pentamidine and clindamycin/primaquine are used for prevention and treatment of PCP, but possible resistance mechanisms have yet to be discovered and reported.

9 Conclusion

In spite of the inability to culture the organisms, it is now clear that mutations involved in sulfa and atovaquone drug resistance have emerged in *P. jirovecii* as a result of selective pressure by the widespread use of PCP prophylaxis. Currently the clinical effect of the described mutations seems modest. DHPS mutations at codon 55 and 57 are implicated in failure of low-dose sulfa prophylaxis, but there is so far no firm evidence that DHPS mutations result in significant resistance to high-dose sulfa therapy. However, it is possible that if additional mutations arise, then high-level sulfa resistance could emerge and lead to diminished efficacy of TMP-SMX. This would lead to the loss of the most efficient and inexpensive therapy for PCP.

The increasing HIV epidemic and use of TMP-SMX in the third world may significantly increase the risk for development of high-level resistance [129]. Therefore, investigations into the mechanisms of drug resistance and

identification of new molecular targets are continuing. The recent sequencing of the genomes of *Pneumocystis* species, especially *P. jirovecii*, has increased our understanding of the biology of the organism and it's metabolic requirements, and has identified a number of new pathways that appear critical to growth and survival of the organism, which thus are potential new targets for drug development. Better understanding of the organism's biology may eventually also lead to the development of a functional culture system [7, 159].

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Part X

Viral Drug Resistance: Clinical

Antiviral Resistance in Influenza Viruses: Clinical and Epidemiological Aspects

71

Erhard van der Vries and Michael G. Ison

1 Introduction

There are three classes of antiviral drugs approved for the treatment of influenza: the M2 ion channel inhibitors (amantadine, rimantadine), the neuraminidase (NA) inhibitors (laninamivir, oseltamivir, peramivir, zanamivir), and the protease inhibitor (favipiravir); some of the agents are only available in selected countries [1, 2]. These agents are effective at treating the signs and symptoms of influenza in patients infected with susceptible viruses. Clinical failure has been demonstrated in patients infected with viruses with primary resistance, i.e., antivirals can be present in the virus initially infecting the patient, or resistance may emerge during the course of therapy [3–5]. NA inhibitors are active against all nine NA subtypes recognized in nature [6], including highly pathogenic avian influenza A/H5N1 and recent low-pathogenic avian influenza A/H7N9 viruses [7]. Since seasonal influenza is usually an acute, self-limited illness in which viral clearance usually occurs rapidly due to innate and adaptive host immune responses, the emergence of drug-resistant variants would be anticipated to have limited effect on clinical recovery in otherwise healthy patients, as has been demonstrated clinically [3, 8, 9]. Unfortunately, immunocompromised or immunologically naïve hosts, such as young children and infants or those exposed to novel strains, are more likely to have mutations that confer resistance emergence during therapy; such resistant variants may also result in clinically significant adverse outcomes [10–13].

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Factors that influence the clinical and epidemiologic importance of drug-resistant influenza viruses include the magnitude of phenotypic resistance, its frequency and rapidity of emergence, its stability and ability of resistant variants to compete with wild-type virus in the absence of selective drug pressure, and the effects of resistance mutations on viral replication competence, pathogenicity, and transmissibility in vivo. Prior to being replaced by the pandemic 2009 A/H1N1 virus, most circulating seasonal A/H1N1 viruses in the 2008–2009 season contained the His275Tyr mutation and were therefore highly resistant to oseltamivir while retaining susceptibility to zanamivir. All currently circulating A/H1N1 and A/H3N2 viruses have mutations conferring M2 inhibitor resistance. There is no data to date indicating that M2 inhibitor resistance is associated with worsened viral virulence, atypical influenza, or enhanced transmissibility. Sporadic viruses with primary resistance mutations resulting in neuraminidase inhibitor resistance have been described. Most but not all NA mutations conferring resistance in clinical isolates have been associated with reduced infectivity, replication, and pathogenicity in animal models of influenza. Such features are important not only in clinical management of individual patients but also are key factors that need to be considered by health authorities and governments when making decisions regarding the stockpiling of antivirals for response to pandemics or other influenza threats [14, 15]. Concerns about antiviral resistance, particularly to NA inhibitors, should not dissuade countries from developing adequate antiviral inventories for pandemic response [14, 16].

The frequency of resistance emergence during therapy is higher with M2 inhibitors than NA inhibitors. Development of resistance during the course of therapy was very common among initially M2 inhibitor-susceptible viruses in the past. Mutations in one of five amino acids in the M2 gene result in cross-resistance to both amantadine and rimantadine. Resistance emergence during therapy with neuraminidase inhibitors generally remains rare for circulating strains of A/H1N1, A/H3N2, and B viruses, with higher rates in children

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and immunocompromised patients. A common feature of patients who develop resistance despite ongoing therapy is high-level replication and longer duration of replication in the presence of antiviral therapy. Resistance to neuraminidase inhibitors results from mutations in the neuraminidase gene, the hemagglutinin gene, or both. The specific mutation determines the degree of resistance and which neuraminidase inhibitor has reduced susceptibility. Further, the frequency and magnitude of NA inhibitor resistance vary with drug, virus, and neuraminidase type and subtype. Resistance has been demonstrated to develop during the course of therapy with avian viruses, particularly A/H7N9, with greater frequency than seasonal human viruses. Compensatory mutations may also occur that improve the fitness and transmissibility of resistant viruses and may play a role in establishing persistent transmission, as was demonstrated with the seasonal A/H1N1 during the 2007–2008 and 2008–2009 seasons.

Several new classes of antivirals, many with novel mechanisms of action, are currently undergoing development. Resistance mutations have been identified for many of these agents from in vitro passage experiments, but clinical evidence of resistance emergence is still in its infancy. The following sections review clinical and epidemiological data on antiviral resistance for the three classes of available anti-influenza agents. Information from experimental animal models of influenza is incorporated to supplement the limited data derived from clinical studies.

2 M2 Ion Channel Inhibitors (Amantadine, Rimantadine)

The M2 ion channel allows the influx of protons into the viral particle which, in turn, facilitates uncoating [17]. M2 inhibitors bind to the M2 ion channel and limit the influx of protons resulting in its antiviral effect. Since the M2 protein is present only on influenza A viruses, M2 inhibitors have no activity against influenza B [17]. There are currently two approved M2 ion channel inhibitors, amantadine and rimantadine. Early studies demonstrated that influenza variants with high-level resistance to amantadine and rimantadine could be selected in the laboratory though in vitro and in vivo passage in virus in the presence of the drug [3, 18]. Studies of resistance helped to determine the mechanism of antiviral action of the M2 inhibitors [19].

Mutations in the M2 inhibitor gene at one of five commonly recognized sites (position 26, 27, 30, 31, or 34 of the M2 protein) in human viruses result in reduced binding of the M2 inhibitors or in enlargement of the pore diameter. The function of the M2 pore of viruses with any of the mutations is preserved in the presence of the inhibitor [3, 20, 21]. Resistance mutations do not affect transmissibility or replication fitness as compared to wild-type viruses; documented transmission from person-to-person has been well established [22]. Resistance affects both drugs in the class equally and appears to be persistent over time [3].

During routine treatment with M2 inhibitors for documented influenza, resistant variants emerge frequently. The clinical implications of resistance became apparent in studies during the 1980s of treated children, in whom a high frequency of resistance emergence was documented, and subsequently of households and nursing homes, where transmission of drug-resistant variants was implicated in failures of drug prophylaxis [23–26]. About 30 % of adults treated with M2 inhibitors will have resistant variants detected during the course of their illness with high frequency (up to 80 %) of resistance emergence in immunocompromised patients, patients hospitalized for influenza, and children [8, 23, 25, 27, 28]. Until recently, the frequency of M2 inhibitor resistance among seasonal isolated was low (1–3 %) [29]. Since 2002, though, the prevalence of resistance to M2 inhibitors among circulating influenza A/H3N2 increased globally, and now the majority of A/H3N2 globally is resistant to this class of drugs [29, 30]. Resistance has resulted from the S31N substitution of the M2 inhibitor. M2 inhibitor resistance has also been documented in several important novel strains of influenza: A/H5N1, A/H7N9, and 2009 pandemic A/H1N1 virus [31–36]. Most clade 1 A/H5N1 viruses and all swine-origin A/H1N1 are resistant to the M2 inhibitors as a result of the S31N substitution, while most (~80 %) of clade 2.1 A/H5N1 are resistant secondary to S31N or V27A substitution [31, 32, 37]. Of note, most of the clade 2.2 and 2.3 A/H5N1 viruses remain susceptible to M2 inhibitors [37]. Since most circulating strains of influenza are currently resistant to the M2 inhibitors, this class is not recommended for the prevention or treatment of influenza currently [2].

2.1 Detection of Resistance

There are currently no rapid tests that can screen for and identify the presence of M2 inhibitor resistance. M2 resistance may be diagnosed using phenotypic assays or gene sequencing. Most phenotypic assays, including plaque reduction, yield reduction, and ELISA, utilize the growth of virus in cell culture exposed to a range of concentrations of the drug of interest; these assays are not widely available. Pyrosequencing methods for rapid analysis of mutations in the M2 gene associated with resistance have been described and are used in several reference laboratories [21, 38]. Neither assay is typically available in most clinical labs. As a result, most clinicians rely on data generated from groups

actively monitoring the resistance among circulating strain—in the United States this is actively done by the Centers for Diseases Control and Prevention (http://www.cdc.gov/flu/professionals/antivirals/antiviral-drug-resistance.htm).

Detection of M2 inhibitor resistance has usually relied on virus isolation from respiratory samples and susceptibility testing of virus in cell culture. Several assays have been described including plaque reduction, yield reduction, and ELISA [39]. Following phenotypic analysis, genotypic M2 inhibitor resistance has been confirmed by nucleotide sequence analysis of the M2 gene and detection of the characteristic mutations. Genotypic detection can be accomplished quickly by the use of PCR restriction length polymorphism (RFLP) analysis of RNA extracted from respiratory samples using commercially available endonucleases for discrimination of point mutations in the M2 gene [38, 40]. Greater sensitivity in detecting resistant clones has been described with reverse transcription-polymerase chain reaction amplification of RNA followed by sequencing of multiple clones [4, 38, 41]. Recently, the rapid pyrosequencing technique has been shown to be a reliable, high-throughput method for detecting genotypic resistance in large numbers of community isolates [4, 29, 38].

2.2 Susceptibility of Field Isolates

Historically, human isolates of influenza A/H1N1, A/H2N2, and A/H3N2 were initially susceptible to amantadine and rimantadine [19, 24, 39, 42]. Even after licensure of the M2 inhibitors, there were low levels of primary resistance in community isolates (see Table 71.1). This began to change when field isolates of A/H3N2 viruses from China were noted to have a significant increase in the resistance to the M2 inhibitors, possibly related to increased use of over-the-counter amantadine after the emergence of severe acute respiratory syndrome (SARS) [38]. During the 2004–2005 influenza season, approximately 70 % of the A/H3N2 isolates from China and Hong Kong and nearly 15 % of those from the United States and Europe showed resistance due to a Ser31Asn mutation, and this frequency increased to over 90 % in the United States during the 2005–2006 season [29, 38]. Since then, most clinical isolates of A/H3N2 were noted to have Ser31Asn mutations in the M gene conferring resistance to M2 inhibitors (Table 71.1). This spread occurred despite the absence of sustained selective drug pressure, possibly because the resistant M gene was incorporated into efficiently spreading HA antigenic variants. Phylogenetic

Table 71.1 Representative studies of M2 inhibitor susceptibility of influenza A field isolates from adults and children

	Site	Period	Method	No. tested by subtype	No. (%) resistant
Belshe et al. [24]	United States	1978–1988	EIA, S	65 H1N1	0
				181 H3N2	5 (2.0 %)[a]
Valette et al. [202]	France	1988–1990	EIA	28 H1N1	0
				77 H3N2	0
Ziegler et al. [203]	43 countries	1991–1995	EIA, S, PCR-RFLP	2017	16 (0.8 %)[b]
Dawson [204]	UK	1968–1999	EIA, plaque	1813	28 (1.5 %)
Suzuki et al. [205]	Japan	1993–1998	Not stated	55	0
		1999–2000	Not stated	179	6 (3.4 %)
Shih et al. [206]	Taiwan	1996–1998	Plaque, S	84	1(1.2 %)
Bright et al. [38]	Global	1994–2005	S	6525	392 (6.0 %)
		1994–2002		H3N2	0.3–1.8 %
		2003–2005			12.3–13.3 %[c]
		1998–2004		589 H1N1	2 (0.3 %)
Bright et al. [29]	United States	2005	S	205 H3N2	193 (92.3 %)
				8 H1N1	2 (25 %)
Saito et al. [207]	Japan	2005–2006	S	354 H3N2	231 (65.3 %)
				61 H1N1	0
Barr et al. [43]	Australia, New Zealand, Asia, South Africa	2005	S	102 H3N2	43 (42 %)
				37 H1N1	0

Abbreviations: *S* M2 gene sequence analysis, *PCR-RFLP* polymerase chain reaction-restriction length polymorphism, *EIA* enzyme immunoassay

[a]All resistant viruses from family members receiving rimantadine

[b]Over 80 % of tested isolates were H3N2 subtype and all resistant ones were of this subtype. Separate analysis found that 9 (4.5 %) of 198 strains from Australia, 1989–1995, were resistant

[c]In 2004–2005 the frequencies of resistance in H3N2 viruses were 73.8 % in China, 69.6 % in Hong Kong, 22.7 % in Taiwan, 15.1 % in South Korea, 4.3 % in Japan, 30.0 % in Canada, 19.2 % in Mexico, 14.5 % in United States, and 4.7 % in Europe

analyses of the HA1 and M2 genes have suggested a common origin of these viruses [43]. This experience clearly indicates that this resistance mutation does not reduce transmissibility and is stable over time.

The frequency of resistance in seasonal A/H1N1 viruses increased from 2005 to 2007, primarily due to the Ser31Asn mutation [29, 30]. Fortunately, the incidence of primary resistance declined in 2008 and 2009 among seasonal A/H1N1 viruses as oseltamivir-resistant viruses predominated [44]. This seasonal A/H1N1 virus, which was replaced by the 2009 pandemic A/H1N1 virus, was primarily resistant to the M2 inhibitors generally due to the Ser31Asn mutation [44]. As a result, all currently circulating strains of influenza A are primarily resistant to the M2 inhibitors, and this class of drug is not recommended for the prevention or treatment of influenza [2].

M2 proteins show considerable evolution in human and swine viruses, and the H3 and H1 subtype viruses have phylogenetically different M2 proteins [45]. This may influence the mutations that are more advantageous for conferring M2 inhibitor resistance. A characteristic feature of A/H1N1, A/H1N2, and A/H3N2 swine viruses circulating in Europe since 1987 has been the presence of Ser31Asn mutation, as well as Lys27Ala in some isolates, that confers resistance to M2 inhibitors [46]. The postulated role of swine as intermediate hosts in the emergence of some novel human viruses and direct interspecies transmission from birds may be another mechanisms for a reassortment event leading to acquisition of an M gene encoding resistance in a human strain [47, 48].

Although the initial human isolates of highly pathogenic avian A/H5N1 viruses in Hong Kong in 1997 were M2 inhibitor susceptible, resistance to this class of drugs has become more prevalent [32, 37]. Most clade 1 A/H5N1 viruses are resistant to the M2 inhibitors as a result of the Ser31Asn substitution, while most (~80 %) of clade 2.1 A/H5N1 are resistant secondary to Ser31Asn or Val27Ala substitution [32, 37]. Of note, most of the clade 2.2 and 2.3 A/H5N1 viruses remain susceptible to M2 inhibitors [37]. Isolates of A/H7N9 infected humans have also had the Ser31Asn mutation conferring resistance to the M2 inhibitors [49, 50].

2.3 Resistance in Posttreatment Isolates

Studies in experimentally infected animals and treated humans have documented the common emergence of resistant variants as the course of infection progresses over time. Following treatment, approximately 70–90 % of amino acid substitutions in resistant viruses occur at position 31, and about 10 % each are found at positions 27 and 30 [40]. The Ser31Asn mutation has been responsible for the resistant A/H3N2 and A/H1N1 variants recently identified globally [29, 38].

2.3.1 Animal Studies

The rapid emergence of resistant variants in M2 inhibitor-treated patients has been found also in studies of experimentally infected animals. In a study of a chicken A/H5N2 virus, resistant viruses are detectable by 2–3 days after starting drug administration and persisted thereafter [51]. A study in ferrets inoculated with a human influenza A/H3N2 virus detected M2 inhibitor resistance mutations in four of nine amantadine-treated animals by day 6 after inoculation; in each instance two or more M2 gene mutations were identified [52].

2.3.2 Immunocompetent Patients

Resistant variants arise commonly and rapidly in M2 inhibitor-treated children and adults with acute influenza (Table 71.2). One study of adults found that resistant virus could be detected in 50 % of six rimantadine recipients by day 3 of treatment, although the nasal lavage titers were lower than in placebo recipients shedding susceptible virus [27]. Another study found that 33 % of 24 adult and pediatric household members receiving rimantadine shed resistant virus on day 5 of treatment; none were positive when tested 5 days later [27]. A larger pediatric trial found emergence of resistant virus in 27 % of 37 rimantadine recipients, including 45 % of those still virus positive on day 7, compared to 6 % of 32 acetaminophen recipients [23]. Resistant virus was detected as early as day 3 in one child but was usually present on days 5–7. A study of Japanese children treated with amantadine found that 30 % of 81 in the 1999–2000 season and 23 % of 30 during the following season had resistant virus detected on day 3–5 after a 3-day course [53]. Resistant

Table 71.2 Recovery of resistant influenza A during M2 inhibitor treatment

Study	Seasons	Patient group	Treatment	No. treated	No. (%) shedding resistant viruses
Hall et al. [23]		Children	Rimantadine	37	10 (27 %) H3N2
Hayden et al. [25]	1987–1989	Children	Rimantadine	21	6 (29 %) H3N2
Hayden et al. [27]	1988–1989	Adults	Rimantadine	13	5 (38 %) H3N2
Englund et al. [28]	1993–1994	Immunocompromised	Amantadine, rimantadine	15	5 (33 %) H3N2
Saito et al. [53]	1999–2001	Children	Amantadine	111	22 (33 %) H3N2
					9 (20 %) H1N1
Shirashi et al. [41]	1999–2001	Children (hospitalized)	Amantadine	15	8 (100 %) H3N2
					4 (57 %) H1N1

variants were detected more frequently in A/H3N2-infected children (33 %) than in A/H1N1-infected children (20 %). Another study employing sensitive molecular cloning detection methods found mutations conferring resistance in 80 % of 15 hospitalized children during or immediately after amantadine treatment [41]. Nine (75 %) of 12 children had 2–4 resistance mutations detected in clones from a single sample, sometimes mixed with wild-type virus. In a randomized study patients hospitalized with influenza were to receive either rimantadine alone or rimantadine plus nebulized zanamivir [54]. Rimantadine-resistant virus was detected in 2/20 (10 %) of rimantadine monotherapy patients, while non-resistant variants were detected in the 21 patients receiving combination therapy [54].

2.3.3 Immunocompromised Hosts

Resistant influenza A viruses may be shed for prolonged periods in immunocompromised hosts, who can serve as a reservoir for nosocomial transmission. One study of adult bone marrow transplant and acute leukemia patients recovered resistant virus in 5 (33 %) of 15 M2 inhibitor-treated patients and in 5 (83 %) of 6 patients with illness who shed virus for ≥3 days [28]. The median time between the first and last virus isolation was 7 days with range up to 44 days. Death associated with influenza occurred in 2 of 5 (40 %) patients with resistant virus, compared to 5 of 24 (21 %) without, and prolonged illness was noted in several with protracted shedding. Other reports have documented prolonged shedding of resistant variants in immunocompromised hosts with or without continued drug exposure, including one transplanted SCID child who shed for 5 weeks and one adult leukemia patient who shed resistant virus for ≥1 week of therapy [55]. Another case report documented recovery of resistant virus >1 month after cessation of a course of amantadine, as well as shedding of mixtures of wild-type virus and variants with different resistance genotypes [56]. Heterogeneous populations of resistant variants with sequential or dual mutations have been found in several immunocompromised hosts [28, 55]. One stem cell transplant recipient shed dually M2 inhibitor and oseltamivir-resistant virus for at least 5 months and probably over 1 year [57]. The prolonged shedding of resistant variants in immunocompromised hosts is consistent with the genetic stability of such variants observed in experimental animal models [51].

2.4 Transmissibility of Resistant Variants

The transmissibility of M2 inhibitor-resistant viruses has been demonstrated in animal models and in several clinical settings. Competition-transmission studies with an avian A/chicken/Pennsylvania/1370/83 A/H5N2 virus compared the transmissibility of wild-type virus with resistant variants possessing M2 substitutions at positions 27, 30, or 31 [51]. Contact birds shedding resistant virus due to earlier incorporation of amantadine in the drinking water of donors (4 days only) were caged with birds shedding susceptible virus, and the virus was allowed to transmit through three more sets of contact birds in the absence of selective drug pressure. Resistant virus was detected from the final set of contact birds in three of four experiments over four cumulative transmission cycles.

2.4.1 Households

Both amantadine and rimantadine are effective for postexposure prophylaxis of illness due to susceptible strains in household contacts, when ill index cases are not given concurrent treatment (Table 71.3). In contrast, two studies have found no significant reduction in secondary influenza illness in household contacts receiving either amantadine or rimantadine for postexposure prophylaxis, when the ill index cases received treatment with the same drug, and one of these documented failures of prophylaxis due to infection by drug-resistant variants, most likely transmitted from the treated index cases [25]. These findings indicate that the strategy of using M2 inhibitors for both index case treatment and postexposure prophylaxis in households should be avoided.

Table 71.3 Influenza prevention in households with postexposure prophylaxis (PEP)

Study	Drug (age of contacts)	Season (virus)	Index case treated	Influenza A illness in contacts No./total evaluable (%)		PEP efficacy (%)
				Active	Control	
Galbraith et al. [208]	Amantadine (≥2 years)	1967–1968 (A/H2N2)	No	0/91 (0 %)	12/90 (13 %)	100
Bricaire et al. [209]	Rimantadine (≥1 year)	1988–1989 (A/not stated)	No	8/151[a] (5 %)	26/150[a] (17 %)	70
Monto et al. [210]	Zanamivir (≥5 years)	2000–2001 (A/H3N2, B)	No	12/661 (2 %)	55/630 (9 %)	82
Welliver et al. [211]	Oseltamivir (≥13 years)	1998–1999 (A/H3N2, B)	No	4/493 (1 %)	34/462 (72 %)	89
Galbraith et al. [212]	Amantadine (≥2 years)	1968–1969 (A/H3N2)	Yes	5/43 (12 %)	6/42 (145 %)	6
Hayden et al. [25]	Rimantadine (≥1 year)	1987–1989 (A/H3N2, A/H1N1)	Yes	11/61 (18 %)	10/54 (19 %)	3
Hayden et al. [213]	Zanamivir (≥5 years)	1998–1999 (A/H3N2, B)	Yes	7/414 (2 %)	40/423 (9 %)	82
Hayden et al. [214]	Oseltamivir (≥1 year)	2000–2001 (A/H3N2, B)	Yes	11/400 (3 %)	40//392 (10 %)	73

[a]Clinical influenza

2.4.2 Chronic Care Facilities

Transmission of M2 inhibitor-resistant viruses is well documented in nursing home outbreaks of influenza A and may be manifested by a persistent or an increasing number of virus-positive patients despite amantadine prophylaxis. The recovery of the same genotype of resistant virus from multiple patients on prophylaxis or from patients or staff not receiving drug indicates ongoing transmission in this setting [26, 58]. This particularly true with multiple isolations of a less commonly observed resistant variant, as was found with nine isolates of a Leu26Phe variant in one nursing home outbreak [58]. The frequency of instances in which amantadine or rimantadine has failed to control outbreaks because of resistance emergence is not well defined, although existing studies demonstrate a range of protective efficacy from 59 to 76 % [59]. Studies have demonstrated patients who developed infections with resistant viruses, typically with the Ser31Asn mutation, in 16–28 % of residents' long-term care where M2 inhibitors were used during an outbreak [40, 60]. Such findings emphasize the importance of proper isolation of treated persons and of using NA inhibitors for treatment of ill persons.

2.5 Pathogenicity

M2 inhibitor-resistant influenza A viruses appear to cause typical influenza illness without obviously enhanced or attenuated symptoms [25, 27]. Illness occurs in both the presence and absence of the drug, a finding that indicates the loss of antiviral effectiveness in vivo. Although severe and progressive infection with resistant virus has been noted in immunocompromised and debilitated elderly patients, this is likely more of a marker of the patients underlying condition than virulence of the resistant virus [57, 61]. In most patients, M2 inhibitor-resistant virus has similar risks of pneumonia, hospitalization, or death compared to those with wild-type illness [39].

While the M gene mutations do not appear to attenuate or potentiate the virulence of human influenza viruses, more subtle effects on biologic fitness cannot be excluded by studies to date. In occasional patients wild-type virus replaces resistant variants after cessation of amantadine [41]. As noted for some avian A/H7 viruses, this reversion in the absence of selective drug pressure suggests diminished replication competence of some resistant genotypes. However, the most common resistant variant with Ser31Asn has no apparent loss of replication competence or transmissibility. In studies in birds and ferrets, influenza viruses with Val27Ala, Ala30Val, or Ser31Asn mutations had no impact of virulence, mortality, febrile responses, peak nasal viral titers, or nasal inflammatory cell counts [22, 51]. In general, it appears that M2 inhibitor-resistant human influenza A viruses that emerge in vivo do not differ substantially in replication ability or pathogenicity from drug-susceptible wild-type viruses, and resistance phenotypes were typically retained in the absence of drug.

In treated patients the emergence of resistant virus may be associated with persistence of viral recovery and in some studies delays in resolution of illness in immunocompetent persons. Although patients who had resistance emergence during the course of therapy had a somewhat longer time to resolution of symptoms, fever, and possibly functional impairment, they still had a more rapid recovery than placebo-treated patients [23, 27]. Patients may have worsening symptoms or viral titers when resistance emerges as well [23].

2.6 Treatment Alternatives

Amantadine and rimantadine share susceptibility and resistance, so that resistance to one M2 inhibitor confers high-level cross-resistance to another one and the entire class of compounds targeting M2 protein. Because of their different mechanism of antiviral action, neuraminidase inhibitors and protease inhibitors (discussed below) retain full activity against M2 inhibitor-resistant viruses and are appropriate choices for both prophylaxis and treatment of suspected M2 inhibitor-resistant infections. Clinical studies suggest that both oseltamivir and zanamivir are successful in terminating institutional outbreaks where amantadine resistance is proven or highly probable [58, 62, 63]. In vitro testing suggests that the synthetic nucleosides ribavirin and favipiravir are also inhibitory for M2 inhibitor-resistant influenza A and B viruses and are a therapeutic consideration [64].

Combination therapy may also be an option. A small study randomized hospitalized adults to rimantadine monotherapy or rimantadine-nebulized zanamivir combination therapy. The combination arm had a trend to less cough and fewer patients with detection of M2 inhibitor resistance mutations over the course of therapy [54]. Recently, a triple combination of amantadine, oseltamivir, and ribavirin has been studied in vitro, in vivo, and in infected patient and appears effective at inhibiting viral replication in vitro and improved outcomes in vivo [65, 66].

3 Neuraminidase Inhibitors

The initial design of the NA inhibitors was accelerated after solving X-ray structures of NA co-crystallized with the chemical compound *2,3-dehydro-2-deoxy-N--acetylneuraminic acid* (DANA) [67, 68]. This transition state

analogue of the NA substrate sialic acid has served as the scaffold for the NA inhibitor derivatives [69]. In vitro studies to determine the genetic antiviral resistance profiles of the NA inhibitors zanamivir and oseltamivir were initiated shortly after their development (reviewed in [70, 71]. Due to differences in drug binding interactions and structural differences in the enzyme active site, NA inhibitors show varying antiviral resistance profiles in NA that depend on virus type and subtype (reviewed in [72–74]). Sequential passage in cell culture to select resistant variants found that changes in HA could confer resistance in vitro also [75–77]. These HA changes are predominately found at the receptor binding site and thought to restore the functional balance between HA receptor-binding and NA receptor-destroying properties [78]. Mechanisms which influence the viral HA/NA functional balance may have played a role in the emergence of oseltamivir-resistant A/H1N1 viruses during the 2007–2008 season [79–82]. The frequency and possible importance of resistance emergence during drug administration have been studied largely in the context of controlled clinical trials conducted in the late 1990s that served as the basis for approval of zanamivir and oseltamivir in 1999 and, more recently, for approval of laninamivir and peramivir [83–87]. Although zanamivir and oseltamivir have been available in many countries since 1999, their use has been quite limited, except in Japan and during the 2009 influenza A/H1N1 pandemic [88]. Clinical importance of antiviral resistance emergence was assessed when comparing clinical outcome of oseltamivir-treated patients infected with a susceptible or oseltamivir-resistant A/H1N1 virus during the 2007–2008 influenza season [89–93]. A retrospective clinical study by Dharan et al. showed that patients infected with an oseltamivir-susceptible virus ($n=182$) had significantly fewer days of fever if treated with oseltamivir ($n=64$) as compared to non-treated patients ($n=93$;$P=0.02$). In contrast, patients infected with an oseltamivir-resistant viruses ($n=44$) did not benefit from oseltamivir treatment ($n=43$; $P=0.5$) [91]. Similar finding was reported in a study by Saito et al. where a reduction of fever on days 3–6 was reported in treated oseltamivir-susceptible A/H1N1-infected patients ($P<0.01$), but not in the patients infected with the oseltamivir-resistant A/H1N1 virus strain [93]. This oseltamivir-resistant A/H1N1 virus variant with an NA H275Y amino acid change emerged first in Norway during the 2007–2008 season and was able to spread rapidly in humans [94, 95]. As it became the dominant variant, it was suggested that the oseltamivir-resistant virus was able to spread more easily in the population than wild type [79, 96]. This was unexpected, as both in vitro and in vivo animal studies had claimed reduced virulence and transmissibility of NA inhibitor-resistant viruses before the beginning of 2007–2008 influenza season [97–99].

3.1 Detection of NA Inhibitor Resistance

Unlike the situation for M2 inhibitors, cell culture-based assays have not been validated for detecting phenotypic resistance in clinical isolates, partly because of the differences in cellular receptor specificity between human respiratory epithelium and available cell culture types (reviewed in [71]). In addition, these types of assays are labor intense and require an additional virus titration step, which make these assays unfavorable for high-throughput surveillance. Humanized Madin-Darby canine kidney (MDCK) cell lines that stably overexpress human 2,6-sialyltransferase (SIAT1) to increase alpha 2,6-linked sialic acids may overcome this first limitation. However, these cells have not been widely utilized to date [100, 101]. Other challenges are the broad variation in morphology of influenza plaques between different influenza types and subtypes and the reduced sensitivity of yield reduction assays. The NA enzyme inhibition phenotypic assays have, therefore, been the preferred assay to screen for clinically relevant NA inhibitor resistance mutations in influenza antiviral resistance surveillance [5, 102, 103]. Both fluorometric (MUNANA) and chemiluminescent (NA-star) type of phenotypic assays are available. Both assays have the same limitations, such as the necessity of a virus propagation step, and may therefore not reliably detect resistant subpopulations and do not detect HA-mediated NA inhibitor resistance [104]. To standardize interpretation and reporting of NA inhibitor susceptibility of influenza viruses, clear definitions were formulated in 2012 using 50 % inhibitory concentration (IC_{50}; the concentration of drug required to inhibit a standardized amount of NA activity by 50 %) fold-change thresholds, compared to the median for viruses from the same type/subtype/lineage showing "normal inhibition" [105, 106].

Besides the phenotypic resistance assay, numerous genotypic PCR-based resistance assays have been developed for detection of previously identified antiviral resistance mutations in NA [107–110]. As compared to the phenotypic assays, these types of assays are rapid and easy to perform, and they allow minor variant detection (~1–5 % of the quasispecies) with no requirement of an additional virus culture step. Unknown resistance patterns in newly emerging influenza subtypes or novel NA inhibitors cannot be identified using PCR-based resistance assays.

The typical NA mutations conferring resistance depends on the drug and NA subtype [72, 74, 111, 112]. For oseltamivir, His274Tyr (based on N2 numbering) confers resistance in N1 [113], whereas Arg292Lys and Glu119Val are the most common antiviral resistance mutations in N2-containing viruses (Table 71.4). Because of the differences in interaction among drugs with the active enzyme site, varying patterns of cross-resistance are found for particular NA mutations.

Table 71.4 Effects of NA mutations that confer oseltamivir resistance on viral fitness measures in clinical isolates of influenza

Virus (ref)	Mutation	Enzyme activity or stability (% of parental virus)	Infectivity in mice/ ferret	Replication in ferret	Transmissibility in ferret
A/H3N2	Glu 119 Val	↓	↓ (>10–100-fold)/–[a]	–[a]	–
Yen et al. [138]					
Herlocher et al. [98]					
A/H3N2	Arg 292 Lys	↓↓ (2 %)	↓ (>100-fold)/↓ (>100-fold)	↓↓	0 or ↓↓
Yen et al. [138]				Reversion to wild type observed	
Herlocher et al. [165]					
Carr et al. [97]					
A/H1N1	His 274 Tyr	–	↓ (>1000-fold)/↓ (≥100-fold)	– or ↓	–
Ives et al. [163]					1–2 days delay
Herlocher et al. [98]					
A/H5N1	His 274 Tyr	NR	NR/NR	↓	NR
Le et al. [130]					
B	Asp198Asn	NR	NR	–	NR
Mishin AAC [228]					
B	Arg 152 Lys	↓↓ (3–5 %)	NR/↓	↓	NR
Gubareva et al. [12]					
Jackson et al. [112]					
2009 A/H1N1 [13, 166, 167]	His 275Tyr	–	–/↓	–/↓	/↓
2009 A/H1N1 [229]	Ile223Arg	↓ (50 %)	–	–	–
A/H7N9	Arg292Lys	↓	–	–	–
Yen et al. [230]					Reversion to wild type observed

Abbreviations: – no change compared to wild type, ↓ decreased, *O* absent, *NR* not reported
[a]Days of fever in ferrets exposed to the parental A/H3N2 virus was greater than in ferrets exposed to the E119V mutant virus (⩾2 days vs. 1 day, respectively; P > .05).

Importantly, zanamivir and laninamivir retain full inhibitory activity against variants with either the His274Tyr or Glu119Val mutation and partial activity against the Arg292Lys variant [114]. Viruses with a His274Tyr are also cross-resistant to peramivir. Antiviral resistance may be caused by a single resistance mutation or a combination of additional mutations, which may enhance the level resistance and/or causes multidrug resistance [115–117].

HA binding efficiency and associated susceptibility to NA inhibitors are affected by amino acid changes in the receptor binding [112]. Consequently, HA mutations have been looked for in clinical isolates usually by comparing the sequence of pre- and post-therapy isolates and in some instances by examining changes in receptor affinity [10, 11]. HA variants that have reduced receptor affinity show cross-resistance in vitro to all NA inhibitors but in general retain susceptibility to NA inhibitors in vitro and in animal models [118–120].

3.2 Drug Susceptibility of Circulating Viruses

With the exception of the influenza seasons between 2007 and 2009 when the oseltamivir-resistant A/H1N1 viruses were circulating, the global incidence of circulating A and B viruses with de novo resistance to the NA inhibitors has been very low since the approval of these drugs (Table 71.5) [9, 103, 106, 121]. A recent study in which 10,641 viruses were collected globally in 2013–2014 by collaborating National Influenza Centers to determine IC_{50} data for NA inhibitors oseltamivir, zanamivir, peramivir, and laninamivir 172 viruses (1.6 %) showed highly reduced inhibition (>100-fold) against at least one of the four drugs and 32 viruses (0.3 %) with only reduced inhibition (between 10- and 100-fold reduction) [106]. Most of these highly resistant isolates were 2009 pandemic influenza A/H1N1 viruses with a His274Tyr amino acid change ($n = 169$). Only a single resistant A/H3N2 virus was detected, which carried a Glu119Val amino acid change. Two influenza B viruses with and Glu119Gly (B/Victoria) and His273Tyr (B/Yamagata) were detected. In a recent global observational multicenter clinical trial (IRIS) with follow-up sampling of influenza-infected patients after admission to a clinic (2009–2013; $n = 1799$), no genotypic resistance was detected at baseline in respiratory specimens of influenza A or B virus-infected patients apart from the A/H1N1 viruses with an inherited His275Tyr amino acid change [103]. In 19 of 1014 patients (1.9 %) receiving an antiviral, emergence of resistance to oseltamivir could be detected during treatment, in most cases children below the age of 5 ($n = 14$; 74 %). In 17 of these cases, a 2009 A/H1N1 His274Tyr amino acid change was detected. In two oseltamivir-treated children with

Table 71.5 Representative studies of oseltamivir and zanamivir susceptibility of field isolates of influenza A and B viruses

Study	Location	Seasons	Assay	No. tested	No. (%) resistant	Mutations detected
McKimm-Breschkin et al. [215]	Worldwide	1999–2002	NAI-FA, NAI-CL, S	139 A/N1	0	
				767 A/N2	0	
				148 B	0	
Hurt et al. [216]	Australia, Southeast Asia	1998–2002	NAI-FA	235 A/N1	0	
				169 A/N2	0	
				128 B	0[a]	
Bovin and Goyette [217]	Canada	1999–2000	NAI-CL	38 H3N2	0	
				40 H2N1	0	
				23 B	0	
Mungall et al. [218]	Worldwide	2000–2002	NAI-CL	567 A/N2	0	
				271 A/N1	0	
				712 B	0	
Monto et al. 2006 [143]	Worldwide	1999–2002	NAI-CL, S	922 A/N2	3 (0.3 %)	Gln41Gly, Gln226His
				622 A/N1	3 (0.5 %)	His274Tyr, Tyr155His, Gly248Arg
				743 B	2 (0.3 %)	Asp198Glu, Ile222Thr
Ferraris et al. [219]	France	2002–2005	NAI-FA, S	788 H3N2	0[b]	
NISN WER [220]	Japan	2003–2004	NAI-CL, S	1180 H3N2	3 (0.3 %)	2 Glu119Val, 1 Arg292Lys
				171 B	0	
Hatakeyama et al. [221]	Japan	2004–2005	NAI-FA, S	422 B	7 (1.7 %)	3 Asp198Asn, 3 Ile222Thr, 1 Ser250Gly
NISN WER [222]	Japan	2004–2005	NAI-CL, S	558 H3N2	0	4 His274Tyr
		2005–2006	S	60 H1N1	0	
				251 H3N2	0	
				178 H1N1	4	
Whitley et al. [103]	Worldwide	2009–2013	NAI-CL, S, PCR	335 H3N2	0	47 His274Tyr
				47 sH1N1	100	
				889 2009H1N1	0	
				518 B	0	
Meijer et al. [121]	Worldwide	2012–2013	NAI-FA, S	2343 H1N1	18 (<0.1 %)	18 His274Tyr
				5109 H3N2	4 (<0.1 %)	3 Glu119Val, 1 Arg292Lys
				3935 B	2 (<0.1 %)	2 His273Tyr
Takashita et al. [106]	Worldwide	2013–2014	NAI-FA, S	5152 H1N1	169 (3.3 %)	169 His274Tyr
				2574 H3N2	1 (<0.1 %)	1 Glu119Val
				2915 B	1 (<0.1 %)	1 His273Tyr

Abbreviations: *NAI* neuraminidase inhibition, *CL* chemiluminescence, *FA* fluorescence, *S* sequence analysis of neuraminidase gene, *PCR* polymerase chain reaction

[a]One B/Perth/211/2001 isolate had ninefold reduced susceptibility to zanamivir and 14-fold to oseltamivir compared to the mean inhibitory concentrations of influenza B strains and contained a mixed population including resistant variants with a Asp197Glu mutation [79]

[b]Four isolates (0.5 %) with NA deficiency were found to be resistant to NA inhibitors in cell culture-based assays

an A/H3N2 virus infection, an Arg292Lys change emerged posttreatment. Although the incidence of NA inhibitor-resistant viruses is currently low, the occasional clusters of 2009 oseltamivir-resistant influenza A/H1N1 viruses with an His274Tyr are a reason for concern [122–124]. Resistance to zanamivir was reported due to an amino change Gln136Lys [125, 126]. The presence of this mutation, however, may be caused by an artifact propagation of the virus in Madin-Darby canine kidney (MDCK) cell cultures [127]. With regard to the highly pathogenic avian influenza A/H5N1 viruses and low-pathogenic avian influenza A/H7N9 viruses, these are susceptible to the NA inhibitors [128]. Like A/H1N1 influenza viruses, amino acid changes at 119, 274, and 294 were found in 2.4 % of human and 0.8 % of avian A/H5N1 virus sequences, which were deposited to GenBank [33]. Additionally, markers of reduced NA inhibitor susceptibility at amino acid positions 116, 117, 150, 222, and 246 were found in 0.8 % of human and 2.9 % of avian A/H5N1 isolates [129]. Although the His275Tyr change has been the major antiviral resistance pattern found in highly pathogenic avian influenza A/H5N1 viruses [130–133], a A/H5N1 isolate was reported [130], with an Asp295Ser amino acid change causing an 80-fold and sevenfold increase of the IC_{50} for oseltamivir and zanamivir, respectively [134–

137]. This Asn295Ser change has also been observed in A/H5N1 virus isolates. The emergence of an Arg292Lys amino acid change in the low-pathogenic avian influenza A/H7N9 viruses circulating in China since 2013 causes high NA inhibitor resistance to oseltamivir and peramivir and reduced resistance to zanamivir [138, 139]. Unlike A/H3N2 viruses carrying the Arg292Lys amino acid change, A/H7N9 virus does not seem to be much attenuated by this change [128, 140]. Like the influenza A viruses, NA inhibitor resistance in influenza B viruses is currently low [141]. Nevertheless, several oseltamivir-resistant B viruses have been isolated from treated or untreated patients [142–144]. Antiviral resistance to neuraminidase may be caused by changes at residues Asp198 and Ser250. In addition, also influenza B viruses have been found with an Ile221 [144, 145]. These mutations cause only a two- to threefold increase in IC_{50} to oseltamivir, zanamivir, and peramivir.

3.2.1 Immunocompetent Hosts

In natural infections, oseltamivir-resistant variants have been detected much more commonly in treated children than adults (Table 71.6). In the past, analysis of samples from over 2500 influenza patients treated with oseltamivir as outpatients indicated that the frequency of resistance detection is about 0.4 % in adults and about 4.5 % in children [146]. Similar observations were made more recently in the IRIS trial where 14 of 19 oseltamivir-treated outpatients with resistance development were children aged below 5. The higher level of replication with longer duration of virus shedding increases the chance of developing antiviral resistance as compared to adults. Two studies in Japanese children reported high frequencies of 16 and 18 % oseltamivir resistance emergence during oseltamivir therapy [8, 147]. The use of weight-based dosing for children in Japan, as contrasted with unit dosing in most countries, is associated with lower drug exposure in young children. This has been postulated to be a major factor in the higher frequency of resistance detected in these studies. Among 54 volunteers experimentally infected with an A/H1N1 virus, oseltamivir-resistant variants with His274Tyr mutation were detected in two subjects in association with apparent rebounds in viral replication [148]. This study found that oseltamivir-treated subjects were less likely than placebo to have late viral isolates showing reversion of the egg-adapted inoculum virus to a human receptor HA genotype. The His274Tyr finding suggests that HA mutations with reduced affinity for human receptors might have a replication advantage over viruses with human receptor preference during oseltamivir use in humans. Interestingly, amino acid changes in the HA of the influenza A/H1N1 viruses prior to the emergence of the oseltamivir-resistant A/H1N1 virus in the 2007–2008 season have been predicted to have facilitated the emergence of the His274Tyr amino acid change [149, 150].

3.2.2 Immunocompromised Hosts

Immunocompromised individuals tend to suffer from influenza longer with more serious complications than otherwise healthy patients [151–154]. Since immunocompromised patients are more likely to acquire influenza [155], showing relatively high influenza-associated mortality [10, 11, 153], effective antiviral is crucial for these patients. Like with influenza and young children, the higher level of replication with longer duration of virus shedding in immunocompromised patients increases the chance of developing antiviral resistance [156]. Several recent clinical studies have reported that the emergence of antiviral resistance among treated immunocompromised patients is not uncommon [151, 157, 158]. Recently, a prospective clinical study aimed to study antiviral resistance in immunocompromised patients ($n = 24$); a resistance prevalence of 17 % (4/24) was reported [158]. In all four cases the NA His275Tyr was detected by RT-PCR of 2009 influenza A/H1N1 virus-infected patients. In other retrospective studies, similar rates have been reported [151, 157]. The NA His275Tyr amino acid change has been described frequently during the 2009 influenza A/H1N1 virus pandemic in case reports of antiviral-treated immunocompromised patients [117, 159]. Amino acid changes at position 223 have also been reported to cause increased levels of resistance (48-fold) to oseltamivir. The impact on therapy is unclear for such moderate increase in oseltamivir resistance; however viruses with the combination of Ile223Arg and His275Tyr are highly resistant to oseltamivir in vitro (1750-fold) [160]. In the past, emergence of resistance in immunocompromised patients has been also described for influenza A/H3N2 and influenza B virus-infected patients treated with oseltamivir and zanamivir with mutations in both the viral HA and NA glycoproteins [8, 10, 12, 161]. Most fatal cases during influenza pandemics and seasonal epidemics are patients belonging to the traditional high-risk groups for developing severe disease, including the very young children, elderly, and immunocompromised patients [162]. Given the high mortality and morbidity, the moderate effectiveness of current antivirals, and the relatively high prevalence of resistance in immunocompromised patient, better treatment strategies are clearly needed for these patients.

3.3 Pathogenicity and Transmissibility of Resistant Variant

Before the 2007–2008 influenza season, it was thought that NA inhibitor resistance development was to go hand in hand with reduction of virus fitness [97, 163]. Mathematical modeling predicted a 10 % relative transmissibility of oseltamivir-resistant variants would result in low levels of resistant viruses circulating in the community [164]. Based

Table 71.6 Frequency of resistance emergence to oseltamivir or zanamivir during treatment

Drug/study	Population	Assay	Virus type	No. isolates tested	No. (%) resistant	Mutations detected
Oseltamivir						
Gubareva et al. [148]	Adults	NAI, S	A/H1N1	54	2 (4%)	2 His274 Tyr
Roberts [146]	Adults	NAI, S	A/H3N2	418	5 (1%)	4 Arg292Lys, 1 Glu119Val
Whitley et al. [223][a]	Children—outpatient	NAI, S	A&B	150 A	10 (6.7%)	8 Arg292Lys, 1 Glu119Val, 1 His274Tyr
				66 B	0	
Kiso et al. [8][a]	Children—outpatient + hospitalized	Cloning + S	A/H3N2	50	9 (18%)	6 Arg292Lys, 2 Glu119Val, 1 Asn294Ser
Ward et al. [224][a]	Children—outpatient + hospitalized	NAI, S	B	74	7 (16%)	7 His274Tyr
	Children—outpatient	NAI, S			1 (1.4%)	Gly402ser
Whitley et al. [103]	Children + adults outpatient	NAI, S	A&B	759 A	19 (2.5%)	17 His275Tyr, 2 Arg292Lys
				256 B		
Hatekayama et al. [221]	Children—outpatient	NAI, S	B	77	0	1 Gly402Ser
Stephensen et al. [225]	Children—outpatient	NAI, S	A&B	43 A	1 (1.3%)	3 His275Tyr, 1Arg292Lys
				19 B		
Harvala et al. [227]	Children—outpatient	NAI, S	A	32 A	4 (7.4%)	5 His275Tyr
					0	
Tramontana et al. [226]	Adults + children hospitalized	PCR	A	30 A	5 (15.6%)	4 His275Tyr
	Adults—hospitalized	NAI, S			4 (13.3%)	
Zanamivir						
Barnett et al. [88]	Adults	NAI, S	A + B	41	0	

[a]These pediatric studies used a 2 mg/kg dose of oseltamivir that has been shown to give reduced drug exposure because of more rapid clearance in children under the age of 5 years. Insufficient drug exposure may have contributed to resistance emergence in these studies

on animal experiments, the reduced fitness and replication competence of certain NA resistance mutations appeared to be depending on virus subtype and resistance mutation. For instance, an oseltamivir-resistant influenza A/H3N2 virus with an Arg292Lysine amino acid change did not transmit between infected and naïve ferrets and showed a 10–100-fold reduction in nasal virus titers [165]. For the Glu119Val oseltamivir-resistant mutant, however, it was found that the mutant was as transmissible as wild type with comparable nasal virus titers in both donor and recipient animals [98]. An influenza A/H1N1 virus with a His275YTyr mutation required 100-fold higher inoculum to infect the donor ferret, but once infected, they transmitted the virus to contact animals with a delay of 1–3 days compared to wild-type virus. Early after the outbreak of the 2009 pandemic, it was questioned whether a His275Tyr oseltamivir-resistant mutant would be attenuated [13, 166, 167]. In vitro replication and in vivo pathogenicity studies were performed using resistant isolates; however, the answers were conflicting. Some researchers found slight attenuation of the early His275Tyr mutant A/H1N1 viruses [166], while others did not find such differences [167]. At most, from these conflicting data, it can be concluded that the differences between a wild-type 2009 pandemic A/H1N1 virus and its His275Tyr-resistant counterpart are too close to call by means of its pathogenicity and transmissibility [74]. Additional compensatory mutations may facilitate the emergence of NA inhibitor resistance mutations, which cause an initial loss of virus fitness [96]. For instance, for the His275Tyr amino acid change in 2007–2009 A/H1N1 viruses, several permissive amino acid changes have been suggested to have facilitated the emergence of this oseltamivir resistance change. The Asp344Asn amino acid change, which appeared before the 2007–2008 season, had increased the enzymatic properties of NA prior to the introduction of the His275Tyr amino acid change [113, 168]. Amino acid changes Val234Met and Arg222Gln maintained high NA expression in vitro, which was reduced if the single His275Tyr was expressed [80, 169]. In A/H3N2, compensatory roles for amino acid changes at position 222 have been assigned to compensate for the loss of fitness due to the Glu119Val oseltamivir resistance mutation [170, 171]. The observed community clusters of 2009 A/H1N1 viruses with a His275Tyr amino acid change do not seem to be attenuated by the His275Tyr amino acid change either [122]. These viruses contain, in addition to the His275Tyr change, changes at amino acid positions 241, 369, and 386. These mutations may also have permissive effects [122].

3.4 Treatment Alternatives

The patterns of NA inhibitor cross-resistance vary by virus type and subtype, such that zanamivir retains inhibitory activity for the most common resistant variants that emerge during the therapeutic use of oseltamivir or peramivir. Zanamivir is fully inhibitory for oseltamivir-resistant variants possessing the Glu119Val substitution in N2 or His275Tyr or Asn294Ser in N1 [102, 172]. Depending on the virus and assay, zanamivir is partially inhibitory for resistant variants with Arg292Lys substitution in N2, in that the loss of susceptibility is about 5–25-fold compared to the wild type [102, 172–174]. There is controversy about the role of peramivir in the management of variants that are resistant to oseltamivir as in vitro and in vivo models have given conflicting results [175–177]. Oseltamivir is not inhibitory for the Arg152Lys mutation in influenza B NA that confers reduced susceptibility to zanamivir [178].

Given these findings, most experts recommend using zanamivir for the treatment of patients who develop resistance or virologic failure to oseltamivir. Inhaled zanamivir has been utilized in a few patients with variable success but has not been studies systematically in oseltamivir-resistant infections; success is less likely in patients with influenza pneumonia [179–182]. Intravenous zanamivir has been utilized most frequently for patients with proven or suspected resistant influenza; while the therapy is effective for some patients, available data precludes assessing the optimal role of this intervention given the severity of illness of many patients at conversion to therapy and significant prior exposure to numerous interventions [183–186]. Other NA inhibitors and zanamivir dimers that have prolonged duration of antiviral effect after topical application are currently under development [187]. These may provide NA inhibitor prevention and perhaps treatment alternatives in the future.

Ribavirin would also be expected to be inhibitory for influenza A and B viruses resistant to the NA inhibitors, but there are no reports of its use in human influenza infections due to such variants. Ribavirin combined with a NA inhibitor exerts additive to synergistic antiviral activity in vitro [188]. In mice experimentally infected with influenza A, the combination of orally administered ribavirin and peramivir was associated with improved survival relative to ribavirin alone but not to peramivir alone [189]. A more recent study found that a combination of ribavirin and oseltamivir was no more effective than ribavirin alone against a lethal influenza A(H1N1) infection but superior to single agents against influenza B [189]. Further studies of such ribavirin-NA inhibitor or T-705-NA inhibitor combinations (see below) are warranted to determine whether this strategy offers the possibility of treating severe influenza, particularly that due to M2 inhibitor-resistant viruses. Recently, triple combinations of amantadine, ribavirin, and oseltamivir have been studied in vitro, in vivo, and in humans with influenza infection [65, 66, 190]. Given the promise of this combination, a prospective phase 2 study is ongoing to assess the safety and clinical efficacy of this combination for the treatment of influenza. Combination therapy has been demonstrated to reduce the development of resistance in clinical studies and therefore may be of benefit in populations at increased risk of development of resistance emergences [54].

4 Novel Agents

4.1 T705/Favipiravir

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is an antiviral drug that is phosphoribosylated by cellular enzymes to its active form, favipiravir-ribofuranosyl-5′-triphosphate (RTP), and selectively inhibits the RNA-dependent RNA polymerase of influenza virus [191]. It is highly active against seasonal strains A/H1N1, A/H3N2, and influenza B; the 2009 pandemic A/H1N1 virus; highly pathogenic avian influenza virus A/H5N1 isolated from humans; A/H1N1 and A/H1N2 isolated from swine; and A/H2N2, A/H4N2, and A/H7N2. The antiviral is active against viruses that are resistant to amantadine, rimantadine, oseltamivir, and zanamivir, in addition to dually resistant viruses (M2 and NA inhibitor resistant) [191, 192]. In studies of serial passage of two seasonal (A/Brisbane/59/2007 and A/New Jersey/15/2007) and two 2009 pandemic (A/Denmark/524/2009 and A/Denmark/528/2009) A/H1N1 viruses in MDCK cell lines in the presence or absence of low concentrations of favipiravir, no favipiravir-resistant viruses were phenotypically or genotypically (PB1, PB2, PA, and NP sequencing) detected. Sequence analysis, though, did demonstrate an enrichment of G → A and C → T transversion mutations, increased mutation frequency, and a shift of the nucleotide profiles of individual NP gene clones under drug selection pressure [193]. Few clinical studies have been published with this novel compound, so the frequency of resistance emergence is not fully understood at this point. The drug is currently licensed in Japan for use selectively when approved by the Ministry of Health; studies of efficacy are ongoing in the rest of the world with the goal of seeking regulatory approval in the near future.

4.2 Antibodies

Recent studies have reported the development of neutralizing antibodies to specifically target conserved regions of the virus HA [194, 195]. HA binding of the antibodies was elegantly shown by X-ray crystal structures of HA-antibody protein complexes [196, 197]. These antibodies differ in

their recognition sites: Some are targeted to the sialic acid RBS and globular head, while others bind to the stalk region [195]. As the stalk region is more conserved between different HA subtypes, cross-reactive immunity against several influenza subtypes may be obtained with broadly neutralizing capacities. Although the antibodies are being developed against conserved regions of HA, mutations do arise at the antibody target sites, which may result in viral escape.

5 Implications and Future Research Directions

Currently, circulating strains of influenza are primarily resistant to the M2 inhibitors but are generally susceptible to the clinically available neuraminidase inhibitors. Sporadic cases of neuraminidase inhibitor resistance have been recognized, and limited regional transmission has been demonstrated [4, 44, 198]. Further, resistance in seasonal A/H1N1 became widespread during the 2008–2009 influenza season. Lastly, NA inhibitor resistance has been demonstrated to emerge during therapy in highly pathogenic avian influenza viruses that infect humans, with the highest frequency in A/H7N9 viruses [35, 36, 49]. As such, most regions of the world are currently limited to a single class of drug, the neuraminidase inhibitor, for the management of influenza infections. The risk that resistance could emerge and result in global spread poses a serious threat and requires the development of novel agents and combinations [128, 187].

Lessons learned from the 2009 pandemic suggest that there is a significantly higher frequency of antiviral resistance emergence in the pandemic virus compared to interpandemic influenza. Further, the clinical and epidemiologic implications of antiviral resistance in a future pandemic influenza virus cannot be predicted with confidence. As a result, the great progress made in developing global systems to rapidly monitor the susceptibility patterns of circulating strains needs to be maintained and potentially expanded to include regions with sparse surveillance [106]. Further, surveillance of resistance patterns in animals may give early warnings about future pandemic influenza viruses.

A number of unanswered questions remain regarding antiviral drug resistance in influenza viruses. With contemporary next-generation sequencing, it is possible to understand the kinetics of the emergence of resistance from minor variant populations to the predominant population in a given host. Such data can inform the optimal timing of screening and intervention. Specific risk factors beyond generic concepts, such as immunocompromised and young age, should be identified that predict the emergence of resistance. From a therapeutic perspective, the optimal approach, including the duration of therapy and the benefit of combination therapy in patients with severe illness or who are predicted to have prolonged shedding, needs to be carefully studied. Currently, there is a significant gap in the capacity to test specimens for resistance, and as a result, many patients with potential resistance may be missed. As a result, there is a desperate need for susceptibility assays that can be utilized broadly in the clinical laboratory. Lastly, there is need for ongoing and expanded surveillance of antiviral susceptibility patterns in human and animal influenza viruses, especially community isolates in countries with higher antiviral use, and for resistance transmission in high-risk epidemiologic settings.

Given the current pattern of antiviral susceptibility in circulating strains, M2 inhibitors should not be utilized for the prevention or treatment of influenza, while any of the neuraminidase inhibitors should be considered whenever therapy is indicated. Such therapy should be started as early as possible to improve the benefit obtained from the use of the therapy. Given its slightly broader activity against most oseltamivir-resistant variants, zanamivir would be the preferred therapy for patients with proven or suspected oseltamivir-resistant influenza. Novel agents, optimally with novel mechanisms of action, need to be developed. Drugs in advance stages of development include the polymerase inhibitor favipiravir [191], the receptor-destroying sialidase DAS181 [199], and nitazoxanide [200]. Neutralizing antibodies and convalescent plasma need to be studied further to optimize the treatment of patients, particularly with novel or highly resistant viruses [201]. Lastly, combinations of antivirals should be studied to understand their ability to prevent and overcome resistance clinically [128].

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Herpesvirus Resistance to Antiviral Drugs

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Jocelyne Piret and Guy Boivin

1 Introduction

Herpesviridae is a large family of DNA viruses including nine human viruses which belong to the α-*herpesvirinae* [herpes simplex virus 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV)], the β-*herpesvirinae* [human cytomegalovirus (HCMV) and human herpesviruses 6 and 7 (HHV-6 A/B and HHV-7)], and the γ-*herpesvirinae* [Epstein-Barr virus (EBV) and HHV-8] subfamilies. These ubiquitous viruses have the ability to establish latency in specific cell types and to reactivate under certain circumstances. Among members of the *Herpesviridae* family, four of them (HCMV, HSV-1, HSV-2, and VZV) will be discussed in this chapter since they are the main targets of antiviral strategies. HCMV is responsible for mononucleosis-like syndromes as well as systemic and organ-specific diseases in immunocompromised patients. HSV-1 and HSV-2 cause orolabial and genital infections as well as keratitis, encephalitis, and neonatal infections. VZV is the causative agent of varicella and herpes zoster.

The discovery of the nucleoside analogue acyclovir (ACV) was made more than 35 years ago; it represents a milestone in the management of HSV and VZV infections. The modest activity of ACV against HCMV has prompted the development of another nucleoside analogue, ganciclovir (GCV), for the management of systemic and organ-specific HCMV diseases. Clinical use of intravenous GCV began in 1984 for the treatment of life-threatening and sight-threatening HCMV infections in immunocompromised patients. In 1988, strains of HCMV exhibiting resistance to GCV in vitro had been already identified. Second-line antiviral agents such as the pyrophosphate analogue foscarnet (FOS) and the nucleotide analogue cidofovir (CDV) have been approved subsequently. In contrast to ACV and GCV, the latter drugs do not require an initial phosphorylation step by viral protein kinases to be converted into their active forms. However, their use is limited by the absence of oral formulations and their toxicity profiles. As all currently available antiviral agents target the viral DNA polymerase (pol), mutations conferring cross-resistance to two or all drugs emerged. There is thus a need to develop new antiviral compounds with different mechanisms of action, appropriate safety profiles, and good pharmacokinetic properties. In this chapter, we review the antiviral drugs approved for the prevention and the treatment of HCMV, HSV and VZV infections, the laboratory methods for detecting antiviral resistance, the clinical significance of drug-resistant strains, and their management.

2 Antiviral Agents for Herpesvirus Infections

Three antiviral agents and a prodrug are currently available for the systemic treatment of HCMV infections [1]. Ganciclovir (Cytovene®, Roche) is a deoxyguanosine analogue and was the first drug to be approved for this indication in 1988. Since then, it has remained the first-line treatment for HCMV infections in immunocompromised patients. Upon entry in HCMV-infected cells, GCV is selectively phosphorylated by a viral protein kinase homologue (the product of the *UL97* gene, pUL97). Subsequently, cellular kinases convert GCV monophosphate into its triphosphate form, which acts as a potent inhibitor of the HCMV DNA pol (the product of the *UL54* gene) by competing with deoxyguanosine triphosphate on the enzyme binding site (Fig. 72.1). Ganciclovir is also incorporated into the viral DNA where it slows down and eventually stops chain elongation [2]. Ganciclovir formulations are available for intravenous (IV) or oral administration for the treatment of HCMV diseases in

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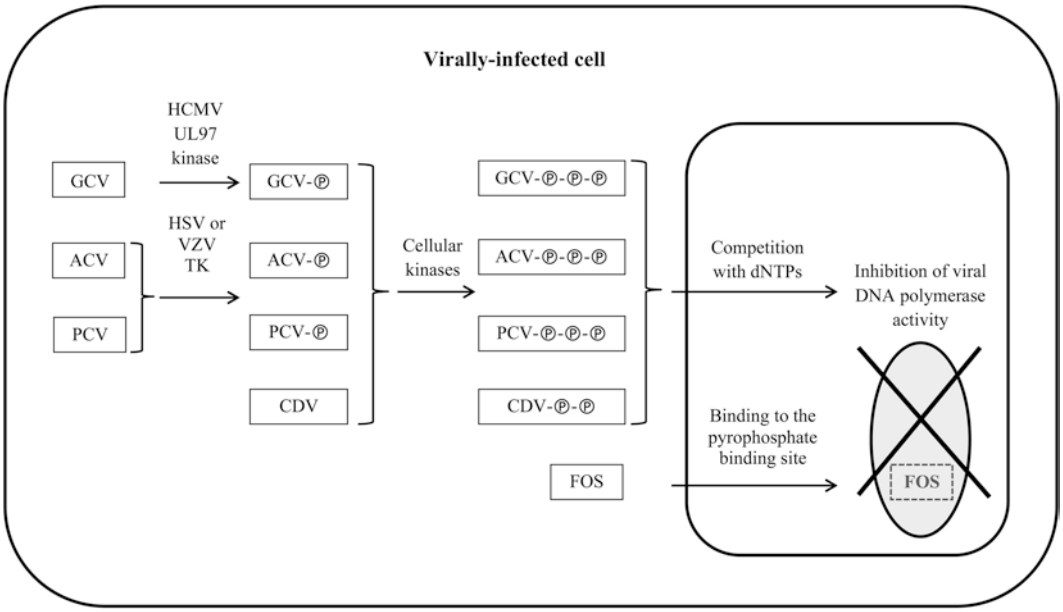

Fig. 72.1 Mechanisms of action of the different classes of antiviral agents. The nucleoside analogues such as ganciclovir (GCV), acyclovir (ACV), and penciclovir (PCV) must be first phosphorylated by the *UL97* protein kinase or viral thymidine kinase (TK) and then by cellular kinases to be converted into their active forms. The acyclic nucleoside phosphonate derivatives such as cidofovir (CDV) must be phosphorylated by cellular kinases only to be active. The resulting triphosphate forms compete with deoxynucleotide triphosphates (dNTPs) to inhibit the viral replication. The pyrophosphate analogue foscarnet (FOS) directly inhibits the activity of the viral DNA polymerase. **Key**: Ⓟ represents one phosphate group

immunocompromised patients as well as ocular implants (Vitrasert, Chiron) for the local treatment of HCMV retinitis. Due to its poor bioavailability (~6 %), efforts were made to develop prodrugs of GCV. Valganciclovir (VGCV, Valcyte®, Roche) is a L-valyl ester prodrug of GCV exhibiting an approximately 10 times improved GCV bioavailability following oral administration compared to the parent drug [3].

The other two compounds approved for systemic treatment of HCMV infections are also potent inhibitors of the viral DNA pol. However, due to their toxicity profiles and the absence of oral formulations, they are usually reserved for patients failing or not tolerating GCV therapy. Cidofovir (Vistide®, Gilead Sciences) is a nucleotide analogue of cytidine (also called acyclic nucleoside phosphonate) that only requires activation (phosphorylation) by cellular enzymes to exert its antiviral activity [4]. Once in its diphosphate form, CDV inhibits the HCMV DNA pol by acting as a chain terminator (Fig. 72.1) [5]. The IV formulation of CDV is indicated for the treatment of HCMV retinitis in patients with the acquired immunodeficiency syndrome (AIDS) and is also occasionally used in transplant patients. Foscarnet (Foscavir®, Astra-Zeneca), a pyrophosphate analogue, differs from the two previous antivirals both by its mechanism of action and by the fact that it does not require any activation step to exert its antiviral activity. Foscarnet binds to and blocks the pyrophosphate binding site on the viral polymerase, thus preventing incorporation of incoming deoxynucleotide triphosphates (dNTPs) into viral DNA (Fig. 72.1) [6]. The IV formulation of FOS is indicated for the treatment of HCMV retinitis in individuals with AIDS and for GCV-resistant HCMV infections in immunocompromised patients.

In addition to the treatment of established HCMV diseases, antivirals have also been used to prevent such symptomatic episodes, especially in transplant recipients. The first strategy, defined as "prophylaxis," consists of administering an antiviral to all at-risk patients during the first 3 months or so after transplantation. However, the occurrence of late-onset HCMV disease which is associated with high rates of graft loss [7] and mortality [8] is an important issue after discontinuing prophylaxis. The second strategy, referred to as "preemptive therapy," consists of using short courses of antivirals only for high-risk patients based on evidence of active viral replication (e.g., detection of early HCMV antigens such as the pp65 protein or a certain assay threshold of viral DNA/mRNA in the blood), optimally before the onset of symptoms [9, 10]. The advantages of preemptive therapy include a lower rate of delayed occurrence of HCMV disease and less drug toxicity [11]. However, patients are more prone to recurrent episodes of DNAemia, and the indirect effects of HCMV infection on graft and patient survival may not be prevented.

Antiviral agents currently licensed for the treatment of HSV and VZV infections include ACV (Zovirax®,

GlaxoSmithKline) and its L-valyl-ester prodrug valacyclovir (VACV, Valtrex®, GlaxoSmithKline), famciclovir (FCV, Famvir®, Novartis) which is the L-valyl-ester prodrug of penciclovir (PCV), and FOS [12, 13]. Acyclovir and PCV are deoxyguanosine analogues that must be phosphorylated by the thymidine kinase (TK) of HSV (encoded by the *UL23* gene) or VZV (encoded by the *ORF36* gene) and then by cellular kinases to exert their antiviral activity [14]. Their triphosphate forms are competitive inhibitors of the viral DNA pol (Fig. 72.1) [15]. In addition, incorporation of ACV triphosphate into the replicating viral DNA chain stops synthesis. Oral ACV, VACV, and FCV are used for short-term therapy of primary and recurrent HSV infections (particularly genital herpes), long-term suppressive therapy of recurrent genital herpes, as well as treatment of herpes zoster. The IV formulation of ACV is indicated for the management of severe HSV (including encephalitis and neonatal herpes) and VZV infections. Topical formulations of ACV and PCV (Denavir®, Novartis) are used for the treatment of herpes labialis and keratitis. The pyrophosphate analogue FOS is usually indicated for ACV- or PCV-resistant HSV or VZV infections [16–18]. Topical and IV formulations of CDV may be used "off label" in the treatment of nucleoside analogues- and/or FOS-resistant HSV infections [13].

3 Human Cytomegalovirus Antiviral Drug Resistance

3.1 Phenotypic and Genotypic Assays to Evaluate HCMV Drug Susceptibility

Two different albeit complementary approaches have been developed to assess HCMV drug resistance. In the phenotypic method, the virus is grown in the presence of various concentrations of an antiviral in order to determine the drug concentration that will inhibit a percentage (more commonly 50 %) of viral growth in cell culture. In this assay, a standardized viral inoculum is inoculated onto susceptible cultured cell lines. The virus is then allowed to grow for a few days (typically 7–10 days) in the presence of serial drug dilutions before staining the cells. The number of viral plaques per antiviral drug concentration is first counted, and the percentage, as compared to control wells without antiviral, is plotted against drug concentrations. The concentration that reduces the number of viral plaques by 50 % (50 % effective concentration or EC_{50}) is then determined. Proposed cutoff values defining resistance to GCV, CDV, and FOS are 6 μM, 2 μM, and 400 μM, respectively [19, 20]. An increase in the EC_{50} value greater than two- to threefold over that of a sensitive reference strain or a baseline isolate is also a widely accepted breakpoint value [21]. Even though efforts have been made to standardize this assay [22], the inter-assay and interlaboratory variability is still problematic. Several phenotypic assays, either based on detection of HCMV DNA by hybridization [23] or quantitative PCR [24] or detection of specific HCMV antigens by ELISA [25], flow cytometry [26–28], immunofluorescence [29], or immunoperoxidase [30], have also been developed to increase the objectivity of the readout. Altogether, these assays are time-consuming, limited by the difficulty of obtaining an appropriate clinical specimen for cell culture, subject to possible selection bias introduced during viral growth of mixed viral populations in cell culture [31, 32] and may lack sensitivity to detect low level of drug resistance or minor resistant subpopulations [31, 33].

In contrast to phenotypic assays, which directly measure drug susceptibility of viral isolates, genotypic assays detect the presence of viral mutations known to be associated with drug resistance. Approximately 80 % of GCV-resistant clinical isolates typically contain one of the seven canonical mutations (M460V/I, H520Q, C592G, A594V, L595S, and C603W) in the *UL97* gene [34]. The limited number of *UL97* mutations responsible for GCV resistance has thus prompted the development of a method based on rapid restriction fragment length polymorphism (RFLP) of PCR-amplified DNA fragments to detect their presence in clinical samples [35, 36]. Typically, the presence of a given mutation will either obliterate an existing restriction site or create a new one. The difference in RFLP patterns can thus be visualized following gel electrophoresis. The major advantages of this assay include its short turnaround time (2–4 days) and its ability to detect as little as 10–20 % of a mutant virus in a background of wild-type viruses [35]. A real-time PCR assay with melting curve analysis using hybridization probes specific for each more common mutations in the *UL97* gene has also been developed [37, 38]. However, melting curves may be affected by natural polymorphisms, and this method does not allow distinguishing different point mutations that occur at the same codon. As GCV resistance mutations also emerge at other codons, DNA sequence of a region typically comprised between codons 400–670 of the *UL97* gene should be determined for a comprehensive analysis. Genotypic analysis of DNA pol mutations can also be performed by sequencing a region of the *UL54* gene typically spanning between codons 300–1000 to cover the large number of mutations reported within all conserved regions of this enzyme [39]. One of the advantages of these assays is that they can be performed directly on clinical specimens [40, 41] thus reducing considerably the time required for data generation (1–3 days). By omitting the need to grow the virus, such methods also minimize the risks of introducing a selection bias. Standard Sanger dideoxy sequencing method can detect an emerging resistance mutation when it exceeds approximately 20 % of the total population. It is thus estimated that a viral load of at least 1000 copies/mL of clinical sample is required

to obtain reliable genotypic profiles [42]. Next-generation sequencing methods use a three-step sequencing process including library preparation, DNA capture and enrichment, and sequencing/detection [43]. Recent advances in high-throughput deep-sequencing technology allows the acquisition of hundreds or thousands reads of gene regions involved in drug resistance and thus improves the detection of emerging mutant subpopulations that are present in less than 10 % of the total viral population [44–46]. Genotypic approaches are objective, but their interpretation is not always straightforward (i.e., discriminating between mutations associated with natural polymorphisms [47–50] and those related to drug resistance). Mutations identified in the *UL97* and *UL54* genes can be linked to drug resistance phenotypes by using a web-based tool (http://www.informatik.uni-ulm.de/ni/staff/HKesler/hcmv) [51]. In order to characterize the role of new mutations not previously linked to drug resistance, recombinant viruses need to be generated by marker transfer experiments of mutated genes in a wild-type virus background [52–54] or by using either overlapping cosmid/plasmid inserts [55] or a viral genome of a susceptible reference strain cloned into a bacterial artificial chromosome [33, 56, 57] prior to testing the phenotypes of mutant viruses in drug susceptibility assays. The introduction of a reporter gene in a permissive cell line [58] or directly in the recombinant virus [59–61] accelerates drug phenotypic testing of mutants and allows a more objective evaluation of viral replication.

3.2 Clinical Significance, Incidence, and Risk Factors for Drug-Resistant HCMV Infections

Shortly after the introduction of GCV, the emergence of drug-resistant HCMV strains was reported particularly in untreated or poorly treated AIDS patients who developed HCMV retinitis at a high frequency (ranging from 20 to 45 %) [62]. Two large studies have evaluated the temporal emergence of GCV-resistant strains during therapy using either phenotypic [63] or genotypic [64] assays. In these studies, GCV resistance (defined by an EC_{50} value ≥6 μM) at the initiation of treatment was a rare event (≤2.7 % of tested strains). Phenotypic evaluation of blood or urine isolates from 95 patients treated with GCV (mostly IV) for HCMV retinitis revealed that 7, 12, 27, and 27 % of patients excreted a GCV-resistant strain after, respectively, 3, 6, 9, and 12 months of drug exposure [63]. On the other hand, a study of 148 AIDS patients treated for HCMV retinitis with oral VGCV has identified the presence of GCV resistance mutations in 2, 7, 9, and 13 % of patients after 3, 6, 9, and 12 months of therapy, respectively [64]. The lower incidence of GCV resistance in the latter study despite the use of sensitive genotypic methods might be explained by differences in the study population, notably improvement in human immunodeficiency virus (HIV) therapy. Due to their less frequent use in clinic, fewer data have been reported on the temporal emergence of FOS- and CDV-resistant HCMV strains in HIV-infected individuals. One small study found an incidence of phenotypic resistance to FOS of 9, 26, 37, and 37 % after 3, 6, 9, and 12 months of therapy using an EC_{50} cutoff value of 400 μM [65], whereas another one reported rates of 13, 24, and 37 % after 6, 9, and 12 months using an EC_{50} cutoff value of 600 μM [66]. The data on CDV resistance (EC_{50} value ≥2–4 μM) are even more limited, but they seem to indicate a resistance rate similar to what has been observed with GCV and FOS [65]. Proposed risk factors for the development of HCMV resistance in this patient population include inadequate tissue drug concentrations due to poor tissue penetration (e.g., the eyes) or poor bioavailability (e.g., oral GCV), a sustained and profound immunosuppression status (CD4 counts <50 cells/μL), frequent discontinuation of treatment due to toxicity, and a high pre-therapy HCMV load [67, 68]. The introduction of highly active antiretroviral therapy (HAART) substantially reduced the incidence of HCMV retinitis in AIDS patients, and this was associated with a concomitant decrease in the rate of GCV resistance from 28 to 9 % evaluated over a period of 2 years in the pre-HAART and HAART eras, respectively [69]. Patients with AIDS, especially those with CD4 counts below 50 cells/μL, remain at risk of developing HCMV retinitis and eventually GCV-resistant infections even nowadays [70].

Thereafter, the more widespread use of oral GCV (with a low bioavailability of 6 %) and the intensification of immunosuppressive regimens resulted in an increased prevalence of HCMV drug resistance in solid organ transplant (SOT) recipients. In this setting, infections caused by HCMV drug-resistant isolates have been associated with an increased number of asymptomatic and symptomatic viremic episodes, earlier onset of HCMV disease, graft loss, and an increased risk of death [71]. Lung transplant recipients appear to have the highest incidence of HCMV resistance development with rates of 3.6–9 % after median cumulative GCV exposures ranging from 79 to 100 days [72–74]. The incidence of resistance increased to 15.8–27 % in seropositive donors (D+)/seronegative recipients (R−) lung transplant patients [73, 74] and occurred as a late complication, i.e., a median of 4.4 months after transplantation [73]. As opposed to what has been reported in lung transplant recipients, the incidence of GCV resistance in other SOT populations has been much lower in D+/R− patients [74, 75] and very occasional in R+ subjects [74]. More specifically, two cohorts of SOT patients including heart, liver, and kidney recipients were evaluated at two US centers [74]. Phenotypic evaluation for HCMV resistance prompted by either clinical suspicion or positive blood cultures indicated that rates of resistance were generally low (e.g., <0.5 %) at one center and varied from 2.2 to

5.6 % at another center depending on the transplanted organ. Another retrospective study evaluated 240 SOT patients including 67 D+/R− patients but excluded lung transplant recipients [75]. In this cohort, GCV-resistant HCMV disease developed only in D+/R− SOT recipients, with resistance rates of 7 % in these patients. HCMV resistance was more frequently seen among recipients of kidney/pancreas or pancreas alone (21 %) than among kidney (5 %) or liver (0 %) recipients. Of note, cases of GCV-resistant HCMV infections occurred at a median of 10 months after transplantation with a median total drug exposure of 194 days (129 days of oral GCV) including 2–3 treatment courses for HCMV disease per patient. Importantly, GCV-resistant HCMV infections accounted for 20 % of HCMV diseases that occurred during the first year after transplantation [75]. Documented risk factors for the emergence of GCV resistance in SOT patients include the lack of HCMV-specific immunity (as encountered in the D+/R− group) [76, 77], lung or kidney/pancreas transplantation, longer drug exposure (prophylaxis > preemptive therapy), suboptimal plasma or tissue drug concentrations (as seen with oral GCV), potent immunosuppressive regimens, a high HCMV viral load, and frequent episodes of HCMV disease [71, 73, 75, 78].

In contrast to GCV, VGCV is highly absorbed after oral administration leading to an improved systemic exposure (about 60 %) that could limit the emergence of drug-resistant HCMV mutants. The clinical efficacy and safety profile of a once-daily (900 mg OD) dose of VGCV were shown to be similar to thrice daily (1 g TID) doses of oral GCV for the prevention of HCMV diseases in high-risk SOT recipients [79]. The first prospective study evaluating the emergence of GCV resistance in SOT recipients used molecular methods to assess the emergence of *UL97* and *UL54* mutations associated with GCV resistance in D+/R− patients (175 liver, 120 kidney, 56 heart, 11 kidney/pancreas, and 2 liver/kidney recipients) receiving HCMV prophylaxis with either oral GCV or VGCV [80, 81]. Among 301 evaluable patients, the incidence of GCV resistance at the end of the prophylactic period (day 100 posttransplant) was very low in both arms (0 % and 3 % for the VGCV and oral GCV arms, respectively). During the first year following transplantation, GCV resistance-associated mutations were found in none compared to 6.1 % of patients at the time of suspected HCMV disease after receiving VGCV and oral GCV prophylaxis, respectively. Of note, however, no lung transplant and a small number of kidney/pancreas recipients were included in this study, which might explain at least partly the low emergence of GCV resistance as compared to previous reports. Interestingly, detection of known GCV resistance mutations was not necessarily associated with adverse clinical outcomes in the latter study [80, 81]. The incidence of drug resistance evaluated by molecular methods in 80 lung transplant recipients who had received IV GCV (D+/R− patients), oral GCV (R+ patients), or oral VGCV prophylaxis was also found to be low [82, 83]. Finally, a low incidence of drug resistance was observed by genotypic testing in adult D+/R− patients (138 kidney, 4 kidney/pancreas, 58 liver, and 25 heart recipients) and pediatric transplant recipients (12 heart, 33 kidney, 17 liver, and 1 liver/kidney recipients) who had received VGCV prophylaxis [84, 85]. The low frequency of drug resistance in SOT recipients receiving VGCV (compared to oral GCV) could be related to an improved GCV exposure and to a better compliance of the patients to the once-daily dosing.

High-risk patients who receive VGCV prophylaxis for 100 days posttransplant might still be at risk of developing late-onset HCMV disease [8]. Extending the prophylactic regimen beyond 3 months may theoretically increase the risk of emergence of drug resistance. Therefore, the impact of extending VGCV prophylaxis from 100 to 200 days on the incidence of resistance was investigated in 318 D+/R− kidney transplant recipients based on genotypic testing [86]. The rates of drug resistance were similar (1.8 % vs. 1.9 %) in patients who had received VGCV prophylaxis for 100 and 200 days suggesting that extending the prophylactic period up to 200 days did not significantly affect the incidence of GCV resistance. Of note, almost all cases of resistance occurred during VGCV prophylaxis and rarely thereafter. Prophylaxis with VGCV for 200 days after transplantation could thus be an interesting option in high-risk kidney transplant recipients.

No clear evidence demonstrates whether a prophylactic or a preemptive approach is more effective in preventing HCMV disease in high-risk transplant patients. Several studies evaluated the effect of these preventive strategies on the emergence of drug resistance based on molecular methods. In a first retrospective study evaluating 1244 renal transplant recipients who had received a once-daily VGCV preemptive therapy, GCV resistance mutations were detected in 2.2 % of the overall population and, more specifically, in 12.5 % of D+/R− patients [87]. Another retrospective study compared the emergence of resistance in D+/R− kidney transplant recipients who had received VGCV prophylaxis for 3 months (32 patients) or VGCV preemptive therapy (80 patients) [88]. HCMV drug resistance was more frequent in the preemptive compared to the prophylactic group (16 % vs. 3 %). The author suggested that, during preemptive therapy, patients may be exposed to suboptimal drug levels which favor an active viral replication state thus increasing the risk of emergence of GCV resistance. It is thus proposed that the prophylactic strategy may be more appropriate than the preemptive therapy in high-risk transplant recipients although further studies are still needed to confirm this point.

Valganciclovir was shown to be noninferior to IV GCV for the treatment of established HCMV disease in SOT recipients [89, 90]. A secondary endpoint of this trial was the

evaluation of the emergence of drug resistance in a cohort including 20 heart, 216 kidney, 23 liver, and 17 lung transplant recipients treated for HCMV disease with a 21-day induction dose of IV GCV or VGCV followed by VGCV maintenance dose for 49 days in both arms [91]. Probable or confirmed drug resistance mutations were low and found to be similar for VGCV (3.6 %) and IV GCV (2.3 %) treatments. Overall, incidence of GCV drug resistance was low in kidneys (3.7 %), intermediate in livers and hearts (4.3–5.0 %), and highest in lungs (17.6 %).

Limited data from small-scale studies suggest that the incidence of GCV resistance in the bone marrow transplant (BMT)/hematopoietic stem cell transplant (HSCT) population might not be as high as observed in SOT recipients and AIDS patients, perhaps because of the more limited immunosuppression exposure. Due to early detection of HCMV reactivation in this setting (prior to engraftment), many centers have adopted a strategy of preemptive therapy in order to reduce morbidity and mortality from subsequent HCMV disease [92]. In a prospective study, molecular methods were used to detect the presence of the most common *UL97* mutations associated with GCV resistance in blood samples of HSCT patients selected on the basis of having a positive HCMV PCR despite ≥14 days of preemptive IV GCV or a second viremic episode within the first 98 days after transplantation. No *UL97* mutations associated with GCV resistance were detected in this cohort of 50 patients (ten of them fulfilling the above criteria for genotypic testing) [93]. However, this was a small study, and resistance would be unlikely after such a short period of preemptive treatment. In another prospective study designed to evaluate risk factors and outcomes associated with rising HCMV antigenemia levels during the first 2–4 weeks of preemptive therapy, 119 HSCT patients receiving preemptive GCV or FOS therapy following a positive pp65 antigenemia test were evaluated [94]. Among these subjects, 47 (39 %) exhibited a significant rise in antigenemia levels despite antiviral administration, and 15 had at least one isolate available for susceptibility testing. Only one GCV-resistant isolate was identified in a patient who had received 4 weeks of GCV therapy [94]. Several other studies [95–97] also reported a low incidence of drug resistance among HSCT recipients who had received preemptive therapy with GCV or VGCV based on genotypic testing. Therefore, the high rate of treatment failure observed in this setting is probably more related to a profound immunocompromised status since immunological reconstitution plays an important role in the final eradication of the infection. In a recent study, a high rate of drug resistance (14.5 %) was exclusively identified in haploidentical-HSCT recipients receiving preemptive therapy with GCV [98]. Ganciclovir resistance appeared after median cumulative treatment duration of 70 days (range 39–330 days) and was associated with severe clinical manifestations. The authors suggested that the continued viral replication may be due to delayed immune reconstitution combined with impaired cross talk between the disparate donor T cells and recipient antigen-presenting cells. Even though short courses of GCV therapy appear to be relatively safe in adult BMT patients, the situation might differ in pediatric patients receiving T-cell depleted unrelated transplants. In a study of 42 such patients [99], 3 showed genotypic evidences of GCV resistance. Of note, in the same study, none of the 37 patients who underwent a similar procedure, but who received their transplant from a mismatched related donor, developed GCV resistance [99]. Rapid emergence of GCV resistance was also documented in 4/5 children with congenital immunodeficiency disorders who underwent T-cell-depleted BMT [100]. In those patients, genotypic evidence of GCV resistance was demonstrated after only 7–24 days (median 10 days) of cumulative GCV therapy.

3.3 Role of UL97 Kinase and UL54 DNA Polymerase Mutations in Drug-Resistant HCMV Clinical Strains

The great majority (>90 %) of drug-resistant HCMV clinical isolates selected from initial treatment with GCV contain one or more mutations in the UL97 kinase, whereas mutations in the UL54 DNA pol are less frequently encountered [101]. The catalytic domain of protein kinases consists of eleven major conserved regions numbered I to XI, with region I having the highest level of homology [102]. The ATP-binding site, the phosphate transfer domain, and the substrate-recognition site correspond to codon ranges located at positions 337–345 (region I), 453–462 (region VIB), and 574–579 (region IX), respectively. Laboratory-engineered UL97-negative HCMV mutant exhibited a severe replicative deficiency compared to the wild-type parental strain highlighting the essential role of this enzyme in the viral replicative cycle [103]. Therefore, only a small number of mutations clustered in a relatively short genomic region of the *UL97* gene have been reported to confer resistance to GCV. Ganciclovir resistance mutations in the *UL97* gene consist in single nucleotide substitutions or in-frame deletions (Fig. 72.2a) [34, 39, 104]. Cumulative analysis of UL97 mutations detected in clinical isolates [74, 105] or in blood samples [64] from 61 AIDS and SOT patients is in general agreement with those of 76 independent UL97 mutants gathered in a single laboratory over years [33]. Those data suggest that mutations A594V (30–34.5 %), L595S (20–24 %), M460V (11.5–14.5 %), and H520Q (5–11.5 %) represent the most frequent UL97 mutations present in GCV-resistant mutants [106]. Additional frequent UL97 mutations associated with GCV resistance include M460I, C592G, and C603W [34, 39, 104]. Other less frequently encountered

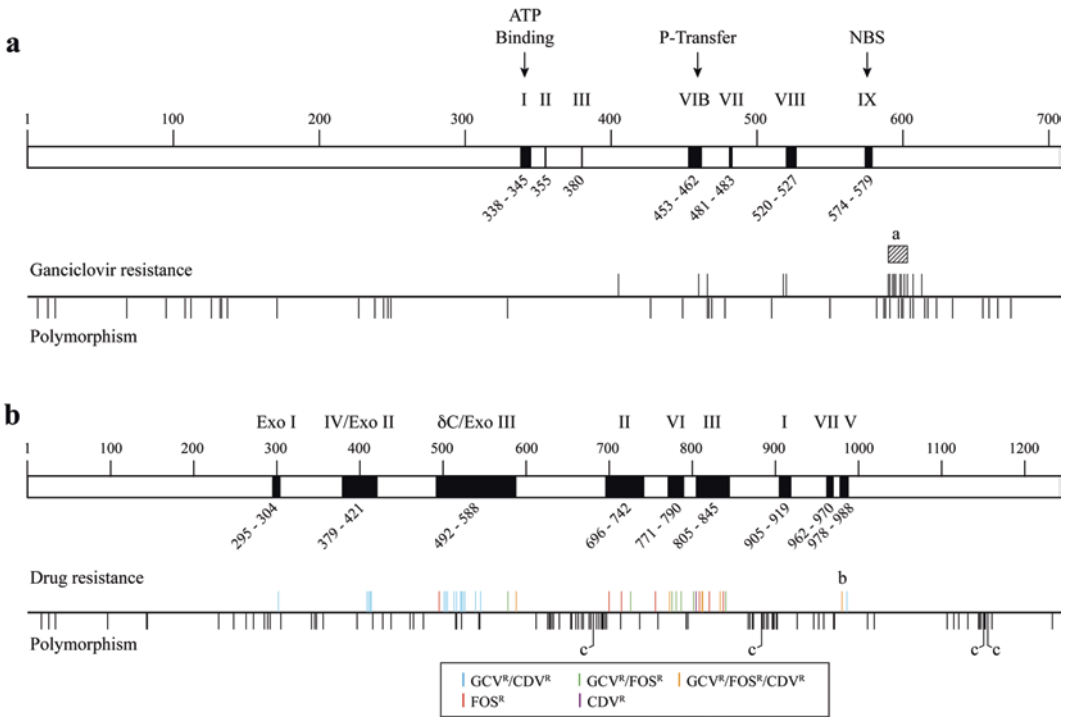

Fig. 72.2 Confirmed drug resistance mutations identified in clinical HCMV isolates. Panel (**a**) shows mutations in the *UL97* gene associated with ganciclovir resistance or natural polymorphism. The ATP-binding site, the phosphate transfer (P-transfer) domain, the nucleoside-binding site (NBS), and some regions conserved among the protein kinase family (i.e., I, II, III, VIB, VII, VIII, and IX) are represented by the *black boxes*. Bars (|) indicate amino acid substitutions associated with ganciclovir resistance (*upper bars*) or with polymorphism (*lower bars*). (*a*) Shaded area corresponds to the codon 590–603 region where different amino acid deletions were identified (i.e., deletions 591–594, 591–607, 595, 595–603, 600, and 601–603). Panel (**b**) shows mutations in the *UL54* gene associated with resistance to ganciclovir (GCV^R), foscarnet (FOS^R), and/or cidofovir (CDV^R) or with natural polymorphism. Conserved regions among the *Herpesviridae* DNA polymerase are represented by the *black boxes*. The roman numbers (I–VII) and δ-region C corresponding to each of these regions are indicated above the boxes. Conserved motifs (Exo I, Exo II, and Exo III) in the exonuclease domain are also indicated above the boxes. Bars (|) indicate amino acid substitutions associated with drug resistance (*upper bars*) or with polymorphism (*lower bars*). (*b*) Amino acid deletion 981–982 that confers resistance to all three antivirals; (*c*) amino acid deletions or insertions associated with polymorphism (i.e., deletions 681–688, 1151, and 1156; insertion 884)

mutations can emerge at codons 460 and between codons 590 and 607 of the UL97 kinase. Based on marker transfer experiments or recombinant phenotyping (Table 72.1), high-level GCV resistance mutations appear to be associated with ≥5-fold increase in EC_{50} values over the parental strain, whereas low-level GCV resistance mutations seem to be associated with <5-fold increase in EC_{50} values. Substitutions or small deletions in the *UL97* gene had no major impact on the viral replicative capacity [33, 110, 114, 115]. Mutation V466G, located outside typical codon ranges, confers a low level of GCV resistance (3.5-fold) and is associated with a significant replicative defect [116]. Amino acid changes associated with natural polymorphisms in the UL97 kinase are mainly clustered in two distinct regions (codons 1–249 and 427–674) [47].

Ganciclovir-resistant HCMV clinical isolates with an altered DNA pol activity result from numerous mutations widely distributed among the different conserved domains of the enzyme, but mostly occur at codons 395–545 and 809–987 (Fig. 72.2b) [34, 39, 104]. The *Herpesviridae* DNA pol belong to the family of α-like DNA polymerases [117] which share regions of homology numbered I to VII. These regions correspond to the degree of conservation among these enzymes, with region I being the most conserved. The *Herpesviridae* DNA pol also contain a δ-region C, which is shared by enzymes related to eukaryotic DNA polymerases δ [118]. Moreover, a 3′-5′ exonuclease domain (containing Exo I, Exo II, and Exo III conserved motifs) maps to the N-terminal region of the herpesvirus DNA pol. DNA pol mutations that emerge under GCV therapy can confer cross-resistance to CDV and, less frequently, to FOS. Cross-resistance to GCV and CDV is associated with mutations located in the exonuclease domains (codons 301, 408–413, 501–545) and in region V (codons 981–987) of the enzyme. Based on marker transfer experiments or recombinant phenotyping (Table 72.2), mutations F412S, D413A, L501I, K513E/N, V526L, and A987G confer a high level of resistance to GCV (≥5-fold increase in EC_{50} values) and cross-resistance to

Table 72.1 HCMV UL97 mutations associated with resistance to ganciclovir confirmed by marker transfer or recombinant phenotyping

Mutation	Fold changes[a] in GCV EC_{50} values	References
L405P	2.5	Chou [21]
M406I	5.0	Chou et al. [33]
M460T	9.3	Chou [21]
M460V	8.3	Chou et al. [35, 60], Marfori et al. [107]
V466G	3.5	Martin et al. [85]
C518Y	12.0	Zhang et al. [108]
H520Q	10.0	Hanson et al. [36], Chou et al. [33]
del591-594	3.0–10.0	Chou et al. [33]
del591-607	6.2	Chou et al. [33]
C592G	2.9	Chou et al. [33, 60]
A594E	3.0	Chou [21]
A594G	13.5	Bourgeois et al. [109]
A594T	2.7	Chou et al. [33]
A594V	8.3	Chou et al. [33, 35, 60]
L595F	15.7	Chou et al. [33]
L595S	9.2	Chou et al. [33, 35, 60]
L595W	5.1	Chou et al. [33]
Del595	13.3	Baldanti et al. [52]
del595-603	8.4	Chou and Meichsner [110]
E596G	2.3	Chou et al. [33]
K599T	5.3	Faizi Khan et al. [111]
del600	1.9	Chou et al. [33]
del601-603	11.0	Marfori et al. [107]
C603R	3.6–8.3	Chou [21], Martin et al. [85]
C603S	1.9	Chou [21]
C603W	8.0	Chou et al. [54], Chou [21]
C607F	1.9	Chou et al. [33]
C607Y	12.5	Baldanti et al. [112], Chou et al. [33]
A613V	2.3	Fischer et al. [113]

GCV ganciclovir, *EC_{50}* concentration of antiviral that reduces viral plaques by 50 %, *del* deletion

[a]Fold changes are calculated as the ratio of EC_{50} values of mutant recombinant virus to the wild-type counterpart. An increase in EC_{50} value ≥1.9-fold higher than that of the wild-type strain corresponds to drug resistance

CDV. Other mutations (i.e., D301N, N408D/K/S, N410K, F412C/L/V, D413E/N, T503I, A505V, K513R, L516R, I521T, P522A/S, del524, C539G, and L545S/W) confer a lower level of resistance to GCV (between 1.9- and 5.0-fold increase in EC_{50} values) and are also cross-resistant to CDV. Resistance to FOS is widely dispersed in the conserved domains of the UL54 DNA pol. However, clusters of mutations are mainly found in regions II, VI, and III and are associated with resistance to FOS alone (i.e., N495K, D588E, T700A, V715M, E756D/Q, and T838A) or to both FOS and GCV (i.e., Q578L, I726V, L776M, V781I, V787L, L802M, A809V, and G841S). Mutation K805Q confers resistance to CDV alone. Importantly, some mutations (i.e., Q578H, D588N, E756K, L773V, V812L, T813S, T821I, A834P, G841A, and del981-982) have been associated with resistance to all three antivirals. Contrasting with the situation with UL97 mutants, isolates with UL54 mutations conferring drug resistance usually exhibit an attenuated or slow-growth phenotype in cell culture compared to their wild-type counterpart as assessed in marker transfer experiments. Among those, mutations T700A and V715M (conserved region II) [53], K513N (δ-region C) [123], and D301N (Exo I motif) [56] were shown to significantly reduce the yield of progeny virus in cell culture supernatants, whereas some others (D413E, T503I, L516R, and E756K/D) were only associated with a modest attenuation of viral replication [56]. Finally, the natural polymorphism is more common in the *UL54* gene than in the *UL97* gene and occurs most often at nonconserved residues (between codons 614 and 697) where little homology exists among herpesvirus DNA pol [48, 49]. The high degree of inter-strain variability in the *UL54* gene complicates the interpretation of genotypic testing in the absence of recombinant phenotyping.

In the case of HCMV mutants selected during GCV therapy, it should be noted that UL97 mutations have been generally shown to emerge first and to confer a low level of resistance (EC_{50} < 30 μM), whereas subsequent emergence of UL54 DNA pol mutations usually leads to a high level of GCV resistance (EC_{50} > 30 μM) with potential cross-resistance [133–135]. However, occasional reports have described mutations restricted to the *UL54* gene only after initial therapy with GCV [81, 96].

3.4 When and How to Monitor for HCMV Resistance

HCMV resistance to antivirals should be suspected in patients failing treatment who have been exposed to an antiviral for substantial periods of time (typically >3–4 months in AIDS patients and >6 weeks in transplant recipients), especially if some risk factors are present (i.e., D+/R− SOT, lung or kidney/pancreas transplant, AIDS patients with CD4 counts <50 cells/μL). Resistance should be suspected in pediatric patients with shorter periods of drug exposure if they had T-cell depletion. Clinical resistance is more likely if active viral replication (high or increasing levels of DNAemia/antigenemia or viremia) persists or recurs despite maximum IV doses of the antivirals [68, 78]. On the other hand, rising antigenemia levels during the first 2 weeks of antiviral therapy in HSCT recipients have not been associated with antiviral resistance, but rather with host and other transplant-related factors [94, 136]. Whenever antiviral resistance is suspected, phenotypic and/or genotypic investigation for resistance should be undertaken. As discussed above, genotypic methods are fast,

Table 72.2 HCMV UL54 DNA polymerase mutations associated with drug resistance confirmed by marker transfer or recombinant phenotyping

Regions	Mutation	Fold changes[a] in EC_{50} values for			References
		GCV	FOS	CDV	
Exo I	D301N	**2.6**	0.5	**3.0**	Chou et al. [56]
Exo II	N408D	**4.9**	1.3	**5.6**	Cihlar et al. [55]
	N408K	**4.2**	0.7	**21.0**	Scott et al. [119]
	N408S	**3.1**	1.0	**7.5**	Hantz et al. [120]
	N410K	**2.9**	0.8	**3.0**	Chou et al. [56]
	F412C	**4.2**	1.2	**18.0**	Chou et al. [54]
	F412L	**4.6**	1.1	**9.4**	Chou [121]
	F412S	**5.3**	0.8	**13.0**	Chou [121]
	F412V	**4.3**	1.1	**15.5**	Cihlar et al. [55]
	D413A	**6.5**	0.8	**11.0**	Marfori et al. [107]
	D413E	**4.8**	0.8	**4.3**	Chou et al. [56]
	D413N	**3.8**	1.0	**10.0**	Chou et al. [45]
Exo III	N495K	1.1	**3.4**	1.1	Ducancelle et al. [122]
	L501I	**6.0**	1.4	**9.1**	Cihlar et al. [55]
	T503I	**2.9**	0.5	**6.1**	Chou et al. [56]
	A505V	**1.9**	1.0	**1.9**	Chou et al. [44]
	K513E	**5.0**	1.4	**9.1**	Cihlar et al. [55]
	K513N	**6.0**	1.1	**12.5**	Cihlar et al. [123]
	K513R	**3.7**	1.1	**10.0**	Chou et al. [45]
	L516R	**2.1**	0.8	**5.1**	Chou et al. [56]
	I521T	**3.1**	0.9	**3.9**	Chou et al. [124]
	P522A	**3.0**	1.0	**4.1**	Chou et al. [124]
	P522S	**3.1**	1.1	**3.6**	Cihlar et al. [55]
	del524	**3.5**	1.1	**9.7**	Hantz et al. [120]
	V526L	**5.5**	1.8	**2.5**	Drouot et al. [125]
	C539G	**3.1**	1.0	**4.4**	Chou et al. [45]
	L545S	**3.5**	1.2	**9.1**	Cihlar et al. [55]
	L545W	**4.9**	1.3	**6.3**	Chou [121]
	Q578H	**3.3**	**4.5**	**2.3**	Chou [121]
	Q578L	**1.9**	**3.0**	0.8	Chou et al. [44]
	D588E	1.3	**2.3**	1.1	Cihlar et al. [55]
	D588N	**3.8**	**3.2–9.0**	**2.7**	Springer et al. [126], Mousavi-Jazi et al. [127]
Region II	T700A	0.9	**4.7**	1.5	Baldanti et al. [53]
	V715M	1.0	**5.5**	1.1	Baldanti et al. [53]
	I726T	**2.0**	1.1	1.7	Chou et al. [44]
	I726V	**1.9**	**1.9**	1.2	Chou et al. [44]
[Regions II–VI]	E756D	1.2	**3.4**	0.7	Chou et al. [56]
	E756K	**3.5**	**>8.0**	**2.2**	Chou et al. [56]
	E756Q	1.7	**4.3**	1.0	Weinberg et al. [66]
Region VI	L773V	**3.0**	**4.4**	**2.5**	Chou et al. [45]
	L776M	**2.5**	**3.5**	1.0	Shapira et al. [128]
	V781I	1.0–**4.0**	**4.0–5.2**	1.2	Cihlar et al. [55], Mousavi-Jazi et al. [127]
	V787L	**2.4**	**4.1**	1.0	Weinberg et al. [66]
[Regions VI–III]	L802M	1.1–**3.5**	**3.2–10.8**	0.9–1.8	Chou et al. [54], Cihlar et al. [55]
Region III	K805Q	1.0	0.2	**2.2**	Cihlar et al. [55]
	A809V	**2.6**	**6.3**	1.7	Chou et al. [129]
	V812L	**2.5**	**4.9**	**2.7**	Cihlar et al. [123]
	T813S	**2.5**	**4.9**	**2.7**	Chou et al. [130]
	T821I	**4.5**	**21.0**	**1.9**	Cihlar et al. [55]
	A834P	**5.4**	**6.4**	**3.0**	Scott et al. [119]
	T838A	1.8	**2.4**	0.8	Springer et al. [126]
	G841A	**3.2**	**4.3**	**2.6**	Chou et al. [130]
	G841S	**2.2**	**2.1**	1.1	Chou et al. [44]
Region V	del981-982	**8.3**	**3.6**	**2.8**	Chou et al. [131]
	A987G	**5.3**	1.2	**11.3**	Sullivan et al. [132]

GCV ganciclovir, *FOS* foscarnet, *CDV* cidofovir, *EC_{50}* concentration of antiviral that reduces the number of viral plaques by 50 %

[a]Fold changes are calculated as the ratio of EC_{50} values of mutant recombinant virus to the wild-type counterpart. An increase in EC_{50} value ≥1.9-fold higher than that of the wild-type strain corresponds to drug resistance (in *bold*)

Regions indicated in brackets are located between conserved regions

more convenient, and provide useful information for selection of an alternative treatment. However, identification of mutations of unknown significance remains problematic, and, for that reason, phenotypic assays may still be necessary. Furthermore, genotypic assays do not quantitate the degree of resistance while phenotypic assays do. The choice of the sample to analyze may also have some importance. Some studies have reported that there is a good correlation between genotypes detected in the eyes and the blood (93.5 %) [137] or between blood and urine isolates (87.5 %) [134] of AIDS patients with HCMV retinitis. However, there have been at least some reports of resistant HCMV strains restricted to specific body compartments [99, 138–140]. This suggests that resistance assessment based solely on blood or urine samples may be suboptimal in some cases [32]. Therefore, genotypic testing of cerebrospinal fluid, bronchoalveolar lavages, or biopsy specimens could be occasionally performed in high-risk patients.

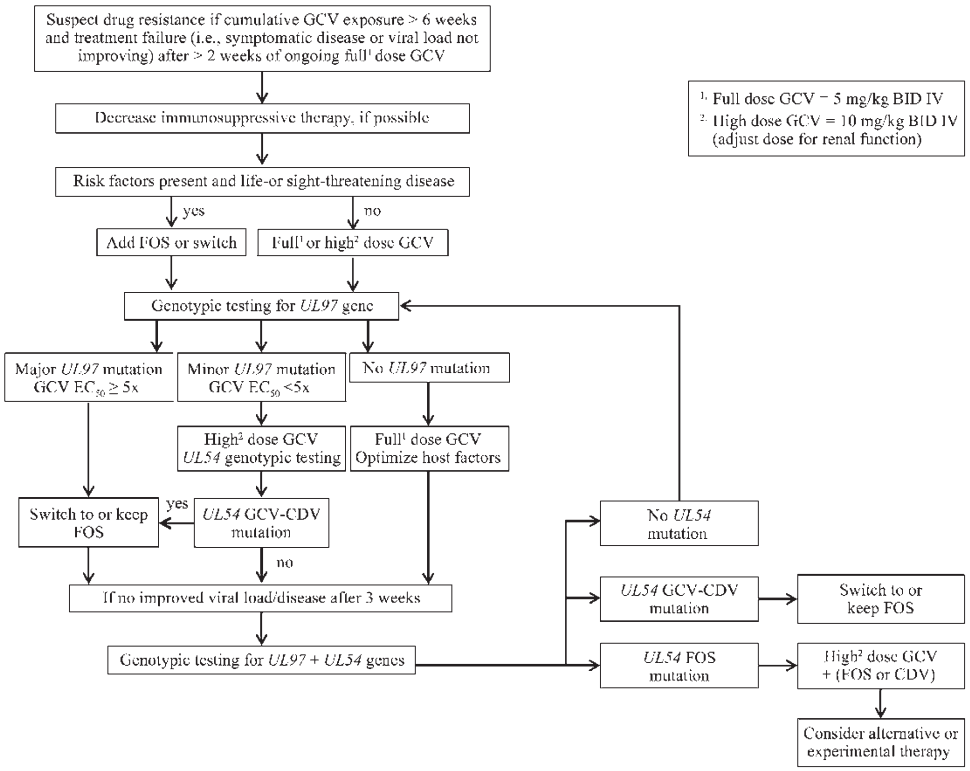

Fig. 72.3 Suggested algorithm for the management of suspected drug-resistant HCMV infections in solid organ transplant recipients. **Key**: *GCV* ganciclovir, *FOS* foscarnet, *CDV* cidofovir, *BID* twice a day, *IV* intravenous, EC_{50} concentration of antiviral that reduces HCMV replication in cultured cells by 50 % compared to the control (without drug) determined in phenotypic assay. Adapted from [141]

3.5 Management of Infections Caused by Drug-Resistant HCMV Strains

Guidelines for the management of GCV-resistant HCMV diseases in SOT have been established during consensus meetings organized by the Transplantation Society International CMV Consensus Group [141] with a suggested algorithm shown in Fig. 72.3. Antiviral drug resistance should be suspected in case of cumulative GCV exposure for more than 6 weeks and stable or rising viral loads (especially DNAemia levels) despite more than 2 weeks after initiating appropriate full dose IV GCV (5 mg/kg of body weight twice daily, adjusted for renal function). Whenever possible, improvement of the patient's immune status (i.e., reduction of immunosuppressive regimen in transplant patients or aggressive antiretroviral therapy in AIDS patients) should be considered. As the modulation of immunosuppression is rarely sufficient to control HCMV, the use of adjunctive immunoglobulins containing HCMV antibodies could be considered, but these agents are expensive, and their supply is limited. At the initial step, a clinical decision about an antiviral switch is empirical but should be based on the evaluation of host risk factors (e.g., D+/R− recipients, lung transplant recipients) and disease severity (sight- or life-threatening disease) [67, 78]. Full or higher doses of IV GCV (5 or 10 mg/kg twice daily, respectively, adjusted for renal function) can be administered to low-risk patients with mild disease [142], whereas FOS alone or combined with GCV can be initiated for high-risk patients with severe disease. Of note, clear evidence of the superiority of GCV and FOS combination over FOS alone has not yet been demonstrated [143]. As resistance mutations to GCV typically emerge in the protein kinase, *UL97* gene sequencing is first recommended. Genotypic assays are performed typically on whole-blood or plasma specimens [144]. Despite the limitations mentioned above, genotypic resistance testing is more practical and rapid than phenotypic assays. Thus, rescue therapy should be ideally based on results of the genotypic assays. In centers where genotypic testing is unavailable or performed infrequently, initial management should avoid the use of drugs with similar pathways of resistance. For instance, patients failing GCV should be given FOS alone or combined with GCV in the absence of any sequencing data due to high frequency of UL54 mutations that confer resistance to both GCV and CDV. If no mutation is identified in the *UL97* gene, full dose of IV GCV (5 mg/kg twice daily, adjusted for renal function) should be continued together with an optimization of host factors. If a high-level GCV resistance mutation (more than fivefold increase in EC_{50} value) is identified in the *UL97* gene, a switch to IV FOS is recommended (or IV FOS should be kept). If a low-level GCV resistance mutation (less than fivefold increase in EC_{50} value) is detected in the *UL97* gene, the patient could be re-induced with higher than normal doses of IV GCV (up to 10 mg/kg twice daily, adjusted for renal function), and drug

resistance mutations should be looked for in the *UL54* gene. If a mutation conferring cross-resistance to GCV and CDV is detected in the *UL54* gene, a switch to IV FOS therapy is recommended (or IV FOS should be kept).

The viral load is typically monitored once weekly by quantitative PCR during the period covering an episode of symptomatic HCMV disease. If there is no improvement in the viral load and a persistence of HCMV disease after a period of 3 weeks, genotypic testing should be repeated to assess the emergence of drug resistance mutations in both the *UL97* and *UL54* genes. If a mutation conferring cross-resistance to GCV and CDV is detected, a switch to IV FOS is recommended (or IV FOS should be kept). If a resistance mutation to FOS is detected, combination of high-dose IV GCV (10 mg/kg twice daily, adjusted for renal function) with IV FOS or CDV (5 mg/kg once a week for 3–4 weeks) should be considered. CDV has a long intracellular half-life that makes infrequent dosing possible. Because of its nephrotoxicity, CDV is routinely administered with probenecid and requires IV hydration. Antiviral therapy is typically continued until viremia is no longer detectable. In case of multidrug-resistant HCMV disease, alternative or experimental therapies should also be considered.

Several nonconventional interventions have been described for the treatment of multidrug-resistant HCMV diseases, although their clinical utility has not been adequately evaluated [145]. Immunoglobulins containing HCMV antibodies and adoptive infusions of HCMV-specific T cells [146] may improve antiviral host defenses. Artesunate, an antimalarial drug, demonstrates in vitro and in vivo activity against HCMV [147] including drug-resistant mutants [148], but its mechanism of action remains unclear. It is suggested that artesunate blocks the synthesis of viral immediate early proteins probably through inhibition of virus-supportive cellular activation pathways [149]. Clinical reports on the treatment of drug-resistant HCMV infections with artesunate are rare and controversial [128, 150, 151]. A recent study indicated that artesunate may be useful for the treatment of mild HCMV diseases due to multidrug-resistant strains but may not be effective against severe HCMV diseases [152]. Leflunomide, a prodrug with immunosuppressive, antiproliferative, and anti-inflammatory properties indicated for the treatment of rheumatoid arthritis, possesses anti-HCMV activity including against GCV-resistant isolates by acting on late-stage virion assembly through the inhibition of viral nucleocapsid and tegument development [153, 154]. Thus, no cross-resistance is expected with the current antiviral agents. The use of leflunomide, alone or in combination with antivirals or HCMV immunoglobulins, has been reported in 17 transplant recipients with complex HCMV syndromes failing to respond to available antiviral agents [155]. Initial clearance of HCMV viremia was observed in 82 % of patients, and 53 % of patients achieved long-term suppression of HCMV recurrences. A review of several case reports shows some efficacy for the use of leflunomide, alone or in combination with standard antiviral agents, particularly in transplant recipients refractory to current therapy [156]. Mammalian target of rapamycin (mTOR) inhibitors are immunosuppressive agents that may affect viral replication by inhibiting cellular pathways critical for HCMV infection and/or by influencing immune-mediated responses [157]. Combination of GCV and sirolimus for the treatment of GCV-resistant HCMV infections has led to favorable outcome with respect to antigenemia level and graft rejection in six kidney and three kidney/pancreas recipients [158]. Salvage therapy with a mTOR inhibitor (sirolimus or everolimus) was effective for the treatment of two SOT recipients with GCV-resistant HCMV infections [159].

4 Herpes Simplex Virus and Varicella-Zoster Virus Antiviral Drug Resistance

4.1 Phenotypic and Genotypic Assays to Evaluate HSV and VZV Drug Susceptibility

Phenotypically, HSV and VZV resistance to ACV is related to one of the following mechanisms: (1) a complete deficiency in viral TK activity (TK-deficient); (2) a decreased production of viral TK (TK low producer); (3) a viral TK protein with altered substrate specificity (TK altered), i.e., the enzyme is able to phosphorylate thymidine, the natural substrate, but does not phosphorylate ACV; and finally (4) a viral DNA pol with altered substrate specificity (DNA pol altered) [160–166]. Both TK and DNA pol mutants resistant to ACV exhibit a decrease in so-called in vivo "fitness" and neurovirulence. Alteration or absence of the TK protein is the most frequent mechanism seen in the clinic, probably because TK is not essential for viral replication in most tissues and cultured cells [161, 164, 167]. However, HSV TK plays an important role in the pathogenesis of infection as demonstrated in animal models [168]. It has been proposed that altered or deficient TK enzyme could not fulfill the greater requirement of thymidine phosphorylation for virus replication in neurons compared to other cells [169]. In this respect, TK low producer mutants show some reduction in pathogenicity compared to wild-type strains but are generally able to reactivate [170]. In contrast, TK-deficient mutants have impaired pathogenicity, establish latency in sensory ganglia with a lower efficiency than wild-type strains, and reactivate poorly [168, 171–174]. However, it has been suggested that some TK-deficient HSV clinical isolates express ultralow levels of enzyme activity that could be sufficient to allow reactivation [170, 175]. Moreover, phylogenetically related strains

sensitive and resistant to ACV can coexist in latently infected trigeminal ganglia of patients which may allow the reactivation of TK-deficient strains [176, 177]. As the HSV DNA pol is essential for viral replication, mutations emerging in this enzyme must be functionally conservative. Mutants with altered DNA pol activity have been less studied, but they seem to exhibit different degrees of attenuation of neurovirulence in mice [178–181].

The TK phenotype can be determined by the selective incorporation of radiolabeled iododeoxycytidine (IdC) and thymidine into infected cells using plaque autoradiography [182]. More recently, a nonisotopic enzyme assay has been developed to assess TK functionality by measuring monophosphate forms of both ACV and thymidine using high-performance liquid chromatography with diode-array detection [162].

Levels of drug resistance (EC_{50} values) are best measured by cell-based (phenotypic) assays. The plaque reduction assay (PRA) is the gold standard phenotypic method to determine the susceptibility of HSV isolates to antiviral drugs and is approved as a standard protocol by the Clinical and Laboratory Standards Institute [183]. Breakpoint values that are widely accepted to define HSV resistance to ACV and FOS are EC_{50} equal to or greater than 9 μM and 330 μM, respectively [183]. No consensus value has been proposed for PCV. Drug resistance can also be defined by an increase in the EC_{50} value greater than three to five times that of the baseline isolate from the same patient.

Susceptibility of VZV to antiviral drugs can be tested in the PRA by using fibroblastic cell lines such as human embryonic MRC-5 fibroblasts [184]. The low rate of VZV isolation from vesicle samples (from 20 to 43 %) and its slow growth in cell culture (typically 5–6 days) limit the use of the PRA in that context [185]. The endpoint for detecting resistance to ACV is a susceptibility index equal to or greater than four times that of a control, known sensitive reference strain such as the Oka strain [186].

An alternative to phenotypic assays is genotyping by sequence analysis. Mutations conferring resistance to nucleoside analogues occur in *UL23* (HSV) or *ORF36* (VZV) gene encoding the TK and/or in *UL30* (HSV) or *ORF28* (VZV) gene encoding the DNA pol. For a comprehensive genotypic analysis, the whole TK gene as well as the conserved regions of the HSV or VZV DNA pol gene sequences should be determined [187]. As some degree of inter-strain variability exists in these genes, mutations conferring drug resistance must be discriminated from natural polymorphisms. In this respect, results of genotypic testing must be interpreted by comparison with mutations already assigned to natural polymorphism or confirmed drug resistance in the literature. Different molecular biology-based systems can be used to generate HSV recombinant viruses and characterize the role of unknown mutations such as the transfection of a set of overlapping cosmids and plasmids allowing rapid site-directed mutagenesis in the gene of interest [188, 189] or the cloning of the viral genome of a control susceptible strain into a bacterial artificial chromosome [190] that can be then manipulated in bacteria.

4.2 Clinical Significance, Incidence, and Risk Factors for Drug-Resistant HSV and VZV Infections

Cases of HSV infections unresponsive to treatment in immunocompetent patients are usually associated with diagnoses of recurrent genital herpes, keratitis, and encephalitis. In general, most unresponsive cases in immunocompetent patients are not due to antiviral drug resistance. Furthermore, the rare instances of resistance in that setting are not associated with prolonged active lesions due to a functional immune system. Studies have shown that 0.1–0.6 % of HSV isolates recovered from untreated, prophylaxed, or treated immunocompetent subjects harbor a resistant phenotype to ACV ($EC_{50} \geq 8.8$ μM) as assessed by a PRA, and this seems to reflect the natural occurrence of TK-deficient mutants in a viral population [191–199]. Except for a few notable cases [200, 201], the occasional recovery of ACV-resistant HSV-2 from immunocompetent hosts has not been associated with clinical failure and proved to be transient [199, 202]. However, a relatively high prevalence (6.4 %) of ACV-resistant HSV-1 isolates has been reported in immunocompetent patients with recurrent herpetic keratitis [203], and some of these cases were clinically refractory to ACV therapy [204–207]. The cornea is an immune-privileged site where a lower immune surveillance could favor the rapid selection of drug-resistant viruses [208]. On the other hand, ACV-resistant HSV strains are more often isolated in immunocompromised hosts, and such isolates have been associated with persistent and/or disseminated diseases [16, 194, 209–214]. Patients with AIDS can develop extensive mucocutaneous lesions usually not associated with visceral or central nervous system infections [215]. In the few clinical surveys reported, the rate of ACV-resistant HSV isolates has varied from 4.3 to 14 % among all immunocompromised groups [194, 195, 198, 211, 215–217]. More specifically, 6.5 % of HSV isolates obtained from patients with cancer were resistant to ACV compared to 10 % from heart or lung transplant recipients and 6 % from AIDS patients [194]. Similarly, 7 % of HSV isolates recovered from AIDS patients were resistant to ACV compared to 5–14 % from diverse SOT and BMT recipients [211]. The prevalence of ACV resistance has ranged from 3.5 to 7 % in HIV-positive patients in several studies [195, 215, 217–219]. Of note, high prevalence rates have been reported in HSCT recipients, with a range of 4.1–10.9 %

[167, 195, 210, 220–224]. An even higher frequency (36 %) of ACV resistance in that population has also been reported [225]. Patients receiving either autologous or allogenic bone marrow have a similar incidence, i.e., 9 %, of HSV infection, but resistance only occurred in allogenic transplants, reaching a prevalence of 30 % in a study [226]. The severity of immunosuppression and the prolonged use of ACV are considered two important factors for the development of drug resistance. The impact of the severity of immunosuppression was underscored in adult patients undergoing lymphocyte-depleted hematopoietic progenitor cell transplant from HLA-matched family donors [225]. All seven evaluable HSV-1 or HSV-2 seropositive patients reactivated at a median of 40 days posttransplant, and the five strains tested were all resistant to ACV. Furthermore, FOS resistance developed rapidly in the three patients treated with this drug [225]. Importantly, the prevalence of ACV-resistant HSV isolates has remained stable in immunocompromised patients over the past two decades [194, 195], and there has been no unequivocal evidence of transmission of a resistant HSV strain from person to person. Drug-resistant HSV mutants have been isolated in some patients in the absence of known history of ACV exposure [227, 228] and likely represent the natural rates of TK mutations.

Only a few FOS-resistant HSV isolates ($EC_{50} \geq 330$ μM or a threefold increase in EC_{50} value compared to the parental susceptible strain) have been reported in the clinic mainly in AIDS patients failing therapy [17, 229–236]. Nine FOS-resistant HSV clinical isolates from HIV-infected subjects for whom ACV and FOS therapy sequentially failed have been described [236]. Interestingly, most of these isolates retained susceptibility or, at the most, borderline levels of susceptibility to ACV and CDV [236, 237].

The emergence of VZV isolates resistant to ACV has not been described in immunocompetent individuals with primary VZV infections or herpes zoster, except for one case report of a patient with an ACV-resistant VZV keratitis [238]. Cases of resistance to ACV have been described in patients with AIDS, SOT, and HSCT recipients as well as hemato-oncological patients with VZV reactivations unresponsive to therapy [184, 239–242]. In these patients, VZV infections not responding to ACV therapy persist in the form of chronic skin lesions and are associated with significant morbidity and mortality due to visceral dissemination. An unusual verrucous form of VZV infections caused by ACV-resistant mutants has also been described in some of these patients [243, 244]. Two cases of immunocompromised children presenting herpes zoster due to the Oka vaccine strain and who developed chronic disseminated drug-resistant VZV infections following ACV therapy have been reported [243, 245]. However, the prevalence of ACV-resistant cases in these different populations is unknown because only case reports have been published so far. In a recent study, it was reported that 27 % of hemato-oncological patients, including HSCT recipients, with persistent VZV infections had mutations probably associated with ACV resistance [246]. Few reports have described the emergence of VZV strains resistant to FOS in immunocompromised patients [17, 229, 231, 242, 247].

4.3 Role of Thymidine Kinase and DNA Polymerase Mutations in Drug-Resistant HSV and VZV Clinical Strains

In HSV clinical isolates, resistance to ACV is mediated in 95 % of the cases by mutations in the *UL23* gene and, in the remaining cases, by mutations in the *UL30* gene [39, 248, 249]. Resistance hot spots in the *UL23* gene consist of either additions or deletions in homopolymer runs of Gs and Cs leading to a premature stop codon [161, 167, 250]. The remaining ACV-resistant clinical isolates have single amino acid substitutions in conserved (especially in the ATP-binding site, the nucleoside-binding site, and at amino acid 336) and nonconserved regions of the TK polypeptide (Table 72.3; Fig. 72.4a) [39, 248, 249]. Globally, each mechanism (additions/deletions or substitutions) accounts for approximately 50 % of ACV-resistant phenotypes in the clinic [161]. However, recent studies reported an increased proportion of additions/deletions which accounts for 62 % [263] or even 80 % [264] of *UL23* gene mutations. Most HSV DNA pol mutations conferring ACV resistance are located in the conserved regions of the enzyme, especially in regions II, III, VI, and VII, the greatest clusters being found in regions II and III (Table 72.4; Fig. 72.4b) [236, 270]. Only a few mutations have been described within the other conserved domains or outside such regions [236]. Most FOS-resistant clinical isolates contain single amino acid substitutions in conserved regions II, III, and VI and in a nonconserved region (between regions I and VII) of the DNA pol [236, 237]. Mutations within conserved regions II and VI are frequently associated with resistance to both ACV and FOS. The mutations S724N (region II) and L778M (region VI) in HSV-1, which confer cross-resistance to ACV and FOS, also cause reduced susceptibility to CDV [188]. Genotypic analyses of drug-sensitive HSV strains reveal a high degree of polymorphism in the *UL23* and *UL30* genes [268, 271, 272].

In VZV clinical isolates, resistance to ACV is mostly associated with mutations in the viral TK (Table 72.3; Fig. 72.4a) and, less frequently, with mutations in the viral DNA pol (Table 72.4; Fig. 72.4b) [39, 249]. The genome of VZV has a lower GC content (46 %) than those of HSVs (68 %), and only a few homopolymer stretches are present in the *ORF36* gene [273]. The string of six cytosines located at codon position 493–498 within this gene emerged as a hot

Table 72.3 Amino acid substitutions associated with acyclovir resistance in the thymidine kinase of clinical HSV-1, HSV-2, and VZV strains confirmed by enzyme assay or recombinant phenotyping

aa changes	Phenotypic test	Confirmation by	TK phenotype (fold change)[a]	References
A. HSV-1				
ATP binding site/site 1 (aa 51–63)				
R51W	PRA, dye-uptake assay	Enzyme assay	Reduced TK	Frobert et al. [251]
Y53C	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
Y53D	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
Y53H	PRA	Enzyme assay	TK-	Sauerbrei et al. [165]
P57H	Dye-uptake assay	Autoradiography	TK low/alt	Gaudreau et al. [161]
K62N	Dye-uptake assay	Autoradiography, recombinant virus	TK low/alt (42.0x)	Gaudreau et al. [161], Sergerie and Boivin [189]
T63I	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
Site 2 (aa 83–88)				
E83K	PRA, dye-uptake assay	Enzyme assay	TK-	Frobert et al. [251]
P84L	PRA	Enzyme assay	TK alt	Malartre et al. [162]
P84S	PRA	Enzyme assay	TK low	Saijo et al. [252]
Site 3 (aa 162–164)				
D162A	PRA	Enzyme assay	Reduced TK	Frobert et al. [253]
R163H	PRA	Enzyme assay	TK-/low	Malartre et al. [162]
Nucleoside binding site/site 4 (aa 168–176)				
L170P	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
Y172C	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
P173L	PRA	Enzyme assay	TK-	van Velzen et al. [207]
A174P	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
A175V	Dye-uptake assay	Enzyme assay	TK alt	Frobert et al. [251], Malartre et al. [162]
R176Q	PRA, dye-uptake assay	Autoradiography, enzyme assay	TK low/alt	Gaudreau et al. [161], Kussmann-Gerber et al. [254]
R176W	PRA	Enzyme assay	Reduced ACV phos., TK-	Burrel et al. [160]
Site 5 (aa 216–222)				
R216C	PRA, dye-uptake assay	Autoradiography, enzyme assay	TK low/alt	Gaudreau et al. [161], Bae et al. [255]
R220H	PRA	Enzyme assay	TK low	van Velzen et al. [207]
R222C	Dye-uptake assay	Autoradiography	TK low/alt	Gaudreau et al. [161]
Site 6 (aa 284–289)				
T287M	PRA, dye-uptake assay	Autoradiography, enzyme assay	TK low/alt TK-	Gaudreau et al. [161] Sauerbrei et al. [166]
C-terminal active region				
C336Y	PRA, dye-uptake assay	Autoradiography, enzyme assay, recombinant virus	TK low/alt (30.0x)	Gaudreau et al. [161], Harris et al. [256], van Velzen et al. [207], Sergerie and Boivin [189]
Nonconserved regions				
S74stop	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
T103P	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
Q104stop	PRA, dye-uptake assay	Enzyme assay	TK low	Sauerbrei et al. [166]
H105P	PRA, dye-uptake assay	Enzyme assay	Reduced TK	Frobert et al. [253]
M121R	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
Q125H	PRA	Recombinant virus	(NA)	Kakiuchi et al. [257]
V187M	PRA	Enzyme assay, autoradiography	TK-	Horsburgh et al. [258]
A189V	PRA	Enzyme assay	TK-/alt	Malartre et al. [162]
G200C	PRA	Enzyme assay	TK low	Sauerbrei et al. [166]
G200S	PRA	Enzyme assay	TK-	Malartre et al. [162]

(continued)

Table 72.3 (continued)

aa changes	Phenotypic test	Confirmation by	TK phenotype (fold change)[a]	References
T201P	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
V204G	PRA	Autoradiography, recombinant virus	TK low/alt (125.0x)	Pan et al. [206]
A207P	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
L208H	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
L227F	PRA	Enzyme assay	TK alt	Malartre et al. [162]
T245M	PRA	Enzyme assay	TK-/low	Sauerbrei et al. [166]
Q250stop	PRA	Enzyme assay	TK-	Sauerbrei et al. [166]
L315S	PRA	Enzyme assay	TK-	Sauerbrei et al. [165, 166]
L364P	PRA	Enzyme assay	TK-, reduced TK	Harris et al. [256], Frobert et al. [253]
B. HSV-2				
Nucleoside binding site/site 4 (aa 169–177)				
R177W	PRA	Enzyme assay	TK alt	Kost et al. [200]
Site 6 (aa 284–289)				
T287M	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
C-terminal active region				
C337Y	PRA, dye-uptake assay	Autoradiography	TK low/alt	Sasadeusz et al. [250], Gaudreau et al. [161]
Nonconserved regions				
S66P	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
A72S	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
I101S	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
Q105P	PRA	Autoradiography, enzyme assay	TK-	Chatis and Crumpacker [259], Tanaka et al. [260]
T131P	PRA, dye-uptake assay	Enzyme assay	TK low/alt	Gaudreau et al. [161]
L158P	PRA	Enzyme assay	TK-	Harris et al. [256]
S182N	PRA	Enzyme assay	TK ultralow	Tanaka et al. [260]
M183I	PRA	Enzyme assay	Reduced ACV phos.	Burrel et al. [160]
G201D	PRA	Enzyme assay	TK-	Harris et al. [256]
R223H	PRA	Enzyme assay	TK alt	Kit et al. [261]
R271V	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
P272S	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
D273R	Dye-uptake assay	Autoradiography	TK-	Gaudreau et al. [161]
C. VZV				
ATP binding site/site 1 (aa 12–29)				
G24E	DNA:DNA hybridization	Autoradiography	TK-	Boivin et al. [239]
K25R	DNA:DNA hybridization	Autoradiography	TK alt	Talarico et al. [241]
Nucleoside binding site/site 4 (aa 129–145)				
D129N	DNA:DNA hybridization	Autoradiography	TK-	Talarico et al. [241]
R130Q	PRA	Enzyme assay	TK alt	Sawyer et al. [262], Roberts et al. [164]
R143G	DNA:DNA hybridization, dye-uptake assay	Autoradiography, enzyme assay	TK-/alt	Talarico et al. [241], Morfin et al. [240]
R143K	DNA:DNA hybridization	Autoradiography	TK alt	Talarico et al. [241]
Other conserved regions				
E48G	Dye-uptake assay	Enzyme assay	TK-	Morfin et al. [240]
T256A	PRA	Enzyme assay	TK-	Bryan et al. [243]
Nonconserved regions				
E59G	DNA:DNA hybridization	Autoradiography	TK alt	Talarico et al. [241]
C90T	–	Enzyme assay	TK low	Levin et al. [245]

ACV acyclovir, *PRA* plaque reduction assay, *TK-* TK-deficient, *TK low* TK low producer, *TK alt* TK altered, *phos* phosphorylation
[a]Fold changes are calculated as the ratio of EC_{50} values of mutant recombinant virus to the wild-type counterpart

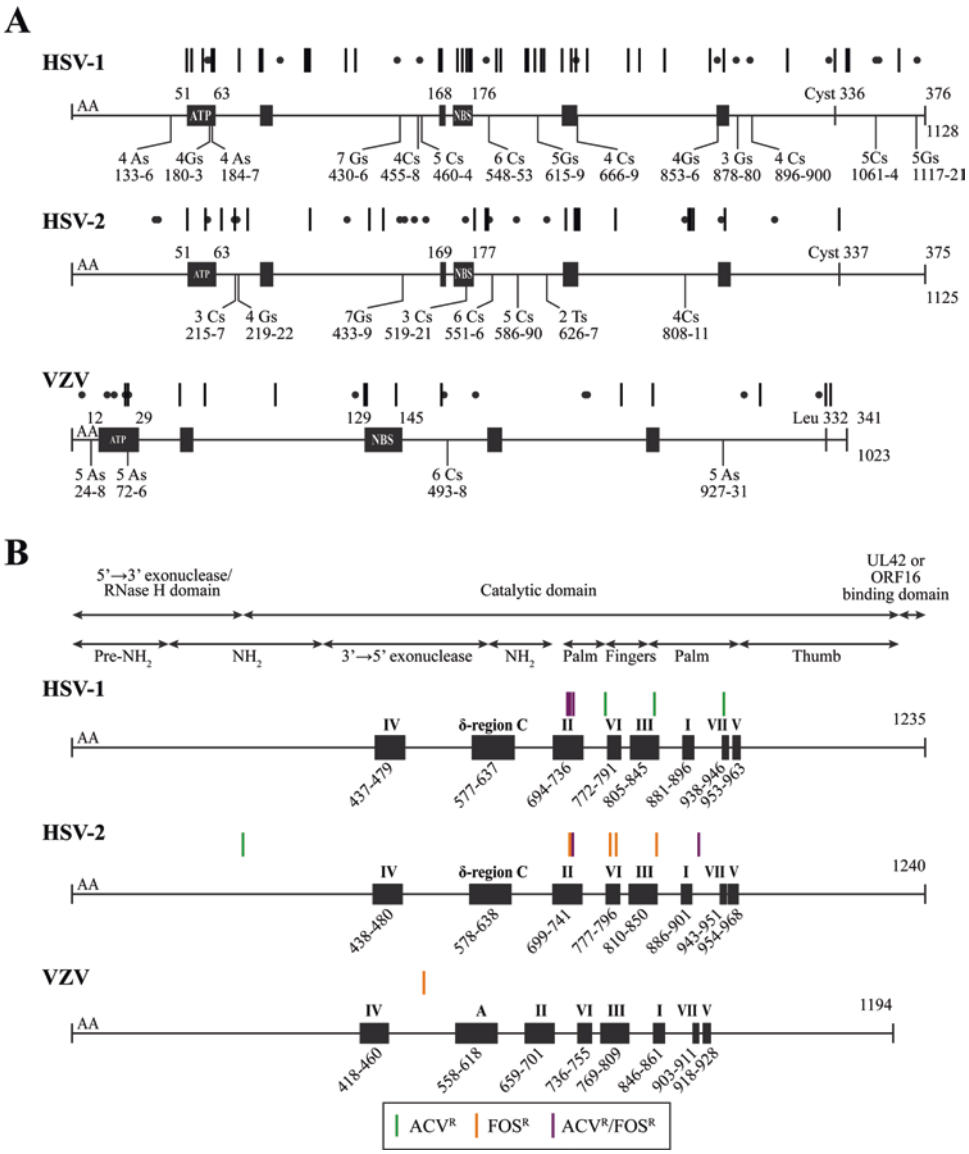

Fig. 72.4 Confirmed drug resistance mutations identified in clinical HSV-1, HSV-2, and VZV isolates. Panel (**a**) shows mutations in the *UL23* gene of HSV-1 and HSV-2 and in the *ORF36* gene of VZV conferring resistance to acyclovir. Conserved regions among the thymidine kinase of *Herpesviridae* including the ATP-binding site (ATP) and the nucleoside-binding site (NBS) are represented by the *black boxes*. Bars (|) indicate amino acid substitutions, whereas dots (*filled circle*) represent nucleotide additions and/or deletions. The homopolymer runs, as well as the nucleotides involved, are indicated below vertical bars. Panel (**b**) shows mutations in the *UL30* gene of HSV-1 and HSV-2 and in the *ORF28* gene of VZV conferring resistance to acyclovir (ACV^R) and/or foscarnet (FOS^R). Conserved regions among the *Herpesviridae* DNA polymerase are represented by the *black boxes*. The roman numbers (I–VII) and δ-region C corresponding to each of these regions are indicated above the boxes. Colored bars (|) indicate amino acid substitutions

spot for the insertion or deletion of nucleotides involved in ACV resistance [239, 240, 246, 273]. Deletions of nucleotides that result in frameshift reading leading to a stop codon at position 231 are often detected in ACV-resistant clinical isolates [240]. In addition, nonsynonymous nucleotide substitutions conferring resistance to ACV are widely dispersed in the *ORF36* gene [184, 186, 239–241, 262, 274]. However, these amino acid changes occur more frequently in the ATP-binding and nucleoside-binding sites of the TK enzyme [240].

A few reports have described ACV-resistant and/or FOS-resistant VZV clinical isolates with mutations in the *ORF28* gene [242, 274, 275]. The amino acid substitutions are mainly found in the catalytic site and in the conserved regions of the DNA pol and may confer cross-resistance to ACV and FOS. The TK and DNA pol of VZV are highly conserved compared with those of HSVs, and only few natural polymorphisms have been identified in the *ORF36* and *ORF28* genes [274].

4.4 Management of Infections Caused by Drug-Resistant HSV and VZV Strains

An algorithm for the management of infections caused by drug-resistant HSV mutants is proposed in Fig. 72.5. The persistence of active lesions due to HSV for 7–10 days after initiation of high-dose oral ACV, VACV, or FCV therapy without appreciable decrease in size, an atypical appearance, or the emergence of satellite lesions is suggestive of treatment failure. When drug resistance is suspected, a change of therapy should be considered depending on the clinical severity of the disease. Most ACV-resistant strains isolated from immunocompromised patients are TK-deficient and are therefore also resistant to VACV and FCV. An initial step in case of treatment failure with oral drugs is to initiate high doses of IV ACV (10 mg/kg of body weight every 8 h adjusted for renal function). If there is no improvement after 7 days, a switch to IV FOS (40 mg/kg every 8 h with reduction in dose for renal

Table 72.4 Amino acid substitutions associated with drug resistance in the DNA polymerase of clinical HSV-1, HSV-2, and VZV strains

aa changes	Phenotypic test	Confirmation by	Drug phenotype (fold change)[a]	References
A. HSV-1				
Region II (aa 694–736)				
R700G	PRA	Recombinant virus	ACV^R (NA), FOS^R (NA)	Gibbs et al. [265]
A719V	PRA	Recombinant virus	ACV^R (19.0x), FOS^R (NA), $CDV^{S/B}$ (NA)	Larder et al. [266]
S724N	PRA	Recombinant virus	ACV^R (9.9x), FOS^R (2.7x), CDV^R (2.1x)	Bestman-Smith and Boivin [188, 237]
Between regions II and VI				
E771Q	PRA	–	ACV^R, FOS^S	Chibo et al. [267]
Region III (aa 805–845)				
N815S	PRA	Recombinant virus	ACV^R (59.0x-233.0x)	Larder et al. [266]
G841S	PRA	Recombinant virus	ACV^R (29.0x)	Larder et al. [266]
Region VII (aa 938–946)				
Y941H	PRA	Recombinant virus	ACV^R (9.4x), FOS^S (1.4x), CDV^{HS} (0.4x)	Bestman-Smith and Boivin [188]
B. HSV-2				
E250Q	PRA	–	ACV^R, FOS^S	Chibo et al. [267]
Region II (aa 699–741)				
A724T	PRA	–	ACV^S, FOS^R, CDV^S	Bestman-Smith and Boivin [237]
A724V	PRA	–	ACV^R	Burrel et al. [268]
S725G	PRA	–	FOS^R	Chibo et al. [269]
S729N	PRA	–	ACV^B, FOS^R, CDV^S	Bestman-Smith and Boivin [237]
Region VI (aa 777–796)				
L783M	PRA	–	ACV^S, FOS^R, CDV^B	Bestman-Smith and Boivin [237]
D785N	PRA	–	ACV^S, FOS^R, $CDV^{HS/S}$	Bestman-Smith and Boivin [237]
Region III (aa 810–850)				
L850I	PRA	–	ACV^S, FOS^R, CDV^{HS}	Bestman-Smith and Boivin [237]
Between regions I and VII				
D912V	PRA	–	ACV^R, FOS^R, CDV^S	Bestman-Smith and Boivin [237]
C. VZV				
Between region IV and region A				
E512K	Ag reduction assay	–	FOS^R	Visse et al. [242]

ACV acyclovir, *FOS* foscarnet, *CDV* cidofovir, *PRA* plaque reduction assay, *Ag* antigen, *B* borderline level of resistance, *HS* hypersusceptible, *R* resistant, *S* susceptible, NA Not available

[a]Fold changes are calculated as the ratio of the EC_{50} values of mutant recombinant virus to the wild-type counterpart

dysfunction) should be considered. In parallel, isolates from the lesions should be submitted for phenotypic susceptibility testing (starting with ACV and FOS and then CDV, if required) and/or genotypic assays of the *UL23*/*UL30* genes if the patient is failing therapy. Standard doses of IV ACV have no clinical benefit if the HSV isolate is resistant to ACV in vitro and IV FOS should be continued. If there is still no improvement of HSV disease after 7–10 days, another option could be to administer continuous infusion of high-dose ACV (e.g., 1.5–2.0 mg/kg per hour) as it is a well-tolerated alternative for severe ACV-resistant or multidrug-resistant HSV infections [276, 277]. Intravenous CDV has shown some efficacy in the treatment of progressive ACV-resistant and/or FOS-resistant mucocutaneous HSV infections in immunocompromised patients [278–281] but is not approved for this indication. A switch to IV CDV (5 mg/kg once a week for 3–4 weeks) could also be considered.

A topical cream containing 1 % FOS was effective in the treatment of mucocutaneous infections unresponsive to ACV [282]. Topical formulations of CDV also demonstrated efficacy in the treatment of drug-resistant mucocutaneous HSV infections [283–286]. Although the use of topical formulations could avoid the adverse effects associated with IV administration of FOS and CDV, they are not commercially

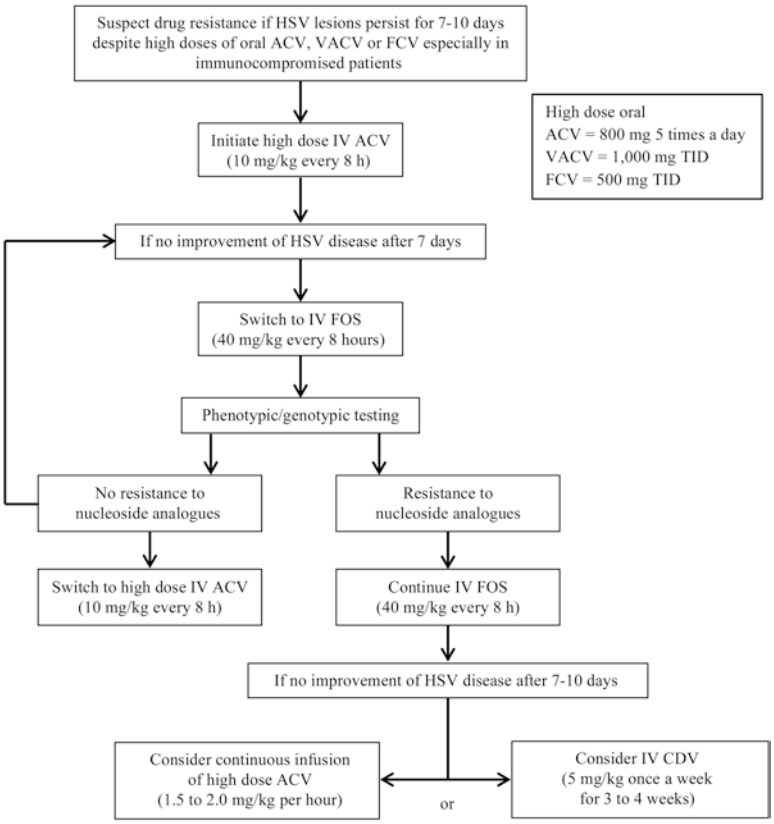

Fig. 72.5 Proposed algorithm for the management of suspected nucleoside analogue-resistant HSV infections. **Key**: *ACV* acyclovir, *VACV* valacyclovir, *FCV* famciclovir, *FOS* foscarnet, *CDV* cidofovir, *TID* thrice a day, *IV* intravenous

available. A topical formulation containing 5 % imiquimod, an immunomodulatory drug, was effective in the treatment of recurrent and severe mucocutaneous lesions due to ACV-resistant and FOS-resistant HSV-2 isolates in HIV-infected individuals [287]. A 1 % topical solution of trifluorothymidine, a fluorinated pyrimidine nucleoside analogue that inhibits thymidylate synthetase, is usually administered in cases of ophthalmic herpetic infections that do not respond to ACV [288].

The persistence of clinical signs of VZV infections for more than 10–14 days after initiation of high-dose oral ACV is suggestive of treatment failure, and it should lead to alternate therapy depending on the clinical severity of the disease [289]. Genotypic testing of the *ORF36* gene encoding for the TK protein could be performed in biopsy of mucocutaneous lesions or other body compartments when necessary [290]. FOS is generally used for the management of VZV infections due to suspected or confirmed ACV-resistant mutants, as described mainly in HIV-infected individuals [17, 291] and some oncology patients [243–245]. The recommended IV dosage is 60 mg/kg every 8 h adjusted for renal function for at least 10 days or until complete lesion healing is observed [289]. Clinical experience with the use of CDV in the treatment of drug-resistant VZV diseases is very limited [292].

5 Conclusions and Future Directions

With the increasing number of immunocompromised subjects and the prolonged administration of antiviral agents, the problem of drug resistance among herpesviruses is not expected to fade. Clearly, some drug-resistant mutants of HCMV and HSV are pathogenic and can result in significant morbidity and mortality among severely immunocompromised patients. The development of fast and efficient methods for detecting viral mutant sequences directly in clinical specimens such as pyrosequencing [293] and ultra-deep pyrosequencing [44–46, 294] will improve the early diagnosis of drug-resistant herpesvirus infections. The use of more powerful recombinant phenotyping techniques [61] and the availability of an Internet database [51] to link individual mutations to their drug susceptibility phenotypes should result in more rational therapeutic strategies.

As all currently available antiviral agents target the viral DNA pol, the development of new anti-herpetic compounds with different mechanisms of action and with adequate safety profiles is an important priority. In that regard, some promising compounds are currently in clinical trials. The orally bioavailable lipid ester prodrug of CDV (i.e., hexadecyloxypropyl-cidofovir; CMX001) could avoid the dose-limiting toxicity of the parent drug and provide a safe alternative

for ACV- and GCV-resistant herpesviruses in immunocompromised patients [295]. Treatment with oral CMX001 (brincidofovir) significantly reduced the incidence of HCMV events in HSCT recipients [296]. Maribavir is a competitive inhibitor of the UL97 kinase [297]. Surprisingly, mutations arising after in vitro selection with this drug most often map to the *UL27* gene and, less frequently, to the *UL97* gene. Of note, mutations found in the *UL97* gene are distinct from those described in GCV-resistant strains [298], and some have been detected outside the conserved kinase domains [19]. Thus, maribavir retains activity against GCV-resistant HCMV mutants. The emergence of resistance to this drug has been reported in some clinical cases [299, 300]. Recently, maribavir faced some limitations in phase III clinical studies [301], but new trials evaluating higher doses are in progress. Letermovir targets the terminase complex of HCMV and interferes with viral DNA concatemer maturation [302, 303]. Accordingly, mutations conferring resistance to letermovir map to the *UL56* gene encoding the HCMV terminase [302, 304]. Successful treatment of a multidrug-resistant HCMV infection with letermovir has been reported in a lung transplant recipient [305]. Preemptive treatment of HCMV infection with letermovir was effective in kidney transplant recipients [306]. Moreover, prophylaxis with letermovir was effective in reducing the incidence of HCMV infection in HSCT recipients [307]. Pritelivir, a potent orally bioavailable helicase-primase inhibitor, reduced the rates of genital HSV-2 shedding and days with lesions in a phase II trial [308]. The bicyclic nucleoside analogue FV-100 and carboxylic nucleoside analogue valomaciclovir were well tolerated and effective for the treatment of herpes zoster in phase II trials [12, 309]. Novel classes of antiviral agents targeting the ribonucleotide reductase, the helicase-primase complex, and the process of viral DNA encapsidation are at earlier stages of development [310].

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Clinical Implications of HIV-1 Drug Resistance

73

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1 Introduction

The human immunodeficiency virus type 1 (HIV-1) is a retrovirus that has an error-prone reverse transcriptase enzyme that results in the production of swarms of related viruses called quasi-species in each infected person. This replication strategy involves production of 10^7–10^9 virions per day, each with a half-life of only 1–2 days [1–3]. HIV-1 utilizes an error-prone reverse transcriptase enzyme that lacks proofreading activity and generates 3×10^{-5} errors per base pair per replication cycle [1]. Since there are roughly 9000 base pairs per virion, every possible single- and double-mutant virus is created every day in every patient in the absence of antiretroviral therapy. This strategy allows the virus to escape immune selection pressure and is very efficient at generating drug-resistant viruses in those patients whose drug regimens do not completely suppress viral replication or who are poorly compliant with a fully suppressive regimen.

Since single base drug-resistant mutants preexist in every patient at low frequencies, they can emerge as the predominant circulating virus in as little as 14 days if monotherapy is utilized. This is seen with drugs such as lamivudine (with an M184V mutation) or non-nucleoside reverse transcriptase inhibitors such as efavirenz or nevirapine (with K103N or Y181C mutations) where single-point mutations cause high-level drug resistance. Alternatively some drugs have a higher barrier to resistance due to the need for multiple mutations to cause high-level resistance, which can take months to develop. A small group of drugs develop low levels of resistance slowly, possibly because their primary resistance mutations are associated with low replication capacities (Table 73.1).

The development of drug resistance is associated with rising plasma HIV RNA levels, declining CD4 cell counts, and disease progression. The prevalence of drug-resistant HIV-1 in treated patients with detectable plasma virus is approximately 80 %. Drug-resistant HIV-1 is transmitted to 10–27 % of newly HIV-infected persons. The rapid emergence of NNRTI resistance after use of single-dose nevirapine to block mother-to-child transmission of HIV-1 has impacted treatment options in resource-limited countries.

Additionally, each virion contains two strands of genomic RNA and the reverse transcriptase enzyme can jump from one RNA template strand to the other when replicating the viral genome [4–6]. If two strains of HIV-1, each resistant to one agent, are circulating in a patient the virus can use recombination to generate a new virus resistant to both drugs. The clinical relevance of recombination in generating drug-resistant HIV-1 has not been established although it has been demonstrated in both in vitro experiments and in clinical viruses [7, 8].

Genotypic and phenotypic resistance assays are commercially available to assist in the management of HIV-infected patients. Due to the current prevalence of transmitted HIV-1 drug resistance, resistance testing is recommended when treatment is initiated and prior to switching drug regimens after virologic rebound has occurred. It is critical that patients be given a drug regimen that contains at least two and preferably three active antiretroviral drugs to ensure complete viral suppression. Combining less than three active drugs often leads to rapid viral breakthrough with resistance to the new class of drugs used. Treatment decisions need to take into account prior drug exposure, drug toxicities on prior antiviral regimens, and resistance test results on both prior and the most recent antiretroviral drug regimen. Where available, advice from an expert with experience in treating patients with drug-resistant HIV-1 should be obtained.

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Table 73.1 Patterns of HIV-1 drug resistance emergence

Level of resistance	High	High	Low
Time course	Weeks	Months–years	Months–years
Mechanism of resistance	Single-point mutation	Accumulation of mutations	Complex or unclear[a]
Drugs	Lamivudine (3TC)	Zidovudine (AZT)	Didanosine (ddI)
	Emtricitabine (FTC)	Abacavir (ABC)	Stavudine (d4T)
	Efavirenz (EFV)	Saquinavir (SQV)	Tenofovir (TDF)
	Nevirapine (NVP)	Indinavir (IDV)	
		Ritonavir (RTV)	
		Nelfinavir (NFV)	
		Amprenavir (APV)	
		Lopinavir (LPV)	
		Tipranavir (TPV)	
		Darunavir (DRV)	
		Etravirine (ETV)	
		Rilpivirine (RPV)	
		Raltegravir (RAL)	
		Elvitegravir (EVG)	
		Dolutegravir (DTG)	

[a]Selected viral mutants may have low replication capacities

2 Epidemiology

With the HIV-1 quasispecies replication strategy it is critical that patients be given a drug regimen which is expected to fully suppress all viral replication. This goal was not achievable for most patients in the early years of HIV-1 therapy when regimens containing only nucleoside agents were available. Many patients developed viruses with progressively higher levels of nucleoside resistance and broad resistance to all agents in the nucleoside class. This nucleoside resistance often limited the durability of responses to subsequent combination regimens which combined nucleoside agents with protease inhibitors or non-nucleoside reverse transcriptase inhibitors (NNRTIs) as they became available. Additionally, the early combination regimens were composed of agents with a high pill burden, multiple doses per day, and significant side effects which reduced patients' ability to take medications as prescribed. Thus, a large population of patients with resistance to multiple classes of HIV-1 agents was created in the late 1980s and 1990s. The more recent development of simple, once- or twice-a-day regimens of highly active antiretroviral therapy (HAART) with NNRTIs, boosted protease inhibitors, or integrase inhibitors appears to improve patient compliance and may result in more durable antiviral responses with reduced numbers of patients harboring viruses with resistance to multiple classes of antiretroviral drugs in the future.

3 Prevalence

A series of studies of the prevalence of drug-resistant HIV-1 in treated patients were conducted in North America and Europe between 1996 and 2013 (Table 73.2) [9–12]. Investigators in the United States evaluated a random representative sample of treated patients and estimated that 63 % of treated patients had viremia of >500 copies/mL [9]. Among viremic patients, the overall rate of any drug resistance was very consistent across the cohorts at 69–80 % of treated patients. Rates of resistance to nucleoside reverse transcriptase inhibitors (NRTI) ranged from 64 to 78 %, non-nucleoside reverse transcriptase inhibitors (NNRTI) from 25 to 61 %, and protease inhibitors (PI) from 31 to 62 %. Three classes of drug resistance (multidrug-resistant viruses) were detected in 13–25 % of viremic treated patients. The most common NRTI mutations detected were M184V associated with lamivudine use and T215Y/F associated with zidovudine use. The rates of NNRTI and PI resistance were driven by general use in the treated population with NNRTI resistance increasing from 1996 to 2003 as NNRTI use became more widespread [9–12]. The most common NNRTI mutation was K103N. PI mutations varied with differential use in the different countries.

Factors associated with the development of HIV drug resistance have included host factors such as advanced HIV disease and low CD4 count at the time of initiation of

Table 73.2 Prevalence and incidence of HIV-1 drug resistance

Location	Years	*N*	Any-R (%)	NRTI-R (%)	NNRTI-R (%)	PI-R (%)	3 class-R	Reference
Prevalence of drug resistance in treated patients with viremia								
USA	1996–1998	1797	76.0	71.0	25.0	41.0	13.0	Richman et al. [9]
Canada	1997–2003	552	69.0	>70	61.0	62.0	NA	Turner et al. [12]
France	1997–2002	2248	80.0	78.0	29.0	47.0	25.0	Tamalet et al. [11]
UK	1998–2000	275	80.0	64.0	36.0	31.0	14.0	Scott et al. [10]
Switzerland	1999–2001	373	72.0	67.0	28.0	37.0	16.0	Yerly et al. [21]
Incidence of drug resistance in newly HIV-infected persons								
North America	1995–1998	264	8.0	8.5	1.7	0.9	NA	Little et al. [18]
North America	1999–2000	113	22.7	15.9	7.3	9.1	NA	Little et al. [18]
New York	1995–1998	154	13.2	11.8	2.6	1.3	2.6	Simon et al. [19]
New York	1999–2001	78	19.7	14.5	6.6	5.1	4.0	Simon et al. [19]
San Francisco	1996–1997	40	25.0	10.0	0.0	2.5	0.0	Grant et al. [20]
San Francisco	1998–1999	94	18.1	4.2	6.4	5.3	0.0	Grant et al. [20]
San Francisco	2000–2001	91	27.4	12.1	13.2	7.7	1.2	Grant et al. [20]
Europe (SPREAD)	1996–1998	217	13.50	13.4	2.3	2.8	NA	Wensing et al. [22]
Europe (SPREAD)	1999–2000	448		9.8	3.1	4.4	NA	Wensing et al. [22]
Europe (SPREAD)	2001–2002	95		6.3	9.2	3.2	NA	Wensing et al. [22]
Switzerland	1999–2001	220	10.5	8.6	0.9	2.3	0.0	Yerly et al. [21]
USA	1997–2007	848	14.9	6.8	7.6	5	2.1	Frentz et al. [30]
USA	2005–2007	228	12.1	4.5	9.8	1.9	2.2	Frentz et al. [30]
USA	2009–2013	405	12.6	3.7	8.4	2	NA	Baxter et al. [139]
Europe	2009–2013	1292	8.8	3.8	3.4	2.9	NA	Baxter et al. [139]

treatment, viral factors such as high baseline viral load and transmitted drug resistance, and drug regimen factors related to adherence, and the potency and composition of the antiretroviral regimen given [9, 13, 14].

4 Transmission

HIV-1 is transmitted predominantly through sexual contact, due to blood exposure, and from mother to child. Factors associated with the risk of HIV transmission include high viral load, concomitant sexually transmitted diseases, host genetic factors, and high-risk behaviors. Persons with drug-resistant HIV-1 can transmit the virus to their partners.

Interestingly, differential transmission of drug resistance mutations has been observed. Viruses containing the M184V mutation in reverse transcriptase or major protease mutations appear underrepresented in newly infected patients compared to the frequency in prevalently infected populations [12, 15]. This could be due to reduced replication capacity combined with lower viral loads in the potentially transmitting patients with these viruses [12, 16].

The sexual transmission of zidovudine-resistant virus was first reported in 1993 [17]. Surveys of HIV-1 drug resistance have subsequently demonstrated different patterns of transmitted drug resistance over time with some evidence of geographic variability [18–29]. Representative studies where prevalent and incident HIV-1 drug resistance can be compared are presented in Table 73.2. In the period from 1995 to 1998 in North America and Europe, the predominant resistance in transmitted HIV-1 was to nucleoside antiretroviral agents (NRTIs) with rates ranging from 8.5 to 13.4 % and low levels of transmission of viruses resistant to NNRTIs (1.7–2.6 %) or protease inhibitors (0.9–2.8 %). In later surveys in North America and Europe from 1999 to 2002, rates of NRTI resistance ranged from 6.3 to 15.9 %, NNRTIs from 6.6 to 13.2 %, and PIs from 3.2 to 9.1 %. A recent review of global transmitted drug resistance (TDR) prevalence showed that transmitted NNRTI resistance has historically been higher in North America compared to Europe and that rates increased in North America after the year 2003 [30, 139]. The incidence of newly infected patients with drug-resistant virus ranges from approximately 10 to 27 % with multidrug-resistant viruses estimated to be present in 0–4 % [19–21, 23, 31]. As was seen with prevalent HIV drug resistance, transmitted NNRTI resistance has progressively increased from 1996–1997 to 2000–2001 [19–21, 23]. A CDC survey of 1082 treatment-naïve newly diagnosed patients who did not have AIDS showed that 8.6 % of these patients had genotypic evidence of drug-resistant HIV-1 and

1.3 % had MDR virus [32]. A subsequent surveillance study conducted in the United States revealed high percentage of transmitted NNRTI resistance in 7.8 % of newly diagnosed individuals [33].

In recent years, TDR has also been described as an emerging health issue in resource-limited countries, although surveillance data has been lacking [30, 34–36]. In these regions antiretroviral therapy has been introduced more recently and has significantly impacted morbidity and mortality, but access to virologic monitoring techniques is often limited. In the absence of modern laboratory monitoring, there is an increased risk of transmission of drug-resistant HIV-1 in a population given the prolonged time between the onset of initial virologic failure and subsequent clinical consequences. Not surprisingly, TDR prevalence in resource-limited countries is directly correlated with the number of years since ART roll-out programs were initiated [30].

Transmitted drug-resistant viruses can persist for long periods of time in the absence of treatment in comparison to the reversion to wild-type (drug-sensitive) viruses that occurs in patients who develop drug-resistant virus on treatment and then stop therapy [37–41]. It is not clear whether these viruses have altered pathogenicity from the available data [39, 42–44]. Transmitted drug resistance has been associated with an increased risk of suboptimal virologic response to the initial regimen and can also impact future treatment options. The median time to virologic suppression is longer in patients with primary drug-resistant viruses who receive combination therapy than in patients who are infected with drug-sensitive virus [21].

The high prevalence of drug resistance in newly infected patients has led to guideline recommendations that all newly diagnosed HIV-infected patients should have drug resistance testing prior to initiating antiretroviral therapy, if testing is available [45–50].

5 Prevention of Mother-to-Child Transmission

Mother-to-child transmission of HIV-1 remains a major problem in the developing world. Initial studies showed that zidovudine given antepartum, and intrapartum to the mother and to the newborn for 6 weeks, could reduce the rate of mother-to-child transmission by 67 % [51]. Subsequently, the HIVNET 012 study in Uganda showed that single-dose nevirapine given perinatally to mother and child could reduce mother-to-child transmission of HIV-1 to 15.7 % compared to 25.8 % at 18 months of follow-up in a breast-feeding population [52]. The simple, effective single-dose nevirapine regimen has been widely used throughout the developing world. Subsequently, it was shown that single-dose nevirapine would induce NNRTI-resistant virus in 20–25 % of mothers and 46 % of exposed HIV-infected infants using population sequencing [53, 54]. If more sensitive measurements of NNRTI resistance mutations are used, higher levels of NNRTI resistance can be detected. If these mothers require treatment with a nevirapine-containing regimen within 6 months after exposure to single-dose nevirapine, treatment responses to nevirapine-containing regimens are significantly reduced [55, 56]. Longitudinal studies have shown that the prevalence of NNRTI-resistant virus in the mothers exposed to single-dose nevirapine declines over time [54, 57] and mothers who require treatment more than 6 months after prior exposure to single-dose nevirapine have treatment response rates similar to women who have not been previously exposed to nevirapine [56]. The development of drug-resistant virus with regimens to prevent mother-to-child transmission of HIV-1 has driven the use of short-term combination treatment in the developed world. As combination antiretroviral therapy becomes more widely available through roll-out programs in resource-limited countries, the ultimate solution will be to provide chronic fully suppressive combination therapy to all HIV-1-infected mothers.

6 Clinical Significance

The emergence of drug-resistant HIV-1 during treatment has been associated with rising plasma HIV RNA levels, declining CD4 cell counts, and reduced responses to subsequent courses of antiretroviral therapy [58, 59]. Development of multidrug-resistant HIV-1 is associated with disease progression and death [60].

Some patients who develop drug-resistant HIV-1 while on a protease inhibitor-containing regimen can maintain low levels of plasma HIV RNA and stable CD4 cell counts for several years [61]. This may be due to reduced levels of replication capacity (viral fitness) in the viruses that emerge on these regimens. Ultimately, many of these patients will experience CD4 decline and HIV-1 disease progression with multidrug-resistant virus.

7 Resistance to HIV Nucleoside Reverse Transcriptase Inhibitors (NRTI)

HIV nucleoside reverse transcriptase inhibitors (NRTI) block HIV replication by chain termination of the growing DNA strand [62]. Resistance to these agents occurs via mutations which selectively block incorporation of the incoming NRTI, such as L74V for didanosine, V75T for d4T, and M184V for 3TC resistance, or alternatively via thymidine analog mutations (TAMs) associated with zidovudine use at positions M41L, D67N, K70R, L210W,

T215Y/F, and K219Q/E that allow the reverse transcriptase to selectively excise the incorporated NRTI by increased phosphorolysis [63, 64]. Generally increasing numbers of NRTI mutations in the reverse transcriptase enzyme are associated with higher levels of drug resistance and broadened resistance to agents in the NRTI class [65]. Multi-NRTI resistance is most commonly produced by sequential accumulation of TAMs with M184V and additional NRTI-resistance-associated mutations [66]. Less commonly virus can develop the Q151M mutation (often combined with A62V, V75I, F77L, and F116Y) or by amino acid insertion(s) at position 69S combined with multiple TAMs to produce broad resistance to agents of the NRTI class [67–70]. HIV-1 NRTI resistance is reviewed extensively in Chap. 33.

8 Resistance to HIV-1 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTI)

HIV-1 non-nucleoside reverse transcriptase inhibitors all bind to a common pocket and block the action of HIV RT in a noncompetitive way [71, 72]. HIV-2 viruses and HIV-1 clade O viruses found in West Africa are naturally resistant to all available NNRTIs [73]. In all other HIV-1 clades, resistance to the first-generation HIV-1 NNRTIs, nevirapine and efavirenz, is generally produced by a single-point mutation at position K103N or Y181C/I resulting in high-level resistance and/or a transient response with rapid viral rebound to these agents [74, 75]. If these agents are continued after viral rebound occurs, additional mutations at positions L100I, V106A/M, V108I, Y188C/L/H, G190S/A, and P225H can be selected [74, 75].

Second-generation NNRTIs, etravirine and rilpivirine, have been introduced into clinical practice to treat patients with NNRTI-resistant virus. These agents are active against viruses with the common K103N mutation. Resistance to etravirine is associated with mutation at positions V90I, A98G, L100I, K101E/P, V106I, V179D/F, and G190S/A, usually in combination with Y181C [76]. Rilpivirine is associated with mutations at positions L100I, K101E/P, E138A/G/K/Q/R, V179L, Y181C/I/V, Y188L, H221Y, F227C, and M230I/L, and interestingly the NRTI M184I mutation can reduce rilpivirine susceptibility when it occurs in combination with either E138K or K101E [77]. Additionally, responses to these agents are reduced when multiple NNRTI-associated resistance mutations are present in the circulating virus. This strongly suggests that patients should not be maintained on a nevirapine- or efavirenz-containing regimen after virologic rebound to prevent development of resistance to the newer second-generation NNRTIs. HIV-1 NNRTI resistance is reviewed extensively in Chap. 34.

9 Resistance to HIV-1 Protease Inhibitors (PI)

HIV protease inhibitors (PI) act by preventing the HIV protease enzyme from cleaving the Gag protein, an essential step of the viral maturation process [78]. Resistance to HIV PIs is a multistep process involving the development of primary mutations in the active site of the protease enzyme responsible for drug resistance and the appearance of secondary compensatory mutations away from the active site which increase the protease enzymatic efficiency [79–81]. Single mutations are generally not sufficient to significantly reduce phenotypic susceptibility for most PIs. Typically, the accumulation of multiple mutations is required to confer resistance to these agents. The accumulation of multiple primary protease resistance mutations (D30N, G48V, I50V, V82A/F/T/S, I84V, or L90M) alters the protease enzyme-binding pocket leading to increasing and broadened PI resistance [82]. Second-generation PIs active against viruses resistant to the first-generation PIs include the agents tipranavir and darunavir.

Mutations can also occur outside of the protease gene contributing to the development of PI resistance. Viral evolution of mutations and insertions at gag cleavage sites may occur after exposure to PIs. These mutations are associated with the restoration of protease enzyme activity and viral replicative capacity that is typically compromised in multi-PI-resistant variants [83–89]. Viruses are able to adapt to the altered drug-resistant protease enzyme by mutating their gag cleavage sites to fit an altered enzyme-binding pocket [90, 91]. When this occurs, the virus becomes "locked" into the altered enzyme configuration since reversion of resistance would require simultaneous reversion of the protease resistance mutations and the gag cleavage site mutations. Gag cleavage site mutations represent another mechanism of PI resistance; however because they occur outside of the protease region they are not generally detected on standard HIV genotypic resistance testing. HIV-1 protease inhibitor resistance is reviewed extensively in Chap. 35.

10 Resistance to HIV-1 Entry Inhibitors

HIV-1 entry inhibitors prevent the HIV envelope proteins gp120 and gp41 from interacting with their cellular receptors and fusing with the host cell membrane.

Enfuvirtide (T-20) blocks the fusion of the viral and host cell membranes mediated by gp41 [92]. The mutations which produce enfuvirtide resistance usually occur at codons 36–45 in the first heptad repeat region (HR1) of gp41 and are not detected with conventional genotypic or phenotypic HIV resistance assays [93]. Patients are generally assumed to have virus sensitive to enfuvirtide if the drug has not been

administered previously and are assumed to have virus with enfuvirtide resistance if they have received enfuvirtide previously and experienced viral rebound on the agent.

Maraviroc, a CCR5 inhibitor, blocks the interaction of the HIV-1 gp 120 envelop protein with the CCR5 molecule on the surface of host cells [94]. The virus gp120 envelope initially binds to CD4 followed by secondary binding to either CCR5 or CXCR4 on the host cell surface. Viruses which are R5-tropic (bind CCR5 to enter host cells) are inhibited by maraviroc while viruses which are X4-tropic (bind CXCR4 to enter host cells) or have a mixed R5/X4 tropism are not inhibited. R5-tropism predominates at the time of infection and during the early stages of HIV disease when patients are asymptomatic. As HIV disease progresses and CD4 cells decline viruses with a mixed R5/X4-tropism or X4-tropism become more common. A tropism assay should be obtained to confirm the presence of R5-tropic virus before maraviroc is given. Viral rebound in the presence of maraviroc therapy is generally assumed to be due to the emergence of X4-tropic virus but this can be confirmed by a tropism assay. Both phenotypic and genotypic tropism assays are currently available. HIV-1 entry inhibitor resistance is reviewed extensively in Chap. 36.

11 Resistance to HIV Integrase Inhibitors

HIV-1 integrase inhibitors block the strand transfer reaction that the HIV integrase uses to insert the HIV genome into host cell DNA [95]. Reduced susceptibility to the first FDA-approved integrase inhibitor, raltegravir, is mediated by two pathways of resistance in the HIV-1 integrase gene: Q148H/K/R combined with either L74M+E138A, E138K, or G140S, or N155H combined with either L74M, E92Q, T97A, E92Q+T97A, Y143H, G163K/R, V151I, or D232N [96]. Integrase mutations associated with elvitegravir resistance include many of the raltegravir-associated mutations and cross-resistance between these agents in common [77]. Additional mutations associated with reduced susceptibility to elvitegravir include S147G and T66I/A/K. Dolutegravir is considered a second-generation integrase inhibitor as it often remains active against HIV-1 strains with reduced susceptibility to raltegravir and elvitegravir. Resistance to dolutegravir is usually associated with Q148H/K/R in combination with G140A/C/S, E138A/K/T, or L74I [77]. Patients experiencing virologic failure while taking integrase inhibitors should have integrase drug resistance testing. In addition, genotypic resistance testing for integrase mutations should be considered in treatment-naïve patients if there is concern for transmitted drug resistance to these agents. HIV-1 integrase inhibitor resistance is reviewed extensively in Chap. 37.

12 Mutational Interactions

Some drug resistance mutations in the HIV genome can interact to result in resensitization of the virus to an antiviral drug to which it was previously resistant. For instance, if a virus is resistant to zidovudine with multiple TAMs and a T215Y/F mutation and develops an L74V mutation due to exposure to didanosine [97] or a Y181C mutation due to nevirapine exposure [98], the virus can show zidovudine sensitivity on a phenotypic sensitivity assay. Viruses with multiple TAMs from nucleoside exposure can demonstrate hypersusceptibility to NNRTI agents and this has been shown to result in better responses to efavirenz-containing regimens when the next round of therapy is given (provided adequate background therapy is available to combine with the NNRTI) [99–102]. Likewise, the M184V mutation enhances susceptibility to tenofovir in the presence or absence of TAMS [77]. Knowledge of mutational interactions can sometimes be used to obtain an enhanced response from a component of a combination regimen in treatment-experienced patients. It should be kept in mind that most of these mutational interactions can be overcome by the virus moving to an alternative resistance pathway so that they are only of clinical benefit if a fully suppressive next regimen can be designed.

13 Viral Fitness (Replication Capacity)

Viral fitness or the ability to replicate in host cells can be reduced due to the presence of drug resistance mutations which decrease a viral enzyme's functional activity as the cost of developing drug resistance. In the patient, the predominant circulating virus is the virus which grows best in the presence of the current drug selection pressure, but this virus can often be rapidly overgrown by wild-type virus if the drugs are stopped. Diminished fitness is seen clinically when a patient's virus rebounds in the presence of a drug regimen but the viral load remains well below baseline levels and the CD4 cell count stays up despite the emergence of drug-resistant virus [61]. Some patients can remain clinically stable for extended periods of time until the virus develops additional mutations which either increase drug resistance or compensate for the drug resistance mutations and allow the virus to replicate more efficiently. When this occurs, CD4 cells will decline and disease progression can occur.

Some drug resistance mutations such as those associated with lamivudine and emtricitabine resistance (M184V in reverse transcriptase) or primary protease inhibitor resistance (D30N) have been associated with decreased viral fitness as manifested by lower viral loads in treated patients who experience viral rebound on therapy and reduced transmission to newly infected patients [12].

Viral fitness or replication capacity is determined by dividing the amount of viral growth of the clinical HIV-1 isolate in the no-drug well of an in vitro drug resistance assay by the amount of growth of the wild-type (drug-sensitive) control virus in the no-drug wells from the same assay. There have been several reviews of the implications of HIV viral fitness on drug resistance, disease progression, transmission, and global epidemic evolution [103, 104].

14 Clades

Most of the current knowledge of HIV-1 drug resistance has been developed from patients infected with clade B virus, which is the predominant strain of virus circulating in North America and Europe [105]. However, most of the patients infected with HIV-1 in the developing world have non-clade B viruses (such as clade A/E viruses in Asia and clade C viruses in sub-Saharan Africa) [105, 106]. The resistance pathways for antiviral drugs are generally similar in non-clade B to those seen in patients with clade B viruses but different primary pathways and profiles can occur [106]. For example, patients exposed to nelfinavir with clade B virus often develop a D30N mutation in their virus whereas those with clade C virus develop an L90M mutation more often than the D30N [107]. Similarly patients who receive nevirapine with clade B virus often develop a secondary V106A mutation whereas those with clade C virus with a 106 mutation usually develop a V106M mutation [108, 109].

The effect of different genetic backgrounds on drug resistance pathways in different regions of the world is currently under investigation. As more information becomes available, resistance algorithms developed in the developed world will need to be expanded to improve interpretation for the non-clade B viruses which predominate in the developing world and now account for up to 24–30 % of new infections in Europe [23, 110–112].

15 Laboratory Diagnosis of HIV-1 Drug Resistance

Zidovudine (AZT)-resistant virus was detected using an MT-2 syncytial assay in 1989 [113]. Soon thereafter, it was shown that phenotypic resistance to zidovudine was associated with mutations in reverse transcriptase at positions M41L, D67N, K70R, T215Y/F, and K219Q/E [114]. As each new antiretroviral drug was developed, viruses with phenotypic drug resistance were detected soon afterwards and the viral genetic mutations associated with drug resistance and/or viral breakthrough were then determined.

Clinical investigators developed a standardized HIV-1 phenotypic drug resistance assay using peripheral blood mononuclear cells which could be applied to the majority of clinical HIV-1 isolates to determine the clinical significance of HIV phenotypic drug resistance [115]. This assay was slow and labor intensive requiring cultivation of HIV-1 in vitro, quantitation of the viral stock to produce a standardized inoculum, and then viral replication in the presence of multiple drug levels to obtain an EC_{50} value (the concentration of drug required to reduce viral replication by 50 % compared to a no-drug control well). The whole process took 4–6 weeks and could only be conducted in a research laboratory. Subsequently, commercial laboratories developed HIV phenotypic resistance assays utilizing recombinant viruses containing PCR-amplified segments of clinical HIV-1 isolates that could be automated and produce highly reproducible results with a 2-week turnaround. Use of HIV-1 phenotypic assays is described in detail in Chap. 35 (Table 73.3).

The development of high-throughput genotypic sequencing allowed the commercial development of sequencing of a PCR-amplified segment containing the HIV-1 protease gene and a portion of the reverse transcriptase gene to detect mutations associated with phenotypic HIV-1 drug resistance and/or viral rebound in the clinic (Table 73.3). Databases of these mutations and listings of these mutations are updated regularly [77, 116, 117]. Interpretative algorithms for resistance resulting from the combinations of drug resistance mutations produced by currently available antiretroviral drug regimens have become complex and are generally generated using computer algorithms. These are then translated into a user-friendly report in which susceptibility to each agent is generally interpreted as sensitive, partially resistant,

Table 73.3 Comparison of genotypic and phenotypic drug resistance testing

	Genotypic drug resistance testing	Phenotypic drug resistance testing
Strengths	Rapid turnaround	Direct measure of drug susceptibility
	Less expensive	Can provide a measure of viral replication capacity
	Widely available	
	Clinically validated in multiple clinical trials	
Weaknesses	Interpretative algorithms are not standardized	Lack of availability of standardized clinical cutoffs
	Indirect measure of resistance	More expensive
	Difficulty interpreting complex mutation patterns	Slower turnaround
	Difficulty interpreting resistance to novel agents	Less widely available
	Cannot detect minority variants (<20 % of all viruses)	Cannot detect minority variants

Table 73.4 Indications for obtaining an HIV drug resistance test

1	Primary/acute or recent HIV infection[a,b,c,d]
2	Initiation of antiretroviral therapy[a,b,c,d]
3	Poor response to initial antiretroviral therapy[a,b]
4	Viral rebound on antiretroviral treatment[a,b,c,d]
5	Pregnancy if detectable plasma virus[a,b,c]
6	Postexposure prophylaxis[c]
7	Pediatric patients initiating antiretroviral treatment[b,c]

[a]IAS-USA recommendations [40, 103]
[b]US DHHS Treatment Guidelines [42]
[c]European Guidelines [41]
[d]British HIV Association [39]

Table 73.5 HIV drug resistance websites

1. Stanford HIV Drug Resistance Database	http://hivdb.stanford.edu/
2. Los Alamos HIV Drug Resistance Database	http://resdb.lanl.gov/Resist_DB
3. Stephen Hughes, HIV Drug Resistance Program, National Cancer Institute (structural database)	http://www.retrovirus.info/rt/
4. HIV InSite—Genotypic Testing for HIV Drug Resistance	http://hivinsite.ucsf.edu/InSite?page=kbr-03-02-07
5. Geno2pheno website	http://www.geno2pheno.org/
6. IAS-USA website	http://www.iasusa.org/content/drug-resistance-mutations-in-HIV
7. WHO Global HIV Drug Resistance Network	http://www.who.int/hiv/topics/drugresistance/hivresnet/en/
8. The French ANRS Resistance Group	http://www.hivfrenchresistance.org/

or resistant. Use of HIV-1 genotypic assays is described in detail in Chap. 38.

Several groups have provided guidance on the use and interpretation of HIV drug resistance assays [46–48, 118]. The patients for whom resistance testing is recommended are listed in Table 73.4. Numerous websites contain current information on HIV drug resistance [119]. Some useful websites are listed in Table 73.5.

There are several important caveats to the interpretation of HIV-1 drug resistance assays. The assays all report the results for the predominant circulating virus at each time point and will not detect minority viral species that are present at levels below 20–25 %. Additionally, virus populations can turn over rapidly if antiretroviral drugs are discontinued or drug regimens are changed. Thus, patients who are considering switching antiretroviral therapy should have a resistance test performed while on the failing regimen and not after stopping drugs for a period of time. Importantly, when considering drugs to utilize in a new antiretroviral regimen for patients who have received prior antiretroviral therapy, the clinician needs to consider all prior drugs given and all prior antiretroviral resistance results since these earlier viruses will continue to be present as archived viral DNA in HIV-1-infected cells and can rapidly reemerge under the appropriate antiviral selection pressure.

16 Treatment of Drug-Resistant HIV-1

16.1 Initial Treatment of HIV-1

Treatment is initiated for HIV-1 infection with different guidelines in different regions of the world [45, 48, 120, 121]. Most guidelines agree that treatment should be initiated for patients with symptomatic HIV-1 disease or CD4 counts less than 350 cells/μL. In North America and Europe, guidelines have moved to earlier treatment with CD4 cell counts <500 cells/μL or universal treatment of all HIV-infected persons. Initial treatment is typically with two nucleoside drugs in combination with either a non-nucleoside reverse transcriptase inhibitor, a protease inhibitor, or an integrase inhibitor. Data showing that approximately 10–27 % of newly HIV-1-infected persons have a virus with genotypic evidence of drug resistance and up to 4 % of these patients may harbor a multiple drug-resistant (MDR) virus has led to the recommendation that all newly HIV-infected persons should have a resistance test prior to initiating therapy. Patients who do not have a brisk antiviral response in plasma HIV RNA to combination antiretroviral treatment during the first 2 months of treatment should be evaluated for treatment adherence and be considered for genotypic resistance testing at that time.

The goal of combination therapy for treatment of HIV-1 disease is to obtain complete suppression of HIV replication which is measured by a plasma HIV RNA level of less than 20 copies/mL. The challenge for the treating physician and patient is to maintain high levels of adherence to taking the drug regimen over decades of treatment since the most common cause of virological rebound is poor adherence or discontinuation of treatment. If the patient has evidence of a rising plasma HIV RNA value, the clinician should carefully review patient adherence to taking the medications, side effects of treatment that could reduce adherence, concomitant medications such as rifampin which can lower the levels of HIV NNRTI and protease inhibitors, and new-onset gastrointestinal disorders such as nausea, vomiting, or diarrhea to determine if there is any modifiable issue which can be resolved in order to fully suppress the virus. If the plasma HIV RNA remains elevated after these measures are taken, a resistance test should be considered to guide the next round of treatment. Allowing patients to remain on a combination antiretroviral regimen despite active HIV-1 replication manifested by detectable HIV RNA levels will result in increasing levels of resistance to the drugs administered and broadened resistance to the remaining drugs from the classes of drugs used in the regimen [122].

17 Treatment of Drug-Resistant HIV-1

Patients who have experienced virologic breakthrough after initial or early rounds of antiretroviral treatment usually have active drugs available to develop an effective combination treatment regimen. It is critical for each new round of therapy to combine at least two and preferably three active antiretroviral drugs together to ensure that a fully suppressive regimen is used. Adding less than three active drugs often leads to rapid viral breakthrough with resistance to the new class of drugs. HIV drug resistance testing has shown short-term clinical benefit in helping to select active drugs for treatment-experienced patients and should be utilized [123–126]. Treatment decisions need to take into account prior drug exposure, drug toxicities on prior antiviral regimens, prior resistance test results and the resistance data while on the most recent antiretroviral drug regimen, and patient wishes (Table 73.6). Where available, advice from an expert with experience in treating patients with multidrug-resistant HIV-1 should be obtained [124, 126].

18 Salvage Therapy for Drug-Resistant HIV-1

The goals of HIV treatment can change for patients who have virus resistant to most or all currently available drugs. The benefits of drug resistance testing may be limited in this group of patients. These patients should be maintained on antiretroviral treatment since discontinuing all treatment results in disease progression. For patients who are asymptomatic with stable CD4 cell counts, the clinician may elect to continue the current regimen, if it is well tolerated, or switch to a simpler, more easily tolerated combination drug regimen if drug toxicities are present. The goal in these patients is no longer complete viral suppression but to maintain immune status (especially a CD4 count above 200 cells/μL) and patient functioning until active drugs become available to develop a fully active antiviral regimen [60].

Structured treatment interruptions (STI) to allow sensitive virus to reemerge and overgrow the multidrug-resistant circulating virus are not recommended. Studies have shown that reemergence of wild-type, drug-sensitive virus is associated with increasing viral loads and CD4 declines potentially resulting in disease progression events [127–129]. Re-initiation of combination therapy after an STI results in a transient improvement of antiviral responses compared to continued treatment but the decreased CD4 counts can remain depressed for more than a year compared to continued therapy [128]. Discontinuation of antiviral treatment has been associated with increased risk of opportunistic disease or death from any cause including cardiovascular, renal, and hepatic disease [130, 131].

Some investigators have tried "mega-HAART" regimens to treat patients using 5–8 antiretroviral drugs [132–134]. While some short-term antiviral benefits have been observed, the toxicity of these regimens has limited their utility in general practice.

Table 73.6 Factors in choosing drug regimens for treatment-experienced patients

1. Number and duration of prior antiretroviral drugs
2. Toxicity while receiving prior antiretroviral drugs
3. Current and prior HIV drug resistance test results
4. Ability to develop a combination drug regimen with at least two and preferably three drugs active against the current circulating virus
5. Patient desires

19 Newer Classes of Antiretroviral Drugs

The availability of second-generation HIV protease inhibitors (tipranavir and darunavir), second-generation NNRTIs (etravirine and rilpivirine), a CCR5 inhibitor (maraviroc), and HIV integrase inhibitors (raltegravir, elvitegravir, and dolutegravir) has greatly expanded the potential treatment options for patients whose virus is resistant to multiple classes of antiretroviral drugs. Combinations of these drugs have made complete viral suppression possible for patients with the most resistant viruses and have led to a standard goal of therapy to achieve undetectable virus (plasma HIV RNA <20 copies/mL) for all stages of HIV treatment. It is critical in these patients to combine 2–3 active antiretroviral drugs together to ensure that a fully suppressive regimen is used. These decisions in patients with limited options for use of NRTI, NNRTI, PI, and integrase inhibitor classes of drugs may benefit from use of both genotypic and phenotypic HIV drug resistance tests [135]. Advice from an expert with experience in treating patients with multidrug-resistant HIV-1 should be obtained, if possible [135].

20 Prevention of HIV-1 Drug Resistance

The most effective method to prevent emergence of HIV-1 drug resistance and block further transmission of HIV-1 is to fully suppress HIV replication with combination therapy in all HIV-infected persons [136]. Once fully suppressive therapy is given, high patient adherence to the prescribed regimen determines the ultimate durability of each drug regimen. Recent advances utilizing daily fixed-dose combination regimens with well-tolerated agents have significantly increased the success rates and durability of initial antiretroviral treatment.

For patients with multidrug-resistant virus, the availability of second-generation NNRTIs and PIs, entry inhibitors, and

integrase inhibitors offers the potential for patients with HIV-1 resistant to multiple classes of antiretroviral drugs to fashion fully suppressive combination drug regimens and obtain durable treatment responses. This should reduce the potential for transmission of multidrug-resistant viruses to the next generation of HIV-1-infected patients.

Data suggest that the availability of fully suppressive combination therapy for HIV-1 can lower the rates of HIV-1 transmission along with both prevalent and incident HIV-1 drug resistance rates [137]. Additionally, early initiation of antiretroviral therapy as well as prevention programs encouraging safe sex practices and needle exchange should reduce the number of new HIV infections in many communities [138].

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Hepatitis B Virus Drug Resistance

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Apostolos Beloukas and Anna Maria Geretti

1 Introduction

There have been major advances in the prevention and management of antiviral drug resistance in the treatment strategies against HBV infection in the past two decades [1]. Currently, interferon alfa-2b and pegylated interferon-a (pegIFN-α), and several oral inhibitors of the HBV polymerase enzyme are approved for the treatment of CHB [1–5]. Polymerase inhibitors, comprising both nucleoside and nucleotide analogues (NAs), are generally more effective than IFN-based treatment and cause fewer side effects. Whilst current therapeutic options for the de novo treatment of chronic hepatitis B (CHB) carry a low risk of selecting for drug resistance, long-term and indefinite treatment is commonly required to sustain viral suppression. The demand for better treatments heightens with the fact that many patients have accumulated HBV drug resistance and cross-resistance through previous, less efficacious therapy, and have limited treatment options. Even more so, large numbers also continue to be at risk of drug resistance due to limited access to effective antiviral compounds in middle and low-income settings. Several novel compounds are under development that may pave the way for therapeutic regimens of finite duration and, potentially, for HBV eradication [5–7].

The overall short-term and long-term efficacy of NA-based therapy for CHB is influenced by several factors, including drug potency and treatment adherence as key determinants (Table 74.1). Even in optimally adherent patients, some NAs, when used as single agents, can be insufficiently able to suppress virus replication, particularly in the context of a high HBV DNA load. Due to the plasticity of the HBV genome, ongoing virus replication in the presence of drug pressure can lead to the emergence of HBV variants carrying mutations that reduce drug susceptibility. Although some HBV drug-resistant mutants may show a significant loss of fitness and replicative capacity, others replicate efficiently, can cause progression of liver disease, and can be transmitted.

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2 Mechanisms of HBV Drug Resistance

2.1 Emergence of Drug Resistance

HBV is a DNA virus that replicates via an RNA intermediate. The viral polymerase enzyme, which also has reverse transcriptase (RT) activity, displays a high error rate and lacks the proofreading function required to repair incorrectly incorporated bases [8, 9]. HBV has a high replication rate, resulting in the production of ~10^{12} virions per day. This combines with a mutational rate of ~10^{-5} substitutions per base and replication cycle [10, 11]. In individuals with ongoing viral replication, approximately $10^{10–11}$ point mutations are estimated to occur per day across the entire viral genome. Some mutations cause a severe loss of function, impairing viral fitness to the extent that the mutant stops replicating. Other mutants display variable degree of fitness impairment. Thus, in an infected host, HBV exists as variety of diverse strains that constitute the *viral quasispecies*. At any given time, and subjected to the influence of modulating factors including immune-mediated and drug-mediated selective pressure, certain mutant species are dominant, whereas others exist only as rarer, low-frequency variants.

HBV mutations that confer reduced drug susceptibility arise spontaneously in the targets of antiviral therapy and circulate within the viral quasispecies prior to the introduction of therapy. Given the overall functional cost, in the absence of drug pressure, HBV drug-resistant variants circulate at low frequency, typically as single mutants, and escape

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detection by both routine and sensitive methods. Under drug-selective pressure, if virus replication continues, the variants acquire a selective advantage and gradually emerge as dominant species. With continued virus replication under drug pressure, the single mutants evolve genetically, acquiring additional primary and secondary mutations, including compensatory changes that restore viral fitness and increase pathogenicity [12–14].

The partially double-stranded circular HBV genome is organized into four overlapping reading frames (ORFs) (Fig. 74.1). Overlapping of the polymerase ORF with the surface ORF has two important consequences. Firstly, there is a reciprocal impact of genetic changes driven by selective pressure, including changes in HBsAg antigenic determinates which create potential for vaccine and diagnostic escape [15, 16]. Secondly, genetic evolution under drug pressure is constrained by the simultaneous impact on polymerase and surface functionality. These constrains are illustrated by comparing the emergence of resistance to lamivudine (LAM) monotherapy in HBV and HIV infection. In HIV-positive subjects, resistant strains emerge as dominant within a few weeks of LAM monotherapy. In contrast, months are required for the emergence of LAM-resistant strains in HBV-positive subjects. Nonetheless, sensitive methodologies have revealed that HBV resistance to LAM can emerge more rapidly than previously appreciated. In a study of patients with HIV and HBV co-infection starting LAM-containing antiretroviral therapy (ART) without additional HBV-active agents, most patients with detectable serum HBV DNA after 6 months had evidence of LAM resistance when evaluated by deep sequencing (Fig. 74.2) [12].

Table 74.1 Determinants of responses to antiviral therapy in chronic hepatitis B

Host	Drug	Virus
Adherence	Potency	HBeAg status
Tolerability	Side effect profile	HBV DNA load
Liver disease status	Genetic barrier	Acquired drug resistance
Immunity	Pharmacokinetics	Transmitted drug resistance
Genetics		

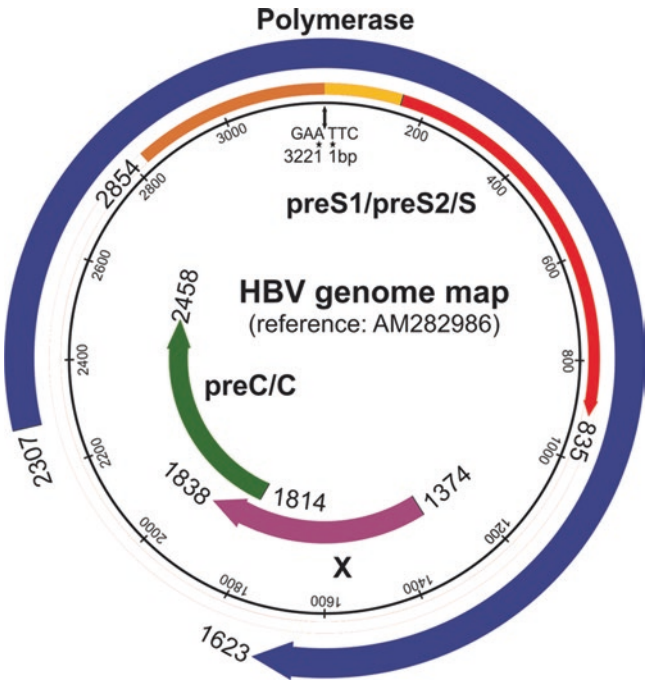

Fig. 74.1 The HBV genome. The relaxed partially double-stranded circular DNA has a size of ~3.2-kilobases and comprises four overlapping reading frames: polymerase (*blue*), surface (preS1, preS2, and S domains; *orange*, *yellow*, and *red*, respectively), X (*purple*), and pre-core/core (*green*). Complete genome numbering starts from TTC and ends at GAA

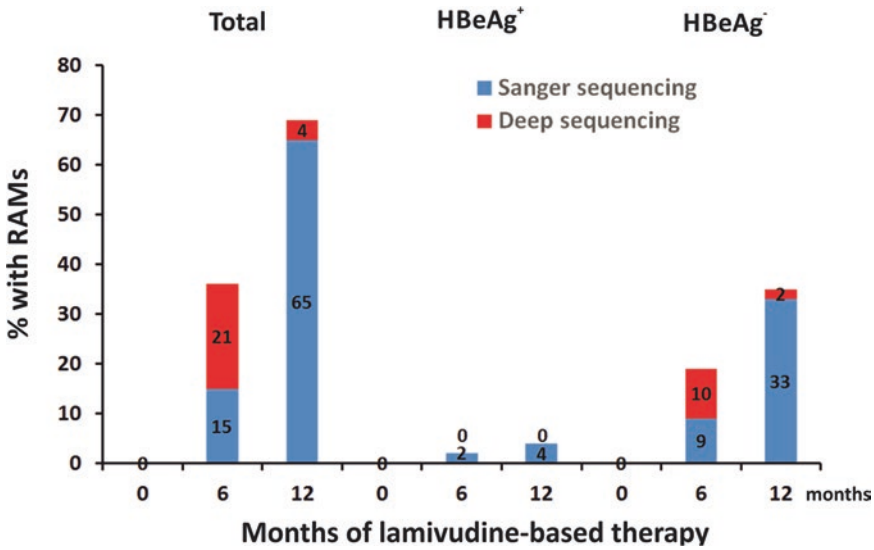

Fig. 74.2 Emergence of HBV drug resistance during lamivudine monotherapy. A total of 133 HIV and HBV co-infected subjects starting lamivudine-containing antiretroviral therapy without additional HBV-active agents underwent testing for the presence of lamivudine resistance-associated mutations (RAMs) in HBV polymerase. Testing was performed at baseline and after 6 and 12 months of therapy using Sanger sequencing and deep sequencing. Resistance rates are presented as total and by baseline HBeAg status (Adapted from [12])

2.2 Persistence of Drug Resistance

HBV RAMs that emerge as dominant during antiviral therapy lose their replicative advantage once therapy is discontinued, and are outgrown by fitter, drug-sensitive ("wild-type") strains. In patients who have discontinued therapy for more than a few weeks, it is usually no longer possible to detect the resistant mutants using routine technology. The disappearance of resistance is only apparent however; treatment-enriched mutants persist as low-frequency circulating variants and rapidly re-emerge if suboptimal treatment is restarted. In addition, HBV establishes an archive of genetic variants within the covalently closed circular DNA (cccDNA) that persists in infected hepatocytes [17]. HBV cccDNA is generated from the relaxed circular DNA genome of incoming virions and persists long-term in episomal form in the nucleus of hepatocytes [18]. Whilst effective in suppressing HBV replication, NAs have limited efficacy in reducing the cccDNA reservoir [19–21]. Current research aims at targeting the reservoir and potentially cure HBV infection [5, 18]. Meanwhile, HBV cccDNA retains a long-term memory of any selected resistant strain, from which new replicating strains can re-emerge [1, 5, 18, 20, 22].

2.3 Nomenclature

A nomenclature for describing HBV drug resistance-associated mutations (RAMs) was established in 2001 [23]. The HBV polymerase gene is divided into four functional units (Fig. 74.3). Resistance is defined by the presence of one or more nucleotide substitutions in the RT domain of the polymerase gene, resulting in amino acid substitutions within the enzyme. Primary or major RAMs play a key role in conferring a drug-resistant phenotype by directly reducing drug susceptibility. Secondary and compensatory RAMs play an accessory role by increasing the level of resistance or restoring the functional defects caused by major RAMs [13]. Mutations are reported with the letters rt followed by the wild-type amino acid, the codon numbered position relative to the start of the rt region, followed by the mutant amino acid. For example, rtM204V describes the major LAM RAM, whereby methionine at RT codon 204 is replaced by valine.

3 Pathways of HBV Drug Resistance

Current treatment strategies for CHB are guided by a number of viral- and host-related parameters and mainly comprise (1) 1 year of treatment with peg-IFN-α or (2) long-term treatment with NAs [1, 2, 4, 5, 24]. Combination strategies of peg-IFN-α plus potent NAs are under evaluation [1]. Peg-IFN-α exerts both direct antiviral and immune modulatory functions by regulating the expression of interferon-stimulated genes (ISGs) [25]. In CHB, IFN-α causes immune activation, inhibition of HBV genome transcription, intracellular destabilization of viral nucleocapsid, and degradation of intracellular cccDNA via APOBEC3A [26–28]. A number of host genetic determinants have been shown to modulate IFN susceptibility, including an effect of viral genotype on the rate and kinetics of HBeAg and HBsAg loss. Generally, patients infected with HBV genotypes A and B show better responses to IFN-based therapy than patients with genotypes C and D, whilst responses to NAs appear to be comparable across different HBV genotypes [29]. Additionally, several mutations in the HBV genome (especially in pre-core and basal core promoter) have been shown to modulate responses to IFN-based treatment [30].

NAs compete with the natural substrate for binding to the active site of the HBV polymerase enzyme [31]. NAs must be phosphorylated to their nucleoside triphosphates or nucleotide di-phosphate derivatives in order to exert antiviral activity. Phosphorylation is mediated by cellular kinases and the initial phosphorylation is the rate-limiting step of the process, considered to modulate some of the differences in efficacy observed among NAs [8, 32]. Once phosphorylated, NAs are incorporated by the viral polymerase in the growing

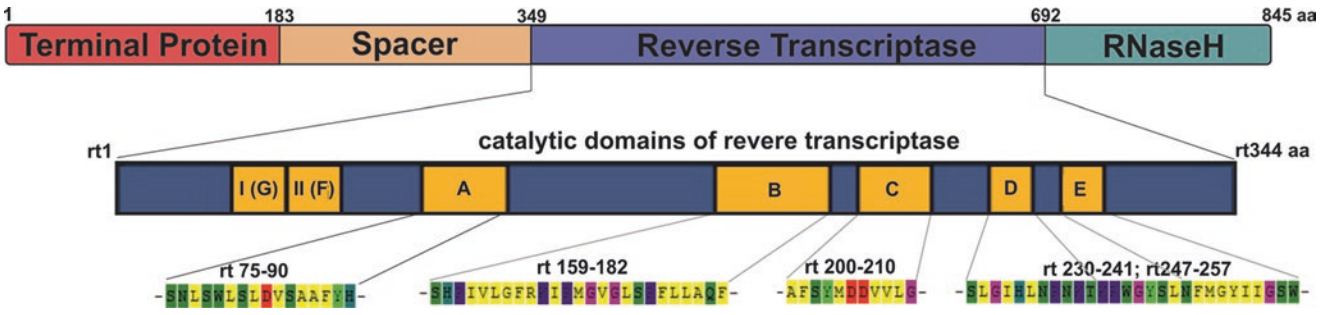

Fig. 74.3 Organisation of the HBV polymerase open reading frame, showing the reverse transcriptase (RT) region and its catalytic domains. The sequence corresponds to HBV genotype A (subtype adw2) (GenBank accession number AM282986); numbering is given according to the standardized nomenclature

Table 74.2 Resistance mutations associated with resistance to nucleoside and nucleotide inhibitors of the HBV polymerase enzyme

Class	Drug	Chemical structure	Genetic barrier	Major or primary RAMs	Compensatory and other RAMs
L-Nucleoside	Lamivudine	2′,3′-Dideoxy-3′-thiacytidine	Low	rtM204I/V/S/Q	rtL80V/I, rtI169T, rtV173L, rtL180M, rtT184S/G, rtS202I, rtQ215S
				rtA181T/V	
	Emtricitabine	5-Fluoro-1-(2R,5S)-[2-(hydroxymethyl)-1,3oxathiolan-5-yl]cytosine	Low		
	Telbivudine	β-L-2′-deoxythymidine	Intermediate		
D-Cyclopentane	Entecavir	2-Amino-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylidenecyclopentyl]-3H-purin-6-one	High (naïve)	rtL180M + rtM204V + [rtT184A/C/F/G/I/L/S or rtS202I/G or rtM250L/V]	
			Low (LAM resistance)	rtL180M + rtM204V + rtA186T + rtI163V	
Acyclic phosphonate	Adefovir dipivoxil	9-[2-[[Bis[(pivaloyloxy)methoxy]-phosphinyl]methoxy]ethyl]adenine	Intermediate	rtN236T	rtI233V
				rtA181T/V	
	Tenofovir disoproxil fumarate	9-[(R)-2[[bis[[(isopropoxycarbonyl) oxy] methoxy]phosphinyl]methoxy] propyl] adenine fumarate	High	rtA194T (+rtL180M + rtM204V/I)	Precore and basal core promoter mutations
				rtP177G + rtF249A	

RAMs resistance-associated mutations, *LAM* Lamivudine

viral DNA chain, and act as chain terminators, inhibiting negative and positive HBV DNA strand synthesis. Based on the similarities between the HBV and HIV polymerase enzymes, it is not surprising that several NAs have dual antiviral activity [33]. Among available agents, LAM, emtricitabine (FTC), and tenofovir disoproxil (TDF) have significant activity against HIV. Entecavir (ETV) has a low residual antiretroviral activity and potential for selection of HIV drug resistance if used in isolation [34]. Adefovir dipivoxil (ADF) at HBV dosing and telbivudine (LdT) are not thought to exert anti-HIV activity.

NAs are classified into three structural groups: (1) L-nucleosides, (2) D-cyclopentanes, and (3) Acyclic (or alkyl) phosphonates (nucleotides). The classification corresponds to distinct pathways of resistance (Table 74.2) [5, 19, 21, 35]. Four major pathways are recognized: (1) the rtM204 pathway with L-nucleosides; (2) the rtN236T pathway with alkyl phosphonates; (3) the rtA181T/V pathway shared between the L-nucleosides and alkyl phosphonates; and (4) the D-cyclopentante pathway.

3.1 L-Nucleosides

L-Nucleosides comprise LAM and LdT, which are widely available worldwide; FTC, which is available in combination with TDF for the treatment of HIV and HBV co-infection; and clevudine, which is currently available in South Korea and the Philippines. The compounds have a similar molecular structure and bind to the same region of the viral polymerase, which results in shared resistance pathways and extensive cross-resistance [5, 8, 14, 19, 21, 35–50].

Resistant mutants generally remain susceptible to ADV and TDF [37, 51] and may retain partial susceptibility to ETV. The rtA181T/V mutation in domain B can emerge in treated patients and reduce susceptibility to both L-nucleosides and acyclic phosphonates [52–57].

3.1.1 Lamivudine

LAM was the first direct-acting antiviral agent to become available for the treatment of HBV infection. LAM was already used for the treatment of HIV infection and in this context codon M184 in the HIV RT catalytic site (YMDD motif) was identified as the major resistance site. Reflecting sequence homology between the two viral polymerases, the major HBV LAM resistance site is located at the corresponding codon 204 in the catalytic site (C domain) of the RT region of HBV polymerase. A single mutation that results in the substitution in YMDD of methionine by isoleucine (rtM204I), valine (rtM204V), or rarely serine (rtM204S) is sufficient to confer high-level LAM resistance. rtM204Q is an additional LAM RAM, conferring moderate drug resistance and displaying higher replication capacity than rtM204I [58].

During LAM treatment, M204I mutants are typically detected first, and subsequently replaced by rtM204V [12]. The mutants display reduced viral fitness. Molecular modelling indicates that rtM204I/V induce both steric hindrance and electrostatic repulsion for the incoming LAM triphosphate [8, 37, 59–61]. The catalytic activity of the polymerase is also reduced as a result, due to altered alignment of the natural substrate with respect to template and primer. With ongoing virus replication under LAM pressure, compensatory mutations occur in domain A (codon 80), domain

B (codons 169, 173, 180), interdomain B-C (codon 184), and domain C (codons 202 and 215) [14, 21, 35, 38, 45–47, 50]. Compensatory mutations are insufficient to confer LAM resistance in isolation, but combined with rtM204 mutations they enhance resistance and improve enzymatic function and HBV replication. The negative electrical charge of rtL180M/C, for instance, decreases binding affinity for LAM-triphosphate, allowing better discrimination between the drug and the natural substrate [14, 60, 62]. In clonal and single genome sequences, LAM resistance and compensatory mutations typically coexist on the same viral genome [12].

3.1.2 Telbivudine

LdT is more potent than LAM in reducing serum HBV DNA levels in vivo [63, 64] and is also more potent than ADV [65]. LdT has an improved genetic barrier to the emergence of resistance relative to LAM, but shares a similar resistance profile, with rtM204I being the RAM most commonly observed in patients with virological breakthrough [19, 35, 42, 48, 49, 66–69].

3.2 D-Cyclopentanes

ETV, a synthetic analogue of 2-deoxyguanosine, displays a high genetic barrier to resistance in treatment-naïve patients, as resistance requires multiple substitutions to emerge [70–76]. ETV has high potency in both HbeAg-positive and HBeAg-negative treatment-naïve subjects [70, 71, 73, 74]. Due to shared resistance pathways however, ETV activity is reduced in LAM-experienced patients, requiring higher treatment doses and overall reducing the genetic barrier so that evolution of further resistance is facilitated [34, 45, 50, 77–81].

3.3 Acyclic Phosphonates (Nucleotides)

Alkyl nucleoside phosphonates comprise ADV and TDF. These compounds are structurally similar and possess a phosphonate group, requiring two rather than three phosphorylation steps to become intracellularly active [82]. Their structural similarity to the natural substrate deoxyadenosine triphosphate and the small, flexible phosphonate linker favour access to the HBV polymerase active site and high affinity for the enzyme [31, 32, 82].

3.3.1 Adefovir

ADV was initially developed for the treatment of HIV infection, but use was discontinued due to renal toxicity [8]. ADF suppresses HBV replication at significantly lower doses than those required to suppress HIV, and is safe at HBV dosing [83, 84] and putatively inactive against HIV. Development of HBV resistance to ADV occurs more slowly than seen with LAM, and is associated with mutations outside the YMDD motif, most commonly rtA181T (B domain) and rtN236T (D domain) [5, 8, 21, 35, 85–87]. The rtN236T mutant shows 7-fold resistance to ADV in vitro, which increases to 18-fold with the rtA181V + rtN236T double mutant [86]. The N236T mutation also has resistance effects for TDF, but confers no resistance to LAM and ETV. Molecular modelling reveals a possible mechanism of action for rtN236T. In wild-type HBV polymerase, the rtN236 amino acid may be hydrogen bonded to the adjacent rtS85 residue, and may interact directly with the γ-phosphate of ADF di-phosphate. The rtN236T mutation disrupts the hydrogen bond, thereby decreasing the binding affinity for ADF [88].

3.3.2 Tenofovir

The use of ADV for the treatment of CHB is declining, reflecting the superior virological efficacy of TDF in both HBeAg-positive and HBeAg-negative subjects [19, 35, 83, 84, 89–94]. TDF in vivo is converted to tenofovir, an acyclic nucleoside phosphonate (nucleotide) analogue of adenosine 5′-monophosphate. TDF is structurally related to ADV but at the standard dose achieves higher intracellular concentrations and displays higher binding affinity for the HBV polymerase enzyme [95]. This results in a greater virological potency and higher genetic barrier to resistance than ADF. The genotypic resistance profile of TDF remains controversial. The rtA194T mutation has been associated with partial TDF resistance and a negative impact on replication capacity of HBV constructs in vitro [96]. The mutational profile comprising rtA194T plus the LAM RAMs rtL180M + rtM204V/I has been proposed to reduce TDF susceptibility by over tenfold [97], although the finding has not been consistently reproduced [98]. The rtL180M + rtM204V/I + rtA194T mutation profile has a significant fitness cost, reducing replicative capacity by >75 %. The fitness defect of both rtA194T alone and in combination with rtL180M + rtM204V/I however is at least partially compensated through mutations in the precore and basal core promoter regions [96, 98], suggesting that patients with HBeAg-negative CHB may be particularly at risk of TDF resistance. The rt181T/V mutation has been shown to confer low-level resistance to TDF (two- to threefold); resistance levels increase with the combination of rt181T/V + rtN236T, which can be co-localized on the same viral genome [54, 86]. A further proposed pathway comprises rtP177G and rtF249A, which confer enhanced resistance to TDF and reduced replication capacity both in vitro and in vivo [99].

Despite these findings, genotypic HBV resistance to TDF has not been seen to emerge in clinical studies, including subjects with slow HBV DNA kinetics [100–102], and subjects undergoing continued treatment for 6 [91], 7 [89], or 8 [103] years. TDF retains activity in LAM-experienced

[94, 104–106] and ETV-experienced [29] subjects, and is also effective, although less so, in patients with suboptimal treatment responses to ADV [5, 29, 94, 107, 108]. It has been proposed however that the double rtA181T/V + rtN236T mutant in particular is associated with inadequate virological response to TDF [107].

3.4 Genetic Barrier

The genetic barrier to the emergence of drug resistance is the expression of the interaction between multiple factors (Table 74.3) [19, 21, 38, 45, 50, 62, 88, 95, 98, 109, 110]. In general terms, the genetic barrier is low with LAM and FTC, intermediate with LdT and ADV, and high with ETV (in naïve patients) and TDF (Fig. 74.4). With LAM monotherapy, prevalence of RAMs is ~70 % after 4–5 years of treatment [21, 41, 111–114]. The rate of HBV replication is a key modulating factor, with a substantially higher risk of resistance observed in subjects with positive HBeAg status and high HBV DNA levels at start of therapy [12, 106] (Fig. 74.2). LdT resistance emerges more slowly, but rates are substantial, reaching 11 % and 26 % after 2 years in HBeAg-negative and HBeAg-positive subjects, respectively [64]. The cumulative incidence of ADV is 29 % after 5 years [83, 84, 115]. In patients receiving first-line therapy with ETV, rates of resistance are 1.2 % after 5 years for both HBeAg-positive and HBeAg-negative subjects [71, 75], increasing to 2.1 % at 7 years [116]. No resistance has been reported in over 400 patients that received first-line TDF for 7 [89] or even 8 [103] years.

Antagonistic and synergist interactions between the resistance pathways of different drugs modulate the efficacy and genetic barrier of a combination regimen. Emergence of ETV resistance is accelerated by previous LAM exposure, and among subjects with LAM RAMs starting ETV, 51 % have ETV resistance after 5 years [75]. In LAM-experienced subjects with resistance, use of ADV add-on therapy with continuation of LAM shows superior virological efficacy to the use of ADV alone, in part reflecting the antagonism between the main pathways of LAM (rtM204) and ADV (rtN236T) resistance, which result in enhanced susceptibility to ADV, reduced emergence of ADV RAMs, and virological benefit, at least in subjects with low baseline HBV DNA levels [117–121].

Table 74.3 Factors that modulate the genetic barrier to resistance in HBV therapy

Factor
• Drug potency
• Intracellular drug concentration
• Interaction between drug and enzyme (e.g. binding affinity, structural flexibility)
• Phenotypic effect of mutations
• Number of mutations required to compromise drug activity
• Fitness cost of mutations
• Ease of emergence of compensatory mutations that restore viral fitness
• Interactions between resistance pathways
• Viral genome sequence
• Pre-existing selection or transmission of resistance
• Baseline HBV DNA load and kinetics of HBV DNA decline on therapy
• Host genetics and immunity

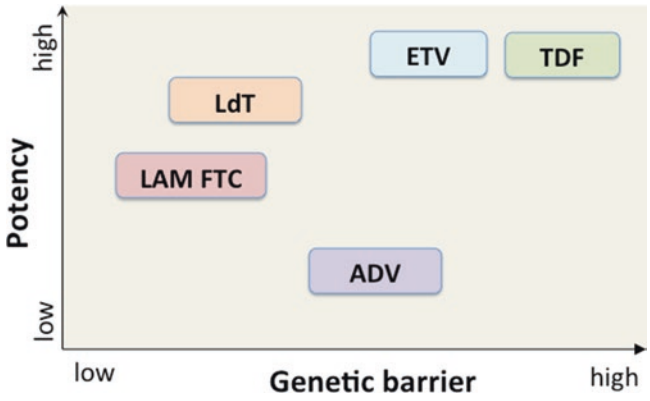

Fig. 74.4 Potency and genetic barrier of available antiviral agents against HBV

4 Tests to Detect Drug Resistance

HBV drug resistance is assessed in clinical practice by demonstrating the presence in the RT domain of the viral polymerase of RAMs that are known to confer a drug-resistant phenotype. Testing is generally recommended in patients experiencing suboptimal treatment responses, as indicated by serum HBV DNA levels [1]. There have been several reports of the transmission of HBV drug-resistant variants; however the prevalence of transmitted HBV drug resistance is too low to support the cost-effectiveness of routine resistance testing prior to starting antiviral therapy [122, 123].

4.1 Genotypic Tests

HBV genotypic tests available for diagnostic use comprise conventional and deep genome sequencing and reverse hybridization mutation-specific assays (MSAs) (Table 74.4). Conventional population (Sanger) sequencing of DNA products amplified by PCR provides accurate results, is widely available, and is applicable to any region of the HBV genome. The method yields a consensus sequence of the dominant quasispecies present in a patient's sample and has a sensitivity ranging from 10 to 20 %. Limitations therefore include

Table 74.4 Methods for detecting HBV drug resistance-associated mutations

Methodology	Detection limit[a]	Target	Advantages	Disadvantages
Population (Sanger) sequencing	10–20 %	Entire gene (RT)	• Current standard of care	• Labour intensive and relatively expensive
			• Both commercial kits and in-house assays available	• Limit of detection leads to underestimation in some patients
			• Moderately portable	• Requires specialized technical skills and laboratory infrastructure
			• Can be performed at low HBV DNA load	
Deep sequencing (e.g. Illumina)	1 %	Entire gene (RT)	• Able to detect low-frequency variants and to estimate the amount (frequency) of variants in a patient's sample	• Assay errors may occur at multiple steps of the process
				• Requires specialized laboratory infrastructure and advanced technical and bioinformatics skills
				• Limited availability in routine care
				• Best suited for centralized testing in high throughput specialist centres
			• Allows simultaneous processing of large number of samples reducing cost	• Testing at low HBV DNA load not generally recommended
Reverse hybridization (INNO-LiPA)	2–10 %	Sentinel RAMs	• Sensitive assay for specific mutations	• Assay development must be tailored for each mutation and across viral genotypes
			• Commercially available and highly portable	• False-positive or false-negative results can occur because of binding site variability
			• Inexpensive and simple to perform	
			• Suitable for resource-limited settings	• Testing at low HBV DNA load not generally recommended

[a]The detection limit describes the sensitivity for low-frequency variants and is a function of the assay and other parameters including HBV DNA load. *RT* reverse transcriptase, *RAMs* resistance-associated mutations

inability to identify linkage of mutations at the individual genome level and limited sensitivity for low-frequency mutants. Deep sequencing is the process of parallel sequencing of millions of individual DNA molecules in a single assay, with thousands of clonal viral sequences being analysed to yield estimates of the number and proportion of unique variants within a sample. Deep sequencing offers increased sensitivity for low-frequency HBV RAMs, providing significant insights into viral kinetics during therapy [12, 124]. Deep sequencing platforms are becoming increasingly affordable and available for routine diagnostic use, although they continue to require extensive expertise in bioinformatics for the analysis of the large sequencing output. Deep sequencing is also vulnerable to errors at various stages of the process [125]. A 1 % interpretative cut-off is generally recommended to distinguish biologically significant findings from spurious detection [126, 127]. In addition, clinical validation studies are required to determine the clinical significance of any low-frequency RAM detected [125].

Among MSAs, the reverse hybridization line probe assay (INNO-LiPA) is commercially available, reliable, relatively inexpensive, and easy to perform. The assay uses a PCR-amplified product for reverse hybridization with specific oligonucleotide probes immobilized on nitrocellulose strips and shows higher sensitivity for low-frequency mutants than Sanger sequencing, generally ranging between 2 and 10 % of the total viral population. INNO-LiPA however remains less sensitive than deep sequencing, and detection is limited to the selected number of targeted RAMs. The major limitation of hybridization-based methods lies in their single-base discrimination. Specificity can be influenced by the sequences neighbouring a polymorphic site, or by interference from secondary structures, and the assay must be tailored for each targeted codon and across viral genotypes. Furthermore, as new RAMs are identified, the assays must be updated accordingly.

In research setting, clonal and single genome sequencing are labour-intensive and costly methods that apply Sanger sequencing to the analysis of single viral genomes [12, 128]. The methods allow detection of linkage of individual mutations on the same viral genome, and the study of the evolutionary pathways of resistant variants. Provided a large number of sequences are analysed, the methods also allow detection of low-frequency variants. Single genome sequencing offers the advantage over clonal sequencing of reducing errors related to in vitro recombination of PCR products.

Restriction fragment length polymorphism analysis and PCR-based methodologies such as allele-specific PCR have been used for research purposes to improve sensitivity of detection of low-frequency RAMs. The methods are generally labour-intensive, technically difficult, and expensive, and only detect known mutations requiring mutation-specific protocols. Assays undergoing development include nanopore and single-molecule long-read sequencing, technologies based on oligonucleotide microarrays (DNA chip) or mass spectrometry, and the amplification-refractory mutation system [129].

4.2 Phenotypic Tests

Phenotypic testing plays a key role in the research of the evolution and significance of HBV drug resistance. The characterization of novel mutations requires in vitro analysis to confirm the effects of the mutation on the viral phenotype, including both drug susceptibility and replication capacity [21, 128]. The methods employ HBV polymerase enzymatic assays and cell-culture methods. Most systems rely on the transfection of recombinant replication-competent HBV DNA into hepatoma cell lines. Testing may be performed with clinical isolates and site-directed mutants, allowing the analysis of the impact of mutations both individually and in combinations.

5 Definition of Virological Responses to HBV Therapy

Based upon the established close relationship between ongoing virus replication and liver disease progression the goal of CHB therapy is to achieve and maintain optimal suppression of serum HBV DNA load, as a key surrogate marker for clinical efficacy, and prevention of disease progression to cirrhosis, liver failure, or hepatocellular carcinoma [1, 4, 5, 130–132]. Further aims, which are difficult to achieve with currently available NA therapy, are to induce loss of HBeAg and anti-HBe seroconversion among HBeAg-positive subjects, and ideally, loss of HBsAg in all treated patients [1, 2, 116, 133]. Patients receiving antiviral treatment undergo regular monitoring of virological responses with HBV DNA assays that are sensitive and specific, offer a wide dynamic range of quantification, and are calibrated to express results in International Units [1, 5, 19, 21, 134].

An optimal virological response (VR) is defined as a serum HBV DNA level below the lower limit of quantification of validated assays, typically <15 or <30 IU/mL. With the highly potent NAs TDF and ETV, rates of virological suppression are >90 % in adherent patients after 3 years [71, 92, 135–137]. In 2006, the National Institutes of Health proposed a set of standardized, HBV-specific definitions to describe suboptimal responses to antiviral therapy, based upon HBV DNA levels measured at key time points after treatment initiation. These definitions remain in clinical use, although they require adjustments when applied to current treatment strategies [1], to reflect differences in antiviral potency and overall resistance risk relative to earlier compounds (Fig. 74.4).

5.1 Primary Non-response

Primary non-response is defined as the inability of treatment to reduce serum HBV DNA levels by ≥1 $\log_{10}$ IU/mL after 12 weeks of treatment or by ≥2 $\log_{10}$ after 24 weeks. It is uncommon with NAs, although seen more frequently with ADV (~10–20 %) than with other NAs because of suboptimal antiviral efficacy [111]. It is recommended that treatment be reviewed promptly, considering adherence as a key determinant, and addressing any concerns related to the anticipated drug efficacy, for instance, in the context of previous drug exposure and likely drug resistance. Patients on ADV monotherapy should be switched to more active therapy. In primary non-responders receiving TDF or ETV who show no evidence of resistance at week 24, continued therapy after 24 weeks may achieve suppression. One study compared the cumulative probability of obtaining a VR in patients with and without primary non-response after 12 or 24 weeks of ETV as first-line. Median time to VR was significantly shorter in primary responders than in non-responders at 24 weeks, but the cumulative probability of achieving a VR at 54 months was similar in the two groups (96 % vs. 100 %) [138]. Time to achieving VR and the cumulative probability of VR over time did not differ between primary responders and non-responders at 12 weeks. A more cautious approach is required when considering continuation of ETV therapy in patients with previous exposure to L-nucleosides due to the risk of resistance.

5.2 Partial Response

A partial response is defined by an initial response as measured at 12 or 24 weeks of therapy, followed by persistently detectable serum HBV DNA levels during continued therapy. Useful reference points include a HBV DNA >2000 IU/mL at 24 weeks or a detectable HBV DNA after 48 weeks of therapy [1]. Review is indicated, and management strategies take into account adherence and anticipated drug efficacy, together with the pre-treatment HBV DNA load, the kinetics of HBV DNA decay after starting therapy, and the likelihood of drug resistance emerging. Patients receiving LAM, LdT, or ADV should be switched to more potent therapy if the response is suboptimal at 24 weeks [1]. Even on potent NAs, some patients with high pre-treatment viral load may need longer to achieve complete HBV DNA suppression. ETV recipients with HBV DNA <1000 IU/mL after 48 weeks of therapy often achieve viral suppression by continuing ETV through at least 2 years total [139]. ETV recipients with higher HBV DNA levels at 48 weeks should be managed by switching to or adding TDF, whereas increasing ETV dose is not usually effective [140, 141]. Management strategies for slow responders to TDF monotherapy are less well defined. A subset of patients may benefit from a change of therapy or treatment intensification, particularly if the treatment history indicates partial resistance is possible, or where immunological function is impaired [101, 142].

5.3 Virological Rebound or Breakthrough

Virological rebound is defined by a confirmed serum HBV DNA increase of ≥1.0 $\log_{10}$ IU/mL relative to the lowest (nadir) level measured during continued treatment in a previous responder. Although the cut-off of 1 $\log_{10}$ is a useful indicator of a significant virological rebound, any HBV DNA increase above the assay quantification limit after achieving suppression should trigger a review. A confirmed viral load rebound typically signals lapses in adherence and drug resistance testing should be considered [5, 19, 21]. Low-level HBV DNA rebound may also reflect poor immunological function. Among HIV and HBV co-infected patients receiving long-term TDF-containing therapy, intermittent HBV DNA rebound is not uncommon; the risk is related to a history of profound immunocompromise as indicated by a low nadir CD4 cell count, but rebound does not appear to result in the selection of TDF resistance [100].

When considering the relevant HBV DNA load cut-off for defining virological breakthrough, it is also important to appreciate that the impaired fitness of emerging variants may initially limit the magnitude of HBV DNA increase. The addition of compensatory mutations that restore replicative capacity is typically signalled by an increase in serum HBV DNA load and serum aminotransferase levels, and potential for progression of liver disease [13, 38, 41].

6 Prevention and Management of HBV Drug Resistance

Development of HBV drug resistance can be largely avoided by starting therapy with drugs that have high potency and a high genetic barrier to resistance—typically TDF or ETV—and with regular monitoring of treatment responses and ongoing re-enforcement of adherence [1, 5, 19, 111, 143, 144]. In a meta-analysis, TDF and ETV as first-line therapy showed no difference in overall virological efficacy and safety over 48 weeks [145]. Long-term data also support the virological efficacy and safety of both treatment options [1, 71, 89, 91, 103, 116, 131]. TDF monotherapy appears to be sufficient even in patients with high baseline HBV DNA load [146], although the combination of TDF plus FTC appears to be more effective than TDF alone in immunotolerant subjects with normal transaminases and high HBV DNA levels (>1.7×10^7 IU/mL) [147]. Combination therapy with TDF and ETV also appears to have a marginal advantage over ETV monotherapy in patients with high HBV DNA levels (≥10^8 IU/mL) [148].

Avoiding the use of LAM, LdT, or ADV as single agents is generally recommended due to the risk of resistance [1, 111, 143]. LdT monotherapy may have a role in selected scenarios, including the prevention of mother-to-child transmission [1]. It has also been argued that LAM monotherapy can be safe and cost-effective as first-line treatment in selected patients with a favourable profile (i.e. low HBV DNA levels, absence of significant fibrosis or cirrhosis), or as a maintenance option after achieving serum HBV DNA suppression with more potent first-line treatment [149, 150]. Further studies are required to provide support for these strategies.

There remain a large number of patients that developed HBV drug resistance prior to TDF or ETV becoming available, and from a global perspective many remain at risk due to regionally limited availability of these more costly compounds [151, 152]. Monotherapy with LAM, ADV, or LdT was frequently prescribed in Europe between 2008 and 2010; among treated subjects undergoing resistance testing monotherapy was frequently associated with the detection of drug resistance, especially HBV RAMs of the rtM204 pathway [151]. The adverse consequences of developing NA resistance have been well documented. Patients treated with LAM or ADV who develop virological breakthrough and emergence of drug resistance frequently experience exacerbation and progression of liver disease [19, 106, 153–157]. In a study of cirrhotic patients treated with LAM, disease progression (as measured by a composite end-point of liver-related complications and mortality) occurred in 13% of patients who developed LAM RAMs compared with 5% of patients who did not develop resistance [158]. Furthermore, sequential rescue therapy increases the risk of developing hard-to-treat multidrug-resistant (MDR) HBV variants [5, 19, 21, 159].

In patients with LAM resistance, add-on therapy with ADV while continuing LAM is superior to switching to ADV alone, and switching to TDF monotherapy is superior to add-on therapy with LAM plus ADV [108] (Table 74.5). TDF alone is as effective for the treatment of patients with the rtM204I/V ± rtL180M as the combination of TDF plus FTC: in a randomized clinical trial, HBV DNA suppression rates over 96 weeks were 89% and 86% with TDF and TDF + FTC, respectively, with no treatment-emergent TDF resistance [105].

TDF is generally less effective for patients with ADV resistance [1, 160]. Patients with the double ADV mutant rtA181T/V + rtN236T may be especially at risk of poor responses [107]. Combination therapy with TDF plus FTC shows superior virological efficacy than TDF monotherapy in this setting [161].

ETV is an alternative treatment option in patients with ADV resistance, with 84% achieving virological suppression after 24 months, although responses are blunted by previous LAM exposure [79, 162]. Whilst ETV use after prolonged LAM therapy failure is not uncommon in clinical practice, ETV monotherapy in L-nucleoside-experienced subjects is associated with a risk of virological breakthrough and evolution of ETV resistance [151] and is not generally recommended [1].

Table 74.5 Treatment strategies for patients with suboptimal responses to HBV therapy

Drug	Strategies
LAM	TDF
	Add-on ADV[a]
ADV (nucleoside-naïve)	ETV
	TDF + FTC
	TDF + ETV
	TDF[b]
ADV (nucleoside-experienced)	TDF + ETV
LdT	TDF
	Add-on TDF
	TDF + FTC
	Add-on ADV[a]
ETV	TDF
	Add-on TDF
	TDF FTC
	Add-on ADV[a]
TDF	Add-on ETV
	ETV

[a]Add-on ADV strategies are generally to be reserved for circumstances when TDF is not available
[b]Reserved for subjects with low HBV DNA load. *LAM* Lamivudine, *TDF* Tenofovir, *FTC* Emtricitabine, *ADV* Adefovir, *ETV* Entecavir, *LdT* Telbuvidine

Combination therapy with ETV and TDF is usually effective in patients with MDR [163]. However combination therapy is not necessarily required in patients with more limited resistance. A multicentre trial investigated patients with ADF RAMs (rtA181V/T and/or rtN236T) randomized to receive TDF monotherapy or TDF plus ETV (1 mg/day) combination therapy [164]. At week 48, the two arms showed similar rates of HBV DNA suppression (62 % vs. 63.5 %; $p=0.88$) and a similar mean change in HBV DNA levels from baseline (−3.03 vs. −3.31 $\log_{10}$IU/mL; $p=0.38$). A second multicentre randomized trial investigated patients with ETV RAMs (rtM204V/I and at least one of rtT184A/C/F/G/I/L/S, rtS202G, or rtM250L/V) randomized to receive TDF monotherapy or TDF plus ETV (1 mg/day) combination therapy [165]. At week 48, the two arms showed similar rates of HBV DNA suppression (71 % vs. 73 %; $p>0.99$) and a similar mean change in HBV DNA levels from baseline (−3.66 vs. −3.74 $\log_{10}$IU/mL; $p=0.81$). In both trials, no patient developed additional RAMs, and safety profiles were comparable in the two groups.

Development of further treatment options is needed to manage certain subgroups of patients. These may include patients with LAM resistance that experience or are at increased risk of TDF toxicity, typically in the form of reduced renal function. These patients are often managed through TDF dose reductions, raising concerns about both ongoing risk of toxicity and sustained virological suppression. There is hope that the novel compound tenofovir alafenamide (TAF) will retain the high efficacy of TDF against HBV combined with an improved renal and bone safety profile. Besifovir is a new nucleotide analogue that has undergone testing in treatment-naïve patients with CHB. Over 96 weeks, besifovir caused over a 5 $\log_{10}$IU/mL decline in HBV DNA levels and suppression rates of around 80 % and similar responses were seen in the comparator arm receiving ETV [166]. There was a low overall incidence of virological breakthrough and no development of drug resistance in both arms. CAdAs (4′-C-cyano-2-amino-2′-deoxyadenosine) are novel compounds that offer hope for the management of drug-resistant HBV [167]. Finally, new treatment strategies are also required to improve long-term control of CHB and allow discontinuation of NA therapy after induction [5–7].

7 The Challenge of HIV-HBV Infection in Resource-Limited Settings

In sub-Saharan Africa (SSA), chronic infection with HBV is an important public health issue characterized by high prevalence, frequent co-infection with HIV, and suboptimally applied ascertainment and management strategies [152, 168]. Among people living with HIV, between 6 and 25 % are co-infected with HBV, and co-infection accelerates fibrosis and increases the risk of liver-related morbidity and mortality. In part as a consequence of reduced HIV-related mortality, cirrhosis and hepatocellular carcinoma (HCC) are increasing in the region. For many years, regimens for first-line ART in SSA have been "HBV-blind" and employed LAM plus zidovudine or stavudine in combination with efavirenz or nevirapine. This approach has led to large numbers of HIV and HBV co-infected patients receiving LAM as the sole HBV-active agent across much of SSA, with the resulting associated risk of drug resistance and liver disease progression. In a typical cohort of HIV and HBV co-infected patients in Ghana, after nearly 4 years of standard LAM-containing ART, over half of patients had detectable HBV DNA, one-third had DNA levels >2000 IU/mL, one-third had HBV LAM resistance by Sanger sequencing, and one in eight had advanced liver fibrosis as determined by transient elastography [106]. In this cohort, the introduction of TDF led to substantial improvements in HBV DNA suppression and promising evidence of reversal of liver fibrosis.

Whilst TDF is now recommended for first-line antiretroviral therapy in all patients with HIV in SSA, availability remains far from universal and much remains to be done to improve the diagnosis and management of CHB in populations with and without HIV. The World Health Organisation [169] has released guidelines for CHB in resource-limited settings that aim to promote the use of simple, non-invasive diagnostic tests to assess the stage of liver disease and eligibility for treatment; prioritize treatment for those with most

advanced liver disease and at greatest risk of mortality; and preferential use of NAs with a high barrier to drug resistance (TDF and ETV). These recommendations provide opportunities to improve the clinical outcomes of persons living with CHB in these settings and reduce HBV incidence and transmission. Implementation remains challenging.

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Part XI

Parasitic Drug Resistance: Clinical

Antimalarial Drug Resistance: Clinical Perspectives

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Bruno Pradines

1 Introduction

Despite efforts to discover new antiplasmodial drugs and to accomplish effective implementation of therapeutic combinations for malaria treatment by health systems, *P. falciparum* fits permanently and develops resistance, including to artemisinin-based combination therapy (ACT). Some mutations allow the parasite to survive in the presence of antimalarial drugs and to become resistant. Thus, other factors favoring the emergence of resistance include the following: (1) misuse of antimalarial drugs by infected people (abusive self-medication, poor compliance) leading to incomplete treatment; (2) unavailability of effective drugs or inadequate deployment of drugs as monotherapies; (3) sub-dosed or counterfeit consumption that allows parasites to survive at suboptimal concentrations of antimalarial drugs and to be selected for their ability to resist; (4) the pharmacokinetics and pharmacodynamics of the antimalarial drugs; and (5) the immunity profile of the community and the individual. Updated epidemiologic data on resistance and the molecular mechanisms involved are presented for each antimalarial drug used. The strategies for delaying the emergence and spread are also presented. The roles of heterogeneous biting and transmission in the establishment and spread of resistance in a population are very important. The role of asymptomatic *P. falciparum* parasites is also important in the evolution of antimalarial drug resistance. Several strategies are considered for controlling the emergence and spread of resistance to antimalarial drugs, such as interruption of asymptomatic carriage with mass drug administration, improvement of surveillance, development of new diagnostics and vaccines, and discovery of new drugs.

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2 Malaria Epidemiology

Malaria remains the most important human parasitic disease. It was transmitted in 103 countries inhabited by approximately 3.4 billion people in 2012 [1]. Of this total, 2.2 billion were at low risk (<1 reported case per 1000 population), of whom 94 % were living in geographic regions other than Africa. The 1.2 billion people at high risk (>1 case per 1000 population) were living mostly in Africa (47 %) and Southeast Asia (37 %). In 2012, malaria caused an estimated 207 million cases and 627,000 deaths, meaning that 1300 young lives are lost to malaria every day. More than 85 % of malaria cases and 90 % of malaria deaths occur in sub-Saharan Africa, mainly in children younger than 5 years old (77 %). Malaria is a protozoan disease transmitted by *Anopheles* mosquitoes. Five species of the genus *Plasmodium* cause all malaria infections (*Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and the monkey malaria *P. knowlesi*). Most cases are caused by either *P. falciparum* or *P. vivax*. Almost all fatal cases are caused by *P. falciparum*.

Between 2000 and 2012, estimated malaria mortality rates decreased by 42 % worldwide and by 49 % in Africa; they are estimated to have decreased by 48 % in children younger than 5 years of age globally and by 54 % in Africa. The annual number of reported malaria cases decreased from 1.5 million in 2000 to 627,000 in 2012. Over the same period, malaria-eliminating countries reduced total malaria by more 70 %, with 17 countries reporting a greater than 90 % reduction.

These successes have been driven by several factors, including increased funding, effective vector control, strengthening of health systems, improved case reporting and surveillance, and improved case management with more effective treatment regimens.

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3 Antimalarial Drug Resistance

Despite efforts to discover new antiplasmodial drugs and to achieve effective implementation of therapeutic combinations for malaria treatment by health systems, *P. falciparum* fits permanently and develops resistance, including against artemisinin-based combination therapy (ACT) (Fig. 75.1).

This resistance can be explained by the large genetic diversity of *P. falciparum* due to a high rate of mutations in its genome and the very large parasite biomass carried by infected people. Even if mutations capable of conferring resistance to a new drug are extremely rare and unlikely, the large numbers of parasites infecting humans lead to emergence of these mutations and their selection by drug pressure. Mistakes in DNA replication introduce random mutations into the genome and allow for the process of evolution. These mutations are the cause of the high genetic variability of *P. falciparum*, and when they are not lethal to the parasite, they can lead to a survival benefit by permitting, for example, escape from the host immune system, resistance to toxic molecules or more rapid multiplication than other clones.

Some mutations allow the parasite to survive in the presence of antimalarial drugs and to become resistant. The mutation is then transmitted to descendants, generating a drug-resistant population. The mutation frequency and the speed of the resistance development depend on the characteristics of the drug, the epidemiological context (intensity of transmission), and the manner in which the drug is used. However, the acquired resistance phenotype after mutation is not always an advantage in the absence of drug pressure. These mutations can have a biological fitness cost. When chloroquine was removed from areas where parasites were chloroquine-resistant, susceptible strains were favored, compared with resistant strains, and replaced many of them [2]. Although susceptible populations reappear at the expense of resistant strains in the absence of selection by chloroquine, a new selection of the resistant population should be expected if monotherapy or chloroquine-based combination therapy is used again [3].

Thus, other factors favoring the emergence of resistance are: (1) misuse of antimalarial drugs by infected people (abusive self-medication, poor compliance) leading to incomplete treatment; (2) unavailability of effective drugs or inadequate deployment of drugs as monotherapies; (3) sub-dosed or counterfeit consumption that allows parasites to survive at suboptimal concentrations of antimalarial drugs and to be selected for their ability to resist; (4) the pharmacokinetics and pharmacodynamics of antimalarial drugs; and (5) the immunity profile of the community and the individual. Thus, resistance has emerged against all antimalarial drugs in most endemic areas. This resistance applies both to old drugs that were used as monotherapy for a long time (chloroquine, amodiaquine, sulfadoxine–pyrimethamine, quinine, mefloquine) and to the new molecules used in combination therapy (atovaquone, lumefantrine).

3.1 Chloroquine Resistance

3.1.1 Chloroquine Resistance and *P. falciparum*

Chloroquine is a 4-amino-quinoline that was synthesized after the Second World War. Effective, rapid-acting and inexpensive, chloroquine was a remarkable antimalarial drug. However, in 1957, the first cases of chloroquine resistance emerged in Asia and South America. This resistance spread rapidly across the two continents and then across Africa, and chloroquine resistance now affects all malaria endemic areas. For more than 30 years, chloroquine was the first-line drug for preventing and treating falciparum malaria.

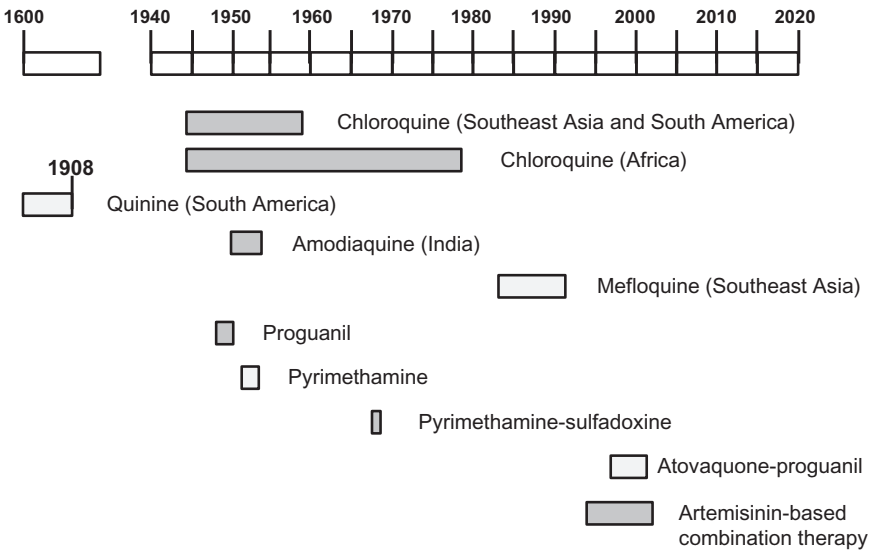

Fig. 75.1 Antimalarial drugs introduction and emergence of resistance in *Plasmodium falciparum*

Chloroquine resistance has been associated with a significant increase in malaria mortality [4, 5]. Chloroquine remains the first-line treatment for vivax malaria.

Characterization of the molecular markers of drug resistance is an important aspect of understanding resistance to antimalarial treatment. Once the genetic changes associated with resistance are identified, drug resistance can be confirmed using molecular techniques. The *pfcrt* gene was first identified in 2000 [6]. To date, at least 20 point mutations have been described, but only one is the reference mutation (K76T), which is a marker of the chloroquine-resistant phenotype [6–9]. This mutation is often associated with other mutations in the *pfcrt* gene, the roles of which have not yet been defined (Cys72Ser, Met74Ile, Asn75Glu, Ala220Ser, Gln271Glu, Asn326Ser, Ile356Thr, Arg371Ile). The odds ratio (OR) for chloroquine failure associated with the K76T mutation was 2.1 (95 % confidence interval [CI]: 1.5–3.0, meta-analysis of 13 studies) over a 14-day follow-up and 7.2 (95 % CI: 4.5–11.5, meta-analysis of 12 studies) over a 28-day follow-up [10]. However, the existence of chloroquine-susceptible strains associated with the K76T mutation suggests that other genes could be involved in resistance to chloroquine. The 76T mutation is necessary, but not sufficient, for influencing chloroquine susceptibility [11].

Polymorphisms within the *pfmdr1* (*Plasmodium falciparum* multidrug resistance 1) gene, which encodes a transmembrane homolog of the PGH1 protein, have been implicated. Field work has shown that the predictive value of chloroquine resistance and of point mutations in the *pfmdr1* sequence resulting in amino acid changes varies depending on the geographic area [12, 13]. Five point mutations have been described: N86Y, Y184F, S1034C, N1042D, and D1246Y. Point mutations, most notably 86Y, have been associated with a decrease in chloroquine susceptibility [14]. However, in some epidemiological studies, the number of chloroquine-susceptible samples has been too limited to provide a statistically meaningful analysis [13, 15]. Using precautions, no relationship or only weak relationships have been established between chloroquine resistance and mutations in *pfmdr1* in *P. falciparum* [12]. However, the risk of therapeutic failure with chloroquine is greater for patients harboring the 86Y mutation, with ORs of 2.2 (95 % CI: 1.6–3.1) over a 14-day follow-up and 1.8 (95 % CI: 1.3–2.4) over a 28-day follow-up [10].

Since the withdrawal of chloroquine, there have been signs of regression of chloroquine resistance in some areas. In China and Vietnam, significant regression of chloroquine resistance has been documented in in vitro and molecular marker studies, while treatment failure rates remain high [16–18]. In Kenya and Malawi, where there is a high level of transmission and almost exclusively infection with *P. falciparum*, there have been signs of a reduction in the prevalence of chloroquine-resistant parasites. Chloroquine was withdrawn from the market in Malawi in 1993 and in Kenya in 1999, when the treatment policies in both countries changed to ACTs. In Malawi, fewer than 10 years after its withdrawal, a re-emergence of chloroquine-susceptible parasites was observed in molecular analyses [19, 20]. Chloroquine was subsequently shown to have 99 % curative efficacy in children with uncomplicated malaria [21]. The prevalence of mutation in the *pfcrt* gene at codon 76 decreased considerably, as did evidence of resistance in vitro [22, 23]. In Kenya, a reduction in resistance to chloroquine was also observed in vitro and with molecular markers, although at a slower rate [24]. It is noteworthy that the prevalence of the Asn86Tyr mutation in the *pfmdr1* gene did not decrease at the same rate as the mutation in the *pfcrt* gene. Decreases in chloroquine resistance have also been observed in vitro and with molecular markers in isolates from Ethiopia, Côte d'Ivoire, and Cameroon [25, 26]. In Senegal, such a decrease was also observed in vitro and with molecular markers in isolates from Senegalese inhabitants in 2009–2011 [27–29], as well as in isolates from travelers returning from Senegal between 2000 and 2011 [25]. However, the chloroquine resistance observed in vitro and with molecular markers is now rising again in Senegal [30–32].

While these results are interesting, caution is nevertheless required. The disappearance of parasites carrying the mutant *pfcrt* gene might be linked to the expansion of wild-type parasites, still present in the subpopulation, replacing the mutant parasites, rather than a reversal of the Lys76Thr mutation [33–35]. Widespread reintroduction of chloroquine is not recommended because it is still too early to predict how long might be needed for chloroquine resistance to reappear or to be reintroduced from neighboring regions. In addition, the rapid dissemination of chloroquine resistance in Dielmo (Senegal), despite strictly controlled antimalarial drug use, argues against the re-introduction of chloroquine, in places where the resistance allele has decreased to very low levels following the discontinuation of chloroquine treatment [3]. Despite the reacquisition of chloroquine susceptibility, any reintroduction would likely result in the rapid re-emergence of resistant strains.

3.1.2 Chloroquine Resistance and *P. vivax*

Chloroquine is the first-line treatment for *P. vivax* in most endemic countries. The clinical efficacy is more difficult to determine in the treatment of *P. vivax* than in *P. falciparum* because recurrent infections can arise from recrudescence, reinfection, or relapses (arising from the dormant liver stages) [36]. Chloroquine-resistant *P. vivax* was first reported in 1989, almost 30 years after chloroquine-resistant *P. falciparum* was first noted, in Indonesia and Papua New Guinea [37, 38]. Papua New Guinea is the epicenter of *P. vivax* chloroquine resistance, and studies conducted there have consistently shown high-grade resistance with early recurrent

parasitemia [39–41]. Treatment failure on or before Day 28 or prophylactic failure have been observed in Afghanistan, Brazil, Cambodia, Colombia, Guyana, Ethiopia, India, Indonesia, Madagascar, Malaysia (Borneo), Myanmar, Pakistan, Papua New Guinea, Peru, the Republic of Korea, the Solomon Islands, Sri Lanka, Thailand, Turkey, Vanuatu, and Vietnam [42]. Several studies in India and Indonesia have shown early treatment failure with rates greater than 10 % and recurrence at Day 28 in between 20 and 100 % of cases [39, 40, 43, 44]. However, antimalarial treatment was not supervised, and drug levels were not measured in several of these studies. True cases of chloroquine resistance (with whole blood concentrations of chloroquine plus desethylchloroquine >100 ng/mL on the day of failure) has been confirmed in Indonesia, Myanmar (Burma), Papua New Guinea, India, and South Korea [45]. The first published reports of chloroquine-resistant vivax parasites in Latin America were from Colombia [46] and Brazil but without blood chloroquine concentration measurements [47]. Consequently, it was only in 1996 that chloroquine resistance was formally documented in a *P. vivax* strain from Guyana [48]. Malaria resistance was then observed in cases from Colombia [49] and was confirmed with chloroquine measurement in two cases from Peru [50]. More recently, two additional reports of in vivo chloroquine resistance in *P. vivax*, with 10.1 and 5.2 % recrudescence despite adequate levels of chloroquine, came from Manaus, a major Brazilian port city in the Amazon Basin [51, 52]. *P. vivax* resistance has also emerged in Ethiopia, with 13 % recurrent parasitemia at Day 28 without chloroquine concentration measurements [53] and between 2.8 and 5.2 % recurrent parasitemia despite adequate levels of chloroquine [54–57].

Parasites carrying the Tyr976Phe mutation of *pvmdr1* showed reduced susceptibility to chloroquine in vitro, compared to wild-type parasites from Indonesia and Thailand [58, 59]. However, this marker was not found to be associated with confirmed clinical chloroquine resistance in Madagascar and Brazil [52, 60, 61].

3.2 Quinine Resistance

Since the adoption of ACTs as the first-line treatment for uncomplicated cases of malaria, quinine has been more commonly used as second-line treatment, and it remains the drug of choice for pregnant women. According to the 2010 *Guidelines for the treatment of malaria*, for the treatment of uncomplicated malaria, oral treatment with quinine should be combined with an antibiotic, such as doxycycline, tetracycline, or clindamycin [62]. Although the World Health Organization (WHO) recommends replacing injectable quinine with injectable artesunate due to the improved efficacy of the latter and higher tolerance of the drug in both adults and children [63, 64], quinine remains a first-line treatment for severe malaria, particularly in Africa. For severe malaria, injectable quinine should be followed by either oral quinine with an antibiotic or artesunate with clindamycin or doxycycline or by a full course of an ACT, once the patient can tolerate oral therapy.

It is difficult to demonstrate resistance to quinine. Despite the efficacy of quinine against chloroquine-resistant *Plasmodium falciparum* isolates, reports of quinine resistance have been increasing. In the 1980s, the frequency of clinical failures increased in Thailand [65–67], Brazil [68], and east Africa [69]. However, the first cases of quinine resistance were described in Brazil in 1908–1910 [70, 71]. Well-documented and confirmed cases have been rare. A few cases were described in French Guiana in 2004 and 2010 [72, 73], in Senegal in 2007 [74], and in Mozambique in 2014 [75].

In the treatment and follow-up of patients, it is important to bear in mind individual differences in the clinical response to quinine. For example, a temporary increase in parasitemia can occur shortly after the first dose, suggesting early treatment failure, although this increase does not tend to affect the treatment outcome [76]. In light of its relatively slow action, as seen by the 48-h parasite reduction rate, the length of treatment should be adjusted to the parasite load [77]. In the event of hyperparasitemia, it might be necessary to extend treatment beyond 7 days or to combine quinine with another antimalarial agent [78].

The in vitro susceptibility of individual *P. falciparum* isolates to quinine has varied widely. Many studies have reported wide ranges of susceptibility to quinine: 25–1253 nM in Comoros [79], 36–1097 nM in the Republic of Congo [80], 5–1291 nM in Senegal [81, 82], and 15–761 nM in Uganda [83]. However, the wide range of quinine susceptibility and recent evidence for quinine treatment failure observed across Africa suggest that the evolution of parasites with reduced susceptibility could contribute to decreased quinine efficacy.

Chromosome 13 in *P. falciparum* contains a candidate gene (*pfnhe-1*), which encodes a putative Na+/H+ exchanger [84]. *Pfnhe-1*ms4760 is highly diverse among parasite isolates. It appears that polymorphisms are more important in Africa and the Indian Ocean region than in India or Asia: in Senegal, 47 different profiles were observed [85]; in the Republic of Congo, 27 different profiles [80]; in Uganda, 40 different profiles [83]; and in the Indian Ocean region, 29 different profiles [86]; whereas in Vietnam, only ten different profiles were observed [87]; in the China–Myanmar border area, ten different profiles [88]; and in India, 16 different profiles [89]. This situation likely reflects the level of transmission in these areas and the level of quinine selection pressure. A repeat polymorphism in *pfnhe-1* microsatellite ms4760 was significantly associated with a poor quinine response, but additional field studies are needed to validate this marker. Conflicting data have been reported regarding

pfnhe-1 polymorphisms. However, investigations of the microsatellite ms4760 polymorphisms in culture-adapted isolates from around the world have shown an association with the quinine susceptibility phenotype [90]. A repetition of the amino acid motif DNNND was associated with decreased susceptibility to quinine, based on the clinical failure of quinine in a traveler from Senegal [74], as well as data from fresh isolates from Vietnam [87] and from culture-adapted isolates from the China–Myanmar border area [88], Asia, South America, and Africa [91]. In cultured-adapted isolates from Kenya [92] and in freshly obtained isolates from Uganda [83], duplication of the DNNND motif was associated with reduced susceptibility to quinine, compared to isolates with one or more than two repeats. Moreover, an increased number of DDNHNDNHNND motifs were associated with increased susceptibility to quinine [84, 87, 88, 90, 91]. Paradoxically, increased numbers of this latter amino acid motif were associated with reduced susceptibility to quinine, based on freshly obtained isolates from Madagascar and 13 other African countries [93]. Moreover, these samples did not exhibit any associations between the number of DNNND repeats and quinine susceptibility. Furthermore, there were no associations between the numbers of DNNND and DDNHNDNHNND repeats and quinine susceptibility, based on freshly obtained isolates from the Republic of Congo [80], Thailand [94], Asia, South America, and Africa [91].

Studies with clones and field isolates have indicated that the *pfmdr1* Asn86Tyr, Ser1034Cys, Asn1042Asp, and Asp1246Tyr mutations might be associated with decreased susceptibility to quinine [95, 96]. Like the response to chloroquine, that to quinine is influenced by mutations in several transporter genes (*pfcrt*, *pfmdr1*, and *pfnhe-1*) [97].

3.3 Amodiaquine Resistance

In the late 1940s and early 1950s, clinical trials for the new and promising 4-aminoquinoline amodiaquine were reported from India, Brazil, the Philippines, Panama, Ecuador, Taiwan, and regions of Africa. Cases of drug resistance were not observed immediately; however, a report of amodiaquine failure was published in 1954 from India [98] and few years later from Colombia [99]. Despite in vivo cross-resistance between chloroquine and amodiaquine [99, 100], amodiaquine is more effective than chloroquine in areas with identified chloroquine resistance [101]. The rates of parasitological or clinical failures with amodiaquine have been lower than those with chloroquine, in Gambia [102], Senegal, Cameroon, Gabon, and Congo [103, 104].

Amodiaquine was therefore chosen by several countries as the first-line drug in combination with artesunate. An amodiaquine failure rate >20 % has been observed in 5 African countries, some of which are currently using artesunate–amodiaquine as the first-line treatment (Burkina Faso, Cameroon, Gabon, Liberia, the Sudan) [105–109]. Parasite strains that are highly resistant to amodiaquine have been reported in Tanzania, and these strains could further compromise the use of artesunate–amodiaquine in Africa [110].

Some chloroquine-resistant isolates have shown cross-resistance with amodiaquine, both in vivo and in vitro. *Pfcrt* and *pfmdr1* alleles interact to yield different levels of resistance to chloroquine and amodiaquine. The *pfcrt* mutations at codons 72–76 observed in South America have been associated with high levels of amodiaquine resistance, whereas *pfcrt* mutations in Southeast Asia and Africa have been linked to greater resistance to chloroquine and moderate resistance to amodiaquine. This difference might be due to the extent of the previous use of amodiaquine in different regions. Amodiaquine resistance might also be modulated by the *pfmdr1* mutations Asn86Tyr and Asn1042Asp [110–112]. However, the role of *pfmdr1* in amodiaquine resistance remains debated. The 86Y mutation was significantly associated with increased in vitro susceptibility to active metabolites of amodiaquine in Senegal [113], while mutant *pfmdr1* 86Y allele showed increased and reduced susceptibility in isolates from Nigeria [114] or no change in isolates from Benin [115].

However, the *Pfmdr1* 86Y mutation has been shown to be associated with treatment failure after monotherapy with amodiaquine [116, 117] or after combination therapy with artesunate–amodiaquine [118]. In a meta-analysis, the *Pfmdr1* 86Y mutation was found to be associated with amodiaquine failure, with an OR of 5.4 [10]. The *pfmdr1* 1246Y mutation has also been found to be associated with in vitro resistance to amodiaquine [119] and with recrudescent infection after treatment with amodiaquine or amodiaquine–artesunate [117, 118].

3.4 Mefloquine Resistance

Mefloquine, an arylaminoalcohol, was discovered at the end of 1970s, and it is still recommended for malaria prophylaxis in areas with multidrug resistance. Mefloquine was widely used to treat uncomplicated malaria as a first line in areas with *P. falciparum* multidrug resistance, such as Thailand [120]. Mefloquine resistance appeared at the Cambodia–Thailand border only a few years after its introduction [121]. There are several probable reasons for this rapid onset. Preexisting strains in the region had markedly reduced susceptibility to quinine. Further, the long half-life of mefloquine might have allowed for exposure to subtherapeutic concentrations. Common use of the low-dose, single-dose regimen (15 mg/kg body weight) in this region might also have contributed to the increase in resistance. The higher dose of 25 mg/kg body weight that is usually recommended

is known to have several adverse effects, particularly vomiting, which can lead to lower blood concentrations and subsequent treatment failure [122].

The *pfmdr1* gene amplification that has been implicated in resistance to mefloquine is acquired relatively rapidly at the Cambodia–Thailand border. Studies conducted in the Greater Mekong subregion (Cambodia and Thailand) showed that increases in copy numbers of this gene were responsible for resistance to mefloquine and to increased risks of treatment failure with artesunate–mefloquine [123]. *Pfmdr1* amplification and deamplification are relatively frequent events related to the rapid evolution of mefloquine resistance when the drug is used as a monotherapy. In vitro susceptibility to mefloquine increased when the *pfmdr1* copy numbers were reduced or when the parasites carried *pfmdr1* mutations [124, 125]. In Southeast Asia, the presence of the Asn86Tyr mutation is a negative marker for gene amplification. It has been shown through heterologous expression that *pfmdr1* mutations at codons 1034 and 1042 abolish or reduce the level of resistance to mefloquine in vitro [126]. Moreover, transfection with a wild-type *pfmdr1* allele at codons 1034, 1042, and 1246 conferred mefloquine resistance to susceptible parasites [95]. However, mutations at *pfmdr1* codons 1034, 1042, and 1246 in *P. falciparum* isolates were not sufficient to explain the variations in mefloquine susceptibility [113, 127]. The significance of the 184F mutation remains less well understood. Indeed, no clear association between the 184F mutation and mefloquine failure has been established. A study showed that Asian isolates with a single 184F mutation exhibited increased resistance to mefloquine [128]. A study from Cambodia demonstrated that isolates with a single 184F mutation had significantly increased IC_{50} values for mefloquine [129]. The *pfmdr1* 86 plus 184 haplotype showed significantly increased in vitro susceptibility to mefloquine in parasites with 86Y plus Y184 from Senegal and Benin [113, 115]. Analyses of *P. falciparum* isolates have shown an association between a mutation at codon 86 and an increase in susceptibility to mefloquine [113, 130–132].

Mefloquine resistance continues to be a concern in the Greater Mekong subregion, particularly in Thailand and Cambodia, where artesunate–mefloquine is still used as a first-line treatment. The national malaria control program in Thailand detected a gradual decline in the efficacy of mefloquine at its sentinel sites, although monitoring of mefloquine monotherapy was last conducted in 2004. Even when the dose was increased from 15 to 25 mg/kg body weight, efficacy increased only temporarily [133]. In Cambodia, after implementation of rapid diagnostic tests and the replacement of artesunate–mefloquine by dihydroartemisinin–piperaquine in Pailin Province, a reduction in mefloquine resistance was detected using molecular markers. The high "fitness cost" linked to mefloquine resistance and the removal of mefloquine pressure led to deamplification of *pfmdr1* copy numbers between 2005 and 2007, resulting in a decrease in the treatment failure rate with artesunate–mefloquine (≤5 %) in 2007–2008 [134–136]. However, a 42-day WHO therapeutic efficacy study, conducted in 2010 in southwest Cambodia, showed 11.1 % late treatment failures with artesunate–mefloquine treatment [137]. In studies from 2008 to 2010, the incidence of Day 3 positive parasitemia among falciparum malaria patients associated with artesunate–mefloquine treatment increased along the Thailand–Myanmar border to surpass 10 % [138]. Approximately 14 % of patients undergoing artesunate–mefloquine treatment remained parasite-positive on Day 3 during 2009–2011 along the Thai–Cambodian border [139].

In Myanmar and Vietnam, the treatment failure rate was as high as 40 % in the late 1990s and early 2000s; however, a low dose of 15 mg/kg body weight was used [140–142]. No recent studies have been reported in which a dose of 25 mg/kg body weight was used.

In Africa, in vitro studies conducted prior to the introduction of mefloquine showed the presence of parasites with reduced susceptibility to mefloquine that were still susceptible to chloroquine [143]. The validation of molecular markers for chloroquine and mefloquine resistance now allows for better understanding of these results. Recently, isolates with increased *pfmdr1* copy numbers have been detected in West Africa and have been associated with mefloquine treatment failure in travelers [144, 145]. However, amplification of *pfmdr1* in Africa has only rarely occurred. Very few isolates with ≥2 copies of *pfmdr1* were identified in Africa in 1993–2014: three in Côte d'Ivoire [145, 146], one in Burkina Faso [145], one in Togo [145], three in eastern Sudan [147], ten in Kenya [148, 149], and ten Senegal [27, 150]. Another isolate was obtained in a patient from Benin who did not respond clinically to mefloquine treatment [144]. In a multicentric study to analyze the polymorphisms in *pfmdr1* after artemether–lumefantrine and artesunate–amodiaquine treatments, amplification of *pfmdr1* was observed in only 2.6 % of isolates from Africa versus 50 % of isolates from Asia [151]. *Pfmdr1* amplification has not been found in many studies in samples collected either before or after treatment for recurring *P. falciparum* infection in Africa. However, the percentage of isolates with increased *pfmdr1* copy numbers increased from 4 % in 2003 to 18 % in 2010 in Ghana [152]. Efficacy studies conducted in Benin (cure rate of 97.5 %) and Nigeria in 2008–2009 (cure rate of 94 %) showed low treatment failure rates [153, 154]. In a multicentric study in Benin, Gabon, Mozambique, and Tanzania of the use of mefloquine as an intermittent preventive treatment in pregnancy (two doses of mefloquine 15 mg/kg), mefloquine reduced the incidence of clinical malaria [155]. In Tanzania between 2004 and 2008, a dose of 125 mg of mefloquine as an intermittent preventive treatment in infancy only protected 38.1 % of infants against malaria [156]. Therapeutic

efficacy in Senegal with mefloquine–artesunate was 96.2 % in children in 2008 and 98.5 % in 2010 [157, 158], compared to 96 % in Mali in 2004–2005 [159]. Selection of the *pfmdr1* N86 mutation was observed in 2009 in Gabon after the introduction of mefloquine–artesunate [160].

Some cases of malaria prophylaxis failures in travelers who correctly took the drug have been observed in Mozambique and Senegal [161, 162].

In South America, mefloquine resistance has remained low, although few therapeutic efficacy studies have been performed. The efficacy of mefloquine at 15 mg/kg was 100 % in 1999–2000 in Peru and in 2001 in Bolivia [127, 163, 164]. The efficacy of mefloquine–artesunate was 98.9 % in the Peruvian Amazon in 2005–2006 [165]. The prevalence of an increased *pfmdr1* copy number was 12 % among 93 samples from Venezuela in 2003 and 2004 [166]. In French Guiana, amplification of the *pfmdr1* gene was associated with in vitro reduced susceptibility to mefloquine, and it was observed at a high rate (mean of 40 %) [167]. However, the proportion of isolates with multiple copies of *pfmdr1* decreased from 2005 to 2008, when in vitro resistance declined, corresponding to the progressive replacement of mefloquine with artemether–lumefantrine in French Guiana [167]. In Suriname, no significant changes in *pfmdr1* copy numbers were observed between 2005 and 2011: 12.5 %, 8.7 %, and 13 % of isolates carried multiple copies of *pfmdr1* in 2005, in 2009, and in 2009–2011, respectively [168, 169].

3.5 Artemisinin and Artemisinin-Based Combination Therapies (ACT)

3.5.1 Artemisinin and Artemisinin Derivatives

In contrast with most previous antimalarial treatments, such as chloroquine and sulfadoxine–pyrimethamine, which are eliminated slowly, artemisinin and its derivatives are eliminated rapidly, and they target all of the blood stages of the malaria life cycle, including early ring forms. This targeting is particularly beneficial for the treatment of severe malaria, when rapid elimination of parasites is critical for patient recovery.

In the 1980s, treatment failures with artemisinin were already reported in China after a 3-day treatment (48 % of recrudescence) [170]. When treatment was extended to 5 and 7 days, the recrudescence rates decreased to 10 % and 2 %, respectively [170]. These data could be explained by the short half-life of artemisinin and its derivatives because not all parasites would necessarily be eliminated after the initial rapid effect of a short treatment with oral artemisinin-based monotherapy. Therefore, monotherapy was not effective unless it was administered over an extended period. In the same manner that parasites that are consistently exposed to a suboptimal dose of treatment develop resistance, incomplete or short treatment with oral artemisinin-based monotherapy could also facilitate the development of resistance, although the short half-lives of these drugs reduce the time window during which resistant parasites can be selected.

In 2007–2008 in Pailin, western Cambodia, where artemisinins have been used for more than 30 years, treatment failure was confirmed in 30 % of patients receiving artesunate monotherapy (2 mg/kg/day for 7 days) and in 5 % receiving artesunate–mefloquine therapy (artesunate at 4 mg/kg/day for 3 days, followed by mefloquine at two doses totaling 25 mg/kg) [135]. This resistance was characterized by slow parasite clearance in vivo. These markedly different parasitological responses were not explained by in vitro *P. falciparum* susceptibility, obtained by a standard 48 h in vitro test. In 2009–2010, in Pursat in western Cambodia, 64 % of the patients treated with artesunate monotherapy (4 mg/kg) had parasite clearance half-lives longer than the geometric mean of the patients in Pailin [171]. Similar parasite clearance half-lives for artemether were found in patients with severe malaria, compared with artesunate in patients with uncomplicated malaria, suggesting that artemether treatment for severe malaria did not accelerate parasite clearance rates, compared with artesunate treatment for uncomplicated malaria [172]. In 2010–2011 in Vietnam, the efficacy of artesunate monotherapy (2 mg/kg/day for 3 days) was 94 %, and 27 % of the patients had a parasite clearance time >72 h [173]. In 2010, artemisinin resistance emerged on the western border of Thailand, with slow parasite clearance half-lives [174]. An open trial of artesunate at a daily dose of either 2 or 4 mg/kg/day for 3 days, followed by a standard 3-day course of ACT, showed that slowly clearing infections (parasite clearance half-live >5 h) were detected throughout mainland Southeast Asia, from southern Vietnam to central Myanmar [175]. No evidence of delayed parasite clearance to artemisinin was shown in Bangladesh or in Africa (Mali, Nigeria, Democratic Republic of Congo, or Kenya) [175, 176]. A method for measuring the parasite clearance in patients was standardized to compare the data and to track artemisinin resistance [177].

P. falciparum isolates with reduced in vitro susceptibility to artemisinin and derivatives were found by 42 h-standard in vitro testing from 2001 in Asia (Thailand, Cambodia, Laos), Africa (Senegal), and South America (French Guiana) [178–180]. However, these in vitro resistance data failed to be associated with in vivo resistance. Artemisinin acts on *P. falciparum* ring stages, which can develop tolerance to artemisinin by a quiescence mechanism [181]. A new in vitro test, the ring stage survival assay, was developed to measure in vitro resistance to artemisinin derivatives manifested by an increase in the ring-stage survival rate after contact with artemisinin [182, 183]. This in vitro resistance was associated with slow in vivo parasite clearance (parasite clearance half-life >5 h) [184–186].

In falciparum malaria, artemisinins are believed to inhibit the sarco-endoplasmic reticulum calcium-ATPase (SERCA)-type, PfATPase 6 protein [187]; however, this protein is unlikely to be the only target [188]. One molecular marker for artemether resistance has been proposed, *pfATPase6* Ser769Asn, but this suggestion was based exclusively on findings from in vitro tests [180], and field studies have not confirmed this hypothesis [189, 190]. In vivo delayed parasite clearance was not associated with *pfATPase6* polymorphisms in artemisinin resistance at the Thai–Cambodian border [191]. Amplification of the *pfmdr1* gene has been associated with significant reductions in susceptibility to artemisinin and derivatives in vitro [123, 124, 150, 192] but not with parasite clearance half-life [193]. So far, none of the known markers, particularly *pfmdr1* copy numbers or mutations, *pfATPase6*, the 6-kb mitochondrial genome (including cytochrome *b*, *COXI*, and *COXIII*) or *pfubp-1* encoding a deubiquitinating enzyme, has been correlated with the artemisinin resistance phenotype observed at the Cambodia–Thailand border [136]. In 2010, a *P. falciparum* strain susceptible to artemisinin became resistant after 3 years of artemisinin pressure [194]. Whole-genome sequences were obtained for both strains. The data indicated that the M476I mutation in the propeller domain of the *Kelch 13* (K13) gene (*PF3D71343700*) was associated with in vitro resistance to artemisinin of this strain [184]. Then, several mutations in K13 (C580Y, R539T, Y493H, and I543T) were associated with in vitro resistance of Cambodian isolates, assessed by ring stage survival assay and delayed parasite clearance half-lives (>5 h) [184]. These mutations were confirmed to be associated with in vitro resistance to artemisinin and with delayed clearance after artemisinin treatment in Southeast Asia [175, 186, 194]. In addition, recent reports have supported a causal role for K13-propeller mutations in conferring resistance to artemisinin and particularly the roles of the C580Y, Y493H, R539T, and I543T mutations due to genome manipulation [195, 196]. These mutations were introduced into the genome of a *P. falciparum* clone and into clinical isolates susceptible to artemisinin, and they consequently increased ring-stage parasite survival in the presence of artemisinin. However, the presence of multiple, population-specific mutations responsible for artemisinin resistance has led to the independent emergence of resistance in multiple geographic locations in Southeast Asia [175, 194].

The polymorphisms associated with artemisinin resistance in Southeast Asia were not detected in other countries in Africa, with the exception of the P553L polymorphism, which was detected in one isolate in Mali [175, 197–200], in Bangladesh [175, 201], in India [202], and in China [203]. In Uganda, the prevalence of K13-propeller polymorphisms was not associated with the persistence of parasites after two days after treatment with artemether–lumefantrine [204]. However, due to the high baseline parasitemia in Uganda, persistent parasitemia two days after the onset of therapy is likely not to be a reliable indicator of resistance in Uganda. No mutations on the K13 gene were detected in parasites from artemether–lumefantrine and dihydroartemisinin–piperaquine failures in Democratic Republic of Congo or Angola [205]. When artemisinin resistance emerges in Africa, it might be due by the spread of resistant parasites imported from Southeast Asia and/or the selection for de novo evolution of resistance (uncommon mechanisms between Asia and Africa). Further studies are needed to characterize better the roles in artemisinin resistance of the mutations in K13 found in Africa, China, Bangladesh, and India.

3.5.2 Artemisinin-Based Combination Therapies (ACTs)

Artesunate–Mefloquine

The artesunate–mefloquine combination was introduced after the spread of resistance to mefloquine in Thailand. It was first available as a co-blister and is now also available as a fixed-dose combination. Currently, eight countries use artesunate–mefloquine as first- or second-line treatment.

In Cambodia in 2000, artesunate–mefloquine became the first-line drug (12 mg/kg artesunate and 20 mg/kg mefloquine given for 3 days). The first report of the emergence of resistance to artesunate–mefloquine was in 2003. Between 2002 and 2004, the efficacy of this combination decreased from 85.7 to 79.3 % in Pailin [206]. Artesunate–mefloquine was introduced in 1995 in Thailand. The efficacy of the same regimen was 78.6 % at the Thai–Cambodian border in 2003 [207]. In Cambodia, after the implementation of rapid diagnostic tests and the replacement of artesunate–mefloquine by dihydroartemisinin–piperaquine in Pailin Province, mefloquine resistance decreased from 9.9 to 14.3 % in 2002–2004 to 0–5 % in 2007–2008 [136]. However, 42-day therapeutic efficacy studies conducted by the WHO in 2006–2008 and 2010 in southern Cambodia showed 18.8 % and 11.1 % failures of late artesunate–mefloquine treatment, respectively [137, 208]. In studies from 2008 to 2010, the proportions of Day 3 positive parasitemia among falciparum malaria patients associated with artesunate–mefloquine treatment increased along the Thailand–Myanmar border to surpass 10 % [138]. Approximately 14 % of patients undergoing artesunate–mefloquine remained parasite-positive on Day 3 during 2009–2011 along the Thai–Cambodian border [139]. In 2008–2009, the 42-day efficacy of artesunate–mefloquine was 72.6 % along the western border of Thailand (Thailand–Myanmar), and parasite clearance was significantly prolonged in patients experiencing treatment failure [2010].

Because of the long half-life of mefloquine, the efficacy of artesunate–mefloquine must be monitored for at least 42 days. Artesunate–mefloquine fails mainly in areas where

mefloquine resistance is highly prevalent. Regardless of whether these failures are due to mefloquine resistance only or to resistance to both mefloquine and artesunate, countries in the Greater Mekong subregion should continue to monitor the efficacy of this combination carefully and should review their treatment policies accordingly. The further spread of mefloquine resistance in areas where there is artemisinin resistance and where artesunate–mefloquine is used as the first-line treatment could jeopardize efforts to contain artemisinin resistance.

Artesunate–Amodiaquine

Amodiaquine was combined with artesunate in clinical trials conducted in Africa [210]. Artesunate–amodiaquine was first available as a co-blister and is now also available as a fixed-dose combination. Currently, 27 countries (25 in Africa) are using artesunate–amodiaquine as a first- or second-line treatment. More than 200 million treatments have been distributed in Africa since the medication became available in 2007 [211].

The efficacy of artesunate–amodiaquine is heterogeneous in Africa, probably due to preexisting amodiaquine resistance [212]. Of the 23 African countries that have adopted artesunate–amodiaquine as the first-line treatment, six (Burkina Faso, the Democratic Republic of the Congo, Eritrea, Gabon, Ghana, and Sierra Leone) have reported a treatment failure rate ≥10 % in at least one study after 28-day follow-up between 1999 and 2009. Overall, 15,017 patients were treated for uncomplicated *P. falciparum* malaria (51 % with artesunate–amodiaquine) at 44 sites in 20 sub-Saharan African countries. The parasite reduction ratio ranged on Day 1 from 77.1 % in Mozambique to 99.2 % in Kenya. The proportion of patients treated with artesunate–amodiaquine who were still parasitemic on Day 3 was 1.5 %, ranging from 0 % at many sites to 55.9 % in the Democratic Republic of Congo. The proportion of patients treated with artesunate–amodiaquine who experienced parasite clearance failure by Day 7 was 0.2 %, mostly from the Democratic Republic of Congo. Studies conducted in Tanzania in 2007 showed that amodiaquine–artesunate had limited clinical efficacy, with 20 % treatment failures after PCR correction [213].

Artesunate–amodiaquine is less effective in Asia than in Africa. In Myanmar, 9.4 % (14 of 155) of patients treated with artesunate–amodiaquine had recrudescent *P. falciparum* malaria in 2008–2009 [214]. In 2005, in rural areas from southern Papua, Indonesia, the cumulative risk of overall parasitological failure by Day 42 was 45 % in patients treated with artesunate–amodiaquine [215]. In 2002–2003, the proportion of late treatment failures with artesunate–amodiaquine was 28.4 % in Afghanistan [216]. However, artesunate–amodiaquine has been effective in Vietnam, with a 2 %, unexpectedly low rate of treatment failure in 2006–2007 and 2008–2009 [217, 218].

Artesunate–amodiaquine was effective in South America, with a 100 % rate of clinical adequate responses in Colombia in 2008–2009 [219].

Artemether–Lumefantrine

In anticipation of the need to protect against resistance to artemisinin and its derivatives, Chinese researchers began studying ACTs in 1981 [220] and registered the first ACT in 1992. This treatment was a combination of artemether and lumefantrine into a single tablet. Currently, 56 countries are using artemether–lumefantrine as first- or second-line treatment.

Artemether–lumefantrine began to lose its efficacy in Africa. In Uganda, the rate of adequate clinical and parasitological response was only 45.4 % in patients treated with artemether–lumefantrine in 2011–2012 [221]. Residual parasitemia, associated with a longer duration of gametocyte carriage, a higher transmission to mosquitoes and a higher risk of recurrence, was detected in 33.3 % of children at Day 3 treated with artemether–lumefantrine in Kenya in 2009 [222]. The proportion of patients with residual parasitemia on Day 1 rose from 81 % in 2005–2006 to 95 % in 2007–2008 in children treated with artemether–lumefantrine on the Kenyan coast [223]. However, by Day 28, the rate of recrudescent primary infection after PCR correction was 1 %, but it rose to 13 % by Day 84. Another study in Kenya showed a cumulative risk of recurrent parasitemia of 20.7 % on Day 42 after the initiation of treatment in 2009 [224]. A proportion of 11.2 % treatment failure was reported in Burkina Faso between 2008 and 2010 [225]. Using intention to treat analysis, the adequate clinical and parasitological response rate was 85.2 % in Malawi in 2004–2006 [226]. Artemether–lumefantrine was efficacious in Ghana, with significant ecologic zonal differences in 2010–2011: the 90.4 % day-28 cure rate observed in the savannah zone was significantly the lowest, compared with 100 % in the forest zone and 93.8 % in the coastal zone [227]. Few cases of clinical failure were reported of artemether–lumefantrine in travelers with imported uncomplicated *Plasmodium falciparum* malaria from Africa: one case in an Italian traveler returning from the Democratic Republic of Congo [228] and one in a Japanese traveler returning from Sierra Leone [229].

The adequate clinical and parasitological response rate was 100 % in Tanzania in 2013 and in Senegal in 2011–2012 after treatment with artemether–lumefantrine [213, 230], compared to 92.3 % in the Democratic Republic of Congo in 2011–2012 [231].

In 2005, the proportion of recrudescence was 17.8 % after artemether–lumefantrine treatment in the western part of Cambodia bordering Thailand [232]. At the Thai–Cambodian border, studies showed a cure rate of only 71.1 % in 2002 and 86.5 % after food supplementation in 2003 [233]. The efficacy of artemether–lumefantrine combination is strongly

influenced by broad variation in the pharmacokinetics of lumefantrine among individuals. Because its absorption is enhanced by concomitant intake of fatty foods [234], treatment failures with this combination might be due to insufficient absorption of lumefantrine. The main determinant of the efficacy of the combination is the area under the curve of the plasma concentration of lumefantrine, or its surrogate, the plasma concentration of lumefantrine, on Day 7 [235]. Even with flavored milk, the treatment failure rate by Day 42 was 25 % after artemether–lumefantrine on the northwestern border of Thailand in 2002 (Karen ethnic group from Myanmar) [236]. Three-day artemether–lumefantrine remained effective in Laos with a 42-day cure rate of 97 % in 2008–2010 [237].

Artemether–lumefantrine remains effective in South America. The clinical adequate response rates were 97.5 % and 99 % in Colombia in 2007–2008 and 2008–2009, respectively [219, 238].

Artemether–lumefantrine remains highly effective in most parts of the world, with the exception of Cambodia. Although no time trends have been observed in any subregions, continuous monitoring is necessary. Artemether–lumefantrine was reported to select for the wild-type *pfmdr1* Asn86 allele in recurrent infections, which could be a marker of reduced susceptibility to lumefantrine. Mutations in *pfmdr1* have also been associated with decreased susceptibility to artemether and lumefantrine drugs separately [160, 239, 240]. The *pfmdr1* N86 allele can predict in vitro decreased susceptibility to lumefantrine, whereas the 86Y mutation was significantly associated with increased susceptibility to lumefantrine [113, 115, 239, 241]. Field studies in east Africa have also shown selection of the 86N allele in recurrent infections after treatment with artemether–lumefantrine, suggesting that 86N could be a potential marker of lumefantrine resistance in vivo [242–245].

Dihydroartemisinin–Piperaquine

Piperaquine is a bisquinoline developed independently in the 1960s by Chinese investigators and the French pharmaceutical company Rhone Poulenc. It was used widely for the treatment and prevention of malaria in China in the 1980s; however, resistance to piperaquine eventually emerged, which led to its use in combination therapy [246]. The most widely studied combination is dihydroartemisinin–piperaquine, which is now one of the five ACTs recommended by the WHO.

Many trials to monitor the safety and efficacy of dihydroartemisinin–piperaquine have been conducted in Africa and Southeast Asia [247–249]. The treatment failure rates in the majority of the studies were <10 %. Dihydroartemisinin–piperaquine began to loss efficacy in Africa. In 2003–2004, the proportion of treatment failures after dihydroartemisinin–piperaquine was 8 % in Rwanda [250]. Residual parasitemia, associated with a longer duration of gametocyte carriage, higher transmission to mosquitoes, and a higher risk of recurrence, was detected at Day 3 in 30.0 % of children treated with dihydroartemisinin–piperaquine in Kenya in 2009 [222]. The proportion of patients with residual parasitemia on Day 1 rose from 55 % in 2005–2006 to 87 % in 2007–2008 in children treated with dihydroartemisinin–piperaquine on the Kenyan coast [223]. However, the adequate clinical and parasitological response rates were 99 % at Day 28 and 96 % at Day 46 in 2010–2011 [251]. In Uganda, the rate of adequate clinical and parasitological response was 87.9 % in patients treated with dihydroartemisinin–piperaquine in 2011–2012 [221]. The risks of treatment failure by Day 28 were 8.9 % and 82.7 % by Day 84 in Uganda in 2009 and 2012, respectively [252]. Recurrent malaria was correlated with low piperaquine concentrations at Day 7 in Burkina Faso [253]. However, the risk of treatment failure with dihydroartemisinin–piperaquine by Day 28 remained low in Burkina Faso in 2005 [254]. Adequate clinical and parasitological responses were observed in 93 % by Day 28 in Zambian children in 2005–2006 [255].

In the brief period since the introduction of dihydroartemisinin–piperaquine, there has been early evidence suggesting declining efficacy in Asia. In 2010, a study conducted in Cambodia showed efficacy of 79 % of dihydroartemisinin–piperaquine [256]. In 2013, at the same location, the efficacy of dihydroartemisinin–piperaquine rose to 65 % [257]. In 2010, the PCR-corrected treatment failure rates for dihydroartemisinin–piperaquine on Day 42 were 25 % in Pailin and 10.7 % in Pursat, while the therapeutic efficacy of dihydroartemisinin–piperaquine remained high (100 %) in Ratanakiri and Preah Vihear provinces, located in northern and eastern Cambodia [258]. PCR-adjusted falciparum efficacy at Day 42 was 75 % on the Thai–Cambodian border in northern Cambodia [256]. In central Vietnam, the efficacy of dihydroartemisinin–piperaquine remained satisfactory (100 % at Day 28 and 97.7 % at Day 42), but the delayed parasite clearance time and rate were indicative of emerging artemisinin resistance [259].

Between 2005 and 2007, the rate of adequate clinical and parasitological response to dihydroartemisinin–piperaquine was 88 % in Papua New Guinea [41].

Dihydroartemisinin–piperaquine remains highly effective in South America. Between 2003 and 2005, the rate of adequate clinical and parasitological response to dihydroartemisinin–piperaquine was 98.4 % in Peru [260].

3.6 Atovaquone–Proguanil

Strictly speaking, atovaquone–proguanil is not a monotherapy but is classified as such because its efficacy relies on the synergistic action of the two components. Early studies of

atovaquone, administered as a monotherapy, showed that resistant parasites were selected rapidly, and the synergistic combination atovaquone–proguanil was developed to delay the emergence and spread of atovaquone resistance [261]. Atovaquone is currently used in combination with proguanil for the treatment and prophylaxis of malaria, but because of its high price, the combination is generally limited to travelers from industrialized countries.

Prophylactic failure with atovaquone–proguanil in travelers has been extremely rare [262–264]. Prophylactic and clinical failures of atovaquone–proguanil against *P. falciparum* have been associated with poor absorption, which can lead to inadequate blood levels. However, resistance can also explain failures of prophylaxis. Few cases of clinical failure of atovaquone–proguanil in travelers with imported uncomplicated *Plasmodium falciparum* malaria have been reported from Africa, including Nigeria [265, 266], Côte d'Ivoire [267], Mozambique [268], Comoros [269], Democratic Republic of Congo [270], Kenya [271], Uganda [264], and Sierra Leone [264]. Treatment failure was confirmed among travelers returning from Nepal [272]. The proportion of treatment failure with atovaquone–proguanil was 1 % in a prospective study of patients treated for uncomplicated malaria in nine travel clinics located in Paris (mostly young men of African origin living in France and infected in West Africa) [273]. This rate was 13.6 % in Israeli travelers [274]. All of these failures were observed in travelers returning from West Africa. In 2004–2005, 97.8 % of Thai patients were cured using a standard 3-day course of atovaquone–proguanil therapy [275].

In previous studies, molecular analysis of recrudescent isolates showed that atovaquone resistance was associated with a single mutation at cytochrome *b* (*cyt b*), which seemed to compromise its efficacy. Mutations in this gene have been reported in Burkina Faso, Cameroon, the Comoros, Côte d'Ivoire, French Guiana, Guinea, India, Kenya, Mali, Mozambique, Nigeria, Senegal, Sierra Leone, and Uganda [276]. A point mutation at codon 268 in cyt b has been linked to atovaquone–proguanil treatment failure [264, 277–281]. This mutation is sufficient for, but not a necessary cause of, atovaquone–proguanil treatment failure [279]. Mutations were also detected among patients who failed treatment due to frequent de novo mutations. Because of the variability in the size of this mutant reservoir, some patients failed to eliminate all of the mutant parasites [282]. In other cases, treatment failures were linked to poor absorption, which could lead to inadequate blood levels [264, 283]. Atovaquone is a lipophilic drug, and its absorption is heavily influenced by the availability of fatty foods.

Atovaquone–proguanil failure requires resistance to atovaquone and to proguanil. Studies of the *pfdhfr* gene have consistently demonstrated the importance of a point mutation at the Ser108Asn codon in the proguanil-resistant phenotype of *P. falciparum*. Additional point mutations at the Asn51Ile, Cys59Arg, and Ile164Leu positions strengthen the resistance of *P. falciparum* to antifolates. The level of resistance increases with the number of mutations [284]. Cycloguanil resistance appeared to be associated with the double mutations Ser108Thr and Ala16Val [285].

The S108N mutation was screened in 71 surveys conducted during or after 2004 from 62 unique sites in 24 countries [286]. Of 9463 samples tested for S108N since 2004, 78 % carried the S108N mutation. Among 62 surveys, only three reported a prevalence of less than 50 % and, like the N51I and C59R mutations, these cases were in Burkina Faso in 2004 [287], Côte d'Ivoire in 2006, and Madagascar in 2006–2008 [288]. There were 24 surveys in which the prevalence was 100 %, and these surveys were conducted in Angola in 2007 [289], Ethiopia in 2004 [290], Kenya in 2004–2006 [291], Malawi in 2005 [292], Rwanda in 2005 [293], São Tome and Principe in 2004 [294], Tanzania in 2004 [295], and Uganda in 2005 [296].

3.7 Doxycycline

Daily administration of doxycycline is currently a recommended chemoprophylactic regimen for travelers visiting malaria-endemic areas with a high prevalence of chloroquine or multidrug resistance [297]. In addition, the French malaria consensus recommends quinine and doxycycline for the first-line treatment of severe *Plasmodium falciparum* malaria in Asia and South America. Doxycycline remains the recommendation for second-line treatment of uncomplicated falciparum malaria or for the treatment of severe malaria, in combination with artesunate or quinine for a 7-day course [298]. Doxycycline in combination with other antimalarial drugs has been studied many times, particularly in areas of multidrug resistance such as Thailand [299–302]. The most described associations have been doxycycline (200 mg)–quinine (10 mg/kg/day) for 7 days, which has been used in Thailand with therapeutic efficacy of 91–100 %.

The main studies of the efficacy and safety of doxycycline prophylaxis were performed in different types of populations that were followed for at least 28 days after the discontinuation of prophylaxis: semi-immune or immune subjects living in endemic areas [303–305] and non-immune travelers, mainly soldiers from different armies [306–308]. The results, which were conclusive, showed efficacy of 91–99 % in semi-immune and immune subjects and 95–100 % in travelers.

Most prophylactic failures of doxycycline against *P. falciparum* have been associated with the use of standard doses, resulting in lower-than-expected serum drug levels [309], inadequately low doses [305], or poor compliance [310–313]. Moreover, doxycycline pharmacokinetic parameters could explain some of these cases. Doxycycline has a short

elimination half-life (16 h), compared to proguanil (24 h), atovaquone (31–73 h), chloroquine (2–3 days), and mefloquine (6–41 days), and a short mean residence time (63 % of the administered dose is eliminated in 27 h) [314]. A surge in the number of malaria cases within 3 weeks after doxycycline prophylaxis discontinuation is often observed after return [303, 314]. Therefore, it is recommended that doxycycline be taken for 4 weeks after returning from an endemic area. However, resistance can also explain failures of prophylactic doxycycline. Cycline resistance in *Plasmodium* was documented as a consequence of drug pressure in a *P. berghei* murine malaria model [315]. The administration of increasing minocycline doses to mice infected with 1×10^7 parasites for 86 successive passages over a 600-day period made it possible to obtain a resistant strain with a median inhibitory concentration (IC_{50}) of 600 mg/kg/day, sixfold greater than that of the susceptible starting strain (100 mg/kg/day).

Although no *P. falciparum* malaria clinical failures with doxycycline have been reported, Bayesian mixture modeling approaches have identified three different phenotypes (low, medium, and high doxycycline IC_{50} phenotypic groups) among clinical *P. falciparum* cases [316, 317]. Using 90 isolates from 14 countries, we demonstrated that copy number increases of *P. falciparum* metabolite drug transporter gene (*pfmdt*, PFE0825w) and *P. falciparum* GTPase TetQ gene (*pfTetQ*, PFL1710c) were associated with reduced susceptibility to doxycycline [318]. This association was later confirmed [317]. In addition, isolates with pfTetQ KYNNNN motif repeats have been associated with in vitro reduced susceptibility to doxycycline and with a significantly greater probability of having an IC_{50} greater than the doxycycline resistance threshold of 35 µM [318, 319].

3.8 Sulfadoxine–Pyrimethamine

Although sulfadoxine–pyrimethamine is actually a co-formulation of two different medicines, it is considered as a monotherapy because the two components act on the same biosynthesis pathway of the parasite. Sulfadoxine–pyrimethamine has been widely used to treat chloroquine-resistant malaria. In contrast to the situation with chloroquine, resistance to antifolates emerged rapidly, after only 1–2 years of intensive use.

In vivo efficacy studies, conducted in 2002 in Benin by the National Malaria Control Programme, according to the WHO protocol, revealed treatment failures rates by region ranging from 3.3 to 45.9 % with sulfadoxine–pyrimethamine, with an overall failure rate of 22.8 % (8.3 % and 24.5 % early and late treatment failures, respectively) [320]. In 2005, Aubouy conducted an in vivo study according to the WHO protocol over 28 days in Benin, showing a very high failure rate of 50 % [153]. In Lambéréné in Gabon, in 2005–2007, 46 % of patients treated with sulfadoxine–pyrimethamine achieved an adequate clinical and parasitological response, and 50 % were late parasitological failures, while 4 % experienced early treatment failure [321]. In two previous studies conducted in 1998 and 2000 in children between 5 and 14 years of age in the extreme south of Cameroon, a clinical and parasitological failure rate for sulfadoxine–pyrimethamine of 13.6 % was reported [322]. In 2003, clinical and parasitological failure rates with sulfadoxine–pyrimethamine of 53.4 % and 56.5 %, respectively, were reported in the west and southwest of Cameroon [323]. In 2004–2006, the clinical and parasitological failure rates on Day 28 for sulfadoxine–pyrimethamine were 29.9 % in north Cameroon and 37.5 % in south Cameroon [106]. In a meta-analysis that included 115 trials of sulfadoxine–pyrimethamine, the treatment failure rates reported from Africa were >20 % in 1999–2002 [324]. In an analysis conducted by the WHO between 2000 and 2007, the median failure rate in eastern Africa (52.8 %) is higher than in the western (18.7 %), central (23.0 %), or southern (23.2 %) subregions.

Since 1978, treatment failures with sulfadoxine–pyrimethamine have been reported in Cambodian refugee camps in southeastern Thailand [325]. In 1979, all 23 patients treated with sulfadoxine–pyrimethamine failed within 21 days at the Thai–Cambodian border [326]. In 1980–1981, treatment failures with sulfadoxine–pyrimethamine ranged from 10 to 68 % in Thailand [327]. In Myanmar in 2002, early treatment failures occurred in 24 % of patients treated with sulfadoxine–pyrimethamine, and treatment failures increased to 81 % by Day 42 [142].

In South America, resistance to sulfadoxine–pyrimethamine was initially observed in few cases in a study of experimentally induced malaria in Brazil [328]. Sulfadoxine–pyrimethamine resistance occurred in 63 % in the Amazon region in the beginning of the 1980s, rising to 90 % at the end of the 1980s in Brazil [329–331]. The first cases of sulfadoxine–pyrimethamine-resistant *Plasmodium falciparum* infection in Suriname were reported in 1981 [332]. In 1982–1983, treatment failures represented 25 % in Colombia [333]. In 2002, in vivo antimalarial drug efficacy studies of uncomplicated *P. falciparum* malaria at an isolated site in the Amazon basin of Peru, bordering Brazil and Colombia, showed >50 % RII/RIII resistance to sulfadoxine–pyrimethamine [334].

In 2002, the WHO recommended the use of ACTs to ensure high cure rates of *P. falciparum* malaria and to reduce the spread of drug resistance. Sulfadoxine–pyrimethamine treatment was gradually abandoned in favor of ACT. In contrast to the situation with chloroquine, resistance to antifolates emerged rapidly, after only 1–2 years of intensive use. Moreover, reductions in resistance have been reported, although they have been rare and poorly documented. The absence of reduction might be a result of cross-resistance between sulfadoxine–pyrimethamine and antibiotics, such as

co-trimoxazole, or of the existence of compensatory mutations in resistant parasites [335]. Furthermore, sulfadoxine–pyrimethamine is still circulating in large quantities in the informal sector, which maintains the drug pressure on the regional parasite populations. After 2 years of use of insecticide-treated nets in a village in Tanzania, the prevalence of wild-type strains was higher than in a nearby control village [336]. Confounding and other factors, such as migration of sensitive parasites into study sites, have weakened the conclusions of such studies [337]. In Peru, the frequency of mutations conferring sulfadoxine–pyrimethamine resistance appeared to decline between 1997 and 2006; however, the studies were not conducted at exactly the same sites or in the same epidemiological setting; e.g., a study in 1997 was undertaken during an epidemic outbreak [338, 339]. Additional field studies are needed to confirm the regression of antifolate resistance [340].

The S108N mutation in the *pfdhfr* gene has been associated with resistance to antifolate drugs [341]. The OR for sulfadoxine–pyrimethamine failure associated with S108N was 3.5 (95 % CI: 1.9–6.3, meta-analysis of ten studies) over a 28-day follow-up [10]. The additional mutations N51I, C59R, or I164L increased the level of in vitro resistance to antifolate drugs and sulfadoxine–pyrimethamine. The OR values for single mutations at codons 51 and 59 were 1.7 (95 % CI: 1.0–3.0) and 1.9 (95 % CI: 1.4–2.6), respectively [10]. A triple mutation (51 + 59 + 108) increased the risk of in vivo resistance to sulfadoxine–pyrimethamine by 4.3 (95 % CI: 3.0–6.3, meta-analysis of 22 28-day studies) [10].

Sulfones (dapsone) and sulfonamides (sulfadoxine) are inhibitors of the *P. falciparum* DHPS [342]. The mutations S436A, S436F, A437G, and K540E are involved in resistance to sulfadoxine [343]. Mutations at 437 and 540 confer some degree of resistance; the 436, 581, and 613 mutations all contribute to a higher degree of resistance [285]. The single mutation A437G and the double mutation A437G + K540E increased the risk of in vivo resistance to sulfadoxine–pyrimethamine by 1.5 (95 % CI: 1.0–2.4, meta-analysis of 12 studies) and 3.9 (95 % CI: 2.6–5.8, meta-analysis of 10 studies), respectively [10].

Several mutations in both the *pfdhfr* and *pfdhps* genes are necessary to induce treatment failure with the sulfadoxine–pyrimethamine combination, such as triple mutations at codons 108, 51, and 59 of the *pfdhfr* gene and double mutations at codons 437 and 540 of the *pfdhps* gene [344]. In population studies, mutations at codon 59 of the *pfdhfr* gene and at codon 540 of the *pfdhps* gene have been strongly predictive of treatment failure. A quintuple genetic mutation could create the conditions needed for the emergence of the *pfdhfr* Ile164Leu mutation and the *pfdhps* A581G mutation [296]. The quintuple mutant of *pfdhfr* (codons 51 + 59 + 108) plus *pfdhps* (codons 437 + 540) increased the risk of in vivo resistance to sulfadoxine–pyrimethamine by 5.2 (95 % CI: 3.2–8.8, meta-analysis of three studies) [10].

The relationship between the parasite genotype and the therapeutic response to sulfadoxine–pyrimethamine is influenced by the parasite, pharmacokinetics, and human factors. When a parasite has wild-type *pfdhfr* without a mutation, the risk for failure is trivial, regardless of the *pfdhps* alleles. In contrast, the risk increases with the number of mutations in the *pfdhfr* gene, particularly when there is an additional mutation in the *pfdhps* gene or when immunity is lacking [341, 345]. Cumulative mutations in the *pfdhfr* gene increase parasite clearance time and the risk for gametocyte carriage. As a result, although sulfadoxine–pyrimethamine remains effective, the emergence of one or two mutations could increase the transmission of malaria and the spread of resistance [346].

The major resistance mutations in *pfdhfr* are widespread and have been thoroughly established throughout Africa. Very few sites found prevalence rates of S108N, N51I, and C59R less than 50 %, and these were Burkina Faso in 2004 [287], Ivory Coast in 2006, and Madagascar in 2006–2008 [288]. Elsewhere in Africa, all recent surveys have recorded a prevalence exceeding 50 % because sulfadoxine–pyrimethamine was used as the first-line treatment for clinical malaria for many years, exerting strong selection on these mutations [286]. The prevalence of the N51I/C59R/S108N triple mutation in *pfdhfr* and the *pfdhfr* N51I/C59R/S108N and a *pfdhps* A437G quadruple mutation was high in all of the areas with *P. falciparum*, even after sulfadoxine–pyrimethamine treatment was gradually abandoned in favor of ACT. The prevalence of the N51I/C59R/S108N triple mutation in *pfdhfr* increased from 40 % in 2003 to 93 % in 2011 in Senegal [347]. Furthermore, the prevalence of the *pfdhfr* N51I/C59R/S108N and *pfdhps* A437G quadruple mutation increased, from 20 to 66 %, over the same time frame, then decreasing to 44 % by 2011. The double mutants *pfdhfr* 108Asn/51Ile were detected at rate of 98.4 % in 2005 and 98.7 % in 2008, 3 years after the withdrawal of sulfadoxine–pyrimethamine in Ethiopia [348]. A significant decrease in the triple *pfdhfr* (108Asn/51Ile/59Arg) mutation was observed from 2005 (78.6 %) to 2008 (56.4 %). The quadruple mutations of *pfdhfr* (108Asn/51Ile/59Arg)/*pfdhps* 437Gly significantly decreased from 78.6 % in 2005 to 53.8 % in 2008; however, this rate remains high. There are eight countries in east Africa where the "quintuple mutant" (*pfdhfr* (108Asn/51Ile/59Arg)/*pfdhps* (437Gly/540E)) has been consistently reported at a prevalence exceeding 50 % [349]: Kenya (nine surveys since 2004), Uganda (three surveys since 2004), Tanzania (seven surveys since 2004), Zambia (five surveys since 2004), Malawi (six surveys since 2004), Ethiopia (three surveys since 2004), Rwanda (two surveys since 2004), and Mozambique (five surveys since 2004).

An increase in the prevalence of the resistance haplotypes DHFR 51I/59R/108N and DHPS 437G/540E occurred under sustained drug pressure, with no change in haplotype prevalence 5 years after a reduction in sulfadoxine–pyrimethamine pressure in Malawi [350]. The DHPS 437G/540E/581G haplotype was observed in 2007, and it increased in prevalence during a period of reduced sulfadoxine–pyrimethamine pressure in 2012.

Sulfadoxine–pyrimethamine has been now used as an intermittent preventive treatment (IPT), given to all children and pregnant women once per month during the transmission season, and it can provide a high degree of protection against malaria. Guidelines for the use of sulfadoxine–pyrimethamine in IPT must consider resistance, and molecular markers came into use in policy for the first time in 2010, when the WHO technical consultation on IPT in infants recommended that the prevalence of the *pfdhps* K540E mutation (indicating presence of the "quintuple mutant" or "fully resistant" genotype) be used as the basis for deciding where to implement sulfadoxine–pyrimethamine-IPT in infants [351]. The WHO recommendation was that where the prevalence of *pfdhps* K540E exceeded 50 %, sulfadoxine–pyrimethamine-IPT in infants should not be implemented.

4 New Strategies Do Delay Emergence and Spread

The emergence and spread of resistance to former first-line antimalarial drugs lead to defining new strategies. Resistance is the result of two processes: (1) drug selection of resistant parasites and (2) spread of resistance.

4.1 Drug Selection of Resistant Parasites

Antimalarial drug resistance is mediated by two processes: (1) the rate at which de novo mutations conferring resistance appear and are selected through drug use within an individual and (2) the spread of these resistant alleles to other individuals. For chloroquine and, more recently, the artemisinin derivatives, unlike other drugs, such as atovaquone and pyrimethamine (when not combined with sulfadoxine), the rate at which de novo mutations confer resistance occur is low. Heritable drug resistance is the result of mutations that can be single point mutations, alterations to multiple loci, or the result of gene duplication [352].

The likelihood that a specific mutation conferring resistance will be present in a treated individual is a function of the mutation rate and the biological fitness cost of the mutation [353]. If the frequencies of two necessary resistance-conferring mutations are both 0.01 %, then parasites with both mutations will have an initial frequency among infections of 0.0001 %. This process underlies the recommendation that all malaria infections should be treated with combinations of two or more drugs.

The total parasite load also plays an important role in the drug selection of resistant parasites. Although the density at which symptoms occur can vary widely, depending on the immune status of the individual, they are generally associated with blooms in parasite biomass. In non-immune individuals, symptoms can occur at densities of 50 parasites/L of blood or between 10^8 and 10^9 asexual parasites [354]. Clinically immune individuals may tolerate higher parasite loads, but parasite loads greater than 10,000 parasites/L or 10^{11} parasites are typically symptomatic regardless of immune status [354]. Most individuals harboring parasites at any one time are asymptomatic, with low levels of parasitemia. However, because individuals who are symptomatic have such high levels of parasitemia, the majority of malaria parasites in the world at any one time are likely to occur in individuals who are symptomatic [355], suggesting that symptomatic individuals are more likely to harbor resistant parasites [356].

The appearance of de novo mutations is only important for drug selection if individuals harboring these mutants use drugs. Increased drug use within a population thus leads inexorably to a greater probability of resistant mutants being drug-selected (i.e., all of the sensitive parasites being eliminated, leaving only resistant parasites), and this relationship has been well documented both in models and experimentally [357]. However, the widespread use of drugs has significant benefits both for the individual (reduced likelihood of morbidity and mortality) and the population.

Drug selection for resistant mutants at the individual level depends on the concentration of drug over time in the blood (pharmacokinetics) and on the inhibitory effects on the malaria parasite at these concentrations (pharmacodynamics). Together, the pharmacokinetics and pharmacodynamics yield the concentration and length of exposure to a drug that will be in contact with parasites; however, antimalarial drugs differ significantly in the lengths of time that they are maintained in the body. Some drugs have short elimination half-life, such as artemisinin (1 h) or doxycycline (16 h), compared to proguanil (24 h), atovaquone (31–73 h), chloroquine (2–3 days), or mefloquine (6–41 days). As the concentration of a drug falls, its therapeutic efficacy also falls. If the dosing is incomplete, meaning that it fails to eliminate all of the parasites effectively, either because of non-compliance or a dosage that is too low (due to misuse of antimalarial drugs, inadequate dosage in obese, abnormal metabolism or counterfeit drugs), parasites that might be inhibited at higher concentrations could survive and recrudesce. Alternatively, new infections can be exposed to sub-therapeutic levels of drugs due to a long drug half-life [77] or because of prophylactic use [358]. At low drug concentrations, parasites with resistance

mutations are able to survive and, over time, to increase their fitness through compensatory mutations [359]. However, there have been some cases of high-level drug resistance arising in individual infections during therapy [360].

4.2 Spread of Resistance

4.2.1 Emergence

Once the susceptible parasites have been eliminated from a patient after antimalarial drug treatment, the resistant parasites must be transmitted to become a problem. The parasites first must survive the immune response for a sufficiently long duration to produce infective gametocytes that will be transmitted to a mosquito vector. Then, the resistant mutation must not be lost during meiosis in the mosquito. In addition, the mosquito must survive sporogony and transmit a viable infection to a new individual.

The rate at which resistance emerges defines the time after a drug is introduced into a population until a specific proportion of clinical infections is caused by resistant parasites.

This measurement implicitly assumes both the initial drug selection of resistance mutations and their subsequent spread within a population. The rate at which resistance emerges depends in part on how resistance is encoded. Resistance due to only one mutation (*pfdhfr* 108Asn) or multigenic resistance, in which each subsequent additive mutation increases the tolerance of the parasite (*pfdhfr* 51I/59R/108N), leads to resistance that emerges more rapidly than if every mutation is needed for resistance [361–363].

The roles of heterogeneous biting and transmission in the establishment and spread of resistance in a population are very important. Heterogeneous biting plays a more significant role than transmission in the emergence of resistance [364]. Individuals and mosquitoes are distributed nonrandomly across the landscape [365], generating spatially heterogeneous biting patterns. These differences in biting patterns are further localized by the movement patterns of both mosquitoes and humans [366]. In addition, mosquitoes are differentially attracted to some individuals [367], and they are more likely to feed on larger individuals (i.e., adults versus children) because they have more surface area for biting [368]. Thus, it is likely that the multiplicity of infection (MOI) is highly variable across a spatial landscape, even at the scale of a village, which could result in differential levels of competition, depending upon the rate at which a host is bitten. Once the resistant parasites are established, the rate at which resistance increases from its establishment level is more rapid when heterogeneous biting occurs, regardless of transmission rate [364]. Heterogeneous transmission slows the establishment of resistance in the populations, but once resistance is established, it expedites the spread of resistance through the population because once resistance becomes established: (1) individuals with low probabilities of being bitten by mosquitoes are less likely to become reinfected with susceptible parasites when they are infected, which generates a reservoir of resistance and (2) individuals with higher transmission rates will quickly spread resistant parasites due to their high rates of being bitten. However, this point is in contrast with other models discussing the role of transmission in the initial emergence of resistance, which have generally suggested that resistance is more likely to emerge in low transmission areas. First, resistance likely emerges in high biomass infections [355] because of the lower levels of clinical immunity in these areas; thus, each infection is more likely to result in a higher parasite load. Second, because immunity is less developed in low transmission areas, mutant parasites are more likely to survive the host immune response and subsequently to be spread [369, 370]. Third, there is more drug treatment per parasite in low-transmission areas than in high-transmission areas [370]. Because individuals in low-transmission areas are less likely to develop immunity, they are more likely to become symptomatic and to treat each infection; it is thus more likely that a resistant parasite will encounter drugs. Fourth, in low-transmission areas, individuals tend to be infected concurrently by fewer genetically distinct parasites, so resistant parasites face less competition within the host and an increased probability of transmission success. Fifth, the higher the transmission rate is, the shorter the period is during which individuals with resistant parasites will harbor them without competition after drug treatment. Chloroquine resistance, as well as resistance to sulfadoxine–pyrimethamine, mefloquine, and artemisinin, arose in low-transmission areas of Southeast Asia.

4.2.2 Spread of Resistance Between Populations

The rate at which drug resistance will spread between populations is a function of the frequency with which resistance is introduced into new populations (exchange between populations, travel), associated with the probability of the resistant parasite becoming established, which is determined by the drug usage and transmission rates. The common hypothesis is that resistant parasites will emerge in a low-transmission area and spread to a high-transmission area. However, because the transmission rate is higher, once parasites have acquired compensatory mutations, allowing them to compete more effectively within the host, drug resistance will spread through the population much more rapidly than in a low-transmission setting [371]. However, in Klein's model, heterogeneous transmission slows the establishment of resistance in the population, but once resistance is established, it expedites the spread of resistance through the population [364].

The role of asymptomatic *P. falciparum* parasites is also important in the evolution of antimalarial drug resistance [372]. Malaria parasites are often carried as asymptomatic infections with no defined clinical symptoms. This asymptomatic parasitemia can exist at microscopically detectable levels; however, it often persists at less than this threshold [373, 374]. In areas with low levels of transmission, the use of ACT in association with the use of insecticide-impregnated bednets has brought the incidence of symptomatic malaria to very low rates [375]. However, attempts to eliminate asymptomatic parasitemia have failed to interrupt the regular malaria outbreaks that follow annual rains [376, 377]. In addition, the widespread use of asymptomatic-targeted therapy carries the risk of selecting resistant parasites that might exist at a low prevalence but that have a survival advantage under such uniform drug pressure. Epidemiological and laboratory experimental evidence has suggested that the therapy-free environment that prevails among asymptomatic parasite carriers can favor wild-type, drug-susceptible parasites and can disadvantage mutant, drug-resistant lineages [19, 378, 379]. Asymptomatic parasites have been observed at higher rates in children [380] and across different endemic settings with low- and high-transmission intensity in Africa [380, 381], Asia [382], and South America [383]. When drug-susceptible and -resistant parasites coexist and persist in asymptomatic individuals, their relative frequencies can be affected by factors such as their relative growth rates (fitness costs of resistance), as well as any competitive interaction between the genotypes.

4.3 Controlling the Emergence and Spread of Resistance

4.3.1 Interruption of Asymptomatic Carriage with Mass Drug Administration

Attempts to use therapy to interrupt asymptomatic carriage can enhance the selective advantage of resistant lineages. In turn, this enhancement can lead to subsequent rounds of selection in the transmission season, when clinical malaria and drug pressure prevail, resulting in a more rapid increase in the frequency of drug-resistant parasites. Such a pattern has been observed with the initial spread of resistance when it first appears, when repeated attempts to clear resistant parasites can lead to intense drug pressure, the acquisition of additional mutations, and increased levels of resistance [384]. Epidemiological and laboratory experimental evidence suggest that the therapy-free environment that prevails among asymptomatic parasite carriers can favor wild-type, drug-susceptible parasites and can disadvantage mutant, drug-resistant lineages [19, 378, 379]. The widespread use of therapy to eliminate asymptomatic parasitemia would not only favor the selection of drug-resistant lineages, but it would also reduce within host multiplicity and limit the expansion of wild-type parasites. Using this viewpoint, Read et al. recently suggested that the use of subcurative malaria therapy that retains some wild-type parasites could lead to a reduction in the resistant genotypes in the field [385]. The risks and benefits of subcurative treatment warrant further assessment. In the absence of obvious ethical concerns with this evolutionary approach, stringent guidelines to decrease the use of malaria therapy for asymptomatic infections should be urgently considered.

4.3.2 Improvement of Surveillance

In countries where malaria is controlled, passive surveillance systems are the cornerstone of detection, providing a standardized method for tracking progress, gathering demographical and epidemiological data, and enabling rapid investigation and appropriate response [386, 387]. When malaria is eliminated, passive surveillance is the frontline method for the detection of importation and local transmission. When reliable, passive surveillance data can be linked to remote sensing data, including altitude, population, weather, and wetness, to produce risk maps to guide the implementation of control and elimination measures [388].

During the elimination phase, active case detection, in which malaria programs are used to determine infections in high-risk groups, becomes crucial for targeting of the asymptomatic parasite reservoir in hotspots and hot-pops. Analysis of the complex interplay of factors (de novo mutations, drug use, malaria transmission intensity, human population, and infection among migrants) for the emergence and spread of resistance might provide evidence of a confluence in areas that we might regard as hotspots, which could serve as sentinel sites for surveillance or could be targeted for comprehensive clinical trials that include pharmacological measurements and molecular surveillance [389]. Operational research should be developed around simple models of detecting treatment failure, including institutional collection and the reporting of posttreatment review outcomes, if feasible.

The objectives of monitoring antimalarial drugs must be: to rationalize the distribution of surveillance sites on the basis of up-to-date malaria risk mapping; to conduct regular, standardized therapeutic efficacy studies and encourage capacity building for antimalarial drug resistance surveillance; to establish a mechanism for the exchange of data, the sharing of expertise and best practices, and the dissemination of the results of therapeutic efficacy studies and their implications; to identify and promote important research, support the collation of research evidence, and disseminate results to inform policy and practice; and collectively to address transnational issues and harmonize efforts within and between countries and to collaborate with other regional and subregional groups and broader global networks.

During the era of failing monotherapy, regional and subregional networks were established to monitor the efficacy of antimalarial treatment in Africa routinely. These networks were useful for the development of standard approaches, maintenance of cross-country quality assurance, and provision of a platform for dialogue between national malaria control programs and regional research groups (with a focus on drug resistance and its monitoring) to change policy effectively. Crucial to the success of drug resistance surveillance is communication between national control programs and research groups. The changing technical needs of efficacy studies include the use of molecular techniques to distinguish recrudescence from new infections, which in most settings requires a technical partnership between regional or national research groups and ministry of health staff, either as a long-term sustained relationship or as a provisional step toward building modern epidemiological competencies within ministries of health. Many stakeholders in Africa agree that malaria drug resistance surveillance should be a long-term, national commitment with common national and international goals [390]. Furthermore, investment in technology is needed to enable increased numbers of studies with pharmacokinetic and pharmacodynamic components. Such investment will require a long-term vision and should be integrated with capacity improvements, particularly of human resources, diagnostic techniques, and infrastructural capacities.

The organization of networks is crucial. For example, artemisinin resistance in Africa is initially likely to occur as a rare event, and individual patient-level pooled analysis across several sites could greatly increase the likelihood of detection. This method is frequently used in epidemiology when single studies are too small to allow for any definite conclusions. In an endeavor to encourage pooled analysis, the Worldwide Antimalarial Resistance Network has called for the formation of study groups on ACT in Asia and Africa. In 2011, the WHO introduced the TRAC project (Tracking Resistance to Artemisinin Collaboration).

4.3.3 Diagnostics

Although microscopy and rapid diagnostic tests (RDTs) are the standard methods for diagnosing malaria at health facilities, new and more sensitive methods are needed to screen populations for the identification of low density sub-patent infections [391]. Ideally, these new diagnostic tests would detect all plasmodia species infections at low density and would combine high throughput and low cost with delivery at the point of care.

Malaria antibody-based surveillance has been increasingly recognized as a valuable complement to classic methods for the detection of infection, especially at low transmission levels [392]. Serological tests could identify areas of persistent malaria transmission, even where parasite-infected individuals are undetected by routine tests.

4.3.4 Vaccines

The development of a malaria vaccine is considered to be one of the most cost-effective measures to counter malaria. The goal of malaria vaccine has been to save lives in the highest-risk groups: young children and pregnant women. In a seasonal setting, if the vaccine could induce sufficient immunity to reduce the basic reproductive rate to less than one member of the population at risk for the duration of the malaria season, and it could be administered in conjunction with other control measures, it might interrupt transmission. Valuable progress has been achieved over the last 30 years in the development of *P. falciparum* subunit vaccines [393]. Seventeen vaccines are currently in clinical trials, but the most advanced malaria vaccine candidate, the RTS,S vaccine based on the *P. falciparum* circumsporozoite (CS) protein, has undergone extensive testing in Africa, where a recent phase 3 trial showed 27 % and 46 % protection against clinical malaria in African infants and children, respectively, but unfortunately, its efficacy waned in a relatively short time [394]. Its efficacy was different, depending on the location and transmission rate. The homologation of the RTS,S vaccine might also pose a challenge to the development of alternative vaccines to increase the efficacy of RTS,S, either in combinations of RTS,S with new antigens or in totally new formulations [395]. It is possible that the most effective combinations might derive from mixing vaccines that are found to be efficacious when tested alone, that target different stages of parasite life and/or that are based on different mechanisms. In particular, one might speculate adding the RH5 antigen or antigens acting in opsonization, such as fragments from MSP2, MSP3, GLURP, and PFF0165, to RTS,S. Although single antigens might be only weakly effective, their combination together with RTS,S is expected to be more effective in controlling the disease and, as a result, reducing transmission by mosquitoes.

4.3.5 Drug Discovery Strategy

Since the discovery in 1891 of methylene blue, the first synthetic antimalarial, multiple chemical classes of molecules have been shown to clear blood-stage parasitemia efficiently in humans. As a consequence of resistance to the existing antimalarial drugs, new drugs are needed, and treatments must consist of combinations of two or more active compounds such that no compound is exposed as a monotherapy to high levels of parasites. During the past decade, new potential antimalarial targets have been proposed, based on the increasing number of plasmodial genomes that have been successfully sequenced [396, 397]. New screening and imaging technologies have generated thousands of new active drugs against Plasmodium asexual blood stages. Some of these drugs are currently in clinical development. However, despite this progress and given the increasing effectiveness and safety of current antimalarial drugs, simply curing

malaria rapidly and efficiently with new candidate drugs is no longer sufficient. There is a crucial need for therapeutics that exceed the treating of acute infections and that have the potential to eradicate the disease [398]. Accordingly, four major goals have been identified: (1) efficient elimination of all human parasites that populate the liver as dormant hypnozoites, notably those of *P. vivax* and *P. ovale*; (2) blocking of disease transmission by targeting parasite sexual stages in human blood; (3) identifying and developing new chemical entities that overcome all known cross-resistance and that minimize the risk of resistance emerging; and (4) delivering molecules that protect vulnerable populations. Future antimalarial combination treatments will need to cure the disease efficiently, by rapid clearance of parasitemia in patients, thereby reducing the risk of resistance and preventing recrudescence. In addition, these new medicines will be expected to block transmission and to eliminate all liver forms of the parasite, including dormant hypnozoites. A selection of these antimalarial drugs is described below.

Tafenoquine

Tafenoquine, an 8-aminoquinoline, is the only anti-relapse drug presently in clinical development. Tafenoquine recently completed a pivotal Phase II trial and demonstrated excellent anti-relapse efficacy from a single 300 mg dose; a Phase III trial has been initiated. This latter clinical trial is a randomized, double-blind treatment study to evaluate tafenoquine in adult patients infected with *P. vivax*. The efficacy, safety, and tolerability of tafenoquine to achieve a radical cure when coadministered with chloroquine were analyzed [399]. A single dose of tafenoquine plus 3-day chloroquine was assessed in patients with *P. vivax* from Brazil, Peru, India, and Thailand between 2011 and 2013, compared with chloroquine plus primaquine. Tafenoquine alone at 600 mg prevented relapse in 91.9 % of cases versus 77.3 % with primaquine and 37.5 % with chloroquine alone at 6 months. Coadministration of tafenoquine and chloroquine had no clinically significant adverse effects and was well tolerated [400]. In 2000–2001, the protective efficacy of tafenoquine against falciparum and vivax malaria was 100 % in the Australian Defence Forces deployed to East Timor [401]. However, tafenoquine belongs to the 8-aminoquinoline class, which is associated with hemolytic anemia in individuals with inherited glucose-6-phosphate dehydrogenase (G6PD) deficiency. Therefore, research is ongoing to develop a point-of-care diagnostic to identify individuals with G6PD deficiency, to support well-tolerated and effective use of drugs for a radical cure of patients infected with *P. vivax*. Thus, the development of new antimalarial drugs that display G6PD-dependent hemolysis is crucial.

Endoperoxide OZ439

The most advanced of the new molecules is a second-generation endoperoxide, OZ439, which was designed to have superior pharmacokinetics to the artemisinins [402]. OZ439 is a member of the ozonide class of antimalarials [403]. Endoperoxide in an ozonide could deliver efficacy equal to that observed with the naturally produced artemisinins. A stabilized ozonide, OZ03, which has a simple structure, was sufficient to demonstrate excellent in vitro potency. The high lipophilicity and low solubility, however, had to be addressed, and this need led to an introduction of polarity and ionizable groups in a region of the molecule that was synthetically tractable yet did not compromise potency. This development initially resulted in OZ277 (arterolane), which was the first clinical candidate and which was licensed to Ranbaxy [404]. Indeed, the combination of OZ277 and piperaquine received approval in India in 2012 under the name Synriam™, and it has been widely used to treat malaria patients in India over the last 6 months. OZ277, however, has lower exposure in patients than expected, and this limitation was hypothesized as being due to instability in infected blood, due to an interaction with ferrous iron. The amide with a phenyl ring, which helped to stabilize the ozonide, was substituted by an ether-linked base. The resultant compound, OZ439, was shown to have improved infected blood stability, it was able to cure mice infected with *P. berghei* from a single 30 mg/kg dose, and it progressed as the potential single dose cure candidate [403]. OZ439 has demonstrated clinical efficacy as a single agent in phase II clinical development [405]. It is now being tested in combination safety studies, and it will start combination efficacy studies. Studies in both healthy volunteers and infected patients have shown significant plasma exposure for as long as 20 days, suggesting it might be possible to use as part of a single-dose therapy for uncomplicated malaria [402]. In patients, OZ439 drives the reduction of parasites at approximately the same rate as artesunate.

Spiroindolone KAE609 (NITD609)

The spiroindolone class was found by screening a library of natural products and "natural product-like" compounds at Novartis. The starting "hit" had an intriguing structure and good potency, and impressively for a starting point, it suppressed parasitemia in the *P. berghei* mouse model of malaria by >99 % with a single dose of 100 mg/kg. Excellent medicinal chemistry was applied to contract the seven-membered ring, to define the stereochemical structure/activity relationship and to replace the lipophilic bromine atom. This process resulted in a second compound that had increased potency yet reduced lipophilicity—the ideal outcome from the standpoint of medicinal chemistry. However, its metabolic stability was still non-optimal; this shortcoming was fixed by judicious positioning of halogens on the tetrahydro-beta-carboline ring. The resulting compound, the spiroindolone NITD609, had even greater potency and excellent pharmacokinetics [406]. NITD609 rapidly inhibited protein synthesis in *P. falciparum* and showed pharmacokinetic properties compatible with once-daily oral dosing, and it had single-dose

efficacy in a rodent malaria model [406]. In addition, NITD609 inhibited the early and late development of *Plasmodium falciparum* gametocytes in vitro in a dose-dependent fashion over a range of 5–500 nM [407]. Using the standard membrane feeding assay, NITD609 is also a very effective drug in reducing transmission to the *Anopheles stephensi* mosquito vector. PfATP4, a Na^+ efflux ATPase, is the target of NITD609 [408]. NITD609 at dose of 30 mg daily for 3 days cleared parasitemia rapidly in adults with uncomplicated *P. vivax* or *P. falciparum* malaria [409]. This first-in-humans randomized, double-blind, placebo-controlled, ascending single and multiple oral dose study was designed to evaluate the safety, tolerability, and pharmacokinetics in healthy volunteers of NITD609, and it showed that NITD609 was tolerated, with transient gastrointestinal and genitourinary adverse events of mild to moderate intensity [410].

Imidazolopiperazine KAF 156 (GNF136)

Metabolite identification and excellent medicinal chemistry, in which the metabolically susceptible position on the piperazine was blocked with two methyl groups, led to the isomer KAF156. KAF156 demonstrated a good overall profile with an ED_{99} in the *P. berghei* mouse of 1.1 mg/kg [411]. Consistent with the previously reported activity profile of this series, the clinical candidate KAF156 showed blood schizonticidal activity with 50 % inhibitory concentrations of 6–17.4 nM against *P. falciparum* drug-sensitive and drug-resistant strains, as well as potent therapeutic activity in mouse models of malaria with 50, 90, and 99 % effective doses of 0.6, 0.9, and 1.4 mg/kg, respectively [412]. When administered prophylactically in a sporozoite challenge mouse model, KAF156 was completely protective at a single oral dose of 10 mg/kg [412]. Finally, KAF156 displayed potent Plasmodium transmission blocking activities both in vitro and in vivo [412]. The first-in-humans, single- and multiple-ascending-dose study in 70 healthy male volunteers showed that KAF 156 was tolerated, with self-limited mild to moderate gastrointestinal and neurological adverse events [413].

Triazolopyrimidine-Based Inhibitor DSM 265

The *P. falciparum* enzyme dihydroorotate dehydrogenase (PfDHODH) is known to be essential for the survival of the parasite. The three-dimensional structure of the enzyme–inhibitor complex was resolved, and the subsequent lead optimization program resulted in the identification of the preclinical candidate. DSM1 was an interesting molecule, but it showed non-optimal pharmacokinetics on repeat dosing and insufficient potency. First, progress was achieved in the improvement of its pharmacokinetics by substituting electron-withdrawing groups in the aniline ring; due to the hydrophobic nature of the binding site, only lipophilic groups have had major success in this case, e.g., DSM191 [414]. A second breakthrough occurred using the X-ray crystal structure; it was clear that limited substitution of the triazolo carbon could be achieved, and combined with electron withdrawal, it could reduce desolvation of the heterocycle and improve potency. These changes advanced DSM265, which has a good potency and safety profile, from preclinical studies [415]. The compound is currently in preclinical development.

Dihydrofolate Reductase (DHFR) Inhibitor P218

Dihydrofolate reductase (DHFR) inhibitors, such as pyrimethamine, have been widely used for the treatment of malaria, although their clinical efficacy has been compromised by resistance. P218 is a next-generation inhibitor of DHFR. P218 has a good pharmacokinetic profile, and it is selective and highly efficacious; initial safety testing of P218 indicated a good safety margin between toxicity in animals and the predicted effective human dose [416]. It was found that mutant residues did not reduce the binding affinity of P218 to PfDHFR [417].

Methylene Blue

Methylene blue (MB) is an old antimalarial drug that is no longer in use. In 1891, Guttmann and Ehrlich were the first researchers to report on the antimalarial properties of a synthetic thiazine dye, methylene blue, when they described the clinical cure of two patients after oral administration of MB [418]. Cardamatis wrote in *Progrès Médical* that he found methylene blue to be very effective in the early stages of severe malaria cachexia, in cases that were resistant to quinine [419]. Currently, there is no methylene blue available globally that complies with European Pharmacopoeia. To date, the pharmaceutical use of methylene blue has been stymied by contamination with organic impurities and heavy metals with recognized toxicity. Provence Technologies and its subsidiary, Provepharm, conducted 4 years of research that resulted in the first European Pharmacopoeia-grade methylene blue: Proveblue®. This drug was obtained from an innovative synthetic and heavy metal-free pathway using pharmaceutical-grade reagents (patent application N°FR06/06330, which has been extended to the international PCT reference number PCT/FR/2007/001193). The total concentrations of metals, including Azure B (the most important impurity in methylene blue), and other impurities in Proveblue® are <20 ppm, <2 %, and <0.5 %, respectively. Proveblue® was previously demonstrated to possess in vitro antimalarial activity against 23 *P. falciparum* strains that were resistant to various antimalarial drugs [420]. Proveblue® exhibited noticeable synergistic effects in combination with mefloquine and quinine and highly synergistic effects associated with dihydroartemisinin [421]. Treatment with 1–10 mg/kg of weight of Proveblue® for 5 days significantly reduced or prevented cerebral malaria in mice [422–424]. The IC_{50} for Proveblue® ranged from 0.88 to 40.2 nM, with a mean of 5.3 nM, against *P. falciparum* from Dakar, Senegal [32]. These data showed that Proveblue® is active in vitro, in

agreement with previous studies of methylene blue with organic, as well as inorganic, impurities in parasites from Nigeria, Kenya, and Thailand [425–427]. Methylene blue is also active against *P. vivax* [427]. Another advantage of the use of Proveblue® is that methylene blue has gametocytocidal properties, and it can reduce the transmission of *P. falciparum* [428–430]. The combination of methylene blue with artemisinin-based combination therapy has been confirmed to be effective against the gametocytes of *P. falciparum*. The gametocyte prevalence of *Plasmodium falciparum* was significantly lower in the artesunate–amodiaquine–methylene blue group than in the artesunate–amodiaquine group on Day 7 of follow-up (36.7 % versus 63.3 %) [431].

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Diagnosis and Treatment of Metronidazole-Resistant *Trichomonas vaginalis* Infection

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1 Introduction

Trichomonas vaginalis is the causative agent of trichomoniasis. There is an estimated prevalence of 276.4 million *T. vaginalis* infections worldwide. Important complications due to *T. vaginalis* infections are increased transmission of HIV, and infant morbidity due to preterm birth, low birth weight, and vertical transmission. Metronidazole and tinidazole are 5-nitroimidazole drugs used for treatment of *T. vaginalis* infections. *T. vaginalis* infections not responding to 5-nitroimidazole drugs used for standard therapy is a concern for adult sexual health due to persistence of infection and its complications as well as the risk for increased spread of disease due to clinical symptom relief following treatment without microbiological cure. At least a low level of metronidazole resistance is likely in 2–6 % of infections globally. Tinidazole resistance is strongly correlated to metronidazole resistance ($r=0.8709$, $P<0.0001$). Despite significant improvements in diagnostics in the past decade, new treatments are lacking. Alternative treatments tested in vitro rarely progress to clinical trials. So far, no consistently effective, non-nitroimidazole treatments are available to combat metronidazole-resistant *T. vaginalis* infections.

2 Trichomonas vaginalis

Trichomonas vaginalis is one of the four protozoan species of the family Trichomonadidae known to parasitize humans. Members of this family are characterized by their variable morphology, being spheroid or ovoid in form in axenic culture, but assuming an ameboid shape on contact with other cells [1, 2]. Trichomonads reproduce by longitudinal binary fission and lack a cystic stage, although large, round "pseudocysts" have been known to form under unfavorable conditions. All Trichomonadidae possess five anterior flagella, four of which are free moving. The fifth recumbent flagellum is anchored along the organism as a part of the undulating membrane. This membrane extends along at least half the length of the organism, and is supported by a noncontractile costa. Motility, described as "bobbing" or "quivering," is characteristic of this family of organisms [3, 4].

T. vaginalis is the only trichomonad known to cause disease in humans. It is the causative agent of trichomoniasis. *T. tenax*, usually found in the mouth, has been implicated in respiratory infection but its pathogenicity has never been confirmed [5]. *Pentatrichomonas hominis* [6] and *Trichomitus fecalis* have generally been isolated from the lower gastrointestinal tract. However, to date only one case of *T. fecalis* has been confirmed, leaving its identity as a human parasite in question [7].

Nutritionally, *T. vaginalis* is a fastidious organism. Lacking pathways for de novo synthesis of purines [8], pyrimidines [9], fatty acids, and sterols [10], the protozoan relies on salvage pathways to provide the necessary components of lipid and nucleotide metabolism. Amino acid synthesis and conversion is also thought to be limited. Carbohydrates are the preferred source of energy for metabolism. However, metabolic pathways for using amino acids, especially arginine, threonine, and leucine, as energy sources also exist [11], and energy generation using arginine probably takes place even if carbohydrates are available [12].

Energy metabolism takes place in the cytoplasm (for amino acids and carbohydrate glycolysis) and in an organelle called the hydrogenosome (for adenosine triphosphate (ATP) production via substrate-level phosphorylation). The hydrogenosome is analogous in structure and function to the mitochondrion in higher eukaryotes, although it lacks cristae and cytochromes [13, 14]. In the hydrogenosome, pyruvate is decarboxylated by an enzyme called pyruvate:ferredoxin oxidoreductase (PFOR). Ferredoxin serves as a terminal

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electron acceptor for PFOR, eventually leading to the production of acetate [15]. The fermentative metabolic processes of *T. vaginalis* also lead to the production of H_2, CO_2, lactate, and glycerol, the proportions of which vary depending on whether the organism grows in the presence or absence of oxygen [16].

A microaerophilic organism, *T. vaginalis* grows well under anaerobic conditions; however, some strains can tolerate oxygen well enough to be grown in ambient air. Optimum conditions are generally considered to be at 37 °C in moist air with 5 % CO_2 for growth in both axenic and tissue culture (this microaerophilic environment is similar to that found in the human vagina). Interestingly, *T. vaginalis* aerotolerance has often been found to reflect a particular strain's susceptibility to metronidazole, the drug most commonly used to treat trichomoniasis [17, 18].

3 Epidemiology

3.1 Prevalence and Transmission

With a prevalence of 276.4 million people infected worldwide, trichomoniasis is the most common nonviral sexually transmitted infection (STI) [19]. It is ubiquitous, being found in all races and cultures, but is especially prevalent among the underprivileged, injection drug users, individuals with multiple sex partners, and those who exchange sex for money [20]. It is estimated that at least one million new cases emerge in the United States yearly, many in African Americans [20, 21]. Globally, *T. vaginalis* infection is most prevalent in Africa and Asia, with infection rates reaching 40–60 % in some populations [19, 22].

Trichomoniasis has long been considered a disease of women, but the disease can also cause significant morbidity in men. Prevalence rates are highest in men with partners diagnosed with vaginal trichomoniasis [23]. Previous studies had shown that less than 5 % of the cases of nongonococcal urethritis are attributable to *T. vaginalis* [24]. However, a recent decline in the rates of chlamydial infection in the United States has been accompanied by an apparent increase in the frequency of *T. vaginalis* infection. Up to 17 % of male patients with nongonococcal, nonchlamydial urethritis are now confirmed to be suffering from trichomoniasis [25]. A similar rate of *T. vaginalis* infections was reported in men with urethritis attending a STI clinic in Malawi [26]. It is not yet clear, however, whether this trend represents a bona fide increase in the rate of trichomoniasis, or an improvement in diagnosis of the disease.

The prevalence of *T. vaginalis* infection in women has been found to vary significantly among different populations. Studies have shown that the rate of infection in women attending family planning clinics is about 5 % [27]. Reports from STI clinics indicate that anywhere from 1 to 40 % of female patients are identified with trichomoniasis [28]. The highest rates of infection are found in sex trade workers and women incarcerated in correctional facilities, where 50–75 % of these groups are infected with *T. vaginalis* [27].

The rate of transmission of *T. vaginalis* differs between sexes. Studies have shown that 15–70 % of men who have contact with an infected female partner will develop infection [29, 30]. Women exposed to the parasite via an infected male partner have a 65–100 % chance of developing trichomoniasis [30, 31]. *T. vaginalis* has been found to be able to survive for short periods of time outside of a host if sufficient moisture is maintained. Viable specimens have been obtained from body fluids (urine, semen, and vaginal exudates) 3–6 h after being emitted from the body [32, 33]. Live trichomonads have also been isolated from warm, damp washcloths 24 h after incubation [34], and from insufficiently chlorinated swimming pool water for up to 48 h [35, 36]. However, there have been no confirmed cases of trichomoniasis caused by exposure to contaminated objects.

Few nonsexual modes of transmission have been documented. Shared bathing water was implicated, though unconfirmed, as a source of infection in adolescent girls in Ndola, Zambia [37]; iatrogenic transmission is suspected in a female patient of a traditional healer who was diagnosed with *T. vaginalis* infection following the healer touching the female patient's genitals using his fingers [38]; lastly, perinatal transmission has been reported in a number of cases wherein clinical presentation is often respiratory disease [39–41].

3.2 Association with Human Immunodeficiency Virus and Other STIs

Patients with trichomoniasis are at an increased risk of contracting other STIs. This can be due to lifestyle risk factors (e.g., poverty or promiscuity), but may also be a reflection of the fact that *T. vaginalis* cytotoxicity towards urogenital tract epithelial cells (and the increase in vaginal pH commonly seen in infections of women) helps to create an advantageous niche for other sexually transmitted infectious organisms [42]. It is also possible that a preexisting STI could increase the likelihood of developing a trichomonal infection upon exposure to the parasite. One clinical study reported that 30 % of women diagnosed with *T. vaginalis* infection were accompanied by at least one other STI [43].

Similar to other STIs, *T. vaginalis* infection significantly increases the risk of contracting human immunodeficiency virus (HIV) (odds ratio 2.74, 95 % CI 1.25–6.00; relative risk 2.57, 95 % CI 1.05–4.02; hazards ratio 2.05, 95 % CI 1.43–4.65) [44]. Reasons for increased risk include damage to the mucosal surface, disruption of normal flora and pH facilitating viral penetration and survival, and an increased

number of immune cells at the genital mucosa enabling infection of these cells by HIV [44–46]. Another issue to consider is that *T. vaginalis* coinfection with HIV significantly increases HIV-1 RNA shedding in women (odds ratio 4.07, 95 % CI 1.78–9.37) [47]. Given that *T. vaginalis* and HIV are endemic in similar areas of the world, this means that prevention of trichomoniasis could be an important step in reducing global HIV/AIDS rates.

4 Clinical Aspects

4.1 Trichomoniasis in Men

Trichomoniasis in men is usually an asymptomatic carrier state [24, 48–51]. When symptomatic infection does occur, it presents as a mild urethritis. Clinical symptoms are similar to nongonococcal urethritis and include small amounts of clear or purulent discharge, and discomfort or a burning sensation during urination or after sexual intercourse. Rare cases of acute male trichomoniasis are characterized by more severe manifestations of urethral symptoms [52]. An extragenital *T. vaginalis* infection causing bilateral conjunctivitis has been reported in an adult male. Cause of the infection was linked to ocular exposure to genital secretions or fluids of a recent sex partner. No diagnosis of *T. vaginalis* in the sex partner was conducted [53].

The incubation period for *T. vaginalis* infection in men is usually less than 10 days, although longer incubation periods do occur [52]. Spontaneous resolution of both unapparent and symptomatic infection is common [49]. Studies using more sensitive diagnostic techniques are required to verify this data. One study showed that 70 % of untreated, symptomatic men had cleared the parasite within 2 weeks [29]. However, it has also been found that some cases of persistent nongonococcal urethritis, particularly those that have responded poorly to antibiotic therapy, may in fact be caused by resilient or resistant strains of *T. vaginalis*.

Prostatitis is the most common complication associated with trichomoniasis. Balanoposthitis, epididymitis, and other inflammations of the external genitalia are also frequently seen. Associations of trichomoniasis with prostate cancer remain undetermined [54, 55]. There is also evidence linking persistent *T. vaginalis* infection to urethral disease and infertility [56–58].

4.2 Trichomoniasis in Women

Unlike infection in men, trichomoniasis in women is usually persistent. Incubation periods range from 4 to 28 days [34]. Establishment of symptomatic infection usually involves a rise in the normal vaginal pH of 4.0–4.5 to a pH of 5.0 or higher (some of the virulence factors of *T. vaginalis* have been found to be inhibited at normal vaginal pH) [59]. This rise in pH is probably attributable to a concomitant decrease in acid-producing vaginal *Lactobacillus*, although the mechanism by which lactobacilli are inhibited or eliminated has not yet been elucidated but may be related to phagocytosis by trichomonads as demonstrated in vitro [60]. The symptoms of trichomoniasis are known to worsen during menses. This is likely a reflection of the fact that iron is an important mediator of many of the parasite's metabolic and pathogenic pathways (particularly cellular adherence) [61]. Nearly all cases of urogenital trichomoniasis are found in women of reproductive age, but it is not known if this is due to the unsuitability of the vaginal environment in premenarche and postmenopausal women, or is simply a reflection of the parasite's niche as an STI.

Asymptomatic infection rates are as high as 80 %, but about 30 % of women with an unapparent infection will develop symptomatic trichomoniasis within 6 months [23, 62]. Symptomatic infection is rated as mild, acute, or chronic. Chronic infection generally shows a similar clinical presentation to the mild form of the disease, but lasts for an extended period (i.e., years) and/or shows antibiotic resistance. Mild *T. vaginalis* infection is characterized by pruritus, dyspareunia, and sometimes dysuria. Small amounts of mucopurulent vaginal secretion are often present. Acute trichomoniasis usually presents with vulvar and vaginal erythema, and 2 % of cases show characteristic small hemorrhagic spots on the vagina and cervix, known as strawberry cervix [63, 64]. Use of a colposcope will increase the diagnosis of a strawberry cervix to about 90 % of patients with acute symptoms [64]. Copious discharge is often yellow or green in color, malodorous, and mixed with mucus [63, 64].

T. vaginalis infection has been implicated as a cause of cervical erosion and in the development of cervical cancer, although carcinogenicity likely can be related to high rates of coinfection with human papilloma virus [65]. Other complications associated with trichomoniasis arise when the parasite invades tissues outside the vagina. Skene's and Bartholin's glands are often infected, and ascending infection has been associated with endometritis and infertility [66]. *T. vaginalis* infection can be especially hazardous for pregnant women, predisposing them to premature rupture of the placental membrane, premature labor, and low birth weight babies [64, 67, 68].

4.3 Diagnosis

Diagnosis of trichomoniasis is difficult to make on the basis of clinical presentation alone. The high frequency of asymptomatic infection contributes greatly to underdiagnosis of the disease as well as the lack of resources for diagnosis particu-

larly in areas with the highest prevalence of trichomoniasis. In addition, the symptoms of *T. vaginalis* infection are often similar to those found in bacterial urogenital infection. As previously mentioned, symptomatic trichomoniasis in men presents as nongonococcal urethritis. Many symptoms associated with trichomonal infection in women are also common to bacterial vaginosis. For example, in STIs with bacterial etiology, vaginal pH is elevated in 90 % of cases [69, 70] and a positive "whiff" test, the presence of a fishy odor when vaginal exudate is mixed with 10 % potassium hydroxide, may be present (as in 50 % of trichomoniasis cases) [69, 71]. As coinfection with other STIs is not uncommon, it is important that specific tests for trichomoniasis be undertaken to prevent misdiagnosis and inappropriate treatment.

Microscopic diagnosis of *T. vaginalis* in women is usually performed after sampling vaginal exudates from the posterior fornix with a sterile cotton-tipped applicator. For men, urethral swabs are the most sensitive sample for culture, although a fresh semen sample or urine is also frequently used [48]. If a sufficient number of trichomonal cells are present (at least 10^4 trichomonads/mL), immediate diagnosis may be possible by microscopic examination of a wet mount, but is not used for diagnosis of *T. vaginalis* in men because wet mount lacks sensitivity [72]. *T. vaginalis* cells are similar in size to leukocytes, but can be identified by their characteristic motility [73, 74]. Unfortunately, the reliability of this test is highly variable, and its sensitivity has been quoted in the literature as anywhere from 40 to 90 % [75]. Additionally, if the test is not performed immediately, specimens are usually kept moist in physiological saline or transport medium. Although this does not (in the short term) affect the viability of trichomonads, it does have a profound negative effect on their motility [76] and thus the ability to recognize the organism on wet mount evaluation.

The diagnosis of trichomoniasis most often used due to affordability and acceptable sensitivity (44–75 %) is cultivation of the organisms in axenic medium [72]. Diamond's TYM (trypticase-yeast extract-maltose) supplemented with serum and antibiotics to prevent growth of bacteria and yeast has been found to yield reasonable results. Alternatively, a commercial InPouch TV (Biomed Diagnostics, California, USA) culture medium is available for detection of *T. vaginalis*. The InPouch TV system has benefits over the aforementioned Diamond's TYM preparation. The InPouch TV system can be stored at room temperature, is contained within a clear plastic pouch that can be examined by microscopy without needing to sample the culture, does not require immediate incubation after adding a sample, and does not require warming before use (Biomed product insert). Vaginal specimens can be inoculated into medium immediately or after storage in saline, and growth of motile trichomonads confirms a positive diagnosis. Diagnosis of *T. vaginalis* via cultivation also has the advantage that cultivated trichomonads can be maintained for further testing (i.e., antibiotic susceptibility). The disadvantage of this technique is that trichomonads do not grow quickly, and a minimum of 3 days for samples from women and 5 days for samples from men should be allowed before rendering a negative diagnosis [72, 77].

A number of fixed staining techniques have also been employed in the diagnosis of trichomoniasis. These include Giemsa [78], acridine orange [79], and the Papanicolaou (Pap) smear [80], among others. Unfortunately *T. vaginalis* cells often lose their characteristic shape on fixation. Studies on the diagnostic utility of the conventional or liquid Pap smear have shown its sensitivity to range from 44 to 96 % [72, 81]. A note of caution with these fixed staining techniques, there is a high frequency of false positive results (probably due to the similarity in size and shape of *T. vaginalis* and leukocytes). Between 20 and 30 % of uninfected women will be falsely diagnosed as having trichomoniasis [82].

Nucleic acid amplifications tests (NAATs) are the most sensitive tests available for the diagnosis of *T. vaginalis* in both men and women [48]. NAATs may not be used in resource-limited settings due to the required cost, infrastructure, and training. The APTIMA *T. vaginalis* assay (Hologic Gen-Probe Inc, California, USA) is the first United States Food and Drug Administration (FDA) approved NAAT that has reported sensitivity ranging from 88 to 100 %, with specificity of 98–100 % [72]. Other validated in-house polymerase chain reaction tests have been reported to have similar sensitivity and specificity [72].

NAATs have important advantages and disadvantages over culture. The need for viable organisms is not required for NAATs. Specimen storage and processing requirements are not as stringent. However, NAATs can detect nonviable organisms and may result in a false report of persistent infection following treatment. The follow-up time from treatment to reduce the number of false positive reports of infection using NAATs was investigated by Williams and colleagues [83]. Three weeks following treatment, 85 % of female vaginal samples were negative for *T. vaginalis* [83]. Resolution of clinical symptoms following treatment and a positive NAAT should not be immediately ruled as a false positive. Subclinical infections due to treatment failure have been suggested to be a source of a persistent infection rather than due to reinfection [84]. No FDA approved point-of-care NAAT tests are available at this time.

Three non-amplified point-of-care molecular tests are available. The OSOM TV Trichomonas Rapid Test (Sekisui Diagnostics, California, USA) and Kalon TV agglutination test (Kalon Biological, Surrey, UK) are two commercially available *T. vaginalis* antigen detection tests. Sensitivities range from 77–98 % and 55–99 %, respectively, with speci-

ficities >90 % [72]. A nucleic acid probe hybridization test, Affirm VPIII Microbial Identification Test (Becton Dickinson, Maryland, USA), provides detection of *T. vaginalis*, *Gardnerella vaginalis*, and *Candida albicans. T. vaginalis* sensitivity of the AFFIRM VPIII test has been reported to be 64 % with specificity of 100 % [72]. OSOM and Affirm VPIII have been approved for use as a diagnostic tool in the United States by the FDA. A major disadvantage of the non-amplified molecular tests is a lack of validation for use in asymptomatic women or for use in men [72]. On the other hand, these tests require little training or added infrastructure.

A new point-of-care molecular diagnostic tool is currently under development and testing on clinical vaginal swabs has been reported. The detection limit was evaluated to be five *T. vaginalis* cells. The sensitivity and specificity were 95.5 % and 95.7 %, respectively [85, 86]. The test targets a genetic biomarker that is present in multicopy within and unique to the *T. vaginalis* genome. Three stages of the test lead to identification of *T. vaginalis*. DNA from the test sample is extracted, and if target *T. vaginalis* DNA is present, then the target biomarker is amplified. The amplified products are identified using an electrochemical endpoint detection method. Results can be realized within 30 min.

Finally, it should be noted that isolation of trichomonads to confirm infection in males is often unsuccessful. It is hypothesized that this is because certain aspects of the male genitalia (e.g., an oxidative environment [87], zinc in prostatic fluid [88]) create an inhibitory milieu in which parasite numbers are greatly limited. In the absence of sensitive tests, it is important to assume that any male partner of an infected woman likely harbors the parasite himself. Concurrent treatment of the sexual partner(s) to prevent reinfection is essential.

4.4 Treatment

Metronidazole has been the drug of choice for the treatment of *T. vaginalis* infection since its development in 1959. Derived from the *Streptomyces* spp. antibiotic azomycin, metronidazole (1-(β-hydroxyethyl)-2-methyl-5-nitroimidazole) is a member of the nitroimidazole family of prodrugs whose metabolic products have been found to effectively eliminate infection by a number of protozoa and Gram-negative bacteria [89]. Other members of this family, including nimorazole, ornidazole, secnidazole, and tinidazole, are used throughout the world for the treatment of trichomoniasis. A nitroimidazole designated EU11100 was synthesized. This drug was shown to be both less toxic than metronidazole and effective at a lower concentration, but to date no clinical trials have been published [90].

Infants who contract *T. vaginalis* during vaginal delivery from an infected mother usually do not require treatment because infection generally resolves within a few weeks as the infant's (maternal) estrogen levels wane. However, if infection becomes symptomatic or progresses past the 6th week of life, metronidazole is generally administered. Treatment is often a single 50 mg/kg dose, or a 10–30 mg/kg dose daily for 5–8 days [91]. Canadian guidelines recommend a dose of 15–20 mg/kg, divided into three doses daily for 7 days, or a single dose of 40 mg/kg (to a maximum of 2 g) for the treatment of trichomoniasis in children.

Oral metronidazole is the treatment of choice for trichomoniasis in adults. The recommended regimens are a single 2 g dose oral metronidazole, a single 2 g dose oral tinidazole, or 500 mg oral metronidazole twice a day for 7 days [92, 93]. The single-dose treatment is preferred, as adherence is better than with multiple doses, and the overall amount of drug taken is reduced. However, the incidence and severity of side effects does increase slightly with the larger single dosage. Metronidazole can also be administered intravenously. This method is often utilized when patients show some intolerance to the drug, as side effects tend to be less severe than with oral treatment. Intravenous metronidazole is administered in a dosage of 500 mg to 2 g over 20 min [91].

A number of topical intravaginal preparations have been used to alleviate the symptoms of trichomoniasis in women. These medications include clotrimazole, nonoxynol-9, and povidone-iodine creams and gels, arsenical pessaries, furazolidone, paromomycin preparations, and both cream and insert metronidazole preparations. There are no topical treatments for trichomoniasis in men [20, 94].

The usefulness of non-nitroimidazole vaginal creams and inserts as a cure is doubtful, and no studies have shown definitive proof of efficacy [95]. However, these treatments are effective for relief of symptoms. The exception is hamycin, a drug related to amphotericin B. Currently in use in India as a topical treatment for trichomoniasis, hamycin has been found to effectively eliminate infection with both metronidazole-sensitive and -resistant strains of *T. vaginalis*. However, both clinical trials and in vitro testing on tissue culture have shown that the level of toxicity displayed by the drug toward eukaryotic cells makes it a poor choice of treatment [96].

Vaginal administration of metronidazole has been shown to be relatively ineffective as a cure, eliminating infection in up to 50 % of cases [97–99]. This is probably due to the fact that trichomonads are not always confined to the vagina, frequently invading Skene's, Bartholin's, and other glands, as well as the urethra [66]. Bioavailability of metronidazole as a vaginal suppository compared to IV infusion range from 20 to 56 % (oral metronidazole bioavailability is >90 % compared to IV infusion) [100]. As such, a topical vaginal medication is inadequate in completely eliminating infection. However, in cases of recalcitrant *T. vaginalis* infection, vaginal preparations are often added to the treatment regimen to

increase the chances of effecting a cure by increasing local drug concentration, and because of their comparatively lower risk of side effects (compared to oral administration) [97, 101]. A recent randomized controlled trial compared oral single-dose 2 g metronidazole versus a high-dose metronidazole and miconazole vaginal suppository (750 mg metronidazole/200 mg miconazole nitrate) twice a day for 7 days to treat *T. vaginalis*. The vaginal suppository resulted in similar efficacy of clinical and microbiological cure versus oral single-dose metronidazole (80 % versus 90 %, respectively) [102]. The sample size was small, but is evidence for a potentially useful combinatorial drug therapy for treatment of *T. vaginalis* infections in women that results in less systemic adverse effects compared to oral metronidazole.

Metronidazole regimens are generally well tolerated, and side effects are rarely of a severity that would necessitate discontinuation of metronidazole therapy. Common side effects include nausea and vomiting, headache, insomnia, dizziness, drowsiness, and rash. Patients taking oral metronidazole have also complained of dry mouth and metallic taste during the course of treatment. More serious side effects such as peripheral neuropathy, palpitation, confusion, eosinophilia, and leukopenia are rare, and seem to be associated with the nitroimidazole family. Cessation of therapy leads to mitigation of side effects, and no long-term adverse events have been identified in humans [28].

Cure rates for oral and intravenous metronidazole therapy of trichomoniasis range from 85 to 95 % on the first course of treatment. This rate increases if sexual partner(s) are treated simultaneously to prevent reinfection [91]. Partner treatment is highly recommended given the frequency of asymptomatic *T. vaginalis* infection.

Single-dose metronidazole treatment of *T. vaginalis* with concomitant bacterial vaginosis or HIV and nevirapine-based antiretroviral therapy have been associated with higher rates of treatment failure [103–106]. Multi-dose treatment with metronidazole, in these cases, should be considered while taking into account patient-specific risk of nonadherence.

5 Metronidazole Resistance

5.1 Mechanisms

Two proposed mechanisms for metronidazole resistance will be discussed. In both mechanisms metronidazole resistance is classified as aerobic or anaerobic. The first mechanism proposed involves metronidazole activation via hydrogenosomes [107–113]. The second mechanism is flavin reductase-based [17, 114–116]. Metronidazole enters *T. vaginalis* by passive diffusion wherein the drug is reduced by single and double electron transfers that result in production of toxic metabolites [107, 117]. Potential toxic radicals could be nitro radicals, nitrosoimidazole, or hydroxylamineimidazole [118]. However, the pathway for reduction of metronidazole to its active metabolites is still under debate. The target of the toxic metabolites is not clear. One target could be DNA, where transient binding of the active drug leads to disruption and breakage of chromosomal strands, and rapid cell death (within 5 h) [119]. The DNA of *T. vaginalis* contains about 71 % adenine and thymine residues, and these AT-rich regions are proposed to be both the site of metronidazole activity and the reason for the drug's specificity [120]. It is also possible that metronidazole metabolites target and disrupt proteins and protein trafficking [17, 109].

The first mechanism of metronidazole resistance in the hydrogenosome involves activity of enzymes proposed to be responsible for metronidazole activation. Within this organelle, the drug competes with hydrogenase (the terminal enzyme of pyruvate decarboxylation) for ferredoxin-bound electrons. Metronidazole is reduced and toxic metabolites via the formation of nitro radicals are produced [107, 117].

Aerobic resistance could be a result of impaired oxygen-scavenging mechanisms that lead to a decrease in the metabolism of metronidazole due to oxygen competition for ferredoxin-bound electrons. Increased oxygen concentration and reduction via ferredoxin leads to a decrease in the amount of metronidazole being reduced (i.e., less production of active metabolites), and the oxidation of metronidazole metabolites back into prodrug by oxygen and oxygen radicals (termed "futile cycling") [112, 121]. Decreased ferredoxin activity has also been implicated in aerobic resistance [113, 122], although oxygen-scavenging deficiency alone may be responsible [123]. Since metronidazole enters *T. vaginalis* through passive diffusion, reduced metabolism of the drug into its active form will result in less overall trafficking into the cell, and lower efficacy. Aerobic resistance is responsible for nearly all cases of clinically resistant trichomoniasis.

Anaerobic resistance develops when hydrogenosomal proteins involved in the reduction of metronidazole are downregulated or absent. Studies using laboratory-produced resistant strains of *T. vaginalis* and the related cattle infectious trichomonad, *Tritrichomonas foetus*, have shown that the transcription of ferredoxin, PFOR, and hydrogenase is drastically reduced or completely eliminated in highly resistant strains [110, 111]. Anaerobically resistant *T. foetus* strains often have modified hydrogenosomes that are significantly smaller than those found in metronidazole-sensitive trichomonads, presumably reflecting their decreased activity [111]. Reduced hydrogenosome size has only been demonstrated in laboratory-induced metronidazole-resistant strains of *T. vaginalis* and not in clinically resistant or susceptible strains [124]. Unlike aerobically resistant trichomonads, which use oxygen to detoxify metronidazole, anaerobically resistant *T. vaginalis* is extremely sensitive to oxygen and may survive only in an anaerobic environment. It is

hypothesized that this is because PFOR and hydrogenase have roles in protecting the trichomonad from reactive oxygen radicals. In addition, *T. vaginalis* possesses hydrogenosomal oxidase- and peroxidase-reducing enzymes that help protect the parasite from cell damage due to toxic oxygen species [108]. Reduction of hydrogenosomal function may lead to a downregulation in the activity of enzymes that protect *T. vaginalis* from oxygen stress. The extreme sensitivity of anaerobically resistant *T. vaginalis* to oxygen likely explains why such strains are rarely involved in disease, as the urogenital environments of men and women are aerobic and microaerophilic, respectively.

A second mechanism of resistance has been proposed because PFOR-mediated activation of metronidazole which requires ferredoxin was unaffected by knock-out of ferredoxin genes and the modified strain remained sensitive to metronidazole [17]. In the second mechanism of metronidazole resistance, Leitsch and colleagues have reported on cytosolic flavin reductase (FR), previously named NADPH oxidase, which is a key enzyme for flavin-mediated redox reactions in *T. vaginalis* [17, 114–116].

Diminished or absence of FR activity has been documented in clinical metronidazole-resistant *T. vaginalis* isolates [115]. Mutations of FR induced in vitro have led to metronidazole resistance in sensitive isolates [17]. Additionally, the authors propose that changes in ferredoxin, PFOR, and hydrogenase activity could be a result of reduced activity of flavin-mediated redox reactions. Therefore, changes observed in the first mechanism described do not induce metronidazole resistance, but are a result of resistance [17, 114].

In a study to determine the role of flavin-mediated redox reactions, resistance to metronidazole was attained following the use of diphenyleneiodonium (DPI) flavin inhibitor on *T. vaginalis* isolates grown under anaerobic conditions. The trichomonads were not viable when treated with DPI under microaerobic or aerobic conditions [114] and so these findings could not be applied to aerobic resistance. Whether thioredoxin reductase that was completely inhibited by DPI or FR that was nearly completely inhibited by DPI was responsible for induced metronidazole resistance was unclear. In another study, assays of clinically resistant isolates have demonstrated reduction of FR activity, rather than thioredoxin reductase of which the activity remained unaffected compared to metronidazole-sensitive isolates [115]. Yet, the clinically resistant isolates were aerobically resistant rather than anaerobically resistant and levels of FR were not consistently directly associated with levels of aerobic resistance [115].

Leitsch and colleagues [116] identified seven full length genes of FR, denoted *FR1-FR7*. FR1 activity was significantly impaired in metronidazole-resistant strains. In a laboratory-induced anaerobically resistant strain, C1res, and a clinical anaerobically and aerobically resistant strain, B7268, FR1 activity was absent. Interestingly, sensitivity to metronidazole under aerobic conditions was mostly restored in B7268 when a plasmid carrying a functional *FR1* gene was transfected. This finding is evidence of a role of FR in aerobic resistance, which was not elucidated by the DPI inhibitor study.

Impairment of oxygen-scavenging mechanisms described above remains an explanation for aerobic metronidazole resistance. The mechanism that leads to impairment of oxygen scavenging is unclear. Flavin reductase and NADH oxidase are the only two known oxygen-scavenging mechanisms of *T. vaginalis*. Metronidazole impairs NADH oxidase function [116]. Thus isolates with impaired FR function and treated with metronidazole under aerobic conditions accumulate intracellular oxygen that causes futile cycling of metronidazole [116, 125]. Futile cycling results in restoration of the parent drug, metronidazole, eliminating toxic metabolites. Still, further studies are required to elucidate the role of flavin-mediated redox pathways and ascertain a direct mechanism of resistance.

Other mechanisms of resistance that have been proposed include malate-dependent electron transport within the hydrogenosome, single nucleotide polymorphisms in nitroreductase genes, and inactivation by hydrogenosomal iron-sulphur flavoproteins [126–128]. Lastly, there is a lack of data to explain differences in cross-resistance of metronidazole and tinidazole.

5.2 Diagnosis of Resistance

Infection with metronidazole-resistant *T. vaginalis* is generally suspected when two standard courses of treatment fail to cure, and noncompliance and reinfection can be ruled out. Current estimates are that 2–6 % of cases of trichomoniasis will be caused by parasites with some degree of resistance to metronidazole [59, 129–133]. Rates in specific regions can be significantly higher; a study of prevalence of in vitro metronidazole resistance in Papua New Guinea reported detection of metronidazole resistance in 17.4 % of 23 cases examined [134]. Low or moderately resistant trichomonads are the cause of most recalcitrant infections, although highly resistant organisms have also been isolated from clinical samples.

Metronidazole susceptibility tests for *T. vaginalis* are similar to drug susceptibility assays for other microorganisms. Susceptibility testing usually follows the procedure reported by Meingassner and Thurner [130]. A number of samples of axenic medium containing a range of metronidazole concentrations (0.2–400 μg/mL) are prepared. The trichomonal isolate is then inoculated into each drug-medium sample and incubated, for at least 48 h. Metronidazole susceptibility can then be assessed by calculating the minimum inhibitory concentration (MIC) and/or minimum lethal

concentration (MLC) of drug for the organism. Inhibitory and lethal concentrations are obtained by observing the parasites for motility after the incubation period. The samples containing immobile trichomonads are then inoculated into fresh drug-free medium, incubated (again for at least 48 h), and reexamined for live cells. The MIC is the lowest metronidazole concentration at which nonmotile parasites survived (i.e., proliferated after the second inoculation). The MLC is the lowest concentration at which all trichomonads were killed (i.e., no growth on secondary inoculation).

In vitro metronidazole susceptibility testing is usually performed under aerobic conditions. This is partly because aerobic testing better reflects the environment in which *T. vaginalis* infection is found, and partly because anaerobic testing does not always accurately reflect clinical presentation [135]. In addition, MIC and MLC values can be over five times higher in aerobic testing compared to anaerobic [22], thereby allowing better discrimination of the resistance results.

Currently, there is no standard in vitro assay for the determination of *T. vaginalis* susceptibility to metronidazole. Different researchers favor various techniques, under different conditions (aerobic vs. anaerobic), to calculate different results (MIC vs. MLC). A survey of the literature on aerobic susceptibility testing shows that a strain of *T. vaginalis* having an MIC lower than 10 μg/mL, or an MLC lower than 50 μg/mL is generally considered metronidazole susceptible. A trichomonad with an MLC of >400 μg/mL (MIC of >50 μg/mL) would represent a highly drug-resistant strain of the parasite. Unfortunately, there is no direct correlation between the results of in vitro susceptibility assays and recommended dosages for clinical metronidazole treatment [136]. In vitro testing does not necessarily reflect the level of in vivo metronidazole susceptibility of a clinical isolate or predict outcome of treatment [132, 137, 138]. Thus there can be difficulty in determining a continuing course of therapy if primary treatment fails [139]. However, in one study the majority of patients treated according to metronidazole susceptibility results were cured following the use of susceptibility testing results [137]. Susceptibility testing is not routinely available in most diagnostic laboratories.

5.3 Standard Treatment After Initial Treatment Failure

Infection caused by metronidazole-resistant *T. vaginalis* can often be cured with increased doses of the drug and an extended course of therapy. Standard dosages following treatment failure include 500 mg oral metronidazole twice a day for 7 days, or 2 g oral metronidazole or tinidazole once a day for 5 days. Not surprisingly, there is a greater rate of adverse events associated with an increased (often double) treatment dose. In an attempt to limit side effects, treatment of refractory infection often combines oral and vaginal metronidazole therapy, or involves intravenous administration of the drug [140]. Some success has also been reported in a combination of standard metronidazole treatment and arsenical or clotrimazole pessaries, or zinc sulfate or betadine (povidone-iodine) douches [139, 141, 142]. Although evidence as to the efficacy of these therapies as cures is somewhat anecdotal, it is known that the treatments do ameliorate the symptoms of acute trichomoniasis.

Cases of highly drug-resistant *T. vaginalis* infection are difficult to resolve, as very high doses of metronidazole are toxic to the patient. With no alternatives to nitroimidazole drugs available, patients suffering from recalcitrant trichomoniasis are sometimes resigned to recurrent infection, relying on palliative measures to control symptoms. Fortunately such cases are infrequent. Overall, the cure rate for refractory trichomoniasis is 80 % for the first course of extended/combined therapy, assuming patient compliance and no reexposure [143]. The Centers for Disease Control and Prevention recommends consultation with a specialist and susceptibility testing for recalcitrant *T. vaginalis* infections [93].

5.4 Alternative Treatments for Metronidazole-Resistant Infections

There are very few therapeutic alternatives for the treatment of *T. vaginalis* infection. The 5-nitroimidazole family of drugs represents the only therapies currently proven to safely and effectively treat trichomoniasis. Of the nitroimidazoles, metronidazole and tinidazole have superior trichomonicidal activity, with most studies showing tinidazole to have a cure rate equal to that of metronidazole, but being effective at a slightly lower dosage (1.5 g single dose) [143–146]. Reports of high dose oral tinidazole in combination with intravaginal treatments such as tinidazole, clotrimazole, paromomycin, or ampicillin have demonstrated cure of recalcitrant infections [147–150].

A comparison of in vitro susceptibility of resistant isolates to metronidazole and tinidazole showed a strong correlation between metronidazole resistance and tinidazole resistance ($r=0.8709$, $P<0.0001$) [151]. Therefore, there is a definite need for non-nitroimidazole-based treatments.

An intravaginal preparation, paromomycin, has reported cure of 15 of 29 patients with recalcitrant trichomoniasis [147, 150, 152–158]. Two patients with metronidazole-resistant *T. vaginalis* infections responded to combination therapy of high-dose oral tinidazole combined with paromomycin cream intravaginally for 2 weeks. Unfortunately, as was the case with hamycin (mentioned previously), side effects have been noted that include pain and ulceration of the genital mucosa, making it unlikely that paromomycin is an ideal treatment alternative [156, 159].

Povidone-iodine has failed to cure recalcitrant *T. vaginalis* in three patients [148, 160, 161]. Cure was reported for two patients [162, 163]. Combination of povidone-iodine pessaries with intravaginal metronidazole cured two patients [162]. Povidone-iodine failed as alternative treatment to overcome nitroimidazole allergy in one case [164]. Eight and 30 % of patients that failed "orthodox" treatment also failed povidone-iodine treatment in a study that compared two durations of povidone-iodine treatment [165].

Arsenic had been used as a treatment for trichomoniasis before metronidazole was available. Acetarsol (arsenical pessaries) cleared metronidazole-resistant *T. vaginalis* infection in 4/6 patients reported from four case reports [141, 142, 147, 157].

Acidification of the vagina using acetic acid or boric acid has been reported in a handful of case reports. Based on five patient cases, acetic acid has not been reported to provide relief of infection [152, 158, 160, 166]. Multiple rounds of boric acid were required for microbiological cure in two patients [152]. One successful treatment with boric acid was reported as an alternative treatment due to metronidazole allergy [164].

Evidence for the use of nonoxynol-9 for recalcitrant *T. vaginalis* infections is limited. Two curative and two failed treatments have been reported [147, 157, 160, 162]. There are three reported failures and one success of combination of furazolidone and nonoxynol-9 as an alternative treatment for allergy to metronidazole and metronidazole-resistant *T. vaginalis* [148, 149, 153, 164]. The best evidence that suggest lack of efficacy comes from a randomized trial that reported 17.6 % cure of metronidazole-sensitive *T. vaginalis* infections using nonoxynol-9 versus 100 % cure rate using metronidazole [167].

Nifuratel and furazolidone are nitrofuran-class drugs. Nifuratel has not been licensed for use in the United States, but is used as a gynecological treatment of trichomoniasis in other regions. The efficacy of nifuratel has been reported in studies from the 1960s and 1970s with variable efficacy (38–80 %) [168, 169]. Recent studies have reported effectiveness in vitro and in vivo [170, 171]. Goodhew and Secor [170] noted contact dermatitis as an adverse reaction. Also, Evans, and Catterall [172] reported three adverse events of facial rash and generalized urticaria. However, a randomized trial by Mendling et al. [171] reported non-inferiority of nifuratel and a comparable safety profile. Furazolidone, despite in vitro activity, is unlikely to provide microbiological cure of *T. vaginalis* infections [20, 137, 143, 148, 149, 153, 164, 173, 174]. Furazolidone is contraindicated for use during pregnancy and is also not approved for use in the United States due to genotoxic and carcinogenic effects [175, 176]. Despite numerous reports of treatment failures with furazolidone, this drug has been used as a last resort in cases of recalcitrant *T. vaginalis* infections that have failed other alternative treatments.

A number of compounds containing nitro groups similar to nitroimidazoles have been investigated for activity against *T. vaginalis*. Nitazoxanide is a 5-nitrothiazolyl proven to be active against a broad spectrum of parasites in vitro. The drug was shown to exhibit trichomonicidal activity against both metronidazole-sensitive and -resistant strains. In addition, the drug has been shown to have low toxicity (at least in vitro) [124, 177, 178]. Nitazoxanide treatment of *T. vaginalis* has no reported successes [148, 173]. Analysis of the nitrothiazole derivative, niridazole, has shown it to possess multiple modes of action that contribute to broad-spectrum antimicrobial activity. Although specific mechanisms of action have not yet been elucidated, both metronidazole-sensitive and -resistant strains of *T. vaginalis* were found to be inhibited by the drug [179]. However, toxicity is a major concern and no reports of niridazole treatment of *T. vaginalis* were found. Sulfimidazole possesses two functional groups: a sulfonamide and a 5-nitroimidazole. In vitro testing has shown the drug to be effective against both aerobic and anaerobic bacteria, and metronidazole-sensitive and -resistant *T. vaginalis*. It should be noted however that MLCs for resistant trichomonads were approximately five times higher than for sensitive strains, potentially reflecting *T. vaginalis* resistance to the activity of the 5-nitroimidazole group [180]. Drugs with sulfonamide groups have had very limited treatment success [160, 181].

Disulfiram, a drug used to treat alcoholism, and its metabolite ditiocarb have shown in vitro trichomonicidal activity against both metronidazole-sensitive and -resistant strains of *T. vaginalis*. This is interesting since metronidazole can induce reactions similar to those of disulfiram, specifically nausea and vomiting, if taken with alcohol [170, 182].

This review does not provide treatment guidelines for patients infected with metronidazole-resistant *T. vaginalis* infections nor has an extensive list of all anecdotal treatments reported in literature. Some of the cases reported above and the dosages of the successful treatment have been summarized by Seña et al. [95]. However the successful case reports may be influenced by the previously failed regimens, combinations of therapy use and patient not returning for a late follow-up who had initial symptomatic improvement.

A vast number of studies, beyond the scope of this chapter and the highlights provided above, have been published that report in vitro susceptibility of *T. vaginalis*. For example, a screening of 1040 drugs from the US Drug Collection Library was conducted by Goodhew and Secor [170]. Two non-nitroimidazole drugs, disulfiram and nithiamide, were identified that had the best efficacy to inhibit growth of *T. vaginalis* in vitro, but were not as effective as metronidazole. Other drugs, plant-derived and microorganism-derived products tested in vitro have been summarized by Seña et al. [95]. Although preliminary in vitro research has been conducted on the trichomonicidal activity of a large number of

drugs, testing rarely proceeds to clinical trials. With rates of metronidazole-resistant *T. vaginalis* infection on the rise and current alternative treatments being unreliable, it is imperative that effective alternative therapies become available.

5.5 Infection Prevention

Infection control of sexually transmitted trichomoniasis is the same as for other STIs. Condoms are effective in preventing the spread of disease, and reduced transmission has been shown in women using either oral (hormonal) or prophylactic vaginal (i.e., nonoxynol-9) contraception [183]. Circumcision has not been proven to be an effective method to prevent *T. vaginalis* infections in males, but male circumcision may indirectly reduce prevalence in females (prevalence risk ratio 0.52, 95 % CI 0.05–0.98) [44, 184].

As *T. vaginalis* parasites can be passed from mother to newborn during vaginal birth, treatment of pregnant women to prevent perinatal infection is an option. Previously there have been concerns about metronidazole teratogenicity, based on studies showing mutagenicity in bacteria and carcinogenicity in mice [185, 186]. This led to the reluctance to treat pregnant women, or to limiting treatment to the second or third trimester. Several meta-analyses have shown, however, that children born to mothers treated with metronidazole showed no increase in birth defects compared to controls [187–190]. Additionally, it would seem beneficial to treat infections because there is a proven association between trichomoniasis and pregnancy complications such as preterm labor and low birth weight infants. Paradoxically, treatment may carry a risk of increased preterm labor. Four studies have been conducted that report on pregnancy outcomes following metronidazole treatment [191–194]. Generalizability of the findings is impeded by each study using a cohort with distinct population characteristics. Nonetheless, a recommendation to treat pregnant women infected with *T. vaginalis* is complicated by one of the four studies reporting a significant increase in risk of preterm labor. The other three studies report no significant change in risk of preterm labor. All four studies report no significant change in risk of low birth weight deliveries [195, 196].

Currently, there is no vaccine available against *T. vaginalis* infection and sufficient criteria for the infection to be reportable have not been met [197, 198]. However, the existence of a successful vaccination model in mice [46, 199], as well as a vaccine already commercially available for prevention of related *T. foetus* infection in cattle [200, 201], gives hope that eventually the disease will be preventable. Given the relationship between trichomoniasis and other STIs, especially HIV, the development of a vaccine would be an excellent step in preventing morbidity and mortality due to this and other sexually transmitted infections.

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Drug Resistance in Leishmaniasis

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Shyam Sundar and Jaya Chakravarty

1 Introduction

Leishmaniasis, a vector-borne disease, is caused by an obligate intracellular protozoan of the genus *Leishmania*, order Kinetoplastida, family Trypanosomatidae and is transmitted by the bite of female sand fly vectors. Clinical manifestations range from self-healing cutaneous ulcers to systemic multiorgan disease. It broadly manifests as visceral leishmaniasis (VL; also known as kala-azar), cutaneous leishmaniasis (CL), and mucocutaneous leishmaniasis (MCL). VL is caused by the *Leishmania donovani* complex: *L. donovani*, the causative organism of VL in the Indian subcontinent and Africa; *L. infantum* (*L. chagasi*) which causes VL in the Mediterranean basin, Central and South America. CL is caused by various *Leishmania* species. Based on its geographical distribution, CL can be divided into Old World (OWCL) which includes southern Europe, the Middle East, parts of southwest Asia, Central Asia, and Africa. OWCL is caused by *L. aethiopica*, *L. donovani*, *L. infantum*, *L. major*, and *L. tropica*. New World cutaneous leishmaniasis (NWCL) occurs in Mexico and Latin America and is caused by multiple species of both the *Leishmania* subgenera: *L. amazonensis*, *L. infantum*, *L. mexicana*, *L. venezuelensis* and the *Viannia* subgenera: *L. braziliensis*, *L. guyanensis*, *L. panamensis*, *L. peruviana*. MCL is caused by New World *Leishmania* species *L. braziliensis* and *L. panamensis*. Diffuse CL, a severe form of CL, is caused by *L. aethiopica* in the Old World and *L. mexicana* and *L. amazonensis* in the New World [1, 2].

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2 Epidemiology

Leishmaniasis is endemic in 98 countries with more than 350 million people at risk. Approximately 0.2–0.4 million VL cases and 0.7–1.2 million CL cases occur each year. More than 90 % of global VL occurs in just six countries: India, Bangladesh, Sudan, South Sudan, Brazil, and Ethiopia. CL is more widely distributed, with about one-third of cases occurring in each of three regions, the Americas, the Mediterranean basin and western Asia from the Middle East to Central Asia. The ten countries with the highest estimated case counts, Afghanistan, Algeria, Brazil, Colombia, Costa Rica, Ethiopia, Iran, Sudan, Syria, and Peru, together account for 70–75 % of globally estimated incidence of CL [3]. HIV-VL coinfection has been reported from more than 35 countries. Initially, most of these cases were from south-western Europe, but the number of cases is increasing in sub-Saharan Africa, especially Ethiopia, Brazil, and South Asia [4–6].

The only proven vectors of human disease are sand fly of species *Phlebotomus* in the Old World (Asia, Africa, and Europe) and *Lutzomyia* in the New World (the Americas) [1]. Transmission is of two types: anthroponotic where the vector transmits the disease from infected to healthy humans and zoonotic where the vector transmits the disease from an animal reservoir to humans. In South Asia and the Horn of Africa, the predominant mode of transmission of VL is anthroponotic, and humans with kala-azar or post-kala-azar dermal leishmaniasis (PKDL) provide the major reservoir for transmission [2, 7, 8]. In the Mediterranean, the Middle East, and Brazil, VL is zoonotic, with the domestic dog as the most important reservoir host sustaining transmission [8]. Most CL have zoonotic transmission except those caused by *L. tropica*, which is predominantly anthroponotic. Reports from Europe have shown that transmission of the infection can occur via needle-sharing in HIV/VL coinfected patients in southern Europe, and threaten to convert an apparently zoonotic disease into the anthroponotic form [9, 10].

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Sand flies inoculate the promastigote form of the parasite into the skin of the host. In the human host, these are taken up by macrophages or the dendritic cells, where they transform into aflagellar amastigotes. The future course of infection and the type of disease produced depend upon the species of *Leishmania* and the immune response mounted by the host.

Visceral leishmaniasis, also known as "Kala-azar," is the systemic and most severe form, characterized by prolonged and irregular fever often associated with rigor and chills, splenomegaly, lymphadenopathy, hepatomegaly, pancytopenia, progressive anemia, and weight loss. If untreated, VL is uniformly fatal. These, coupled with poor sensitivity of immunological tests in these patients, pose considerable diagnostic difficulty. Patients with VL may develop a chronic form of dermal leishmaniasis characterized by indurated nodules or depigmented macules, and is called PKDL [11]. PKDL is quite common (occurring in >50 % patients with VL) in Sudan, and may occur concurrently with VL [11, 12]. In the Indian subcontinent it occurs only in a small proportion of patients, 6 months to several years after an episode of VL [13]. Spontaneous healing occurs in most patients in Sudan; however, in India treatment is considered necessary. Treatment of PKDL is difficult and requires prolonged courses of antileishmanial drugs irrespective of the geographical location. Patients with PKDL serve as an important reservoir of infection, and VL outbreaks have been linked to PKDL [14]. Leishmaniasis is also emerging as an important opportunistic infection in HIV-infected patients. In Ethiopia 10.4–40 % of VL patients were coinfected with HIV in different centers [15, 16]. HIV and *Leishmania* infection reinforce each other. HIV patients are more likely to develop visceral leishmaniasis (due to reactivation of a dormant infection or clinical manifestation after primary infection). Patients characteristically have high disseminated parasite loads. Visceral leishmaniasis negatively affects the response to antiretroviral treatment and is difficult to cure in coinfected patients, especially those with CD4+ counts <200 cells/μL, who typically relapse [1], thus making them another potential source for the emergence of drug resistance. The clinical features are usually similar to a classic VL patient; however at times there may be involvement of unusual sites, e.g., infiltration of skin, oral mucosa, gastrointestinal tract, lungs, and other organs, especially in patients with low CD4+ counts.

Cutaneous leishmaniasis is a major health problem in some countries [17–22]. In the Old World, it is caused by *Leishmania major*, and manifests as a papule, which enlarges and ulcerates producing painless ulcer with raised and indurated margin. Most patients have 1–2 lesions, which heal spontaneously, but occasionally lesions may be multiple and disabling with disfiguring scars, which create lifelong aesthetic as well as social stigma. *L. tropica* may cause persistent, spreading scarring lesions associated with exaggerated cellular hypersensitivity (leishmaniasis recidivans or lupoid leishmaniasis), and is a difficult problem to treat [23, 24].

New World CL is mainly zoonotic and is most often caused by *L. mexicana*, *L.* (*V.*) *panamensis*, *L.* (*V.*) *braziliensis*, and *L. amazonensis*. A wide range of forest animals act as reservoirs, and human infections with these species are predominantly rural. Diffuse cutaneous leishmaniasis (DCL), a rare syndrome produced by *L. aetheopica* and *L. amazonensis*, develops because of defective antigen-specific cell-mediated response [25, 26]. The lesions are chronic, disseminated, and nonulcerative. They never heal spontaneously, and relapses following treatments are quite frequent. MCL (espundia) produces extensive destructive lesion of nasopharyngeal mucosa. The disfiguring lesions lead to mutilation of the face. It is commonly caused by *Leishmania* species of the New World, such as *L. braziliensis*, *L. panamensis*, and *L. guyanensis*, but mucosal lesions have also been reported in the Old World due to *L. donovani*, *L. major*, and *L. infantum* in immunosuppressed patients [27, 28].

Usually, each species is true to type; but occasionally a dermatotropic species (e.g., *L. tropica*) may cause visceral disease, or the viscerotropic *L. infantum* may cause self-healing skin lesions [29]. Viscerotropic and dermatotropic strains of *L. infantum* can be distinguished by isoenzyme analysis. But this distinction breaks down in the face of HIV coinfection, in which many hitherto unknown zymodemes have been identified *L. braziliensis*, almost uniquely, has the capacity to produce secondary mucosal lesions of the nose and mouth [17].

3 Antileishmanial Agents and Drug Resistance

The armamentarium of antileishmanial is small and includes pentavalent antimonials, amphotericin B deoxycholate and its lipid formulation, paromomycin, miltefosine, azoles, and pentamidine. For several decades, pentavalent antimonials (Sb^v) have been the standard first-line drug for the treatment of leishmaniasis. In the recent years, drugs like oral miltefosine, and paramomycin and newer therapies like single-dose liposomal amphotericin B (L-AmB) and combination therapies have been added in the treatment of VL. At the same time, as these new therapies are becoming available, drugs like Sb^v and miltefosine are being threatened by the development of resistance [30].

3.1 History of Geographical Spread of Resistance

For more than 80 years, pentavalent antimonials (Sb^v) have been the sheet anchor for the treatment of leishmaniasis in every endemic region of the world. In most parts of the

world, about 98–99 % previously untreated patients with VL respond well to Sb^v. However, the endemic region for VL in North Bihar, India, has the unique distinction of being the only region in the world from where widespread failure to Sb^v has been reported [31, 32]. Sb^v had been the drug of choice for the treatment of VL in this region for several decades. Till the late 1970s, a small daily dose (10 mg/kg; 600 mg maximum) for short duration (6–10 days) was considered adequate, when unconfirmed reports suggested 30 % treatment failure with this regimen from four most severely affected districts: Muzaffarpur, Samastipur, Vaishali, and Sitamarhi [33]. An expert committee revised the recommendations to use Sb^v in two 10-day courses with an interval of 10 days [34], and improvements in cure rates (99 %) were noted [35]. However, only a few years later, another study noted 86 % cure rates with this regimen [36]. In 1984, a WHO expert committee recommended that pentavalent antimony be used in doses of 20 mg/kg/day up to a maximum of 850 mg for 20 days, and the repetition of a similar regimen for 20 days in cases of treatment failures [37]. Four years later, Thakur et al. evaluated the WHO recommendations and reported that 20 days' treatment with 20 mg/kg/day (maximum 850 mg) cured only 81 % of patients; however, on extending the treatment for 40 days, 97 % of patients could be cured [38]. Three years later, the same group noted a further decline in cure rate to 71 % after 20 days' treatment, and recommended an extended duration of treatment in nonresponders [39]. Jha et al. [40] found that extending the therapy for 30 days could cure only 64 % patients in a hyperendemic district of Bihar. From these findings it became clear that antimony refractoriness was on ascendancy, but the reports were sketchy and not under strictly controlled conditions. In two studies carried out under strictly supervised treatment schedules, we observed that only about one-third of the patients could be cured with the currently prevailing regimen [31, 41]. The incidence of primary unresponsiveness was 52 %, whereas 8 % of the patients relapsed. Incidentally, only 2 % of the patients from the neighboring state of (Eastern) Uttar Pradesh (UP) failed in the treatment [31]. There were reports of antimony resistance spreading to the Terai regions of Nepal, especially from the district adjoining the hyperendemic areas of Bihar, where up to 30 % of the patients seems to be unresponsive, though in Eastern Nepal a 90 % cure rate had been reported [41]. Thus, it was reconfirmed that a high level of Sb^v unresponsiveness existed in Bihar and its adjoining region while, the drug continued to be effective in other areas. In a study to determine whether acquired drug resistance was present in Bihar, *L. donovani* isolates were taken from responders and nonresponders. In vitro amastigote-macrophage assay showed that isolates from patients who did respond to sodium stibogluconate treatment were threefold more sensitive, with 50 % effective doses (ED 50) around 2.5 μg Sb/mL compared to isolates from patients who did not respond (ED 50 around 7.5 μg Sb/mL) [42]. The significant differences in amastigote sensitivity supported the concept of acquired resistance in Bihar.

HIV/VL coinfected patients respond poorly to Sb^v, as the drug needs an intact immune system to be effective, and the response is not as good as in immunocompetent patients. Initial parasitological cure with Sb^v could be as low as 37 % [43], and eventually most of the initially cured patients tend to relapse. A recent study from Ethiopia revealed only 43.9 % of HIV-VL coinfected patients were cured with SSG [44]. These relapsing patients may provide a human reservoir for resistant *Leishmania* with consequent emergence of primary resistance.

There is considerable variation in sensitivity to Sb^v among primary isolates from untreated patients with cutaneous leishmaniasis, which correlates with patients' response to treatment [45]. Primary resistance is quite uncommon, but resistance develops in patients with VL, CL, and MCL who have relapsed. Chances of response to further courses of antimonials diminish once there is a relapse after the initial Sb^v treatment [46].

Pentamidine isethionate was used as a second-line drug after widespread Sb^v failure in Bihar, with nearly 100 % cure rate for more than a decade despite being toxic [47]. Its most dreaded toxic effect was insulin-dependent diabetes mellitus in a significant proportion of patients [48]. In later studies, a decline in the efficacy from 100 to ~70 % was noticed. In the face of increasing unresponsiveness, and the associated serious toxicity, pentamidine fell into disrepute and its use was abandoned for the treatment of VL in the Indian subcontinent [49, 50]. While it is no longer used for VL, it is being used for NWCL due to *L. guyanensis* and *L. panamensis* with excellent results [51–53].

Amphotericin B (AmB), a polyene antibiotic, was used as a first-line therapy in areas with Sb^v resistance in Bihar, India. It has excellent cure rates (~100 %) at doses of 0.75–1.00 mg/kg for 15 infusions on daily or alternate days. It has been used extensively in Bihar with uniformly good results [54, 55]. To minimize the adverse events of amphotericin B, various lipid formulations have been introduced where deoxycholate is replaced with other lipids leading to less exposure of the free drug to the organs. Tolerance is greatly improved and adverse effects, including nephrotoxicity, are minimized which enables delivery of large doses of the drug over short periods of time. The dose requirement of liposomal amphotericin B (L-AmB) varies from region to region; while in the Indian subcontinent a small dose induces high cure rates, a higher dose is needed for Eastern Africa, the Mediterranean region, and Brazil [56–58].

Unresponsiveness and relapses after AmB occurs rarely, except among HIV-infected patients who tend to relapse frequently [59, 60]. In a HIV-VL coinfected patient with multiple

relapses treated with AmB, no resistance to AmB was seen in clinical isolates analyzed in vitro [59]. A rare case of Amphotericin B unresponsive *L.donovani* infection was reported from India. In vivo studies showed more than three times greater inhibition of AmB sensitive parasites compared to parasites with AmB relapse at a normal AmB dose in mice of 1.5 mg/kg body weight. Four single nucleotide polymorphisms (SNPs) were detected in this patient in the cysteine proteinase B gene affecting alignments in deduced amino acids [61]. The use of amphotericin B and its lipid formulations have dramatically increased, especially in the Indian subcontinent. Single dose of 10 mg/kg of liposomal Amphotericin B has recently been recommended as the preferred treatment for VL in the Indian subcontinent [1, 62]. With the increasing use of AmB in lipid formulations that have longer half-lives, the possibility of resistance cannot be ignored.

Miltefosine is an alkyl phospholipid (hexadecylphosphocholine) and the first oral antileishmanial agent registered for use in India from March 2002 following a Phase III trial in which 50–100 mg/day dose for 28 days resulted in a long-term cure rate of 94 % [63]. The efficacy of the drug varied with region; a study from Ethiopia showed that the final cure among non-HIV-infected patients 6 months after treatment in the miltefosine group was only 75.6 % [64]. The drug was chosen for the elimination program in India, Nepal, and Bangladesh for its ease of use and applicability in the control program [65]. The main concern with this drug was its long half-life of nearly a week which makes it vulnerable to develop resistance. Added to that frequent gastrointestinal adverse events, quick recovery (within 10 days most patients feel better) and absence of a directly observed treatment program are likely to drive the patients to prematurely discontinue the treatment and suboptimal compliance will ultimately lead to the development of parasite resistance [66]. Recent studies from the Indian subcontinent have shown that its efficacy has declined to 90.3 % and the relapse rate had doubled [67]. In a study from Nepal, 10.8 % and 20.0 % patients relapsed within 6 and 12 months after miltefosine treatment, respectively [68]. In another phase IV study from Bangladesh, enrolling 977 patients a final cure rate of 85 % could only be achieved [69]. Studies of miltefosine for OWCL are scarce; however, it has been used for NWCL, especially of *L. guyanensis* and *L. panamensis*, with good results [2].

Paromomycin (PM) is a broad-spectrum aminoglycosidic aminocyclitol belonging to the neomycin family. A Phase III trial in which a dose of 15 mg/kg PM sulfate (11 mg base) for 21 days gave a cure rate of 95 %, and was approved by the Indian government in August 2006 for the treatment of patients with VL [70]. Encouraged by this, a large Phase III study was done in Sudan, Ethiopia, and Kenya comparing the efficacy of PM alone at the dose shown to be efficacious in India against sodium stibogluconate (SSG) alone (20 mg/kg/day for 30 days) and a combination treatment of SSG and PM for 17 days. The overall efficacy of PM alone was significantly lower than SSG and it had to be discontinued [71]. Topical preparations of paromomycin, a soft paraffin-based ointment containing 15 % of paromomycin and 12 % methylbenzethonium chloride (MBCL), are effective against both Old World and New World CL [72]. A meta-analysis of 14 randomized controlled trials showed that in Sb^v-controlled trials, the efficacy of topical PM was not significantly different from that of intralesional Sb^v in the Old World CL (relative risk [RR] = 0.70; 95 % CI: 0.26–1.89), whereas topical PM was inferior to parenteral Sb^v in treating the New World CL (RR = 0.67; CI: 0.54–0.82) [73]. Clinical resistance to PM has not been reported most probably due to its limited use; however, being an aminoglycoside PM is at an increased risk of developing resistance, thus it is imperative to monitor treatment and avoid its use as monotherapy.

Azoles block ergosterol synthesis of *Leishmania* parasites. Ketoconazole, itraconazole, and fluconazole have all been used for CL in several studies. For NWCL due to *L. braziliensis* fluconazole, at the dose of 8 mg/kg per day, cured 100 % patients [74]. Ketoconazole at 600 mg/day for 28 days was 76 and 89 % effective in *L.* (*V.*) *panamensis* and *L. mexicana* CL in Panama and Guatemala but not effective for *L. braziliensis* [75, 76]. For OWCL Ketoconazole at a dose of 600 mg/day for adults and 10 mg/kg/day for children for 4–6 weeks obtained 89 and 80 % efficacy in Iran and Kuwait while it was ineffective in Turkey [77–79], while fluconazole showed 81 % cure rate for *L. Major* [80]. Clinical resistance to azoles in leishmaniasis has not been reported.

3.2 Clinical Significance of and Epidemiological Reasons for Drug Resistance

The reason for the emergence of antimony resistance was the widespread misuse of the drug which included free availability of drugs, use of drug by unqualified medical practitioners and inappropriate and inadequate dose. It was a common practice to start with a small dose and gradually build up the dose over a week. Drug-free intervals are given on the belief that it will prevent renal toxicity. Many a times, the daily dose of drug was split into two injections, given twice daily. These practices resulted in build-up of subtherapeutic blood levels of the drug, leading to progressive tolerance of the parasite to Sb^v. It was observed that only a minority (26 %) was treated according to the prescribed guidelines, and irregular use and incomplete treatments were common occurrences [81]. Almost half of the patients, receiving pentamidine as a second-line drug, had not received adequate antimony treatment before being labelled as refractory to Sb^v. These facts indicated large-scale misuse of antileishmanial drugs in Bihar, contributing to develop-

ment of drug resistance. There were several manufacturers of Sb^v in India, and quality of products was inconsistent, resulting in occasional batches being substandard and toxic, this added to the problems associated with Sb^v therapy causing serious toxicity and deaths related to the drug [82, 83].

Leishmania do not develop resistance to Sb^v spontaneously, unless they are subjected to drug pressure. In an experimental model, the parasite that was maintained in vitro passage in NNN media and posterior passage in hamster did not lose sensitivity to Sb^v [84]. However, resistance can be induced in the promastigote by repeated in vitro passage of the parasite with step-wise increase in concentration of Sb^v in the culture media [85]. The in vitro sensitivity also decreases progressively in relapsing patients [86]. There are clear indications that Sb^v resistance is a consequence of the exposure to a subtherapeutic dose of Sb^v. Though the in vitro data suggest that increasing the dose of Sb^v could overcome the unresponsiveness to a great extent, unfortunately further increase in the quantity of the drug would seriously jeopardize the safety of the patients [87]. Primary resistance emerges where man is the reservoir of infection, transmission is anthroponotic and intense, and there is a large biomass of parasite. In human-to-human (anthroponotic) transmission such as in the Indian and African subcontinents, once resistance gets established, it spreads exponentially and organisms sensitive to the drug get eliminated quickly, whereas the drug-resistant parasites continue to circulate in the community. There are no reports of either primary resistance or decline in the efficacy of Sb^v from other endemic foci of VL with canine reservoirs such as in Brazil and Southern Europe.

As for miltefosine, although the relapse rate of patients has increased in the Indian subcontinent, decrease in the susceptibility of parasites to miltefosine in vivo, a precursor of the emergence of drug resistance, has not yet been observed [68, 88, 89]. The plasma concentrations in cured and relapsed Nepalese patients were similar, indicating a similar exposure to miltefosine [68]. Another recent study from Nepal revealed that increased infectivity of the parasite is associated with miltefosine relapse [90]. Young age and male gender were associated with increased risk of VL relapse after miltefosine, suggesting that the mechanism of relapse is mainly host-related, i.e., immunological factors and/or drug exposure [91]. Achieving a sufficient exposure to miltefosine was found to be a significant and critical factor for VL treatment success, suggesting an urgent need to evaluate the recently proposed optimal allometric miltefosine dosing regimen [92]. Dorlo et al. demonstrated that children are significantly less exposed to miltefosine than adults when receiving a similar 2.5 mg/kg/day dosage of miltefosine [93] and proposed a new dosing algorithm to solve this apparent difference in drug exposure between age and body-size groups.

Unresponsiveness and relapses after AmB occurs rarely, except among HIV-infected patients who tend to relapse frequently [59, 60]. Rare cases of Amphotericin B unresponsive *L.donovani* infection has been reported from India [61, 94]. Single dose of 10 mg/kg of liposomal Amphotericin B has recently been recommended as the preferred treatment for VL in the Indian subcontinent [1, 62]. With the increasing use of AmB in lipid formulations that have longer half-lives, the possibility of resistance cannot be ignored.

Clinical resistance to PM has not been reported most probably due to its limited use, but being an aminoglycoside PM is at an increased risk of developing resistance.

3.3 Mechanism of Drug Resistance

Understanding the mechanism of drug resistance is crucial for preventing, monitoring, and reverting it. Unfortunately, little is known about the mechanism underlying the drug resistance as seen in human VL. However, there has been some insight into the possible mechanisms of resistance and characterization of probes for its detection using resistant mutants developed in the laboratories largely applying drug pressure.

Antimonial resistance is multifactorial. Reduced uptake of the drug, increased intracellular thiol levels, sequestration and rapid drug efflux are some of the mechanism known for antimony resistance. In *Leishmania*, aquaglyceroporin1 (AQP1), member of the aquaporin superfamily has been shown to facilitate uptake of Sb^{III} [95, 96]. Overexpression of AQP1 in *L. major* (LmAQP1) produces increased susceptibility to Sb^{III}, whereas gene deletion renders the parasite resistant [95, 97]. Studies on clinical isolates from India and Nepal indicated downregulation of AQP1 [98–100]. While, AQP1 RNA levels remained unaltered in resistant isolates of *L. braziliensis* and *L. guyanensis* [101, 102] and did not show consistent downregulation in another study from India [103].

Arsenite- or antimony-resistant laboratory mutants of all *Leishmania* species exhibit significantly increased levels of intracellular thiols, namely cysteine, GSH, and trypanothione (TSH), suggesting a role for thiols in resistance [99, 104]. The synthesis of two precursors GSH and spermidine determines the level of TSH. The γ-GCS gene encoding γ-glutamylcysteine synthetase, which catalyzes the rate-limiting step in GSH biosynthesis, has been found to be amplified in arsenite-resistant *L. tarentolae* [105], while the gene ODC which encodes ornithine decarboxylase, an enzyme involved in the regulation of spermidine biosynthesis, was also overexpressed [106, 107]. However, in clinical isolates results were variable. γ-GCS was neither amplified nor upregulated in *L. donovani* isolated from Sb^V-resistant patients from India [108, 109], but downregulation of γ-GCS

was observed in Nepalese isolates [99, 100]. In *L. guyanensis*, γ-GCS was overexpressed in therapeutic failure isolates [110]. Similarly, the precursor protein of spermidine biosynthesis, ODC was amplified at the genetic and protein levels in Indian *L. donovani*-resistant isolates [98, 109] and in *L. braziliensis* [101], but the gene was downregulated in isolates from Nepal [99]. Studies have shown that antimony-resistant isolates downregulate the expression of γ-GCS of macrophages, probably by downregulating host NFkB, which is known to regulate γ-GCS expression [110]. This would result in the reduction of intramacrophage GSH levels and promote an intracellular oxidative environment, thereby minimizing the intramacrophage reduction of Sb^{v} to its toxic form Sb^{III} [111]. This indicates that SAG resistance in *L. donovani* is associated with manipulation of both host and parasite thiol levels.

Another pivotal enzyme of the thiol metabolism responsible for maintaining the intracellular reducing environment through trypanothione is Trypanothione reductase (TR). Studies have shown increased RNA levels as well as enzyme activity of TR in Sb^{v}-resistant isolates of *L. donovani* [98] and Sb^{v}-resistant clinical isolates of *L. braziliensis* [101].

The ATP-binding cassette (ABC) superfamily of proteins has been widely reported to export xenobiotics [112, 113] outside the cell. These include the P-glycoprotein (P-gp) and multidrug resistance related protein (MRP). The first ABC transporter identified and characterized in *leishmania* was MRPA which was shown to confer antimony resistance by sequestering thiol-metal conjugates in an intracellular vesicle [114] rather than increased efflux. Upregulation of MRPA gene was observed in *L. donovani* isolates only from India [98, 108, 109] but neither from Nepal nor in *L. braziliensis* and *L. guyanensis* [101, 102].

However, *L. infantum* isolates from a HIV/VL coinfected patient on miltefosine maintenance showed a gradual decrease of the miltefosine susceptibility with the IC_{50} values of miltefosine increasing over time, from 5.00 to 50.10 μmol/L. and on sequencing the entire LdMt gene a new SNP, L832F, was found in the miltefosine-resistant strain which reverted back to the wild-type allele 3 years after withdrawal from miltefosine [115].

Miltefosine resistance can be easily induced in vitro. The transport of miltefosine over the parasite cell membrane is thought to be facilitated by a putative *L. donovani* miltefosine transporter (LdMT) and the protein LdRos3. It was shown that decreased miltefosine accumulation and defective inward translocation was the major determinant of decreased susceptibility [116], which was demonstrated to be mediated through inactivation of LdMT and LdRos3 [117–119]. It was shown that decreased miltefosine accumulation and defective inward translocation was the major determinant of decreased susceptibility [116], which was demonstrated to be mediated through inactivation of LdMT and LdRos3 [117–119]. In clinical isolates, low expression of the LdMT-LdRos3 complex was correlated to the natural nonsusceptibility to miltefosine of *L. braziliensis* strains. Increased efflux of miltefosine has also been implicated in miltefosine resistance, mediated through the overexpression of an ABC transporter: the *Leishmania* P-glycoprotein-like transporter (*Leishmania* ABCB1 or LtrMDR1) [120, 121] and *Leishmania*-specific ABC subfamily G-like transporters (LiABCG6 and LiABCG4 half-transporters) [122, 123]. Miltefosine elicit its effects by mitochondrial dependent-programmed cell death associated with generation of reactive oxygen species (ROS). It has been reported that *Leishmania donovani* mitochondrial iron superoxide dismutase-A (LdFeSODA) overexpression protects parasites from miltefosine by protecting the mitochondria of *Leishmania* from oxidative stress, thereby inhibiting programmed cell death [124]. Gene expression levels for LdFeSODA was 5.3-fold higher in MIL-resistant phenotypes upon drug treatment than the sensitive strains [125].

An amphotericin B-resistant clinical isolate which demonstrated eightfold-higher 50 % lethal doses (LD50) than an amphotericin B-sensitive strain altered membrane composition where ergosterol was replaced by cholesta-5,7,24-trien-3β-ol in the membrane of the resistant parasite, ATP-binding cassette transporters, and upregulated thiol metabolic pathway [94, 126]. Similar findings were observed in a laboratory-derived AmB-resistant *Leishmania* promastigote, where ergosterol was replaced by a precursor, cholesta-5,7,24-trien-3-ol [127].

In a line selected for resistance to paromomycin showed reduced accumulation of the drug associated with a significant reduction in the initial binding to the cell surface. The drug induced reduction in membrane potential and inhibition of protein synthesis were less pronounced in the resistant strain in comparison to the wild type [128]. In another study comparative proteomic analysis of the wild type and the paromomycin-resistant *L. donovani* strains showed upregulation of the ribosomal proteins in the PM-resistant strain (PRr) which could be one of the mechanisms utilized by the resistant parasites as a defense against PM. Upregulation of proteins that may have a role in intracellular survival and vesicular trafficking in the PRr strain was observed. Ultrastructural analysis by electron microscopy demonstrated increased number of vesicular vacuoles in PRr strain when compared to the wild-type strain. Vesicular sequestration of PM into the vacuoles by these resistant parasites might be involved in conferring the resistance phenotype [129].

Pentamidine-resistant promastigote clones of *L. donovani* and *L.amazonensis* were shown to have 18- and 75-fold reduced uptakes, respectively, and increased efflux [130].

3.4 Treatment Alternatives

With the growing treatment resistance in the Indian subcontinent there was a look out for alternative treatment which were effective, less toxic, simple to administer even in the periphery, and cost effective. In March 2007, a preferential pricing agreement with WHO (agreement between Gilead and WHO) reduced the price of L-AmB for endemic regions of developing countries to $18 per 50 mg vial [131]. Encouraged by this preferential pricing and the low dose of L-AmB required to cure VL in India a single dose of 10 mg/kg of body weight L-AmB was compared to the conventional amphotericin B deoxycholate administered in 15 infusions of 1 mg/kg, given every other day during a 29-day hospitalization. Cure rates at 6 months were similar in the two groups: 95.7 % (95 % confidence interval [CI]: 93.4–97.9) in the liposomal therapy group and 96.3 % (95 % CI: 92.6–99.9) in the conventional therapy group [62]. The preferential pricing, along with a single day of hospitalization, makes a single infusion of the liposomal preparation an excellent option for this region. This regimen was further tested in primary health centers in Bangladesh where the cure rate at 6 months was 97 % [132]. There was high acceptability of this regimen in Bangladesh; however, strengthening of infrastructure was required for its implementation in the sub-district level as the drug requires a cold chain [133]. Encouraged by the success of the single-dose L-AmB therapy in the Indian subcontinent a randomized controlled trial was done to compare the efficacy and safety of single dose of L-AmB 7.5–10 mg/kg body weight, or multiple doses, seven times 3 mg/kg on days 1–5, 14, and 21 in East Africa. However, the trial was terminated after the third interim analysis because of low efficacy of all the regimens. Definitive cure was 85 %, 40 %, and 58 % in patients treated with multiple doses, single doses of 7·5 or 10 mg/kg, respectively [134].

The growing resistance of the parasite to monotherapy and the use of multidrug therapy in diseases like tuberculosis, HIV, etc. suggested that multidrug therapy should be tested for the treatment of VL. The rationale behind use of multidrug therapy were increased activity through use of compounds with synergistic or additive activity acting at different sites, shorter duration of therapy, and lower dose requirement, thereby reducing chances of toxic side effects and cost, and preventing the emergence of drug resistance. In an experimental study, Seifert and Croft demonstrated activity enhancement index (AEI) of different drugs in vivo, where the highest potentiation of miltefosine activity was achieved with amphotericin B (AEI of up to 11.3). No significant interaction was observed when miltefosine was combined with SSG (AEI of up to 2.38). The potentiation of miltefosine in vivo was also achieved with the combination of miltefosine and PM (AEI of up to 7.22) [135].

The combination of Sb^v and PM has been extensively used in Southern Sudan by Médecins Sans Frontières (MSF), initially in patients who relapsed after conventional Sb^v and since 2002 as first-line therapy for VL.A large retrospective field evaluation by MSF showed that the initial cure rates and survival of patients on 17 days combination therapy with PM plus Sb^v was 97 % compared with 92.4 % among patients with 30-day Sb^v monotherapy [136]. In a recent large multicenter trial, this combination for 17 days had comparable efficacy to SSG treatment [137]. This combination is now the preferred regimen in this region.

Multidrug therapy has been studied in India. In a randomized, non-comparative, group-sequential, triangular design study, 181 subjects were assigned to treatment with 5 mg/kg of L-AmB alone, 5 mg/kg of L-AmB followed by miltefosine for 10 days or 14 days or 3.75 mg/kg of L-AmB followed by miltefosine for 14 days. When it became apparent that all regimens were effective, 45 additional, nonrandomized patients were assigned to receive 5 mg/kg of L-AmB followed by miltefosine for 7 days. Final cure rates were high (>95 %) and similar in all the groups. These results suggest that single infusion of L-AmB (in most instances, administered in an outpatient setting) followed by a brief self-administered course of miltefosine could be an excellent option against Indian kala-azar [138].

In a subsequent large Phase III study in the Indian subcontinent, three drug combinations (single injection of 5 mg/kg L-AmB and 7-day 50 mg oral miltefosine or 10-day 11 mg/kg intramuscular PM; or 10 days each of miltefosine and PM) were tested for the treatment of VL. All the combinations showed an excellent cure rate (>97 %) [139].

These trials established that the combination therapies are safe and effective options in the Indian subcontinent. They also require shorter duration of hospitalization which will lead to decongestion of the overcrowded treatment centers. Encouraged by this new treatment approach, an exploratory study with miltefosine alone and combinations of single dose of L-AmB (10 mg/kg) with SSG (20 mg/kg) for 10 days and L-AmB (10 mg/kg) with miltefosine for 10 days is being done in East Africa [140].

3.5 Treatment Guidelines

As the efficacy and required dosage of the antileishmanial agents vary in different areas, in 2010 WHO published the treatment recommendation based on these regional differences. For VL single dose of L-AmB and combination therapy are the preferred treatment options in the Indian subcontinent. The combination of Sodium stibogluconate with Paromomycin for 17 days is treatment of choice in East Africa and Yemen, whereas L-AmB up to a total dose of

18–21 mg/kg remains the choice in Mediterranean Basin, Middle East, and Central Asia. In India, Amphotericin B 60–80 doses over 4 months or miltefosine for 12 weeks are the recommended regimens for PKDL. In East Africa, PKDL is not routinely treated, as the majority of cases (85 %) heal spontaneously within 1 year. Only patients with severe or disfiguring disease, those with lesions that have remained for >6 months, those with concomitant anterior uveitis and young children with oral lesions that interfere with feeding are treated, with either SSG (20 mg/kg/day per day) for up to 2 months or a 20-day course of L-AmB at 2.5 mg/kg/day. For HIV-Leishmaniasis coinfection, lipid formulations of amphotericin **B** infused at a dose of 3–5 mg/kg/day or intermittently for 10 doses (days 1–5, 10, 17, 24, 31, and 38) up to a total dose of 40 mg/kg are recommended. Antiretroviral therapy should be initiated and secondary prophylaxis should be given till the CD4 counts are >200/μL. For HIV-CL coinfection, it is imperative to look for visceral involvement as immunosuppression due to HIV facilitates dissemination and may lead to disseminated CL and to VL. In the OWCL, local wound care with careful follow-up are indicated for patients with confirmed or strongly suspected infection with *L. major*; fewer than four lesions requiring immediate treatment; lesions <5 cm in diameter; no potentially disfiguring or disabling lesion (face, joints, toes, and fingers); no immunosuppression and possibility for follow-up. If at least one criterion is absent, local therapy should be given. Systemic antimonials are given for severe, complex lesions and in those with HIV infection. Fluconazole or a combination of antimonial and pentoxiphylline can be given for CL due to *L. major*. The recent PAHO guidelines recommends local treatment for NWCL in patients with single lesions up to 3 cm except in head or periarticular region in patients without immunosuppression but with possibility of follow-up. Systemic therapy is indicated for severe lesions and mucosal disease. Systemic antimonials are the drug of choice for NWCL and MCL. For CL caused by *L. panamensis* and *L. guyanensis*, miltefosine and pentamidine is also recommended [141].

3.6 Infection Control Measures

The epidemiology of different forms of leishmaniasis is quite diverse, with different ecological characteristics, different species of sand fly, and different reservoir hosts. Consequently, control strategies need to be tailored to the epidemiological characteristics of the disease. It is impossible to device a single control strategy. However, for any form of leishmaniasis, whether anthroponotic or zoonotic, early case detection and effective treatment will limit the disease-related morbidity and mortality. In anthroponotic foci, it also provides an effective control measure by reducing the reservoirs of infection. Access to antileishmanial drugs is an important issue, and availability of antileishmanial drugs in the endemic areas needs to be ensured, which along with tools for early diagnosis can effectively reduce the disease burden and thus transmission, more so in anthroponotic foci. It could also prove to be an important strategy to prevent emergence of drug resistance. Intense surveillance including active case detection and health education to raise the level of awareness among exposed population and promote community control measures are important for both vector and human reservoir control.

Vector control measures with residual insecticide spray can effectively control the disease in anthroponotic foci of VL. A classic example of the efficacy of this strategy is the near disappearance of VL cases in India in the 1960s when insecticides were used extensively as a part of the National Malaria Eradication Programme. Indoor residual spraying is one of the main means for controlling endophilic sandfly vectors and should be targeted to localities with active transmission. When exophilic or peridomestic sandfly species are involved, outer surfaces of domestic animal shelters and structures close to such dwellings (potential sandfly resting sites) must be sprayed. Personal protection against sand fly bite like insecticide-treated bed nets have been used with mixed results to prevent both VL and CL [142–144] but it is still an effective relatively cheap, sustainable method for sandfly control.

Control of reservoir hosts has been recommended as a component of control strategies for zoonotic visceral and cutaneous leishmaniasis. For zoonotic leishmaniasis, vector control through residual insecticide spraying of houses and animal shelters is restricted to the domestic and peridomestic areas such as Central and South America (*Lutzomyia longipalpis*). Regarding reservoir control, dogs being the main domestic reservoir, humane destruction of infected dogs, identified after annual screening of blood samples by serology, may be a way to control the disease. Nevertheless, the strategy of elimination is not satisfactory, as it provides only a transient effect, and there is always a concern over delay between sampling, diagnosis, and culling of dogs. More effective diagnostic tools may allow culling without delay. In the absence of a reliable tool for detecting infected dogs, dogs may be treated with topical insecticide, which will protect them from infection as well as prevent sand flies from biting the dogs. Another ingenious method tried has been applying deltamethrin-treated collar to dogs [145, 146]. It gives long-term protection against sand fly bite. However, these modalities of disease control, notwithstanding their limitation, are rarely used comprehensively in underdeveloped or developing countries where the disease is endemic.

Vaccination against different forms of leishmaniasis is a viable alternative for the control of the disease. Autoclaved whole parasites with BCG with or without alum have been

tested in randomized clinical trials in Iran and Sudan against CL and VL, respectively; however, they failed to provide adequate protection [147, 148]. Now second-generation vaccine consisting of recombinant proteins and genetic vaccines are being tested for both CL and VL. However, successful vaccination against leishmaniasis still remains a distant reality.

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Occurrence, Measurement and Clinical Perspectives of Drug Resistance in Important Parasitic Helminths of Livestock

78

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1 Introduction

Helminthiases are amongst the most important diseases worldwide that affect sheep, cattle, and horses [1–10]. With anthelmintic treatment being a cornerstone of modern livestock helminth control, anthelmintic resistance is one of the key limitations of continued productivity and sustainability of livestock production. The impact of the resulting clinical or subclinical parasitism has been recognised with regard to sheep production for many decades [3, 11–15] and resistance to multiple anthelmintics is now common in sheep gastrointestinal nematodes [4, 11, 14]. In Australia alone, sheep nematodes are estimated to cost the national grazing industry in excess of $300 million annually [10]. The impact of anthelmintic resistance is now also becoming increasingly recognised with respect to cattle [4, 16–18] and horses [4, 19–22], and helminth parasites of livestock cause more than 55 % of all farm animal diseases in Europe [2].

An understanding of the general principles, specific mechanisms and measurement of anthelmintic resistance in both the laboratory and the paddock is critical in allowing continued profitable livestock production. This review concentrates on the occurrence, detection and clinical significance of resistance in the major nematode and trematode parasites of sheep, cattle and horses. The general principles regarding the development of anthelmintic resistance are discussed in light of an understanding to assist the slowing of worsening spread and to support effective and sustainable helminth control. There is also discussion of methods to detect, measure and monitor anthelmintic resistance.

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2 Parasite Biology

The major gastrointestinal nematode parasites (GIN) of ruminants worldwide are summarised in Tables 78.1 and 78.2. These GIN have a similar direct, non-migratory life cycle, with parasitic immature and adult stages within the definitive host and free-living egg and larval stages. The third-stage larva is infective to the definitive host. Prepatent period is generally between 14 and 21 days in sheep and 21 and 28 days in cattle. The major GIN of horses are summarised in Table 78.3. More detailed information about each parasite's life cycle, biology and epidemiology can be found in a variety of excellent parasitology texts (e.g. 58, 59, 76).

Fasciola hepatica is a trematode parasite of the liver of sheep, cattle and horses, amongst other hosts. It is also zoonotic and is of particular importance as a parasite in human Asian populations. The life cycle is indirect and migratory, with aquatic snails of the *Lymnaea* genus as an intermediate host. *F. hepatica* prefers a warm, wet climate. Optimum temperature for development of stages outside the definitive host is above 15 °C.

Adult fluke, 25–40 mm long, reside in the bile ducts. Eggs are passed to the environment via host faeces and a miracidium emerges that is infective to *Lymnaea*. Following development in the snail, many cercaria emerge and eventually encyst on vegetation as metacercaria that are infective to the definitive host. Immature fluke migrate inside the host from the small intestine to the liver through the peritoneum. The prepatent period for *F. hepatica* is 70–84 days.

F. hepatica can be severely pathogenic. Acute disease and deaths can result from large numbers of migrating immature flukes damaging liver parenchyma, and can also be accompanied by infection with *Clostridium novyi*. Adult fluke can

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Table 78.1 Major nematode parasites of sheep

Parasite	Key epidemiological features
Haemonchus contortus	– Adults, 20–30 mm in length, found in the abomasum
	– Highly pathogenic with fourth-stage larvae and adult worms sucking blood. Clinical signs in definitive host include anaemia, exercise intolerance, submandibular oedema and ascites
	– Highly fecund with mature female worms capable of producing up to 10,000 eggs per day
	– Prefer warmer, wet climates. Typically a summer rainfall parasite. Optimum ambient temperature for larval development between 25 and 30 °C
	– Hypobiosis in abomasal wall as third-stage larvae possible
Teladorsagia (previously *Ostertagia*) *circumcincta*	– Adults, 6–10 mm in length, found in the abomasum
	– Moderately pathogenic. Parasitise gastric glands. Clinical signs in definitive host are most commonly weight loss and intermittent diarrhoea
	– Mature female worm produces 50–100 eggs per day
	– Prefer temperate and moist or subtropical climates. Optimum larval development between 16 and 23 °C
	– Hypobiosis in abomasal wall as third-stage larvae possible
Trichostrongylus spp.	– Adult *T. colubriformis* and *T. vitrinus*, 4–7.5 mm in length, found in the small intestine. Adult *T. axei*, 3–8 mm in length, found in the abomasum
	– Mild to moderately pathogenic. *T. colubriformis* and *T. vitrinus* damage small intestinal mucosa, causing villous atrophy and inflammation. *T. axei* damages gastric mucosa causing inflammation and occasionally ulceration. Clinical signs in definitive host are most commonly weight loss and diarrhoea
	– Mature female *T. colubriformis* and *T. vitrinus* produce 100–200 eggs per day. Mature *T. axei* produces 50–100 eggs per day
	– *T. colubriformis* prefers warm moist climate, with optimum larval development between 25 and 28 °C. *T. vitrinus* prefers a cool moist climate, with optimum temperature larval development between 8 and 18 °C. *T. axei* prefers a temperate moist climate, with optimum larval development between 12 and 22 °C
Nematodirus spp.	– Adults, 10–25 mm in length, found in the small intestine
	– Generally very low pathogenicity except for *N. battus*, which in large numbers disrupts and erodes the small intestinal mucosa. Clinical signs for *N. battus* include diarrhoea, anorexia and polydipsia
	– Relatively low fecundity with mature female producing 25–30 eggs per day
	– Prefer cool, moist climates. Optimum larval development between 11 and 13 °C. *N. battus* is found mostly in the British Isles but may also occur in Norway, Sweden, the Netherlands and Canada

Table 78.2 Major nematode parasites of cattle

Parasite	Key epidemiological features
Ostertagia ostertagi	– Adults, 6–10 mm long, found in the abomasum
	– Hypobiosis in abomasal wall as fourth-stage larvae common
	– Moderately to very pathogenic, with two clinical forms both involving destruction of gastric glands. Type I disease occurs when fourth-stage larvae do not undergo hypobiosis but mature and replicate. Type II disease occurs with large-scale emergence of hypobiotic fourth-stage larvae from gastric glands. Main clinical signs include profuse watery diarrhoea and tissue oedema
	– Mature females produce about 200 eggs per day
	– Prefer cool, moist climates and subtropical regions with winter rainfall. Optimum larval development between 13 and 21 °C
Cooperia spp.	– Adults, 4–8 mm long, found in the small intestine
	– Mildly pathogenic, causing damage to the small intestinal mucosa. Clinical signs in definitive host include anorexia, depressed growth, diarrhoea and possibly submandibular oedema. Failure to control *Cooperia* can result in significantly reduced live weight gains in beef calves. *C. pectinata* and *C. punctata* have a greater impact on productivity than *C. oncophora*
	– Mature females produce 100–200 eggs per day
	– Prefer temperate to subtropical climates. Optimum larval development between 16 and 21 °C
	– Hypobiosis in intestinal wall as fourth-stage larvae possible
Haemonchus placei	– Similar life cycle and biology to *Haemonchus contortus*
	– Pathogenicity moderate to very high

Table 78.3 Major nematode parasites of horses

Parasite	Key epidemiological features
Cyathostomins	– Direct, non-migratory life cycle with free-living egg and larval stages
	– Third-stage larvae infective to definitive host
	– Prepatent period about 40 days. Larvae develop in wall of large intestine. Hypobiosis as fourth- and fifth-stage larvae occurs and can persist for years. Encysted larvae can comprise up to 90 % of burden in temperate areas of the northern hemisphere
	– Adults, 7–25 mm long, found in the caecum and colon
	– Adults cause mild disease but larval development from the mucosal pool causes inflammation and gland hypertrophy. Clinical signs are nonspecific and include ill thrift, anaemia and possibly diarrhoea. High burdens can seriously compromise the health of the affected horse
	– Prefer temperate climates
Strongylus spp.	– Direct, migratory life cycle with free-living egg and larval stages
	– Third-stage larvae infective to definitive host
	– Prepatent period is 200–332 days. There is larval migration within the vessel walls of the host's gastrointestinal blood supply
	– Adults, 14–24 mm long, found in the large intestine and caecum
	– Moderately pathogenic, except for *S. vulgaris* which is very pathogenic. Clinical signs include diarrhoea, pyrexia, anorexia, depression and weight loss. Larval migration can cause arteritis and thrombosis of intestinal blood vessels and may lead to bowel infarction and necrosis
	– Prefer temperate to warm and moist conditions
Parascaris spp.	– Direct, migratory life cycle with free-living egg and larval stages
	– Egg containing second-stage larvae infective to definitive host
	– Prepatent period is 70–84 days. Larval migration is hepato-pulmonary
	– Adults, 150–200 mm long, found in the small intestine
	– Mild to moderately pathogenic. Infection more common in horses under 2 years. Migrating larvae damage liver and lungs, and heavy infections of adult worms may cause impaction and perforation of small intestine. Clinical signs can include diarrhoea, colic and coughing, though most infections are subclinical
	– Mature female is highly fecund, producing up to 200,000 eggs per day
	– Eggs are extremely resistant in the environment and develop in all climates
Oxyuris equi	– Direct, non-migratory life cycle with free-living egg and larval stages
	– Egg containing third-stage larvae infective to definitive host
	– Prepatent period about 150 days
	– Adults, 9–150 mm long, found in the colon and rectum
	– Mildly pathogenic. Mature female lays eggs around the anus, causing intense perineal pruritis. Clinical signs include dull hair coat, hair loss, inflammation and scaling of the skin over the rump and tail head and weight loss due to restlessness and impaired feeding
	– Mature female is highly fecund, producing up to 50,000 eggs per day
	– Perineal environment provides ideal warm, moist microclimate for larval development. Spread to other horses favoured in a stable environment

cause chronic damage to bile ducts. Clinical signs include anaemia, exercise intolerance, jaundice, weight loss, reduced appetite and submandibular oedema.

3 Anthelmintic Classes, Modes of Action and Mechanisms of Resistance

Anthelmintics are designed for the purpose of treating infections with parasitic helminths. The major anthelmintic groups used in livestock worldwide include benzimidazoles, triclabendazole, imidazothiazoles/tetrahydropyrimidines, macrocyclic lactones, amino-acetonitrile derivatives, organophosphates, salicylanilides/substituted phenols and spiroindoles.

Each class of anthelmintic has a distinct mode of action and acts at a specific biochemical target site or sites. This specificity is the reason for their selective toxicity (killing helminths of particular phyla but not killing hosts) because even sites with equivalent function differ sufficiently between species. Importantly, resistance develops in a parasite to one chemical class at a time and the change renders them resistant to all members of that class. Resistance is inherited. In the simplest case, where an individual parasite is resistant due to a single genetic feature, a population of parasites will contain a certain proportion of resistant individuals. As that proportion increases then resistance is said to increase and this may be reflected in survival following treatment, survival in a test for resistance or an increase in the frequency of

a gene linked to resistance. Selection processes for each anthelmintic class are independent, but multiple resistance (resistance to more than one anthelmintic class) can occur in individual parasites.

Knowledge of resistance mechanisms can assist in developing tests for resistance. For example, if the molecular basis of resistance is known and it is the only or most common mechanism in a species, then molecular tests can potentially be developed to detect or describe the particular resistance. Even if they are not a confirmed mechanism of resistance, molecular changes may still provide useful markers. In another example, if a drug acts by causing paralysis, tests using egg hatching or larval migration may be useful if the resistance is expressed in eggs and larvae. The most common resistance mechanisms are changes in drug target molecules at the site of action and such changes may reduce the affinity of the drug for target sites in resistant compared with susceptible parasites. However, other mechanisms are possible such as changes in the structure or expression of proteins that function to lower drug levels in parasites through enhanced drug efflux or metabolism. Differences in genes associated with resistance may be referred to single-nucleotide polymorphisms (SNP). A difference in a single codon may confer a single amino acid difference in a protein sequence and lead to changes in the effect of the drug. A recent summary of the status of research into mechanisms is provided in Kotze et al. [23]. Only a summary of points pertinent to testing is provided here.

A large number of investigations have been conducted into resistance mechanisms; however very few observations have been found to provide conclusive explanations. One challenge that requires consideration is that resistance induced in the laboratory does not always arise through the same mechanisms as field resistance (where parasites are exposed under normal selection and survival conditions). As a result, the proposed mechanisms may be incorrect when applied to field resistance. Other challenges are that genetic variability in natural parasite populations confounds analysis and that worms respond to treatment by up- or down-regulating genes in ways that are protective rather than reflecting resistance mechanisms. Approaches to elucidating molecular mechanisms have relied largely on candidate gene approaches where researchers explore theories based on phenotypic observations. Whole-genome approaches are now available where lines of parasites can be compared using high-throughput sequencing and bioinformatic analysis. Nevertheless, proving the relevance of a mechanism is a difficult task.

The following is a brief summary of the current understanding of the modes of action (MOA) and mechanisms of resistance for the key anthelmintics currently used for the control of GIN and trematode parasites of livestock. The link between phenotype and genotype is emphasized here because of possible use of this knowledge in tests for resistance.

3.1 Benzimidazoles

Benzimidazole (BZ) carbamates have been used for parasite control in a number of species for several decades. The individual benzimidazoles show varying spectra and levels of activity. Albendazole is used in cattle and sheep for abomasal and intestinal nematodes, and is active against adult liver fluke at higher doses [24]. Ricobendazole has a spectrum of activity equivalent to albendazole [25]. Fenbendazole is used in sheep and cattle for susceptible abomasal and intestinal nematodes, and in horses for control of cyathostomins, *Strongylus*, *Oxyuris* and *Parascaris* at higher doses. Fenbendazole is the sulphide parent compound of oxfendazole, which is used for control of the same parasites [25]. Oxibendazole is used for control of susceptible GIN in cattle, sheep and horses [25].

These compounds bind to parasite β-tubulin causing microtubule depolymerisation and inhibiting a range of vital cellular processes. The discovery that tubulin from resistant parasites has lower binding affinity for BZs [26] led to exploration of the tubulin genes. Resistant isolates of many nematode species show a variable number and location of SNPs which confer amino acid changes in β-tubulin isotype 1. The following have been reported: (the notation for these SNPs in susceptible amino acid is followed by the position, and then the resistant amino acid) position 167 (F167Y), position 198 (E198A) and position 200 (F200Y). Generally, only one of these 'resistance' polymorphisms occurs in an individual worm and homozygous genotypes confer the resistance phenotype. In highly resistant parasite populations these polymorphisms appear in between 50 and 100 % of parasites which suggests that there are also other sites that modify resistance.

3.2 Triclabendazole

Though chemically a member of the BZ class of anthelmintics [63], triclabendazole has some unique structural differences and is only effective as a narrow spectrum treatment against the immature and adult stages of susceptible *Fasciola* in livestock [64]. Triclabendazole is the most widely used flukicide in sheep and cattle and is also effective in horses [36] though not often officially registered for this host species.

The exact mode of action of triclabendazole is uncertain [65], though it does not appear to interfere with tubulin like other benzimidazole compounds. Triclabendazole also has a stronger affinity for albumin than other benzimidazoles [66]. Suggested effects include interference with calcium transport in fluke, damage to fluke tegument, uncoupling of oxidative phosphorylation and interference with fluke protein synthesis or reproduction [65, 67, 68]. Recent research suggested influence on Pgp efflux of triclabendazole from resistant fluke [68].

3.3 Imidazothiazoles/Tetrahydropyrimidines

Levamisole has a broad spectrum of activity against susceptible mature stages of many major GIN of sheep and cattle, but shows less activity against immature forms and is ineffective against hypobiotic larval stages [25]. Levamisole has a relatively narrow safety margin, and is not approved for use in horses. Pyrantel and morantel are active against luminal parasites and are used widely against GIN of horses.

Tetrahydropyrimidines and imidazothiazoles have a common mode of action as cholinergic agonists at neuromuscular junctions causing spastic paralysis. The phenotype of resistant parasites involves reduced contractile response to these drugs as well as to acetylcholine [27]. These pharmacological differences suggest a change in the acetylcholine target site which is a pentameric membrane cation channel composed of ligand-binding and structural subunits. Following on from earlier studies, recent work has shown that a number of resistant isolates from several countries have a truncated transcript of *Hco-acr-8* likely due to an insert (indel) of 63 base pairs that is absent from susceptible worms [28]. A PCR-based test which selectively amplifies a product which includes the indel is proposed as a means of identifying resistant parasites. There is still work to do to validate the test and to elucidate the mechanism which probably relates to the likely role of *acr-8b* as an acetylcholine receptor subunit.

3.4 Macrocyclic Lactones

Macrocyclic lactones (MLs) are extremely potent and relatively safe parasiticides, active against immature and adult stages of ruminant GIN, including hypobiotic larval stages as well as tissue-dwelling parasites [29]. Several are also ectoparasiticides [29]. The ML class includes two subclasses: avermectins (AVM) (e.g. abamectin (ABA), ivermectin (IVM), doramectin (DOR), eprinomectin (EPR) and selamectin (SEL)) and the milbemycins (MIL) (e.g. moxidectin (MOX) and milbemycin (MILB)). MOX has been found to persist in adipose tissue, and this is thought to explain why MOX has been found to persist in plasma for significantly longer than ivermectin in cattle, sheep and horses [30, 31]. IVM, ABA and MOX are the only MLs used in horses and control susceptible *Parascaris*, *Oxyuris*, large strongyles and adult cyathostomins. In addition, MOX is effective against encysted stages of the cyathostomins, although efficacy is not complete [29, 32–35].

Macrocyclic lactones are agonists of glutamate-gated chloride channels (GGCC) in pharyngeal and body musculature of nematodes where they cause hyperpolarisation and relaxation. The subclasses may differ in some chemical characteristics, but are broadly thought to act in the same way and do share side resistance in cases of field resistance across several species. Further, some isolates of AVM-resistant parasites differ phenotypically, suggesting that there may be more than one mechanism responsible for resistance. The large number of putative mechanisms of resistance has led to suggestions that ML resistance is multigenic. In contrast, it is known that resistance developed extremely quickly against ivermectin, in less than eight generations, and so a single major gene seems a more likely explanation. Another confusing issue is that IVM appears to act at several sites.

When susceptible and resistant adult parasites from several species were studied in vitro the inhibitory effects (relaxation of body muscle) of the MLs were weaker in resistant isolates [36] and this effect occurred over a period of less than 1 min. Potential GGCC target sites have been cloned and expressed and ML binding to these proteins occurs, but changes in affinity or consistent molecular changes have not been found and so receptor site changes are unlikely to be involved in resistance mechanisms. Effects on drug transport as a mechanism of resistance have also been explored. The p-glycoproteins (Pgp) are transmembrane drug efflux proteins known to pump IVM out of mammalian cells. The hypothesis is that enhanced drug efflux can confer resistance. Whilst studies on transcription of various Pgp genes have been performed and several efflux inhibition studies attempted, unequivocal evidence of enhanced Pgp efflux as a mechanism of resistance to MLs in nematode parasites is lacking. Early descriptions of IVM-resistant *H. contortus* showed abnormal amphidial neuron morphology [37] and this suggested changes in chemoreceptor function. Some IVM-R *C. elegans* [38] have a defective dye-filling phenotype associated with the *dyf-7* gene. The *H. contortus* homologue, *Hco_dyf-7*, from a number of resistant isolates contains several SNPs that differ from susceptible worms, although none of the SNPs code for amino acid differences [39]. Whilst a mechanism is not clear it may involve drug exclusion or drug removal in resistant worms. These findings give hope for a molecular test for future application.

3.5 Amino-acetonitrile Derivatives

The amino-acetonitrile derivative (AAD) monepantel is one of the newest anthelmintics available for GIN control in sheep [40, 41], with broad spectrum activity against adult and larval GIN [42, 43] and high relative safety for sheep [44, 45]. Monepantel is a cholinergic agonist known to cause hyper contraction and paralysis by acting at a unique site on nematode body muscle membrane receptors [40, 46]. Resistance has been explored in experimentally selected isolates of *H. contortus* and SNPs in the *mptl-1* cholinergic receptor subunit are linked to the resistance phenotype [47–49].

Field isolates of monepantel-resistant nematodes have now been reported [50, 51] and it is anticipated that molecular data from these field cases could shed light on mechanisms of resistance.

3.6 Organophosphates

Organophosphates inhibit acetylcholinesterase, leading to accumulation of acetylcholine at nerve endings and therefore disrupted neurotransmission through hyperstimulation of nicotinic and muscarinic receptors. This results in neuromuscular spastic paralysis and death of parasites [52], but can also pose safety risks for hosts. Current sheep treatments contain naphthalophos, which is effective against both adult and inhibited *Haemonchus* and is moderately effective against other GIN [53, 54], or pyraclofos in combination with albendazole [40]. A single case of naphthalophos resistance has been published [55], however little further investigation or records of other field cases have occurred.

3.7 Salicylanilides/Substituted Phenols

Salicylanilides and substituted phenols (SA) include closantel (CLS), nitroxynil (NIT), niclosamide (NIC) and oxyclozanide (OXY) [56]. These chemicals are strongly bound to plasma albumin, which may account for their high efficacy against blood-feeding parasites [57], especially *Haemonchus* and *Fasciola*, and prolonged anthelmintic effect in some instances [58].

Salicylanilides and substituted phenols uncouple oxidative phosphorylation causing energy depletion in parasites [59–61]. Toxicity can be a risk following large doses [62]. Whilst resistance has occurred, there have been no studies to further elucidate mechanisms.

3.8 Spiroindoles

Derquantel (2-desoxoparaherquamide) is a semi-synthetic derivative of paraherquamide used for GIN control in sheep [69]. Spiroindoles are toxic to horses [70]. Derquantel is newly available commercially only in combination with abamectin and this product has shown excellent efficacy against most adult and immature trichostrongyloids of sheep [69, 71, 72]. It has variable efficacy against *Teladorsagia* and immature *Haemonchus* [69, 71, 73].

Derquantel acts as a competitive, but selective, cholinergic antagonist, blocking cation channels in nematode muscle cell membranes [69, 74, 75] leading to relaxation and rapidly induced flaccid paralysis of parasite musculature [59, 69, 70]. There are no published cases of field resistance yet.

4 Development of Anthelmintic Resistance

From a practical viewpoint, anthelmintic resistance is generally thought of as being present when there is an ‘increased frequency of individuals within a population able to tolerate doses of a compound compared to the frequency in a normal population of the same species’ [76]. Anthelmintic resistance is also heritable, passed from one parasite generation to the next, with the inheritance pattern of the gene(s) responsible being a key factor influencing both the speed of development and spread through a helminth population [77].

As anthelmintic treatments are likely to remain a cornerstone of effective livestock parasite control for the future, it is important to understand the general principles and mechanisms of resistance, in order to help sustain remaining anthelmintic efficacy for longer. There also appears little hope of long-term reversion of resistant helminth populations back to susceptibility after a period of no further exposure to the selective anthelmintic [78].

Sutherst and Comins [79] describe three components to the genesis of resistance. The first is establishment. This is largely a random event influenced by the population size and diversity and the mutation rate for the gene(s) in question. The second step is development. In this process, the use of the selective agent (the anthelmintic) allows resistance to develop but the prevalence of resistant alleles is too low for resistance to be clinically apparent. In the third step, dispersal, there is further selection and spread of the resistance genes through the wider population of the organisms. During this phase, clinical resistance (also termed field resistance) first appears. The processes of development and dispersal are influenced by biology, management and chance events, such as linkage disequilibrium and gene dispersal via an intermediate host. These processes are driven by the drug selection, reflected in survival and subsequent reproduction of parasites following drug treatment.

Factors influencing the development and dispersal of anthelmintic resistance with regard to livestock parasites have been well reviewed [10, 14, 77, 80–86].

Inappropriate dosing is one of the simplest ways to accelerate the development and dispersal of anthelmintic resistance. Under-dosing individual hosts can be a problem [87] and was considered common in some major production systems due to underestimation of animal liveweights when calculating dosage [88]. There has been considerable effort in making sheep producers aware of this risk, but the practice change of weighing at least a sample of animals from each group and dosing appropriately for the liveweight of the heaviest individual is still far from widespread [89, 90]. There could also remain a risk with cattle and horse producers [85, 91].

Excessive treatment frequency also exposes parasite populations to further selection for anthelmintic resistance, without accompanying benefits of improved productivity, health and/or welfare [16, 76, 92–96]. This has long been rec-

ognised as a potentially significant issue for sheep enterprises, and, more recently, equine nematode control [91, 97, 98].

Delivery method can also influence the risk of the development and dispersal of anthelmintic resistance. Oral formulations are most common for small ruminants, despite the potential risk of influence from activation of the oesophageal groove reflex [99–102]. Many studies have demonstrated oral treatments as more effective than topical and injectable formulations of the same active [103–106], despite at times lower anthelmintic concentrations in the plasma of treated hosts. Lanusse et al. [84] discussed the implications of the specific targeted parasite with regard to choice of delivery route, and this could explain the apparent increased effectiveness of oral treatments in some circumstances. There are also particular concerns with topical treatments in cattle [16, 107, 108], with influences on bioavailability from host coat characteristics, prevailing climate and licking behavior of the cattle in some cases [101, 108–112]. Persistent exposure of helminth populations to anthelmintics through slow-release and long-acting formulations is another potential risk [16, 76, 113–117].

Fasting sheep before anthelmintic treatment, to slow digesta flow and improve gut residence time, can have beneficial effects on the efficacy of less soluble anthelmintics [101, 118, 119], though this is not a sensible strategy for products with lower margins of safety, such as levamisole and naphthalophos [115].

Administration of a combination of effective anthelmintics, each with a different mode of action, has been suggested as another means to help prolong anthelmintic efficacy [12, 76, 84, 115, 117, 120–131]. Combination products are commercially available in some countries [69] and administering more than one effective anthelmintic is a way to deliver a higher combined efficacy [22, 132–136] and/or delay the development of anthelmintic resistance [126–128]. Modeling studies showed that the speed of selection for anthelmintic resistance is reduced by combining highly effective anthelmintic actives [12, 127, 128]. Combinations exert a greater efficacy against resistant genotypes, especially those carrying genes for resistance to only one of the actives in combination. This results in fewer resistant survivors to treatment, and subsequently a greater dilution of survivors by the remaining susceptible population in *refugia* [12].

Another strategy whereby administration of more than one anthelmintic with different modes of action is important is quarantine drenching [16, 76, 85, 115, 117, 137]. Whilst apparently not completed effectively in many livestock enterprises [90, 116, 138], treatment of new animals coming into a population or those returning from environments where infestation with parasites was possible, with a combination of highly effective anthelmintic groups, is a vital part of an overall worm control strategy. This should reduce the risk of introduction of new resistance alleles in incoming stock. Enhanced benefits are also possible by placing such stock into a high-worm *refugia* environment following treatment [115, 117].

The concept of *refugia* is more complicated for livestock producers, but is recognised as one of the currently most critical influences on anthelmintic resistance development and dispersal [12, 76, 83, 85, 116, 117, 139–150]. Livestock producers require a major mind-shift [83, 151] when comparing *refugia*-based worm control advice to previous recommendations of moving treated stock to low-contaminated environments to prolong treatment benefits [139, 152]. However, helminth control programmes can result in high selection pressure for the development and dispersal of anthelmintic resistance if eggs from parasites surviving treatments are able to develop in an environment where there is little non-selected helminth egg contamination to dilute them [140, 152].

Increasing *refugia* within a helminth control programme can be achieved in two ways. Targeting treatments, by selecting only those hosts who are clinically or subclinically affected by helminths, can prevent treatment of every individual within a group. This allows the parasites from untreated hosts to continue to contribute genetics, which are less selected for anthelmintic resistance, to subsequent helminth generations.

The FAMACHA® system of basing sheep treatments for *Haemonchus contortus* on the clinical anaemia of individual sheep is one such strategy [153, 154]. Targeted or selective treatment strategies have also been discussed for non-haemophagic sheep parasites [146, 147, 155, 156] and cattle [157] and horse GIN control [19, 21, 83, 91, 158, 159]. However, the actual choice of which animals within each group to leave untreated, without an obvious indicator such as clinical anaemia, can be far from simple [19, 21, 83, 85, 147, 155, 160–162]. Some authors have suggested leaving whole groups of animals untreated within an enterprise, and using this whole group as a moveable source of *refugia* [163].

Another way to reduce selection pressure for increased anthelmintic development and dispersal is by avoiding treatments at key epidemiologically selective times of low *refugia* due to prevailing environmental conditions. For example, the so-called summer drenching of sheep in environments with an extremely winter rainfall-dominant climate, such as Western Australia, can result in heavy selection for anthelmintic resistance [10, 76, 82, 161].

With all worm control strategies that promote increased *refugia*, care is needed to monitor helminth burdens and ensure that grazing environments do not become excessively contaminated with parasites [146] and/or animals that are left untreated succumb to the effects of the remaining parasites [161].

5 Measurement of Anthelmintic Resistance

The ability to measure and monitor anthelmintic resistance is a key component of sustainable livestock helminth control. The measurement of resistance is often scored as survival of parasites following a treatment and so parasite numbers (or values that reflect parasite numbers) are commonly recorded. These values are then analysed statistically (e.g. using *t*-tests or *F*-tests) and comparative efficacies generated. Resistance may also be recognised as a reduction in the period of protection that a persistent treatment provides. This provides different types of data and may be amenable to survival analysis.

The ultimate aim is a simple, rapid, inexpensive assay that can be applied by the animal's side to simultaneously assess the efficacy of all relevant anthelmintics against all of the nematode species of interest.

In practice, this has, thus far, proved very difficult to achieve. Currently there are a range of methodologies, which are applied in various circumstances, many of which have significant limitations. The pressure is on to further develop in vitro and genetic tests, however there still remains a lack of knowledge of the basic genetics of resistance mechanisms in many instances. Test sample isolation from livestock faeces, the preferred method to promote practical collection by livestock producers, also continues to pose challenges.

It is also important to remember that while reduced apparent efficacy may suggest anthelmintic resistance, this is just one potential cause of treatment failure. Other causes include misdiagnosis of another mimicking aetiological agent, inappropriate drug choice for the causal parasite, rapid reinfection after treatment, recrudescence of the same infection and product quality or administration issues [164, 165]. Another example is where faster metabolism in goats results in poorer efficacy of antiparasitic compounds compared with sheep [166]. There are also suggestions of differences between donkeys and horses with regard to moxidectin treatment [167].

5.1 In Vivo Bioassays

5.1.1 Treat and Slaughter Studies

The preferred definitive test for anthelmintic resistance assessment in all livestock species is a controlled sacrifice, or treat and slaughter, study [164, 168–176]. This test involves artificial infection of worm-free definitive hosts, individual treatment of confirmed infested animals at the recommended dose for the respective anthelmintic(s) and counting of the total number of surviving helminths after the slaughter of the hosts and completion of total worm counts.

This allows controlled assessment of true efficacy against immature and adult stages of the test parasites. Inclusion of positive as well as negative control groups can also allow generation of dose–response curves and then also effective dose 50 (ED_{50}) and resistance factor (RF) calculations.

The main disadvantages of this method include the cost of animal purchase and disposal, the ethics of euthanasing animals and the creation of worm-free test animals. Accordingly, it is now preferred to perform slaughter trials only when a novel isolation is made, because in many cases the relationship and correlations between in vivo and in vitro assays for anthelmintic resistance in parasites have been established [177].

A similar principle has also been applied to assess anthelmintic efficacy against *T. colubriformis* and *H. contortus* using guinea pigs [178] and jirds [179], respectively. Although these techniques are not likely to be used for field detection, they are useful experimental models because several dose rates of drug can be tested more quickly and cheaply than in sheep.

5.1.2 Faecal Egg Count Reduction Test

The most commonly used practical assessment of anthelmintic resistance in the field is the faecal egg count reduction test (FECRT). This test can be used to determine the prevailing anthelmintic resistance status of the common, significant nematodes, and hence help guide the choice of effective treatments. It has also been used to survey the prevalence of anthelmintic resistance [168].

There has been much detailed discussion of the best methodology for this approach [77, 168, 175, 180–190]. In summary for small ruminants:

- The test involves the identification of a suitably infested group of host species. Younger animals (3–6 months old) are preferred to avoid complications due to host immune status, and tested animals should not have been treated with test products during at least the previous 6 weeks.
- Groups of 10–15 identified animals are randomly allocated to each treatment. Another group is also identified as untreated negative control animals.
- Typically a subsample of the heaviest animals is then weighed and each animal individually dosed at the manufacturer's recommendation for the heaviest animal in their respective treatment group. Animals in the negative control group remain untreated.
- Animals are re-sampled between 10 and 14 days after treatment, with individual faecal samples identified according to treatment group. It is important to collect these samples before infestations after treatment could complete patency.
- Standard McMaster faecal worm egg counts are completed, using a floatation methodology, on each faecal sample.

Arithmetic group means of individual worm eggs counts are then compared between each treatment group and the

negative control group, to calculate the percentage reduction in worm egg count as a result of each treatment. This equates to the 'efficacy' of the applied treatment. Individual worm egg counts are preferred over composite testing [191, 192]. Arithmetic means are also preferred, as they have been found to provide a better, less biased estimate of efficacy, compared to geometric means, especially when a portion of the pre- or post-treatment counts are zero [193].

Logarithmic error calculations can be used to calculate confidence limits of the reduction percentages. An alternative method to calculate confidence limits when efficacy and/or nematode aggregation is high has also been suggested by Dobson et al. [194]. The generally accepted definition of 'resistance' has been when arithmetic mean reduction is less than 95 % and the lower 95 % confidence interval is below 90 % [189]. The use of these two criteria has been thought to result in a 95 % confidence of detecting clinical resistance [189], though there have been some concerns about the validity of results when only slight loss of efficacy is beginning to occur. It has even been suggested that if only one of the accepted criteria is met then a finding be made of 'suspected resistance' [195].

With development and testing of new anthelmintics in mind, Dobson et al. have also proposed to reframe the FECR as a binomial proportion if efficacy is estimated to be 100 % [194]. Where n and x are the total number of eggs counted (rather than eggs per gram) for all pre- and post-treatment animals, respectively, p (the proportion of resistant eggs) $= x/n$ and per cent efficacy is $100 \times (1 - p)$ (assuming equal treatment group sizes and detection levels, pre- and post-treatment).

Reduction results can be further enhanced by including culture of the remaining faeces collected post-treatment [168, 175, 196]. This allows attribution of the post-treatment egg count within each group to each of the parasite genera identified via identification of the cultured third-stage larvae, and this can be particularly important when treated sheep contain at least some highly fecund *Haemonchus contortus* [168, 197]. Replacement of the McMaster method by other floatation and/or centrifugation methods could also enhance sensitivity in some circumstances, for example, when test animals are only excreting low egg numbers [183, 198].

Software tools have been developed to further assist calculations and minimise errors in the FECRT. RESO5 is an anthelmintic efficacy calculator which uses calculations based on those published in a report of the Working Party for the Animal Health Committee of the Standing Committee of Agriculture [199]. It provides upper and lower confidence limits along with the mean FEC reduction, and indicates whether resistance is present. The original RESO program, as developed by Leo Wursthorn and Paul Martin of CSIRO, has undergone various additions and modifications which have been integrated into the most modern addition at this time [11]. The 'eggCounts' package, designed by Torgerson et al. within the software package R, is based on hierarchical Bayesian framework and incorporates both sampling error and overdispersion between animals to rigorously analyse the results of faecal egg counts [200].

To a large extent the interpretation of FECRT relies on a correlation between egg counts and nematode numbers. This relationship is not always strong [168]. Also, some anthelmintics can suppress egg production, rather than kill resistant worms [168], and this can result in an overestimation of treatment efficacy. Zero egg counts post-treatment that suggest that the worms are drug susceptible may mask cases where worms resume egg production more than 14 days after treatment. Sensitivity can be improved by using reduced dose rates of drugs, but this approach requires a good knowledge of resistance phenomena.

Another major generally accepted potential limitation of the FECRT is that it can only detect clinical resistance, often not until the frequencies of resistance alleles reach 25 % or more in the nematode population [140]. Thus genetic changes within nematode populations are not detected until significant. This is often suggested to reduce the likelihood of the possibility of long-lasting nematode population reversion to anthelmintic susceptibility following periods without exposure to anthelmintics previously considered unusable due to resistance levels [201–204].

Finally, another barrier to wider field adoption of this test is low producer enthusiasm for multiple yardings of sheep and treatment and faecal sampling of individual animals. In an attempt to counter some of this reluctance, a simplified test, DrenchCheck-Day10, has been promoted to sheep producers in Australia (www.wormboss.com.au; accessed on 15 May 2015).

Application of FECRT specifically for cattle has been discussed by several authors, and it is generally agreed that accurate determination of anthelmintic resistance is more difficult for bovines [4, 16, 205–211]. Nuances in nematode egg output [80, 205, 212–214] and influences of anthelmintic formulation and delivery route can all influence resistance test results for bovine nematodes [181]. It is recommended that tested anthelmintics are delivered orally to cattle [181] and strongyle egg detection is made as sensitive as possible [207, 211]. Larger treatment group sizes, individual pre-treatment worm egg counts, multiple post-treatment faecal sampling times, differentiation of worm egg counts to nematode genera and further evaluation of appropriate calculation methodology could help improve confidence in results [17, 209, 210, 215].

The specific application of FECRT for horses has also been discussed in detail [4, 20, 91, 159, 216–221]. Similar issues, with regard to relatively lower individual worm egg counts and overdispersion and greater aggregation of worm egg counts, occur in horses as for cattle [159], and this, again,

emphasises the importance of maximising the sensitivity of egg detection methods. Interpretation can also be more complicated for equine nematodes, and must consider original, lower than typical for sheep and cattle, efficacy levels for some anthelmintics against sensitive populations when originally registered [221]. This all suggests the need to consider modification of traditional efficacy guidelines for defining anthelmintic resistance in equine nematodes [19, 220, 221], and/or investigating more appropriate calculations to validly assess worm egg count reduction under some circumstances [159, 216, 222–224].

Another detail to consider in the evaluation of equine nematode anthelmintic resistance is the egg reappearance period [221, 225–227]. Reappearance of nematode eggs in horse faeces, at an interval after treatment, has been suggested as a useful guide to the early detection of the development of anthelmintic resistance [228–230].

There are also complications to be considered in the application of FECRT for evaluation of anthelmintic resistance in *Fasciola hepatica* [164]. The FECRT technique was originally developed and endorsed for nematodes [169, 181] but has been used for flukes, despite the validity being questioned at times. The lower sensitivity of faecal egg detection for *Fasciola*, intermittent egg shedding by adult flukes and possibility of ongoing release of eggs from the gall bladder after effective treatment of adult flukes can all complicate accurate interpretation of the results [231–238]. Triclabendazole resistance also can become evident in the immature stages of the fluke, that are not producing eggs, and hence these can avoid detection when using an FECRT. Consideration of these factors can still allow worthwhile application of the FECRT for *Fasciola hepatica* [239], including the testing of composite faecal samples [240], however the coproantigen reduction test, as is discussed later, is gaining popularity [235, 237, 241]. Application of PCR testing to better assess treatment effects on *Fasciola* has also been evaluated [242].

5.2 In Vitro Assays

In vitro methods of detection of anthelmintic resistance have received considerable attention, as potentially less expensive and time-consuming and more reliable, though usually more technically demanding, techniques to assess anthelmintic resistance.

By measuring the effect against a representative sample of parasites at each range of drug concentrations, a plot of response to treatment against dose can be generated. From this, the effective concentration 50 (EC_{50}) can be calculated. Modern computer analysis (e.g. GraphPad PRISM) has enabled more accurate curve fitting, as well as automated calculations of EC_{50} and standard errors. The EC_{50} is the effective concentration or dose of drug that affects 50 % of the parasite population. Similar expressions include the ID_{50} (inhibitory dose), LD_{50} (lethal dose), LC_{50} (lethal concentration) and CD_{50} (curative dose). The ratio of EC_{50} values between resistant and known susceptible populations of the same species is known as the resistance factor (RF).

5.2.1 Egg Hatch Assay

The egg hatch assay (EHA) was initially developed for the detection of thiabendazole resistance in sheep nematodes [243] and has been evaluated many times since for the assessment of benzimidazole resistance in sheep nematodes [244–252]. It has also been investigated for cattle [208, 253] and horse nematodes [152, 218, 254–258] and the detection of albendazole and triclabendazole resistance in *Fasciola hepatica* [242, 259–261].

The technique is described in detail by Taylor et al. [171] and Coles et al. [181]. In principle, fresh, clean, undeveloped helminth eggs are collected and incubated in the presence of a range of concentrations of the anthelmintic of interest. Following incubation, the proportion of unhatched eggs compared to larvae is calculated, after correction for egg mortality without the presence of anthelmintic, and a dose–response curve can be generated [258]. If possible, the known discriminating dose of the anthelmintic, which would result in 99 % or more of hatch inhibition in susceptible nematode isolates, is included to help increase test sensitivity [140, 181, 246]. There are concerns that EHA will not detect resistance until at least 25 % of the nematode population is resistant [140].

5.2.2 Larval Based Assays

The best characterised in vitro assay is the larval development assay (LDA) [177]. This test relies on the development of eggs, in a liquid or agar culture media, to first-, then second-, and then third-stage larvae in the presence of a range of concentrations of anthelmintics of interest. The proportion of eggs, L1 and L2 of the total eggs and larvae is calculated after correction for the number of undeveloped stages in the negative control replicates.

Advantages of the LDA include the following: a single, relatively simple composite faecal sample collection, no need to test susceptible isolates, simultaneous assessment of several anthelmintics is possible, dose–response data can be generated and sensitivity can be greater than 95 %. General disadvantages include that the validity of the assay as a measure of resistance must be confirmed under a range of conditions, trained laboratory staff are required and species identification of larvae is needed.

A commercial product, with anthelmintics pre-applied to a 96-well plate, was available as the Drenchrite® Test (Microbial Screening Technologies, Smithfield, Australia). This and other LDA methods have been utilised successfully to

assess a range of GIN from sheep and goats for benzimidazole resistance and levamisole resistance, and also macrocyclic lactone resistance in *Haemonchus contortus* [244, 245, 251, 262–271]. The test however lacks adequate sensitivity for useful assessment of ivermectin resistance in *Teladorsagia* [272]. LDA have also been investigated for assessment of anthelmintic resistance in cattle nematodes [273] and in horse nematodes, with some good and some less convincing results [216, 254, 256, 258, 274, 275].

The larval mortality assay (LMA) assesses the ability of fresh, exsheathed third-stage nematode larvae to survive a range of serial dilutions of anthelmintics of interest, and has been shown to be able to assess benzimidazole resistance in sheep nematodes [276, 277].

The larval migration inhibition assay (LMIA) assesses the ability of freshly cultured, exsheathed third-stage nematode larvae, in the presence of serial dilutions of anthelmintics, to migrate through sieves that will only allow the passage of viable larvae [171, 276]. The proportion of non-migrated larvae compared to total larvae can be used to calculate the effective anthelmintic concentration to inhibit migration of 50 % of the larvae (EC50), which can then be compared to known standards. Agar barriers have also been added to the test to improve sensitivity in some cases [283]. The Micromotility Meter™ [36, 278, 279] and the Worminator system [280] have also been suggested as possible ways to automate motility measurements. These have shown promise with third-stage larvae of *Haemonchus contortus* and adult *Cooperia oncophora* [36].

The LMIA was originally developed for sheep nematodes and found to be able to determine resistance to thiabendazole, levamisole, closantel and ivermectin [276, 281–283]. It has also been shown to have application for moxidectin resistance in sheep nematodes [269] and benzimidazole, ivermectin and moxidectin resistance in cattle nematodes [36, 190, 207, 273, 284]. Preliminary considerations have also occurred for horse nematodes [258].

The larval feeding inhibition assay (LFIA) assesses the effect of exposure of fresh first-stage larvae to serial dilutions of anthelmintic concentrations on the feeding activity of the larvae [78, 285, 286]. This approach has shown promise for detection of ivermectin and levamisole resistance in sheep nematodes [247, 285] and has also been used for adult worms [287]. There appears little success with horse nematodes [258].

5.3 Molecular Based Assays

5.3.1 DNA-Based Assays

DNA-based tests offer the potential for very sensitive detection of resistance. They could also offer the opportunity to search for potential resistance before anthelmintic treatment [288]. However, in order to design and use gene probes, the genetic basis of resistance must be known, and this is far from certain in most of the significant anthelmintic resistance examples for livestock.

Benzimidazole resistance is the only current example where genetic markers have been characterised and utilised to develop a useful assay for resistance. Resistance was first shown to be linked to the expression of tyrosine, rather than phenylalanine, at codon 200 of the isotype1 beta-tubulin gene [289, 290]. Other work has since identified additional candidate mutations at codon 167 [291] and codon 198 [292–294]; however these do not seem as consistent as resistance markers [291, 295, 296].

The marker at position 200 has been used to develop PCR-based resistance identification techniques in *Haemonchus contortus*, *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* [181, 297–300], and *Haemonchus* spp. and *Ostertagia ostertagi* in cattle [295, 296, 301]. Investigations in horses have shown variable results [21, 219, 257, 302–305]. The latest work has investigated real-time PCR or pyrosequencing approaches to favour more practical field application of this molecular approach [248, 285, 306–309].

Recently a truncated transcript of *Hco-acr-8* was consistently identified in levamisole-resistant *Haemonchus contortus* [28]. This was absent from susceptible *Haemonchus* investigated in the same work, and thus was suggested as a potential marker genetic marker for levamisole resistance. Recent investigations into the *Hco_dyf-7* gene have also suggested this as a potential future marker for macrocyclic lactone resistance in *Haemonchus contortus* [39].

5.3.2 Immunological Based Assays

Given the concerns about the reliable application of FECRT for the detection of triclabendazole resistance in *Fasciola hepatica*, there has been some attention regarding the potential of ELISA tests to assist resistance detection. The development of a commercial coproantigen ELISA test (Bio-X Diagnostics, Jemelle, Belgium), based on the MM3 monoclonal antibody, offered more sensitive diagnosis that was also more responsive to successful fluke treatment than serological ELISA testing [310]. This test has shown promising results in a coproantigen reduction test, with post-treatment sampling 14 days after triclabendazole administration, in both sheep [235, 311, 312] and cattle [241].

6 Occurrence of Anthelmintic Resistance

6.1 Resistance in Sheep Helminths

Parasitic nematodes of sheep generally show greater levels and spectra of resistance to anthelmintics than those of cattle and horses (Table 78.4).

Table 78.4 Geographical distribution of reported anthelmintic resistance in important helminth parasites of sheep

	Benzimidazoles	Levamisole/ Morantel	Macrocyclic lactones	Aminoaceto-nitrile derivatives	Organophosphates	Triclabendazole	Salicylanilides
Haemonchus contortus	Au [11, 14], Am [309], Eu [227], Af [13], NZ [313], As [96]	Au [11, 14], Af [314], Am [315], Eu [227], As [96]	Au [316], Af [317], NZ [313], Am [318], As [96]	Am [319]	Au [55]		Au [14], Am [315], Af [320], As [96]
Teladorsagia circumcincta	Au, Am, Af [320], Eu [321], NZ [313]	Au [11], NZ [313], Eu [321]	Au [11, 14], NZ [313], Eu [322]	NZ [50]			
Trichostrongylus spp.	Au [11], NZ [313], Eu [323]	Au [11], NZ [313] Af [314, 320], As [324], Eu [322]	Au [325], NZ [313], Eu [321]	NZ [50]			
Fasciola hepatica	Am [174], Eu [327, 328]					Au [329], Eu [165, 237]	Eu [328]

Au, Australia; *NZ*, New Zealand; *Am*, Americas; *As*, Asia; *Eu*, Europe; *Af*, Africa

Table 78.5 Geographical distribution of reported anthelmintic resistance in important helminth parasites of cattle

	Benzimidazoles	Levamisole	Macrocyclic lactones	Triclabendazole	Morantel
Ostertagia ostertagi	Am [210], Au [331], NZ [330]	Eu [16], Au [331], NZ [330], Am [332]	Am [206], Au [333], NZ [330], Eu [205]	–	
Cooperia spp.	NZ [16], Am [334]	Am [332], Eu [16]	Au [331], Eu [321, 335], NZ [16], Am [213]	–	
Haemonchus placei	Am [295, 336]	Au [16], Am [337]	Am [211], Au [16]	–	As [16]
Fasciola hepatica				Au [241], Eu [338], Am [173]	

Au, Australia; *NZ*, New Zealand; *Am*, Americas; *As*, Asia; *Eu*, Europe

Table 78.6 Geographical distribution of reported anthelmintic resistance in important nematode parasites of horses

	Benzimidazoles	Pyrantel	Macrocyclic lactones
Cyathostomins	Am [339], Eu [340], Au [326]	Am [91], Eu [340]	Am, Eu [326]
Parascaris equorum	Au [341]	Au [341], Am [326]	Au [341], Am [342], Eu [326]
Oxyuris equi		Eu [343]?	

Au, Australia; *Am,* Americas; *Eu*, Europe

6.2 Resistance in Cattle Helminths

Despite widespread interest in anthelmintic resistance development in sheep and goats for decades, the investigation of anthelmintic resistance in cattle nematodes has only begun comparatively recently (Table 78.5). The prevalence of resistance in cattle GIN has proven to be surprisingly high in some regions.

6.3 Resistance in Horse Nematodes

The investigation of anthelmintic resistance in equine GIN has also only begun comparatively recently (Table 78.6).

In 1999, Coles et al. [344] reported suspicions of pyrantel resistance in *Strongylus vulgaris*; however no cases have been reported since.

7 Conclusion

Anthelmintic resistance is redefining best practice livestock helminth control. The traditional approach of regular prophylactic treatment without resistance monitoring has proved to be an unsustainable method of control.

The development and sustainable utilisation of novel anthelmintics are important for controlling parasite populations into the future, but a carefully planned approach combining chemical and non-chemical based control strategies is also required to slow the development of resistance and maintain the utility of remaining effective drugs now. Better understanding of resistance mechanisms and better practical and cost-effective tests to allow earlier detection of anthelmintic resistance changes in a parasite population will also be very valuable.

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Part XII

Measurements of Drug Resistance

In Vitro Performance and Analysis of Combination Anti-infective Evaluations

79

Robert W. Buckheit Jr. and R. Dwayne Lunsford

1 Introduction

Antiviral or antimicrobial drug resistance and drug toxicities have provided great impetus to develop combination drug therapies that will suppress the emergence of resistant organisms and allow lower, less toxic doses of drugs to be administered. The evaluation of the activity of combinations of two or more anti-infective compounds has gained significant prominence in light of the innate ability of many infectious organisms to rapidly acquire drug resistance. Pathogens react to the administration of anti-infective agents by the outgrowth of preexisting infectious clones with resistance-engendering mutations and by accumulation of new mutations to allow escape from the suppressive effects of therapeutic drug regimens [1]. Resistance emerges through the error-prone mechanisms of the replicative machinery and through the transmission of resistance elements [2, 3], rendering monotherapeutic drug strategies problematic. Combination chemotherapy significantly decreases the risk that resistance will arise. In addition, combination chemotherapy may ameliorate toxicity by permitting lower and less toxic or nontoxic concentrations of synergistic drugs to be utilized.

In convergent combination therapy [4, 5], the drugs used in the combination target the same functional protein or enzyme, and there is the possibility that lower doses of the individual drugs might be used. A specific therapeutic regimen of several drugs that target multiple essential steps in the replication of the organism is sometimes referred to as divergent drug therapy [5]. This strategy benefits from the possibility that organisms resistant to one of the drugs in the combination therapy will remain completely sensitive to the others, whereas cross-resistance may also emerge, in the case that the drugs inhibit the same replication target. In some cases, targeting the same enzyme or protein may still be considered a divergent therapy since the target may include multiple sites for anti-infective action.

Combination therapies have taken great strides forward with the advent of highly active antiretroviral therapies (HAARTs) for patients with human immunodeficiency virus (HIV) infection, yielding therapeutic regimens routinely involving three to four drugs and creating additional challenges of compliance and drug–drug interactions. An example would be the use of nucleoside and non-nucleoside reverse transcriptase inhibitors which target distinct functional and structural components of the viral reverse transcriptase in treatment of infection by the human immunodeficiency virus (HIV) [6].

Another advantage of combination therapy strategies is that multiple infectious organisms can be targeted [7]. The prevalence of coinfections involving HIV is increasing [8], and it is critical to understand the effects of the HIV therapeutic agents when used in patients that are also administered as direct-acting antiviral agents targeting hepatitis C virus (HCV) or other opportunistic bacterial and fungal infections, including tuberculosis. Similarly, respiratory infections often include both viral and bacterial components [9], and thus it is important to understand the effects of drug interactions on the efficacy and toxicity of the agents targeting the individual agents.

Also of interest is the lack of approved combination therapy for infectious agents such as HBV, herpes viruses, and influenza viruses; the issue is attributed to either the small number of approved antiviral agents being within the same mechanistic class of inhibitor, the overall treatment expense, or the lack of standardization in analysis of the combination results. It is hoped that the in vitro evaluation of combination drug interactions will provide a quantitative and prioritized rationale for the development of specific combination therapies that will enhance the efficacy of therapy, reduce the incidence of drug resistance, and allow for less toxic and demanding therapeutic regimens.

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Finally, though most in vitro combination assays involve the evaluation of efficacy, the evaluation and understanding of combined toxicity and possible antagonistic antiviral interactions are also of paramount importance as the therapy moves into the clinic [10]. For both efficacy and toxicity, the dose–response curve that is evaluated in vitro must define the drug interactions over a broad checkerboard pattern of drug concentrations. This thorough analysis allows the investigator to define the interactions at multiple drug ratios and identify the dose–response areas where different and distinct efficacy and toxicity interaction regions exist. For example, the combination nucleoside strategy of AZT and ribavirin employed in HIV therapy results in two completely different regions of interaction between the drugs, with a region of extreme synergistic antiviral activity giving way to a region of significant antagonistic antiviral activity [11]. Since the combination drug concentrations employed in the in vitro assays can only be truly evaluated at those concentrations that yield less than or equal to the maximal 100 % protection as evaluated from replication or growth of the infectious organism, the combination assays often are performed at concentrations which are much lower than those that would be utilized in the clinic and thus are inadequate for truly evaluating toxicity or efficacy effects at high concentrations. Separate assays at appropriate drug concentrations should always be performed to evaluate toxicity effects in parallel with efficacy evaluations.

In the discussion below, the methodology routinely used to define combination anti-infective evaluations is described. Evolving from the early use of isobolograms and the evaluation of combination chemotherapy strategies for use in cancer patients [12, 13], the combination interaction evaluations used in the past two decades for anti-infective research have primarily involved one of two methods: the three-dimensional surface models as described by Prichard and Shipman [14] and the median dose–effect equation developed by Chou and Talalay [15]. A detailed discussion of the primary methodology considerations and analysis alternatives for the performance of combination anti-infective assays will be provided, followed by a discussion of assay modifications that should be employed to fully define the effects of a drug combination regimen. The novel variations of standard combination assays described provide a greater understanding of the effects of combination therapy in the cellular and tissue environments where the interactions will occur.

From the perspective of effective and efficient drug development, it is critical to understand both the benefits and limitations of the assay methodologies used to evaluate drug combination interactions and the meaning of the results that are obtained. The goal is to choose the appropriate biological assay for use in the evaluations as well as the correct statistically relevant analysis option to define the interaction of the compounds. For combination assays, the in vitro analyses are reasonably straightforward, though adequate assay repetition must be used in order to truly and quantitatively determine the interaction of multiple chemotherapeutic agents. Translating in vitro data to in vivo utility is difficult in light of the natural pharmacokinetic variation in drug concentrations that occur in patients, but several pharmacokinetic models have been described that allow a greater understanding of the relevance and predictability of the in vitro results [16–18]. It is also important to appreciate how these combination data will be viewed by regulatory agencies prior to clinical testing. From a regulatory viewpoint, it is fair to say that the absence of synergistic toxicity and/or antiviral antagonism should be carefully evaluated and confirmed as synergistic, or additive results will be dependent on the dose and regimen used in the clinic and may not be predictable from in vitro assays [19].

The methodology and analysis tools described below are useful for both in vitro and in vivo evaluations and are applicable across the complete spectrum of anti-infective organisms for which combination therapy strategies must be developed.

2 Methods

2.1 Definition of the Dose–Response Curve and Selectivity Index for the Drugs Evaluated

Determination of the efficacy and toxicity of drug interactions requires the appropriate cell-based or biochemical/enzymatic assay and accurate statistical evaluation. The assays utilized for combination drug evaluations should be chosen carefully based on the proposed use of the drugs in the clinical setting. In some cases, both cell-based and biochemical assays [20, 21] are required to fully understand the combined effects of the drugs. Evaluation in multiple cell types, including fresh and established human cells, may be necessary depending on the target-cell specificity of the infectious organism.

The starting point for all in vitro evaluations of combination drug interactions is the precise determination of the dose–response curve for each of the individual agents that will make up the combination therapy in the appropriate assay model. The assay or assays will yield efficacy values at the 25 %, 50 %, 90 %, 95 %, and 99 % level (EC_{25}, EC_{50}, EC_{90}, EC_{95}, and EC_{99}, respectively). It is these efficacy concentrations that will be used in the combination assay methodology to set the correct dose–response surface to be evaluated in a checkerboard pattern of drug concentrations. Additionally, it is important to understand the concentrations of the test compound that cause direct cytotoxicity or cytostasis, yielding 25 %, 50 %, 90 %, 95 %, and 99 % inhibition of cell growth

(IC_{25}, IC_{50}, IC_{90}, IC_{95}, and IC_{99} concentration values, respectively). Upon definition of the efficacy and toxicity values for a drug across its dose–response curve, the selectivity (or therapeutic) index of the drugs can be calculated (SI_{25}, SI_{50}, SI_{90}, SI_{95}, or SI_{99}); the SI is obtained by calculating the ratio of the IC concentration to the EC concentration at a defined level of protection (IC*x*/EC*x*) where *X* is defined as the percent level of protection achieved [20].

The evaluation of the interaction of two drugs requires the selection of a dose–response curve for each of the test agents that begins at doses below a concentration yielding any biological effect, increasing in concentration until complete inhibition of the replication of the infectious organism is achieved. Once the dose–response curves for each individual component of the combination therapy have been defined, the combination of the two drugs in a checkerboard pattern will yield a broad dose–response surface in three dimensions with the drug concentrations forming the *x* and *y* axes and the biologic effect on the *z* axis. The individual dose–response curves form one part of the complete dose–response surface that can be evaluated.

In performing combination assays, it is important to recognize that in most in vitro assay systems, the end point boundaries range between 0 and 100 % inhibition, and thus combination drug effects cannot be quantified where the additive or synergistic interaction of the two compounds would be expected to exceed 100 % inhibition. The concentrations of the agents to be tested must be carefully chosen so that the activity of the two drugs is not evaluated at a large number of points where additive inhibition exceeds 100 %. Similarly, the combination interaction cannot be quantified when an antagonistic interaction results in the level of efficacy falling below 0 % protection, or where combination toxicity effects result in percent toxicity exceeding 100 %. In addition, the interaction of the two drugs may be different at different drug ratios, with the possibility of defining distinct regions of synergy, additivity, and antagonism across the entire dose–response surface. In general each of the test compounds will be evaluated over a range of concentrations that yield a progression of activity from 0 through 100 % with constant incremental increases in drug concentration. The most sensitive measure of compound interactions over the complete dose–response surface occurs when the incremental increases in drug concentrations are small (two- to threefold) from tested dose to the next higher tested dose.

Finally, the design of the combination assay is dependent on how the data will be analyzed at the conclusion of the experiments. For some analyses, such as the original Chou and Talalay methodology (see below), the assay configuration will involve selection of a ratio of the two drugs to be evaluated and the testing of the effects of the drug combination in fixed multiples of that ratio. Recently, it has been recognized that drug interactions must be observed over a very complete dose–response surface [22], and so most analyses are performed with a checkerboard of drug concentrations where every possible combination of concentrations of the two drugs is tested together, yielding a complete three-dimensional combination dose–response surface. Over the years the methodology employed to define the effects of two compounds used together has dramatically improved. The discussion below provides an overview of methodology that has been employed when investigators evaluate combination drug efficacy and toxicity.

It should be noted and emphasized here that the greatest problem with the performance of combination assays is overall assay reproducibility. The size of the combination assays can be extremely large (over 450 data points per assay for three-dimensional models such as MacSynergy II), and data are accumulated across multiple microtiter plates, yielding some level of data variability from assay to assay (inter-assay variability) and plate to plate (intra-assay variability). For combination drug analysis, it is thus important to develop and optimize assays with minimal inter- and intra-assay variation [23]. Our experience indicates that the overall interpretation (synergy, antagonism, additivity) is highly reproducible. Variability is usually observed in the peak level of synergy or antagonism and in the concentration of each drug that results in the peak of synergy or antagonism. In general, combination assays must be replicated in order to precisely and quantitatively define the interaction of two drugs; in our experience that has meant repetition of a given drug combination assay, a minimum of three to five times before the relative level of synergy and the concentrations employed to achieve maximal synergy can be discussed with confidence. Despite the use of microtiter plate formats, these assays require a substantial amount of test compound compared to routine anti-infective evaluations. For high-throughput screening prior to precise definition of the most potent combinations, single-plate combination assay formats may be used, especially under conditions in which test compound or target cells are limiting.

2.2 Analysis of the Interaction of the Drugs Used in Combination

The benefits of combination chemotherapy have long been recognized, and experience with the treatment of HIV infection has driven the utility of combination strategies to new levels of development with three to four drugs forming the core of current highly active antiretroviral therapies (HAART) regimens [24, 25]. The methodology used to analyze the results of combination testing also has evolved [11, 14, 22, 26–31]. A variety of statistical methods have been developed, all with inherent advantages and disadvantages. Over the entire course of preclinical development of an anti-infective

agent, each of these evaluation techniques may be best used depending on the type of assay employed. Some algorithms (such as the three-dimensional MacSynergy II programs) are best suited for extremely large sets of data, with many concentrations in replicate over a wide dose–response surface, whereas others are well suited for situations in which the number of data points available for analysis may be limited, such as in animal model testing (Chou and Talalay median dose–effect equations). The primary problems encountered in developing the models for the evaluation of combination interactions result from the fact that the combination dose–responses represent a three-dimensional issue analyzed in two dimensions and from the fact that no agreement on the definition of additive or synergistic interactions has been obtained [22]. Fortunately, the increasing use of automation and highly complex analysis performed by personal computers has allowed three-dimensional dose–response curves to be easily visualized and evaluated [14, 32].

In simplest terms, a three-dimensional combination drug assay has two independent variables (the concentrations of the two drugs being evaluated) and one dependent variable (the anti-infective activity of the drug combination). The activity of the drug combination can be visualized as a three-dimensional surface with the drug concentrations on the *x* and *y* axes and the biological effect of the combination on the *z* axis. At the zero concentration points for each individual drug, the two-dimensional dose–response curve for a single drug can be observed in the three-dimensional dose–response surface. Evaluation of this three-dimensional surface and defining in statistical terms how the compounds interact have been accomplished by a variety of methodologies discussed in more detail below.

The basic dose–response surface can be evaluated by connecting the 50 % inhibitory levels across the dose–response surface to create an isobol at the 50 % inhibitory value, with the line that is produced representing all combinations of the two drugs that achieve 50 % inhibition of the replication of the infectious organism. The isobol, or line of equal elevation, was originally derived from cartography and is simply the contour line representing various levels of inhibition of the organism [33–41]. The isobologram is the two-dimensional contour plot that results [35, 36, 40]. The shape of the contour lines forming the isobologram represents the three-dimensional dose–response surface and thus provides the definition of the interaction of the two compounds as synergistic, additive, or antagonistic [14, 31, 33, 42–46]. Typically, isobolograms are plotted at the 50 % inhibitory concentration; however any fixed inhibition value can be utilized, and in most cases, multiple isobolograms should be evaluated to understand the interaction of two drugs across the entire surface, since the complete dose–response surface may include regions of synergy, additivity, and antagonism.

In the development of the various analysis models, certain statistical principles were utilized as the basic assumptions underlying the evaluation of the data. For example, several of the programs, notably those defined by Chou and Talalay, based their approach on the median-effect principle [15, 47, 48]. The Loewe additivity model is the basis of the null reference model [39]. Loewe additivity assumes that two drugs should be indistinguishable from each other with respect to antiviral effects in a combination assay. Thus, in this analysis one assumes that if a given concentration of two drugs inhibits replication by a defined amount, any fractional concentration of one drug (Drug A) combined with the complementary fractional concentration of the second drug (Drug B) should inhibit replication by the same amount. Loewe additivity can be expressed as

$$1 = \mathrm{D_A} / \left(\mathrm{IC}_p\right)_\mathrm{A} + \mathrm{D_B} / \left(\mathrm{IC}_p\right)_\mathrm{B}$$

where D_A and D_B are equal to the concentrations of Drug A and Drug B in the mixture that elicits *p* percent effect and $(IC_p)_A$ and $(IC_p)_B$ are equal to the concentrations of Drug A and Drug B in the combination that elicits the same *p* percent effect on the replication of an organism.

Prichard and Shipman based their MacSynergy II analysis program [14] on the Bliss independence null reference model [49]. This model is based on statistical probability and assumes that two drugs should act independently to affect virus replication. Thus if Drug A affects the replication of a population of organisms to a defined level, then the addition of Drug B should affect the remaining population of organisms to the level it would have affected in the absence of Drug A. Bliss independence can be expressed as

$$Z = X + Y\left(1 - X\right)$$

where *X* is equal to the fractional inhibition produced by the dose of Drug A alone and *Y* is equal to the fractional inhibition achieved by Drug B alone and *Z* is equal to the predicted fractional inhibition.

Each of these models offers robust mathematical data interpretation. In the sections below, the various methods that may be employed to evaluate in vitro combination testing results will be described in greater experimental detail. Generally, the models that have been developed to evaluate drug combinations include the fractional product method, the multiple dose–response curve method, isobolograms, the combination index method, the differential surface analysis method, and parametric surface fitting methods [14, 15, 26, 32, 38, 43, 45, 46, 50–69].

2.2.1 Multiple Dose–Response Curves

The simplest method of interpretation of the effect of a second drug (Drug B) on the activity of a single agent (Drug A)

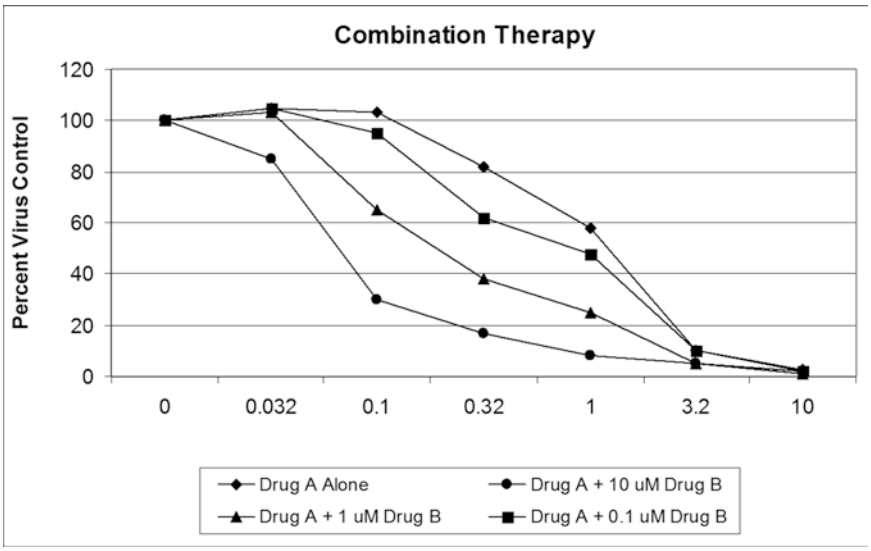


Fig. 79.1 Evaluation of combination interactions by multiple dose–response curve method. A representative example of the antiviral dose–response curve obtained with a single drug (Drug A) alone and with the addition of a single concentration of a second drug (Drug B). The various dose–response curves with increasing concentrations of Drug B may be compared to each other and that obtained with Drug A alone to evaluate the combination drug effect

is to evaluate the effect of a single concentration of Drug B on the dose–response curve of Drug A (Fig. 79.1). This evaluation superimposes the dose–response curves of Drug A obtained in the presence of Drug B, and increases or decreases in biological activity are observed as shifts in the dose–response curves due to the presence of the second agent. A wide variety of research papers have been published using this simple evaluation of the combination effects of two drugs [54, 59, 60, 65], and although the methods do not employ any statistical evaluation of the data that allow confirmation of the precise interaction as additive, synergistic, or antagonistic, the data evaluation does permit simple interpretation of positive or negative effects of the two drugs. Using this methodology, it is impossible to discriminate between slightly synergistic, slightly antagonistic, or additive interactions, although highly synergistic or highly antagonistic definitions are possible. Multiple dose–response curve evaluations are quite simple to perform, especially with a highly sensitive and reproducible assay system, but they obviously suffer from a lack of rigorous and statistics-based data evaluation and the ever-present issue of investigator bias in the interpretation of results.

2.2.2 Isobolograms

The classic method for detecting and characterizing departures from additivity between combinations is the isobologram methodology (Fig. 79.2) [39, 45, 56, 58, 63]. This method was originally introduced by Fraser [35, 36]. The use of the isobologram technique for analyzing drug combinations was extended by the work of Loewe and Muischnek [39], Loewe [38], and Berenbaum [33] (also see reviews by Gessner [37], Wessinger [41], and Berenbaum [34]). The isobologram is essentially a contour plot of a constant dose–response over the dose–response surface compared to a plot of the same contour under the assumption of additivity. Thus,

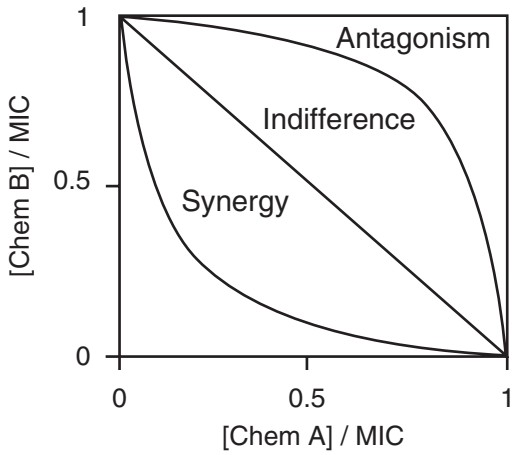


Fig. 79.2 Evaluation of combination interactions by isobologram method. A representation of the classical isobologram method for the evaluation of compound interactions

for a two-drug combination assay, the isobologram analysis compares the concentrations required to achieve a certain dose–response (such as 50 % inhibition of replication) to the line of additivity, formed by joining the 50 % inhibition concentrations of the two drugs when used alone as calculated experimentally. If the observed isobol falls below the line of additivity, the two drugs interact in a synergistic manner; if they fall above the line of additivity, the drugs are antagonistic. The predominant problem associated with the use of isobolograms to predict drug interaction is data variability. Isobolograms can be used to calculate the predicted interactions of two- or three-drug combinations.

2.2.3 Combination Index Method

Another widely used and accepted method for the analysis of anti-HIV data is the combination index method of Chou and

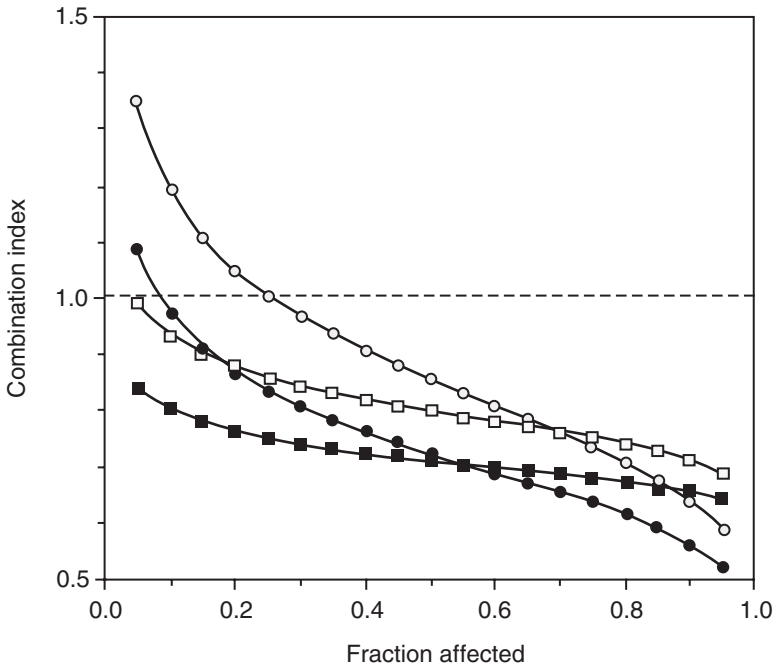


Fig. 79.3 Evaluation of combination interactions by median dose–effect equation method. Representative compound interactions as evaluated by the Chou and Talalay median dose–effect equation

Talalay (Fig. 79.3) [15, 26, 32, 51, 61]. As originally proposed, the experimental design of the Chou–Talalay method required that the total concentration of two drugs be altered, while fixed concentration ratios for the two drugs were maintained. The popularity of this method lies in the fact that relatively few samples are required for computer-based analysis and prediction of the nature of the drug interactions. However, since fixed drug ratios examine only the drug interactions along diagonal lines across the dose–response surface, it is possible the drug ratios chosen by the investigator do not reveal localized areas of synergism and/or antagonism on the drug dose–response surface plot. This means that several fixed-ratio drug combination experiments must be conducted to examine all diagonal lines across the dose–response surface. Recent adaptations of the combination index model now allow for the analysis of checkerboard patterns of drug concentrations as opposed to fixed ratios. The statistical model of Chou and Talalay is reported to be most useful when data points are limited, such as in animal studies. The limitation of the Chou and Talalay method remains the lack of confidence intervals in the statistical analysis of the data.

Through utilization of Monte Carlo mathematical modeling techniques, a probabilistic model, called ComboStat, simulating processes influenced by random factors (e.g., experimental variability associated with repetition of drug combination studies) was developed [51]. Upon application of this mathematical model, statistically relevant confidence intervals have now been assigned to the combination index values produced by the Chou–Talalay method. Using this methodology, it is possible to accurately interpret the Chou–Talalay drug combination index and statistically discriminate between mild synergism/antagonism and additivity. Unfortunately, the use of ComboStat, like the original Chou and Talalay program, requires drug combination studies with fixed drug concentration ratios. As mentioned above, this approach only examines drug interactions at diagonal lines across the dose–response surface, and local domains of synergism and antagonism can be missed unless all diagonals on the dose–response surface are examined.

2.2.4 Three-Dimensional Surface Analysis

The Prichard and Shipman MacSynergy II model evaluates combination data with assumptions based on same-site or different-site modes of action [14, 53, 62, 66, 67]. The more rigorous evaluation assumes the compounds being evaluated act at the same site to inhibit the replication of the infectious organism. The MacSynery II algorithm utilizes the data obtained with each drug alone to calculate the expected level of inhibition of the drug combination at each drug concentration in a checkerboard pattern, generating a three-dimensional surface of expected activity (Fig. 79.4). The actual data points determined experimentally are derived from the antiinfective assay and are plotted as the Antiviral Surface Plot. The expected activities are subtracted from the experimentally determined values at each data point, resulting in the generation of a three-dimensional synergy plot. If the expected and realized activities at each point are identical, a flat plane results indicating the interaction of the two drugs is additive. If the realized activity is greater than the expected level of activity, positive values are obtained, resulting in regions extending above the plane. These points represent the drug concentrations at which the activities of the drugs

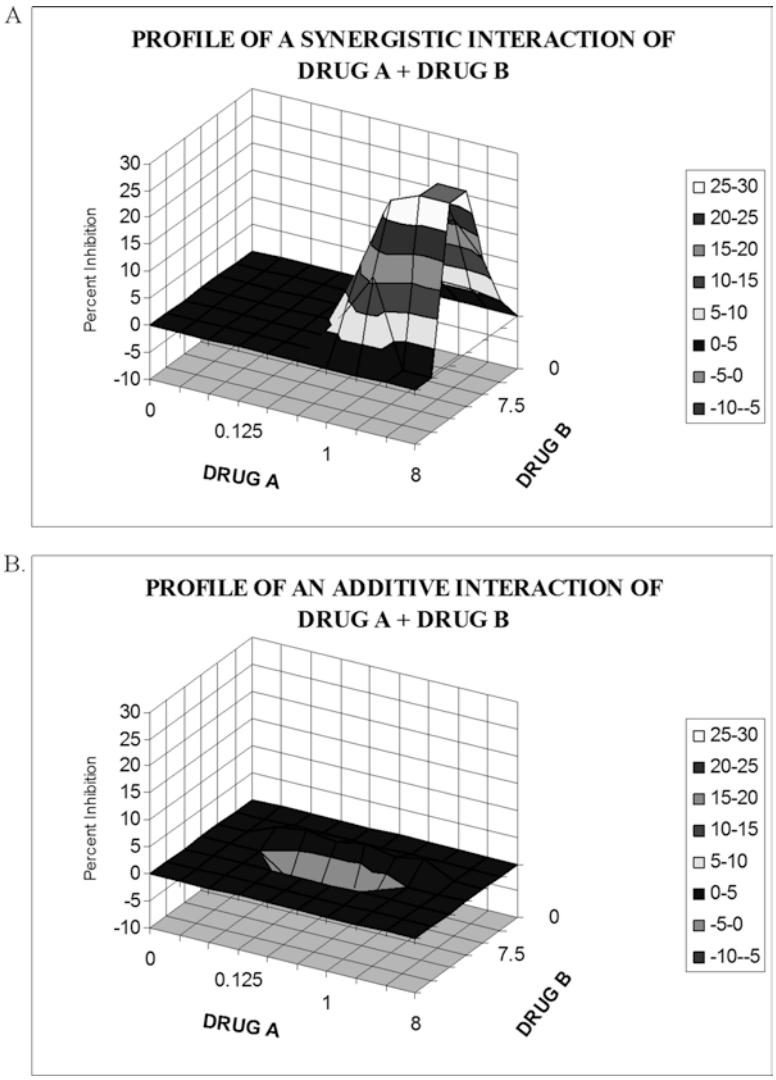


Fig. 79.4 Evaluation of combination interactions by three-dimensional surface method (MacSynergy II). Representative examples of MacSynergy II-based three-dimensional synergy plots illustrating synergistic (**a**), additive (**b**), antagonistic (**c**), or both synergistic and antagonistic (**d**) drug interactions when evaluated in cell-based combination assays

together are greater than that expected or are synergistic. An antagonistic interaction occurs when the realized level of protection is less than that expected; negative values are plotted three dimensionally as regions extending below the plane. The concentrations of the two compounds yielding maximal synergistic activity can be visualized easily with the Antiviral Contour Plot. MacSynergy II also calculates the volume of the synergy peaks or antagonism depressions, and these are used to quantify the amount of synergy or antagonism. The synergy volumes are calculated at the 95, 99, and 99.9 % levels of confidence.

2.2.5 Parametric Surface Fitting

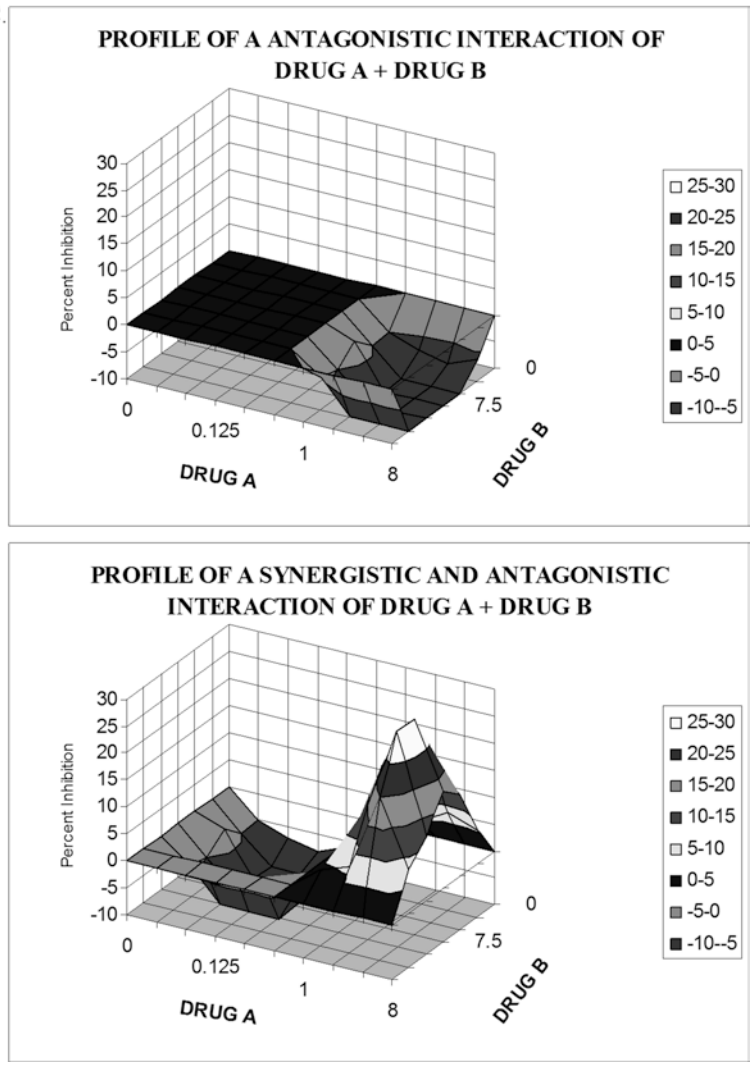
Parametric surface fitting is another three-dimensional modeling technique that uses response surface methodology to fit equations to the experimental data (an example is the COMBO software package) [43, 52, 57, 64, 70]. Mathematical parameters are used to define the surface as additive, synergistic, or antagonistic. The parametric surface fitting uses the Loewe additivity equation. Two models have been developed. Unfortunately, both are difficult to utilize and have the inherent problem that the equations were designed to fit a smooth three-dimensional surface, yielding results that are too simplistic for an irregular and complex three-dimensional surface like that obtained from antiviral combination assays.

2.3 Additional Considerations in Design of Combination Drug Evaluations

In addition to choosing the correct assay and an appropriate means of analysis, there are other considerations in developing a combination therapy regimen for clinical testing and use. These considerations are based on the proposed use of the combination therapy, the potential presence of other infections or drugs, the target of the therapy, and the poten-

Fig. 79.4 (continued)

C.



tial for greater than two drugs being utilized. These considerations and their importance will be discussed below.

2.3.1 Combination Efficacy and Combination Toxicity

Evaluation of the combination interactions of two or more compounds should include the evaluation of effects on both anti-infective efficacy and cellular toxicity. In most cases, the drug concentration ranges chosen for the combination evaluation extend from a low dose with no biologic effect through a high dose that yields at or near 100 % replication inhibition efficacy. These concentration ranges rarely touch on concentrations that are toxic to the host cells, and thus combination toxicity cannot be appropriately evaluated. Thus, in these assays, the toxicity portion of the dose–response curve is not observed, although in some cases synergistic toxicity may be observed when significant combination toxicity is present or when the selectivity index for the individual compounds is extremely narrow. Combination toxicity should be evaluated over a dose–response curve for the individual compounds that extends from a low concentration with no observed toxic effect on the host cells to a high concentration that results in significant toxic effects. These assays are possible after the complete dose–response curves for the individual compounds have been defined. All of the analysis methodologies described above for the evaluation of the combination assays may be used to predict combination toxicity effects. It is possible for anti-infective synergy to be observed that can be explained by a reduction in the toxicity of the two test compounds when used together. For example, we have shown that the efficacy of ribavirin and interferon-α is synergistically enhanced by the addition of a third compound being developed as an anti-HCV clinical therapeutic; the increased antiviral efficacy of the combination is explained by the action of the third compound in reducing the toxicity of ribavirin, thus enhancing its antiviral interaction and synergistic activity with interferon. Similarly, combinations of anti-HIV NCp7 zinc finger inhibitors with many approved anti-HIV

drugs have yielded synergistic anti-HIV activity with the antiviral efficacy derived from the reduced toxicity of the combination of test compounds. In vivo, triple-drug therapy of pegylated interferon with ribavirin and a second-generation HCV protease inhibitor has led to shortened therapy with high efficacy associated with more favorable tolerance and safety profiles, particularly in patients with cirrhosis [71]. Combination therapy of two direct-acting HCV inhibitors was recently FDA approved in order to eliminate the use of interferon and ribavirin [72]. Evaluation of these HCV drugs in combination with HIV HAART is still in clinical trials for use in HIV/HCV coinfections [73].

2.3.2 Mutually Exclusive and Mutually Nonexclusive Evaluations

Analysis of combination interactions using some of the available programs, such as MacSynergy II, requires the user to determine if the analysis should assume that the drugs inhibit the same or different anti-infective targets. These combination parameters have been defined as mutually exclusive and mutually nonexclusive combinations. As a combination therapy strategy, these two therapeutic regimens have also been described in the literature as convergent (same site) or divergent (different site) anti-infective therapies. In our evaluations we have determined that the choice of analysis options may be even more complex than the simple definition of the target enzyme, protein, or replication pathway. For example, the combination of AZT and ritonavir is a mutually nonexclusive therapy, targeting two different steps in the virus replication cycle and two distinct HIV proteins. However, the combination of the nucleoside RT inhibitor AZT with the non-nucleoside RT inhibitor Sustiva could be evaluated using either the mutually exclusive or mutually nonexclusive equations, since they target the same enzyme (RT) but at completely different sites on the enzyme. In many cases, compounds are evaluated in combination assays before the mechanism of action is known or compounds may have a primary and a secondary mechanism of action. We have found that the use of the mutually exclusive evaluation equations provides a more robust evaluation of the interaction of the test compounds.

2.3.3 Performance and Evaluation of Three-Drug Combination Assays

With the increasing incidence of transmission of drug-resistant organisms, more than two drugs are often given to patients simultaneously. The numbers of drugs that can and should be used in combination in the clinical setting requires methodology for evaluating combinations of greater than two test drugs. Prichard and Shipman first described the use of MacSynergy II for evaluating the interaction of three drugs in combination [74]. In these assays, the combination dose–response surface was evaluated for the two-drug combination of acyclovir and 2-acetylpyridine thiosemicarbazone, generating a dose–response surface including 45 data points, each defined in triplicate to allow calculation of the 95 % confidence interval for each data point. This dose–response surface was replicated five times, and each replicate included a single dose of 5-fluorodeoxyuridine. For each of the five replicate dose–response surfaces, the activity defined for the two-drug combination was subtracted, yielding the change in activity that resulted from the addition of the third drug. As with the two-drug interaction analysis using MacSynergy II, the synergy volume can be calculated, and the concentrations of the drugs that yield synergistic interactions can be directly defined. Using the checkerboard pattern of evaluation, regions of different interactions can also be observed and quantified.

2.3.4 Combination Testing with Resistant Organisms

One of the primary driving forces for the use of combination therapy strategies in the clinic is to suppress the selection and replication of drug-resistant organisms. The component drugs of the combination therapy should each have the capacity to inhibit the replication of viruses that are resistant to the other drugs used in the regimen. In some cases, the drugs used must be able to suppress the replication of resistant viruses that were selected to drugs within the same class of inhibitor. The in vitro evaluation of the interaction of two or more drugs should be extended to include evaluation of the ability of the combination of drugs to inhibit drug-resistant viruses, especially the variety of multidrug-resistant (MDR) [75] viruses that have begun to circulate in the patient population. With an estimated 10 % of new infections involving the transmission of resistant organisms [76, 77], this has become increasingly important for anti-HIV therapy. Therapeutic combinations must be evaluated for inhibition of drug-resistant organisms when searching for drugs for pathogens including 3TC-resistant hepatitis B virus strains, amantadine-resistant and oseltamivir-resistant influenza virus strains, protease inhibitor-resistant HCV, antibiotic-resistant bacteria, and drug-resistant tuberculosis. A number of research reports have demonstrated the ability of a combination of drugs to inhibit a drug-resistant virus to one of the components of the drug regimen. In most cases synergistic or additive antiviral interactions are observed with drug-resistant strains when the test concentration of the drug to which the virus has become resistant is increased relative to the level effective against wild type. These results would suggest that clinical resistance can be overcome by increasing the dose or by defining and using the highest possible concentration of a drug to essentially sterilize the patient of replicating virus.

Table 79.1 Combination drug-resistant virus selection using dose escalation method

	Amino acid change (no. of passages in cell culture) with the following Compound 1			
Compound 2	None	Calanolide A	Costatolide	Dihydrocostatolide
None	–	T139I	T139I/L100I	L100I
3TC	M184V	M184V/L100I (5)	M184V/L100I (6)	M184V/L100I (5)
Diphenyl sulfone	Y181C	V108I (6)	Y188H (6)	ND[a]
E-BPTU	Y181C	K103N/V106I (6)	ND	ND
α-APA	Y181C	ND	K103N (14)	ND
UC10	K101E/Y181C	Y188H (6)	K103N (5)	K103N (5)
TSAO	Y181C	K101E (6)	K101E (6)	K101E (6)
Diaryl sulfone	Y181C	Y188H (13)	Y188H (11)	Y188H (8)

Amino acid changes in virus genome determined by dideoxy sequencing following in vitro drug resistance selection of Compound 1 alone or in combination with Compound 2. The virus passage number sequenced is in parentheses

2.3.5 Combination Resistance Selection Evaluations

A variety of resistance selection strategies are available to select for drug- or antibiotic-resistant organisms. These same strategies can be used to select for viruses or bacteria that are resistant to a combination of drugs either sequentially or in a true combination fashion (Table 79.1). We have observed that the pattern of resistance-engendering mutations changes dramatically when a combination of agents are used in the selection strategy. Resistance selection strategies usually employ a high fixed concentration of the drug used for selection (or combination of drugs) or use the technique of serial passage of the microbe in the presence of increasing concentrations of the test compound. Since these techniques are routinely employed to select for resistant organisms, the methods do not always provide additional data on the relative anti-infective impact of the combination strategy. Techniques have been developed for the passage of the organism in the presence of drug that are highly standardized with regard to selection pressure (i.e., the EC_{50} or EC_{90} concentration from passage to passage). We have employed a virus transmission sterilization assay with single and multiple drugs to rapidly select for drug-resistant virus strains in the presence of a variety of fixed high concentrations of the test drugs.

2.3.6 Combination Assays to Evaluate Treatment for Multiple Infectious Organisms

Another important consideration in the design of a combination therapy strategy is the effect of the individual drugs on those drugs being used to treat other infectious disease organisms. This is especially important when considering therapies for transplant patients undergoing immunosuppression (neutropenia), for immune-compromised AIDS patients, and in situations involving viral and bacterial coinfection. The combination assay strategies discussed in this chapter can be utilized for evaluating the effects of the agents on other indications. For example, an antiviral agent designed to treat HIV infection can be evaluated in antibacterial or antifungal assays to determine if the addition of the antiviral agent has any positive or negative effect on the efficacy and toxicity of antimicrobial agents. Conversely, antimicrobial agents should be evaluated for their effects on the HIV therapy. Since the drugs may not be active against the target organisms used in the assay (e.g., the antimicrobial agent versus HIV), it is important to utilize therapeutically relevant concentrations of the agent as opposed to trying to define a concentration that is actually active against the nonspecific organism. The use of checkerboard drug concentration format for these assays allows the broadest possible dose–response surface to be evaluated.

2.3.7 A Special Case: Potentiation and Suppression

In some cases, the two test agents may include one that does not have any detectable activity against the organism being tested or may not be active in the particular assay being employed. In this case, discussions of synergistic and antagonistic interactions of the agents are not completely correct. Combination assays and analysis programs can be performed on these combinations of agents exactly as described above, but the results of the assays should be expressed in terms of potentiation (or enhancement) and suppression (or inhibition) of activity, depending on whether the result was synergistic or antagonistic as defined by the analytical end point of the assay. The terms potentiation and suppression are generally correct when discussing combinations of agents active against different organisms. Compounds that have activity in chronic infection models but not in acute infection models against the same organism might be evaluated in potentiation assays with compounds that only exhibit efficacy in the acute infection models. As discussed previously, it is important to carefully choose the drug concentrations to

be evaluated so that concentrations are therapeutically relevant, even if a particular drug is inactive, and that a broad dose–response surface is evaluated.

2.3.8 Biological Relevance of the Test System to the Therapeutic Strategy

When considering the effectiveness of a combination therapy, it is critical to select the appropriate system and assay for use in the evaluations. Since most combination therapies will be utilized for the therapeutic treatment of systemic infections, the assays to be utilized should have relevance to the biology of the infection. Thus, appropriate cell lines and virus strains should be used, and the assay may be modified to more closely mimic the therapeutic environment through the addition of serum proteins and other additives. Combination products may also find utility as vaginal or rectal microbicides (prevention or preexposure prophylaxis regimens) or for the treatment of wounds or other topical and mucosal infections. In these systems, the cells used for the assays, the isolates chosen for evaluation, and the additives used to mimic the infectious environment will be modified to reflect the therapeutic use of the compound. Determining the appropriate end point measurement of antiviral effectiveness is also important, as in HSV-1 labialis infections where lesion development and progression in recurrent disease is dependent primarily on the proinflammatory host response rather than virus replication as in the initial infection. Combination therapy of a topical antiviral with an anti-inflammatory agent is under investigation for recurrent HSV infection, where antiviral agents have only shown moderate effect on lesion healing time unless you treat early in the prodromal stage [78]. For some regimens, it is also important to consider the method of formulation of the final product and to perform the combination evaluations under those conditions. For example, the final form of a topical vaginal microbicide often includes excipients that may have therapeutic or toxic effects and that may potentiate or suppress activity of the drug of interest [79]. We have also clearly shown that appropriate formulation of drug products may substantially enhance the absorption/permeation of a drug into target cells or tissues, yielding enhanced antiviral effectiveness (and possibly enhanced toxicity).

2.3.9 In Vitro Pharmacologic Models and Evaluations

Combination methods have recently been developed to take into account pharmacodynamics of drug exposure [18]. In these model systems, the concentrations of the drugs in contact with the cells are continuously modified to approximate the plasma concentrations of the drugs in a human being. Thus rather than culturing cells and virus in the presence of fixed concentrations of the two drugs, in the pharmacodynamic model each drug concentration is fluctuated as it would be in the patient, allowing the investigator to model antiviral and toxic effects more realistically than in tissue culture systems. Though this model is an advancement that can aid in prioritizing combination therapies for clinical use, these methods are very expensive and time consuming and are not practical for routine and high-throughput evaluation of combinations of compounds. In addition, these are still in vitro assays: metabolism of the compounds, generation of metabolites, and interaction with tissues do not occur, and thus they do not completely reflect in vivo use.

3 Virologic Evaluations

3.1 Virus Replication and Functional Cell-Based Assays

The most relevant cell-based assays include clinical strains of virus and fresh human cells. For HIV, assays using fresh human PBMCs, monocyte/macrophages, and dendritic cells, as well as assays with tissues such as cervical explants for microbicide testing, have been developed [6, 20, 80]. For a number of other viral agents, a variety of in vitro screening assays involving measurement of cytopathic effects, virus replication, or plaque-formation assays can be performed [81–93]. In these cases, the end points of the assays are quantitative readouts of virus production and typically involve measurement of a viral enzyme, measurement of a viral capsid protein, or measurement of infectious virus. These values can be entered into the analysis programs defined above as actual raw data values or the values expressed as a percentage of the virus or cell control. Although many of these assays are suitable for high-volume screening, in general the variability in fresh human cell populations requires that many replicates of these assays be performed unless a highly standardized and reproducible infection of the primary cells can be achieved. In addition, the cost of both the assay and the availability of adequate cell numbers or tissues can affect the number of replicates that can be performed.

3.2 Assays Measuring Cytopathic Effects

For anti-infective testing for most organisms, a simple, reproducible, and cost-effective solution to high-throughput combination antiviral evaluations is to utilize assays that quantify virus-induced cytopathic effects (CPE) and the ability of test agents to suppress these cytopathic effects [81–93]. A number of tetrazolium dyes and other colorimetric reporters can be used to quantify viability in the cell cultures, and the differential between virus and cell controls can be used as the measure of percent protection. These percent protection (or percent cell viability) values can be easily imported

directly into the analysis programs and the combination interaction quickly evaluated. One drawback of these assays is that the virus replication is not measured directly, but rather an effect of decreased viral replication is measured (which should, in most cases, be proportional to the level of virus produced). The compound not only has to suppress virus production but also has to suppress the CPE, which may not be a natural feature of viral infection in the patient. In addition, virus-induced CPE assays routinely use laboratory-derived strains of virus and established human cells that may not accurately mimic infection in patients. Despite these caveats, CPE assays are the assay of choice for high-throughput combination evaluations in light of their extreme reproducibility (low intra- and inter-assay variability) and low cost. CPE assays are available for nearly all infectious organisms routinely screened in anti-infective development programs. Viral plaque reduction assays are a similar but more labor-intensive approach since the plaques produced by infection must be microscopically counted, introducing greater cost, variability, and level of assay difficulty than assays using reporter dyes.

3.3 Enzymatic and Biochemical Assays

Biochemical assays that directly quantify the ability of a test compound to inhibit the target enzyme or block binding to a target protein are (in most cases) the simplest and least expensive of the various combination assay formats [20]. Biochemical or enzymatic assays effectively reproduce antiviral mechanism of action assays. The readouts of these assays usually have radioactive, colorimetric, fluorescent, or chemiluminescent end points, and the values obtained can be compared to a positive and negative control allowing percent inhibition values to be calculated. These values can be directly imported into the programs for analysis of the combination interaction. Although these assays are usually rapid, inexpensive, easy to perform, and extremely reproducible and quantitative, they have several disadvantages. First, they do not take place in the intact cell. These assays do not require the test agents to actually penetrate the cell membrane and accumulate at the site of action. A second issue of biochemical importance is metabolism by the intact cell, such as the phosphorylation required for nucleoside analogs. Antagonistic effects on metabolism would be missed in a biochemical assay. Third, biochemical assays do not provide information on combination toxicity obtained through cell-based assays. Finally, quite often the enzyme that is targeted by one component of the combination therapy is not targeted by the other, and therefore the combination biochemical assay merely informs the investigator whether or not the inactive drug potentiates or interferes with the activity of the active drug in the limited context of the biochemical assay.

3.4 Chronic and Acute Infection Assays

A special case of cell-based assays involves testing of agents in cells that are chronically infected with an infectious organism and that constitutively or latently produce virus. Though most approved antiviral agents target steps that occur early in the infection cycle, assays with chronically or latently infected cells quantify the effects of test agents on late stages of virus production such as transcription, translation, virus assembly, maturation, and release from the infected cell. The strengths and weaknesses of these cell-based models are identical to those presented above for virus replication-based assays. Though the throughput and reproducibility are much higher than that observed for primary human cell assays, the chronic systems typically require more expensive systems for end point detection. In any event, for compounds that target late stages of infection, it is important to test the combination efficacy and toxicity in both acute and chronic infection models in combination with agents that are more than likely active only in the acute infection models. These assays will essentially confirm that the chronic infection inhibitor will not interfere with the acute infection inhibitor and vice versa.

3.5 Cell-Free and Cell-Associated Virus Transmission Assays

The bulk of antiviral assays performed measures the amount of virus that is produced from infected cells which serves as the inoculum for successive rounds of viral infection. The activity of antiviral agents alone and in combination is measured by reduction of the amounts of virus produced from these infected cells via measurement of viral proteins, enzymes, and infectious progeny. It has been shown that infectious virus may be transmitted from an infected to an uninfected cell directly, without an extracellular phase (or perhaps an extremely short duration of time between release and reinfection). Antiviral assay methodology to measure cell-to-cell transmission has been developed which includes the cocultivation of infected and uninfected cells with measurement of virus-induced syncytium formation and rapid progeny virus production from the co-cultured cells [94]. Data from these assays may involve semiquantitative or quantitative measurement of syncytium formation (or virus-induced cytopathic effect) and measurement of virus burst from the co-cultured cells.

4 Microbiologic Evaluations

The concept of using antimicrobial drugs in combination dates back to the early days of chemotherapy. Combination therapies historically were used either as a means to extend

the therapeutic spectrum against diverse genera and organisms of unknown sensitivity or as a means to stem the tide of selection for drug-resistant strains during extended treatment regimens. Representative examples are the well-known combination of trimethoprim and sulfamethoxazole used for multiple bacterial indications [95], multidrug therapy for tuberculosis [96], and eradication of *Helicobacter pylori* in peptic ulcer disease [97]. Other examples that fall into the combination category range from the streptogramin drug Synercid® (a mixture of quinupristin and dalfopristin 30:70 w/w for parenteral administration, Monarch Pharmaceuticals) indicated for vancomycin-resistant *Enterococcus faecium* to Augmentin® (multiple formulations of amoxicillin and the beta-lactamase inhibitor clavulanate, GlaxoSmithKline Pharmaceuticals) used primarily for community-acquired pneumonia (CAP), bronchitis, and otitis media. Amoxicillin/clavulanate is unique in that it combines an antibiotic with an inhibitor of a common resistance mechanism (secreted beta-lactamase).

4.1 Methods to Study Antibiotic Interactions

Several in vitro methods have been devised to measure the interaction between two or more antibiotics in bacterial culture systems. The primary goal of these studies was to determine whether the drugs acted in synergy to increase killing efficiency above that seen with either agent alone or whether they were antagonistic to each other and thus could have the potential to decrease efficacy and adversely affect clinical outcome. All methods provide either a direct numerical readout such as the fractional inhibitory concentration index (FICI) of a checkerboard test or measurable changes in growth dynamics and viable cell count as seen in time-kill assays from broth cultures.

4.1.1 Checkerboard Testing

This system is an extension of standard broth microdilution methodologies used for the determination of minimum inhibitory concentration (MIC) [98, 99]. Presently, no officially recognized checkerboard testing standard exists. However, starting inoculum densities and scoring of bacterial growth at the end of the assays generally follow the MIC microdilution protocols of the Clinical and Laboratory Standards Institute (CLSI; formerly the NCCLS). Checkerboards are simple arrays of serial dilutions of each drug in two dimensions across microtiter plates. Individual MIC values for each drug are determined against the test organism prior to the assay. Starting drug concentrations are selected such that they bracket the respective MICs by three or four dilutions. After dilution, the plates are incubated and each well is read as for a standard MIC assay. Once wells are scored for growth inhibition, fractional inhibitory concentrations (FICs) are calculated by dividing the MIC of the first drug in combination with the MIC of that drug when used alone. The same process is carried out for the second drug. Both FIC values then are added together to create the fractional inhibitory concentration index (FICI) for the combination. FICI values ≤ 0.5 indicate synergy, whereas values >4.0 indicate antagonism. Values between these two end points represent no significant interaction. Since previous literature sources have made claims as to the significance of intermediate FICI values falling between 0.5 and 4.0, the editorial board of the Journal of Antimicrobial Chemotherapy in 2003 instituted the requirement that these values be used for manuscript submission and required that intermediate values should be labeled as no interaction [100]. These recommendations appear to have broad acceptance in the field. Despite this acceptance the checkerboard MIC test suffers due to lack of reproducibility and the fact that the assay only measures bacteriostatic effects. Variability in the MIC evaluation as well as testing a bacteriostatic agent in combination with mostly bactericidal agents may be the cause for the overestimation of synergy experienced with the checkerboard test. Confirmation of these combination MIC assays should be obtained with a quantitative time-kill assay [101].

4.1.2 Time-Kill Testing

Although not as simple to configure as checkerboard arrays, time-kill assays provide both a kinetic readout of bacterial kill rates over the course of the experiment as well as an indication of synergy, antagonism, or indifference after 24 h of antibiotic exposure and are often used to confirm the results determined in the checkerboard assay. These tests are based on the macroscale broth method used for the determination of bactericidal activity as specified by the CLSI [102]. Broth cultures are configured with test organism, and drugs are added either alone or in combination at fractions or multiples of the MIC (generally ranging from 0.25 to 2 times the MIC) [103]. Cultures can be monitored over the course of exposure to examine bacterial growth/kill kinetics and at the end of the assay period for determining synergy, indifference, or antagonism. In this system, as determined by Eliopoulos and Moellering, an interpretation of "synergy" required a ≥ 2 $\log_{10}$ decrease in cfu/mL by the drug combination when compared with its most active constituent after 24 h and a ≥ 2 $\log_{10}$ decrease in the cfu/mL below the starting inoculum. Likewise, the drug combination was considered to be "antagonistic" if there was a ≥ 2 $\log_{10}$ increase in cfu/mL, and "no interaction" was the interpretation of a <2 $\log_{10}$ change in cfu/mL. White et al. defined synergy as a combination that produced ≥ 2 $\log_{10}$ reduction in colony forming units (CFU) compared to the most active of the two drugs when

used alone [103]. Likewise, ≥ 100-fold increase in CFU indicated antagonism, whereas <10-fold change indicated indifference.

4.1.3 E-Test Strip

The Epsilometer or E-test strip (AB Biodisk, Solna, Sweden) has been utilized for synergy testing [103]. In this configuration, an E-test strip for each drug is placed onto an agar plate inoculated with the test organism. The strips are laid onto the agar surface in a crossed pattern such that the perpendicular intersection of the two strips contact at the precise point on the scale of the individual MIC for each drug. Following incubation, a zone of inhibition radiates out from that point of intersection. The MIC of each drug in combination is read off each scale by noting where the zone of inhibition contacts each strip distal to the point of intersection. FICI values are calculated by the same process as that used for the checkerboard test. Frequent agreement between the E-test, checkerboard, and time-kill assays was found in this study [103], but there was sufficient variability and discordance between the tests to suggest that neither one could be used alone when evaluating new drug combinations. Therefore, when testing new antibacterial agents or combinations of currently approved drugs for new indications, multiple assays should be performed and compared.

4.2 Combination Testing and Prediction of Clinical Outcome

Despite the availability of testing methods for possible interactions between antibiotics used in combination, the final determination, as with any therapy, is whether or not there is a favorable therapeutic outcome. Few examples of synergistic combination therapy exist in the literature, and generally these tend to describe special situations such as therapies for Gram-negative sepsis in neutropenic patients or enterococcal endocarditis [104–106]. Even recent guidelines for combination therapy in normal adult community-acquired pneumonia (CAP), where a macrolide-class drug is recommended together with a beta-lactam, are directed toward increasing spectrum in order to cover atypical organisms rather than for any synergistic pharmacodynamic consideration [107]. Investigators must consider the pharmacokinetic properties of the individual agents. Will combining two drugs with vastly different serum half-lives (such as a macrolide and a beta-lactam) have relevance at the actual site of infection? What about differences in tissue distribution at those sites [108]? One can also argue that static testing methods such as the checkerboard assay have little relevance to the dynamic environment encountered in vivo and that alternative models may be more relevant for predicting clinical outcome [109]. At best, assays such as the checkerboard and time-kill can help predict whether any overt antagonism may exist between two antimicrobial drugs and whether the possibility remains for synergism in vivo.

4.3 Combination Antibiotic Products for the Treatment of Biofilms

Combinations of antibiotics have been found to be effective in the treatment of chronic bacterial infections such as staphylococcal infections which are associated with biofilm formation. The formation of biofilms has been shown to significantly decrease the susceptibility of pathogenic bacteria to antibiotic treatment relative to the individual planktonic organism [110–113]. Biofilm formation has thus been suggested to be a mechanism of antibiotic resistance and combination approaches to attack both the infectious microorganisms, and the structure of the biofilm may be necessary.

As mentioned above the activity of combinations of antibiotics is most commonly evaluated in vitro using standard susceptibility tests based on broth microdilution and diffusion. These methods, however, do not take into consideration the specific requirements for the evaluation of the activity of products against the complex structure and often heterogeneous makeup of the biofilm. Although valuable information for combination biofilm inhibition studies can be obtained using the checkerboard MIC testing and time-kill analysis as described above, additional tests are needed which will take into account the different population of bacterial cells that constitute the biofilm [114]. Biofilm susceptibility assays can be performed using a variety of methods [115–117]. In addition to defining problems in the feasibility of multiple comparative biofilm inhibition studies, for a variety of reasons, the different methodologies used for evaluating combination antibiotic products targeting biofilm formation are not amenable to comparing results obtained from different studies [114]. These significant differences in methodology include varying times of exposure [113], different antibiotic concentrations [118], varying growth states of the bacteria (i.e., adherent [119] vs. suspension [112, 120]), degree of adherence of different bacteria, the support surface used to grow the biofilm, age of the biofilm, and the medium and specific growth conditions (rich medium versus minimal essential medium) in which the organism was grown.

It is critical in the development of new and novel therapies involving combinations of antibiotics that the individual and composite population of cells responsible for biofilm formation are also being targeted and that the assay variables described above are taken into consideration when deciding on the appropriate methodology to use.

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Antimicrobial Susceptibility Testing Methods for Bacterial Pathogens

80

Fred C. Tenover

1 Introduction

Gone are the days when the antimicrobial susceptibility pattern of a bacterial isolate could be predicted simply on the basis of its species identification. Although *Streptococcus pyogenes* isolates remain susceptible to penicillin, one has to continually ask—for how long? With the discovery of strains of *Staphylococcus aureus* that are highly resistant to vancomycin [1], the emergence of carbapenem-resistant *Enterobacteriaceae*, and strains of *Acinetobacter* species that are pan resistant [2, 3], the role of antimicrobial susceptibility testing in guiding therapy for infectious diseases is becoming increasingly important [4]; it is a key function of clinical microbiology laboratories. Results guide physicians in their selection of appropriate antimicrobial therapy for patients with infections. Yet, ironically, many of these novel resistance phenotypes are not easily detected using the automated susceptibility testing methods so prevalent in today's clinical laboratories [5, 6]. The ability of the clinical laboratory to detect emerging resistance profiles is often directly related to the extra efforts expanded to catch novel resistance mechanisms. Although resistant bacteria were common previously only in intensive care units of hospitals, multidrug resistance has become an issue among strains of community-acquired pathogens such as *Salmonella*, *Shigella*, and even *Neisseria gonorrhoeae* [7–9]. To complicate matters even further, resistant organisms that arise in the community are now also spreading into healthcare settings [10, 11]. Thus, it is imperative that changes in resistance patterns of a wide range of bacterial pathogens be monitored continually to insure optimal treatment both of the individual patients and for maintaining the efficacy of empiric therapy regimens. Antimicrobial susceptibility test methods include disk diffusion and minimal inhibitory concentration (MIC) methods, such as broth microdilution, agar dilution, and agar gradient diffusion. MIC tests often utilize semi- or fully automated platforms to decrease time to results and improve workflow. Microbiology laboratories often employ supplemental tests to maximize detection of unusual or borderline-resistant phenotypes or emerging resistance mechanisms that may be missed by standard methods. Qualitative results (susceptible, intermediate, or resistant) for antimicrobial agents may be accompanied by quantitative values for MIC test to help guide dosing regimens. Molecular-based tests, such as polymerase chain reaction assays and film arrays, are used with increasing frequency to provide rapid results, often within 1 h, for resistance genes or mutations associated with antimicrobial resistance to improve antimicrobial therapy. Such assays have gained widespread acceptance for methicillin-resistant *Staphylococcus aureus* (MRSA) in positive blood cultures, vancomycin resistance genes in enterococci, and multidrug-resistant strains of tuberculosis from patients with respiratory disease. This chapter will explore in detail the methods used for antimicrobial susceptibility testing of bacterial pathogens.

2 Antimicrobial Susceptibility Testing Methods

The two major phenotypic methods of determining the susceptibility of a bacterial isolate to an antimicrobial agent are disk diffusion and minimal inhibitory concentration (MIC) testing. In the United States, based on informal surveys, approximately 85% of susceptibility test results are produced using automated methods, while the remainder is mostly the result of disk diffusion testing. However, clinical laboratories also utilize a series of screening and confirmation tests to detect subtle resistance mechanisms and insure the accuracy of antimicrobial susceptibility test reports (Table 80.1). More recently, molecular methods to detect antimicrobial resistance genes and mutations associated with resistance phenotypes have been introduced into clinical

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Table 80.1 Phenotypic screening and confirmation tests

Test name	Resistance phenotype detected	Organism groups
Aminoglycoside resistance, high level	Synergistic activity with ampicillin, penicillin, or vancomycin	Enterococci
Cefoxitin disk test	*mecA*- and *mecC* -mediated oxacillin resistance	Staphylococci
D-Zone test	Inducible clindamycin resistance	Staphylococci, streptococci
Extended-spectrum beta-lactamase screening and confirmation tests	Extended-spectrum cephalosporin resistance	*Escherichia coli*, *Klebsiella* species, *Proteus mirabilis*
Carba NP test	Carbapenem resistance	*Enterobacteriaceae* and *Pseudomonas* species
Modified Hodge test	Carbapenem resistance	*Enterobacteriaceae*

microbiology laboratories. While the most commonly used test is likely the direct detection of MRSA in nasal, wound, or positive blood culture bottles using real-time polymerase chain reaction (PCR) assays [12], a variety of other testing platforms, including microarrays, film arrays [13], and peptide-nucleic acid fluorescent in situ hybridization assays (PNA-FISH) [14] are also used in clinical microbiology laboratories to detect antimicrobial-resistant organisms.

2.1 Disk Diffusion

The disk diffusion method has one of the more colorful histories among clinical microbiology tests that includes luminaries in anti-infective research such as Alexander Fleming, John Sherris, and William Kirby, international collaborative studies headed by the highly influential microbiologist Hans Ericsson, and even a US Supreme Court decision [15–17]. The method as we now know it consists of placing paper disks saturated with inhibitors of bacterial growth (i.e., antimicrobial agents) on a lawn of bacteria seeded on the surface of an agar medium, incubating the plate overnight, and measuring the presence or absence of a zone of inhibition around the disks. In the early 1950s, there was little standardization of disk content, inoculum size, or incubation conditions among laboratories performing the tests. Oftentimes multiple disks, each with a different concentration of the same antimicrobial agent, were used to assess susceptibility. Ericsson and colleagues developed a standardized single disk method that was widely used in Scandinavia [18]. This served as the basis for an international collaborative study that eventually produced a standardized method. Studies conducted at the University of Washington in the mid-1960s resulted in the technique often referred to as the "Kirby-Bauer method," which was published by Bauer and colleagues in 1966 [19]. This method standardized the variables of disk size, inoculum size, temperature, and time of incubation. Results were reported qualitatively as susceptible, intermediate, or resistant. Around this same time, several companies manufactured disks for testing in the United States, but the amount of drug present in the disks varied significantly from lot to lot. The US Food and Drug Administration accepted the responsibility for monitoring the content and potency of each lot of disks manufactured in the United States. A challenge to that authority by a disk manufacturer made its way to the US Supreme Court in 1962. In their decision, the Supreme Court not only reaffirmed the responsibility of the FDA to monitor each batch of disks for potency but noted that manufacturers of antibiotic disks had a legal obligation to describe how the disks were to be used [20]. The US Supreme Court recommended the single disk method of Bauer et al. as the standardized testing method of choice. The rejection rate of antimicrobial disk lots by the FDA dropped from 66 % in 1958 to only 5 % in 1962. The disk diffusion method described by Bauer et al. has been continually expanded and improved by the Clinical and Laboratory Standards Institute (CLSI, formerly known as the National Committee for Clinical Laboratory Standards) in the United States. Several other international societies (e.g., the British Society for Antimicrobial Chemotherapy and the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST)) have similar techniques. Alternative disk-based methods including the Roscoe NeoSensitabs and the Australian Calibrated Dichotomous Sensitivity (CDS) method are also used in some countries. Instruments that measure the zones of inhibition using cameras can speed the process of reading disk diffusion plates. These instruments can also transform the zone diameter readings into approximate MIC values.

2.2 Minimal Inhibitory Concentration (MIC) Testing

The goal of MIC testing is to provide a quantitative result (in μg/mL) along with a categorical interpretation (susceptible, intermediate, or resistant) that can guide antimicrobial therapy more precisely, particularly for infections in body sites where antimicrobial agents achieve lower concentrations than in serum (e.g., cerebrospinal fluid and bone). MIC testing can be performed by one of several methods including agar dilution, broth microdilution, and agar gradient dilution or by one of several automated methods. Quantitative MIC results are also useful when long-term therapy is required, as for bacterial endocarditis and osteomyelitis.

2.2.1 Agar Dilution

The agar dilution method involves preparing a series of agar plates containing the antimicrobial agent to be tested in increasing concentrations, usually in doubling dilutions (i.e., 1, 2, 4, 8, 16, 32 μg/mL, etc.). A suspension of the organism to be tested is prepared to equal the turbidity of a 0.5 McFarland standard [approximately 1×10^8 colony-forming units (CFU) per mL], and 1–5 μL of this suspension is placed on each of the series of plates with increasing concentrations of the antimicrobial agent using a Steers replication (delivering approximately 5×10^4 CFU per spot). Thirty different bacterial isolates (plus quality control organisms) can be tested simultaneously on each agar plate. Non-fastidious organisms are incubated at 35 °C for 16–18 h usually in ambient air, while fastidious organisms, such as *Streptococcus pneumoniae*, are incubated from 18 to 24 h, typically in a CO_2-enriched atmosphere. The agar dilution method, while laborious due to the time required to prepare each set of agar plates for each antimicrobial agent to be tested, is often cost-effective for laboratories that test large numbers of bacterial isolates against a limited set of antimicrobial agents. The testing medium is usually Mueller-Hinton agar for non-fastidious organisms and Mueller-Hinton agar containing 5 % sheep blood for fastidious organisms. The exceptions are *Haemophilus influenzae* isolates, which requires HTM or MF-H media, and *Neisseria gonorrhoeae*, which requires GC medium.

2.2.2 Broth Microdilution

Broth microdilution is the standard method used in most reference laboratories in the United States and abroad. The method typically tests twofold dilutions of multiple antimicrobial agents in 96-well disposable plastic trays. The test medium is typically cation-adjusted Mueller-Hinton broth or for fastidious organisms, cation-adjusted Mueller-Hinton broth containing 5 % lysed horse blood. A suspension of the organism to be tested is prepared in saline or Mueller-Hinton broth to the turbidity of a 0.5 McFarland standard [approximately 1×10^8 CFU/mL]. The suspension is diluted 1:20 in saline and 1–5 μL of this suspension is transferred to the 96-well tray containing doubling dilutions of the antimicrobial agents to be tested (usually between 8 and 12 antimicrobial agents per tray) using a disposable plastic inoculator (the inoculum size varies with the size of the pins in the inoculator). The final inoculum size is 5×10^5 CFU/mL or 5×10^4 CFU/well.

2.2.3 Automated Susceptibility Testing Methods

A series of commercially available automated and semiautomated methods are available to assist laboratories in testing and reporting the results of antimicrobial susceptibility tests. Most of the methods combine bacterial identification and susceptibility testing reagents in a single panel or card to enhance the speed with which antimicrobial susceptibility testing results can be reported. Many systems also incorporate software programs to interpret the results and prepare reports that can be linked readily to laboratory information systems, which in turn deliver the results to the patients' electronic medical record. The goal of the automated methods is to reduce the time necessary to produce accurate identification and susceptibility test results and facilitate the testing of multiple antimicrobial agents. Indeed, results may be available for some bacterial species in as little as 6 h, versus the 16–18 h often required for disk diffusion testing or standard MIC tests. For staphylococci, the results of oxacillin and vancomycin tests often require prolonged incubation times (often 24 h) to achieve accurate results. Some software programs employ "expert systems" to enhance reporting by recognizing and flagging unusual results, such as ampicillin-susceptible *Klebsiella pneumoniae*, where the bacterial identification and susceptibility pattern are conflicting with typical results for wild-type *K. pneumoniae* populations, or rare results, such as carbapenem-resistant *Enterobacteriaceae* or vancomycin-resistant *Staphylococcus aureus*.

Overall, automated systems work well, although they have traditionally shown problems with certain resistance phenotypes including oxacillin-resistant *S. aureus* strains [21] and *Pseudomonas aeruginosa* strains that are resistant to beta-lactam agents, such as piperacillin [22].

2.2.4 Agar Gradient Dilution

Agar gradient dilution incorporates MIC testing into a format similar to the setup of a disk diffusion test. The antimicrobial agent is microencapsulated on the back of a plastic strip and, when placed on the surface of an agar plate, the antimicrobial agent diffuses off of the strip into the agar medium in a rapid and predictable fashion forming a gradient. The agar gradient strips evaluate the inhibitory potential of a single antimicrobial agent over a large range of concentrations. Several strips containing different antimicrobial agents can be arranged on a single agar plate. The agar gradient method is particularly useful for testing fastidious microorganisms such as *Campylobacters* [23], pneumococci [24], and anaerobic bacteria [25, 26] where only a limited number of antimicrobial agents need to be tested. Agar gradient strips are available from several commercial manufacturers.

3 Interpretive Guidelines

Once a disk diffusion zone of inhibition has been measured or an MIC for an antimicrobial agent has been determined, and the microbiologist has affirmed that the quality control results indicate that the testing system has performed appropriately, the results of the susceptibility test have to be interpreted. For most antimicrobial agents, the results transmitted to the patient's chart will be either "susceptible," "intermediate,"

or "resistant." If an MIC method was used, the results transmitted may include the quantitative MIC result as well. However, for some antimicrobial agents, such as daptomycin when testing staphylococci, the results transmitted will be either "susceptible" or "non-susceptible." This is because at the time of the drug was approved for use by the US Food and Drug Administration and when interpretive criteria (i.e., breakpoints) were established by the CLSI, there were inadequate numbers of resistant strains available on which to establish intermediate and resistant breakpoints [27]. The lack of interpretive intermediate and resistant breakpoints often poses a challenge for the automated methods which, depending on the system, will either leave the interpretation field blank for a non-susceptible result or place an "N" or "NS" (for non-susceptible), or an "NI" (for non-interpretable), in the interpretation field—a result that may be confusing to the physician reading the laboratory report. Some microbiology laboratories will override these "non-S, I, or R results" and simply report them as resistant to avoid confusing physicians.

Recently, a new interpretive category called "susceptible dose dependent" (SDD) was introduced for reporting results for cefepime for bacterial infections. Although this category has been used for reporting results for antifungal susceptibility testing for several years, its application for cefepime results for bacterial infections is novel. It is meant to bring clarity to the intermediate category by indicating to the physician that the organism causing the infection may still respond to cefepime if high doses of the drug are used.

The categorical interpretations used for disk diffusion and MIC test results are drawn from one of several standard-setting organizations. In the United States, breakpoints for antimicrobial agents are set initially by the Food and Drug Administration. After the FDA establishes their interpretive criteria, data are gathered and reviewed by CLSI, which independently established breakpoints. Usually, the breakpoints coincide, although for some agents, they may differ. The description of the reference disk diffusion method and the interpretive criteria for antimicrobial agents approved in the United States, and several antimicrobial agents available only outside of the United States, are available in the CLSI M2 document (Performance Standards for Antimicrobial Disk Susceptibility Tests). The M2 series is revised every 3 years. The agar and broth dilution reference (MIC) methods are described in CLSI document M7 (Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically), which also is revised every 3 years. A separate document containing the interpretive criteria for both disk diffusion and MIC testing, quality control ranges, and methods for preparing and diluting antimicrobial agents is published each year in January (the M100 series). Similar documents are published online by the European Union Committee for Antimicrobial Susceptibility Testing (EUCAST) (see http://www.srga.org/Eucastwt/bpsetting.htm), the British Society for Antimicrobial Chemotherapy (see http://www.bsac.org.uk/), and other organizations. A document outlining interpretive criteria for susceptibility tests conducted with infrequently isolated or fastidious bacteria (M45) is also published by CLSI [28].

4 Resistance Phenotypes That Require Specialized Testing

4.1 β-Lactam Agents

Resistance to penicillins, cephalosporins, and carbapenems among gram-negative organisms is usually mediated by β-lactamases, either intrinsic or acquired, that hydrolyze the β-lactam ring of the antimicrobial agent, which detoxifies the drug. Although other mechanisms of resistance, including efflux and porin changes limiting access of beta-lactam agents may occur, there is no specialized testing to detect these mechanisms. Among gram-positive organisms, in addition to β-lactamases, β-lactam resistance can be mediated by changes in the affinity of the penicillin-binding proteins (PBPs) for the antimicrobial agent. Among staphylococci this is usually mediated by acquisition of a novel PBP (i.e., PBP 2a), while in pneumococci and viridans streptococci, reduced affinity is usually the result of remodeling of the PBP genes by incorporating foreign DNA to form mosaic genes. Beta-lactam resistance in both gram-positive and gram-negative organisms poses unique challenges for antimicrobial susceptibility testing methods.

Detection of oxacillin (or methicillin) resistance in staphylococci is difficult primarily because oxacillin-resistant strains tend to grow more slowly and often show heteroresistance, i.e., only a fraction of the bacterial population actually manifests the resistance phenotype [29]. Methicillin resistance can be mediated by either the *mecA* or *mecC* gene, the latter (originally designated as mec_{LGA251}) having been described recently from both humans and animals [30]. Various strategies have been used over the years to increase the likelihood of detecting the resistant subpopulation including growing the strains at 35 °C instead of 37 °C, adding 2 % NaCl to the testing medium, and incubating the test for a full 24 h [21]. More recently, based on studies by Felten et al. [31], Skov et al. [32], Swenson and Tenover [33], and others, CLSI has described a cefoxitin-based disk diffusion test that accurately predicts the presence of the *mecA* and *mecC* genes among both *S. aureus* and coagulase-negative staphylococci (CoNS). The test can be read in 16–18 h and replaces the use of the oxacillin disk for disk diffusion testing for both *S. aureus* and CoNS and now is also the preferred drug for MIC testing according to CLSI and EUCAST.

Among the *Enterobacteriaceae*, the major susceptibility testing challenges are to detect the presence of extended-

spectrum beta-lactamases (ESBLs) and carbapenemases. Before 2012, CLSI and EUCAST used detection of these enzymes to modify the interpretation of MIC and disk diffusion results from S to R in an attempt to insure accuracy. However, during the last several years, both organizations have instituted lower MIC breakpoints and larger disk diffusion zone diameters in lieu of testing for enzymes to try and improve accuracy of reporting by increasing the sensitivity of detecting resistant strains directly without having to use supplemental tests. Detection of the enzymes (i.e., the mechanism of beta-lactam resistance), which was seen as confusing to physicians and often was ineffectively implemented in laboratories, is now used primarily for infection control activities and epidemiology. Some critics have objected to this approach noting that the lowered breakpoints have not solved the problems of detecting resistant isolates [34].

ESBLs are primarily derivatives of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes (although there are multiple other less common ESBLs) that mediate resistance to aztreonam and third-generation cephalosporins (such as cefotaxime, ceftriaxone, and ceftazidime) [35–37] and, in some cases, fourth-generation cephalosporins (such as cefepime and cefpirome) [38]. (An up-to-date list of β-lactamases can be found at http://www.lahey.org/Studies/.) Since the ESBLs do not hydrolyze all of the extended-spectrum cephalosporins at similar rates, some organisms may show resistance to some cephalosporins but susceptibility to others, even though the latter cephalosporins will not be clinically effective [39, 40]. To identify strains of *Escherichia coli*, *K. pneumoniae*, and *Proteus mirabilis* that contained ESBLs, organisms were tested with cefotaxime and ceftazidime, by either disk diffusion or broth microdilution, in the presence and absence of clavulanic acid, i.e., a β-lactamase inhibitor. If the zones of inhibition increased by 5 mm or more in the presence of clavulanic acid, or the MICs decreased by 3 or more doubling dilutions in the presence of clavulanic acid when compared to the results in the absence of clavulanic acid, the strain were said to contain an ESBL [41]. Thus, the results for all penicillins, cephalosporins, and aztreonam (but not cephamycins, such as cefoxitin or cefotetan) were reported as resistant if ESBLs were detected. β-lactam-β-lactamase inhibitor combinations, such as piperacillin-tazobactam, were reported as they tested (either susceptible, intermediate, or resistant), since they may still have been effective clinically against some ESBL-producing strains of *K. pneumoniae* or *E. coli* [42]. However, interpretive changes are no longer made if the lower breakpoints have been instituted by the laboratory. Similar strategies to identify plasmid-mediated AmpC β-lactamases using boronic acid have been described [43, 44]; however, these tests were never been promulgated by CLSI and thus did not impact the reporting of penicillin or cephalosporin results.

Carbapenemases have emerged within the Ambler class A beta-lactamases including KPC, GES, SME, and IMI; the class B metalloenzymes, including NDM, VIM, and SPM; and among the class D beta-lactamases including OXA48, OXA181, and OXA232. The Modified Hodge Test (MHT) was introduced to detect these enzymes but proved to be ineffective for some carbapenemases, such as NDM. Using MHT was part of CLSI guidelines for several years before lower breakpoints were established. Now, it is primarily used for epidemiologic purposes unless a laboratory has been unable to implement the new lower breakpoints for carbapenems, in which case the MHT is still useful. Other phenotypic tests that can be performed on isolate colonies to detect carbapenemase activity include the carbaNP assay, which is a colorimetric test for carbapenem hydrolysis that is simple and provides results often in less than 2 h [45].

4.2 Macrolides, Azalides, Lincosamides, and Streptogramins

The macrolides, which include agents such as erythromycin and clarithromycin, and the azalides, such as azithromycin, are commonly administered oral (and parenteral) drugs used for the treatment of many bacterial clinical infectious syndromes. Resistance is due either to inactivation of drug (mediated by erythromycin esterases or phoshorylases), efflux of the drug out of the cell, or by modification of the site of action [46]. The latter mechanism, in which the 23S RNA of the 50S ribosome unit is methylated at a specific adenine residue, which prevents binding of the antimicrobial agent to the ribosome, leads to high-level resistance to macrolides but also affects lincosamides (such as clindamycin) and streptogramins (such as pristinamycin), since all three classes of drugs act by binding to the same site on the bacterial ribosome. The so-called MLS_B resistance phenotype (for macrolide–lincosamide–streptogramin$_B$) is typically observed in staphylococci and streptococci. Strains of staphylococci and streptococci that test as erythromycin resistant but clindamycin susceptible may contain an inducible *erm* gene encoding MLS_B resistance or an efflux gene such as *msrA* (in staphylococci) or *mefA* (in streptococci). Since mutations in the *erm* genes can lead to inducible clindamycin resistance, and thus clindamycin treatment failure, it is important to differentiate these two resistance mechanisms in the clinical laboratory to enhance the accuracy of reporting (efflux-mediated resistance cannot mutate to clindamycin resistance). The D-zone test, which is a disk diffusion-based assay, uses an erythromycin disk that is placed 15–25 mm away from a clindamycin disk on an agar plate seeded with a lawn of the test organism [47]. Blunting of the zone of inhibition between the erythromycin and clindamycin disks (which forms a "D" shape) indicates the presence of an

inducible *erm* gene. A circular zone of inhibition (normal zone) indicates a negative test. If the D-zone test is positive, the results for clindamycin are reported as resistant [48].

4.3 Aminoglycosides

Aminoglycosides are commonly used in conjunction with β-lactam agents (or vancomycin in gram-positive-associated infections) to treat serious bacterial infections, such as endocarditis, because the two groups of drugs frequently act synergistically, especially against enterococci [49]. Resistance to aminoglycosides is typically mediated by enzymes that modify the drug so that uptake into the bacterial cell is impaired [50]. These include acetylases, adenylylases, and phosphorylases. In addition, there are 16 s rRNA methylases, multiple efflux pumps, and cell wall permeability barriers that also mediate aminoglycoside resistance.

The number of genes encoding variants of the aminoglycoside-modifying enzymes is remarkably large and diverse. To determine whether there is likely to be synergy between an aminoglycoside (i.e., gentamicin or streptomycin) and a cell wall-active agent (either ampicillin or vancomycin) specifically for treating enterococcal infections, special disk diffusion and MIC tests to detect high-level aminoglycoside resistance have been established by CLSI [41]. The presence of high-level resistance to either aminoglycoside will negate the likelihood of synergistic activity with a cell wall-active agent.

4.4 Sulfa Drugs and Trimethoprim

The sulfa drugs and trimethoprim both inhibit the enzymatic pathway that synthesizes dihydrofolate. The two drugs are usually tested together in a 19:1 ratio of sulfamethoxazole to trimethoprim [41]. Because MIC tests using this combination of drugs often result in trailing endpoints (i.e., a gradual reduction of growth instead of a clear break between the wells of an MIC plate showing growth and those with no growth), the well showing ≥80 % inhibition of growth is usually chosen as the MIC.

4.5 Glycopeptides

Glycopeptide resistance can be mediated by a series of genes that effectively remodel the cell wall of an organism by altering the D-alanine-D-alanine binding site of vancomycin to D-alanine-D-lactate or D-alanine-D-serine through introduction of an altered ligase enzyme (e.g., *vanA*). The family of acquired vancomycin resistance genes now includes *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, and *vanM* [51–53]. The *vanA* resistance determinant has been recognized among enterococci [54] and *S. aureus* isolates [55], the latter due to acquisition of Tn*1545* and its variants [56]. A second mechanism of resistance noted among glycopeptide-intermediate *S. aureus* (GISA) strains (also called vancomycin intermediate strains or VISAs) is the thickening of the cell wall in conjunction with metabolic changes that make *S. aureus* isolates no longer susceptible to glycopeptides [57–59]. While detection of *vanA*-mediated resistance in *S. aureus* has been a challenge for automated MIC methods [60] and in some cases for GISA isolates, GISA isolates have not been detected in the clinical laboratory using disk diffusion [61]. Clinical laboratories typically augment their testing for glycopeptide resistance in staphylococci (whether the use an automated susceptibility method or disk diffusion) by inoculating a Brain Heart Infusion agar plate containing 6 μg/mL of vancomycin with approximately 10^6 CFU of the staphylococcal strain to be tested [61]. This is a very sensitive method for detecting GISA strains as well as VRSA. A modified Etest method may be used to detect GISA isolates [62].

Recently the *vanG* resistance determinant was detected in *Streptococcus agalactiae* [63].

4.6 Fluoroquinolones

Fluoroquinolones are used widely to treat a variety of infections around the world. Resistance to fluoroquinolones typically arises by alterations in the target enzymes (DNA gyrase and topoisomerase IV) and through changes in drug entry and efflux [64]. The discovery of plasmid-mediated horizontally transferable genes encoding quinolone resistance (e.g., *qnrA*, *qnrB*, and *qnrS*) perhaps explains some of the rapid emergence of resistance to these drugs [65, 66]. Likewise, AAC(6′)-Ib-cr, a variant aminoglycoside acetyltransferase capable of modifying ciprofloxacin and reducing its activity, also seems to provide low-level quinolone resistance [67]. It appears that low-level resistance to fluoroquinolones (by whatever mechanism) may be responsible for clinical failures when treating *Salmonella typhi* [68] and non-typhoidal *Salmonella* infections [69]. Strains isolated from patients who failed therapy typically show ciprofloxacin MICs that are elevated (0.25–1 μg/mL compared with typical MICs of 0.003–0.06 μg/mL) but still in the susceptible range; however, the nalidixic acid MICs are usually resistant (MIC > 16 μg/mL). Although, a nalidixic acid screen test was recommended by CLSI for several years to detect strains with low-level ciprofloxacin resistance, lower ciprofloxacin MIC breakpoints have been implemented to provide more reliable data for detecting low-level fluoroquinolone resistance.

4.7 Oxazolidinones

Oxazolidines, such as linezolid, have broad activity against many gram-positive organisms [70]. Resistance to the oxazolidinones among staphylococci and enterococci may be due to modification of ribosomal RNA often through a G to T substitution at position 2765, or one of several other mutations [71, 72], or by acquisition of the *cfr* gene. The *cfr* determinant can mediate resistance to the drugs in the $PhLOPS_A$ classes (i.e., phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics) [73]. Detection of resistance to linezolid, particularly among staphylococci, can be difficult by agar-based methods such as disk diffusion and agar gradient dilution, which tend to lack sensitivity to detect some resistant isolates. Studies at CDC have indicated that broth-based MIC methods typically have better sensitivity for detecting resistance [74].

4.8 Lipopeptides

Daptomycin is an example of a lipopeptide that is rapidly bactericidal for most gram-positive bacteria [75–78]. Testing daptomycin typically requires the presence of 50 mM Ca++ in the broth or agar medium to achieve accurate results. Disk diffusion testing lacked adequate sensitivity to detect reduced susceptibility to daptomycin in clinical studies. Thus, the disk diffusion test was removed from the CLSI documents. However, the agar gradient method was shown to work well [79].

5 Molecular Tests to Detect Resistant Bacteria

5.1 General Considerations

Molecular assays, such as real-time PCR, microarrays, line probes, and film arrays, offer both rapid turnaround times and high sensitivity for identifying antimicrobial resistance genes or mutations associated with resistance in bacterial isolates, even in bacteria directly in clinical samples. Such results may be used in conjunction with other rapid technologies for bacterial identification to guide therapy or to decide whether to implement on a patient contact isolation precautions [80, 81]. Several commercial methods for identifying gram-positive cocci in blood culture bottles test for the presence of *mecA* or *mecC* in addition to identifying *S. aureus* to indicate the presence of MRSA [12, 82]. Additional molecular assays for use on positive blood culture vials include the presence of the vancomycin resistance genes, *vanA* and *vanB*, for detection of resistance genes in enterococci, while other assays test for the presence of the bla_{KPC} carbapenem resistance gene, which may be present in gram-negative organisms [13].

5.2 Detection of Resistance in *Mycobacterium Tuberculosis*

Commercial PCR assays that use molecular beacon technology can detect mutations in the ribosomal RNA polymerase gene *rpoB* that are associated with rifampin resistance in *M. tuberculosis*. Rifampin resistance is frequently a marker of multidrug resistance in *M. tuberculosis*. The PCR assays can be directly on expectorated sputum samples or concentrated pellets prepared for mycobacterial culture and produce results in <2 h [83]. Line probes that utilize reverse hybridization also can be used to assess both mutations associated with rifampin and isoniazid resistance, as well as resistance to second-line drugs, such as fluoroquinolones. These can also be used directly on specimens and report results in approximately 5 h [84].

5.3 Molecular Assays for Detecting Colonization with MRSA, Vancomycin-Resistant Enterococci, and Carbapenem-Resistant Organisms (CRO)

Screening patients being admitted to hospitals or other healthcare institutions for nasal colonization with MRSA has become relatively common in the United States, Europe, and elsewhere around the world as part of enhanced infection control programs to limit its spread. Screening for MRSA can be accomplished by plating material from nasal swabs directly on selective agar media that inhibit the growth of most organisms, while allowing MRSA to produce clearly identifiable colonies. However, this often requires 18–72 h depending on the medium used, whether an overnight broth enrichment step is included, and the number of confirmatory tests undertaken by the laboratory to prove that the organism growing on the agar is MRSA [85]. A more rapid approach uses molecular amplification tests, such as PCR, that simultaneously target the *mecA* (or *mecC*) gene and a chromosomal DNA sequence that is unique to *S. aureus*, thereby linking the resistance gene specifically to the *S. aureus* strain that carries it [86, 87]. PCR-based assays for MRSA detection often can be completed in <2 h from the time the nasal swab specimen arrives in the laboratory, versus the 18–72 h often required to complete an agar-based identification test. Amplification-based assays are more expensive to perform, but the rapid turnaround time can be important when trying to control the spread of MRSA in a hospital setting [88].

Molecular assays for VRE and CROs to identify colonized patients to aid in infection control efforts have been instituted in a variety of healthcare settings. These assays, which target both *vanA* and *vanB* vancomycin resistance

genes [89], or multiple carbapenem resistance genes (e.g., bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA48}, and bla_{IMP}), are often more sensitive than conventional agar screening media for detecting resistant organisms, with results available often in <1 h. For CROs, no single agar media is effective [90]. For enterococci, while there is a high correlation between the results of molecular assays for detection of *vanA* in rectal swab samples and positive cultures for VRE isolates containing *vanA*, the correlation of PCR assay results and cultures for *vanB*-positive samples is lower, i.e., *vanB*-positive enterococci containing the *vanB* gene have not been recovered for several PCR-positive samples. This probably reflects the fact that organisms in bowel flora other than enterococci, such as *Clostridium* species, can harbor the *vanB* resistance determinant [91]. Correlation of results for detection of CROs by culture and molecular methods, however, is very high [92].

6 Summary

The goal of antimicrobial susceptibility testing is to provide practitioners with data that will assist them in choosing the optimal antimicrobial agent to treat an infection in a patient and to implement appropriate infection control measures to curb the dissemination of certain epidemiologically significant multidrug-resistant organisms. Susceptibility testing in most clinical microbiology laboratories represents a combination of phenotypic assays that provide at least qualitative results (susceptible, intermediate, or resistant) for a series of antimicrobial agents and often quantitative results (MICs) that can guide dosing regimens. Molecular-based tests, such as real-time PCR, may provide rapid information on the presence of resistant bacteria in wound specimens or positive blood culture bottles. Rapid identification of patients colonized with MRSA, CRO, ESBL, or VRE can assist in infection control decisions. Finally, rapid detection of *M. tuberculosis* and rifampin resistance-associated mutations directly in clinical samples can optimize therapy especially for multidrug-resistant TB strains.

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Drug Resistance Assays for *Mycobacterium tuberculosis*

81

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1 Introduction

Timely detection of patients harboring drug-resistant *Mycobacterium tuberculosis* strains is of paramount importance for effective treatment and also for preventing epidemics of drug-resistant tuberculosis. Bacteriologic methods currently in use for detection of drug resistance are the agar proportion method and the use of automated liquid medium systems. In addition, genotypic methods are increasingly employed as screening tests and complement conventional antimicrobial susceptibility testing. Accurate detection of clinically meaningful minimal inhibitory concentrations is the prerequisite for pharmacokinetic and pharmacodynamic correlations.

2 Drug-Resistant Tuberculosis as a Public Health Problem

Drug resistance has been recognized from the beginning of antituberculous chemotherapy [1]. Over the last decades, an alarming increase in drug-resistant tuberculosis (TB) cases has been observed, including multidrug-resistant (MDR) strains being resistant to the two most effective first-line drugs isoniazid (INH) and rifampin (RIF). The Global Project on Anti-Tuberculosis Drug Resistance Surveillance, launched by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease in 1994, has been continuously expanded and now has data available for 144 countries that harbor 95 % of the world's TB cases. Whereas the overall portion of MDR-TB cases worldwide remains relatively steady at 3.3 % for new cases and 20 % for previously treated cases, percentages in Eastern European and Central Asian countries that share the major burden of MDR-TB disease reach 35 % for new cases and 75 % for previously treated cases, respectively [2]. Treatment for MDR-TB disease is more complex and of longer duration and requires accurate determination of resistance profiles to reach successful outcomes [3]. Therefore, timely and reliable laboratory diagnosis of drug resistance is essential to meet this challenge.

3 Definitions and Terminology in the Field of Tuberculosis

3.1 Drug-Resistant Strain

A drug-resistant strain is defined as a strain with the capacity to grow in the presence of higher concentrations of a drug that is usually effective against *M. tuberculosis*, compared to a "wild-type" strain obtained from a patient never treated with anti-tuberculosis drugs [4, 5].

3.2 Critical Concentrations

The critical concentration for each drug has been defined as the single concentration that allows distinction between a resistant and susceptible strain. Initially this was defined for egg-based Löwenstein-Jensen medium [5] and subsequently for agar media [6, 7]. The critical concentration is defined as the concentration which prevents growth from the vast majority of susceptible colonies but still allows growth of resistant strains. The critical concentration may not reflect precisely the concentrations that are actually interacting with the bacterial inoculum, because drugs are partially inactivated

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during the media preparation process. Therefore, critical concentrations do not reflect the concentrations attainable *in vivo* and no such correlation should be attempted. Directly related to the concept of critical concentration is that of critical proportion, which takes into account a Gaussian distribution of antimicrobial susceptibility in a given culture. This explains that even in cultures from antimicrobial susceptible isolates, a proportion of bacilli are expected to grow in the presence of a drug at the critical concentration. If the proportion of colonies are beyond the expected critical concentration (1 % for most of the first-line drugs), the strain is found to be resistant.

3.3 Direct and Indirect Antimicrobial Susceptibility Tests

For direct testing, the drug-containing medium is inoculated with a processed clinical specimen such as sputum (usually containing a sufficient number of bacteria as determined by microscopy). Indirect testing is performed on a pure culture grown from the clinical specimen.

3.4 Primary (Initial) Drug Resistance

Resistance to any drug detected in a strain obtained from a newly diagnosed patient without prior history of antimicrobial therapy is called primary drug resistance. It indicates that the patient has been infected with a drug-resistant strain.

3.5 Secondary (Acquired) Drug Resistance

Drug resistance detected in a strain obtained from a patient who has been treated before or is still undergoing treatment with the corresponding anti-tuberculosis agent(s) is suggestive of resistance acquired during treatment.

3.6 Multidrug Resistance

Resistance to **both** RIF and INH, with or without resistance to other drugs, is called MDR.

3.7 Extensively Drug Resistance

Resistance that extends beyond the MDR phenotype that includes both resistance to fluoroquinolones and at least one injectable second-line agent is called extensively drug resistance (XDR).

3.8 Poly-resistance

Resistance to any two or more drugs that does not fulfill criteria for MDR may include resistance to RIF or INH, if the isolate is not resistant to both RIF and INH and thus is not MDR.

3.9 First-Line Drugs

The initial treatment regimen for patients newly diagnosed with tuberculosis is composed from the first-line drugs: INH, RIF, pyrazinamide (PZA), and ethambutol (EMB).

3.10 Second-Line Drugs

All other available anti-tuberculosis drugs represent a backup for selection in cases of drug resistance or intolerance to the first-line drugs: capreomycin, amikacin, kanamycin, moxifloxacin, levofloxacin, ethionamide, para-aminosalicylic acid (PAS), clofazimine, and cycloserine. Recent additions with less well-established efficacy and/or limited clinical data are bedaquiline, delamanid, linezolid, amoxicillin/clavulanate, imipenem/cilastatin, meropenem, and clarithromycin.

4 Indications for Antimicrobial Susceptibility Testing (ASD)

In resource-rich countries, antimicrobial susceptibility testing for clinical *M. tuberculosis* isolates is standard of care in order to ensure optimal drug therapy. However, in many areas of the world which share the major burden of TB, this standard cannot be met. Therefore, the WHO has outlined a programmatic approach that allows for targeted testing directed at individuals at particularly high risk for drug-resistant TB. The following risk factors have been identified: failure of new TB regimens with persistent sputum acid-fast bacilli (AFB) smear positivity at months five or later, delayed sputum conversion, exposure to a known drug-resistant TB case, relapse and return after lost follow-up, exposure to institutions with high drug-resistant TB prevalence, comorbid conditions associated with malabsorption, and HIV in some settings [8].

5 Agar Proportion Method

The major advantage of performing antimicrobial susceptibility tests in agar plates is related to the transparency of the medium, which makes it possible to observe the growing colonies at the beginning of their formation. Therefore, final

results can be reported within 2–3 weeks for most isolates, instead of 4–6 weeks or more when using egg-based media. There are two types of agar medium that can be used for either **direct** or **indirect** test: Middlebrook 7H10 and Middlebrook 7H11. Middlebrook 7H11 agar provides better growth of drug-resistant TB strains compared to Middlebrook 7H10 [9]. Detailed description of their preparation and use can be found in the appropriate publications [6, 7, 10]. These media are made from commercially available 7H10 or 7H11 agar base.

Some strains may have genetically predetermined low-level resistance to INH [11]. Therefore, two concentrations of INH shown in Table 81.1 are needed to distinguish between low and high levels of resistance to this drug. A higher concentration of streptomycin in addition to the critical concentration of this drug (as shown in Table 81.1) is usually employed in agar media.

For a direct test, after the digestion-decontamination procedure, the concentrated sputum sediment is inoculated into plates, 0.1 mL per quadrant. For an indirect test, two sets of plates are used: one inoculated with 10^{-3}-fold and the other with 10^{-5}-fold dilutions of the bacterial suspension adjusted to the optical density of the McFarland #1 standard.

Table 81.1 Critical concentrations (μg/mL) for testing *M. tuberculosis* in solid and liquid media

Drug	L-J	7H10 Agar	7H11 Agar	MGIT960	VersaTREK
References	[8]	[8]	[8]	[8]	[47]
Isoniazid[a]	0.2	0.2/1.0	0.2/1.0	0.1/0.4	0.1/0.4
Rifampin	40.0	1.0	1.0	1.0	1.0
Ethambutol	2.0	5.0	7.5	5.0	5.0
Pyrazinamide	–	–	–	100.0	300.0
Streptomycin	4.0	2.0	2.0	1.0	–
Amikacin	30.0	4.0	–	1.0	–
Kanamycin	30.0	5.0	6.0	2.5	–
Capreomycin	40.0	4.0	–	2.5	–
Ethionamide	40.0	5.0	10.0	5.0	–
Cycloserine	30.0	–	–	–	–
PAS	1.0	2.0	8.0	4.0	–
Ofloxacin	4.0	2.0	2.0	2.0	–
Levofloxacin	–	1.0	–	1.5	–
Moxifloxacin[b]	–	0.5/2.0	–	0.5/2.0	–
Linezolid	–	–	–	1.0	–

L-J: Löwenstein-Jensen medium
7H10 Agar: Middlebrook 7H10 agar
7H11 Agar: Middlebrook 7H11 agar
[a]Isoniazid: Some laboratories may test an additional higher INH concentration in order to differentiate between low- and high-level INH resistance
[b]Moxifloxacin: Two concentrations are proposed. In programs using both ofloxacin/levofloxacin and moxifloxacin, possible testing is for moxifloxacin only at both concentrations, or test ofloxacin/levofloxacin at higher concentration. In programs using ofloxacin/levofloxacin, only test these drugs. In programs using only moxifloxacin, test at higher concentration of moxifloxacin

The plates are incubated at 35–37 °C for 3 weeks, protected from light, in an atmosphere of 5–10 % CO_2 for 7H10 and 7H11 agar plates; however, CO_2 is not essential when performing the indirect method. The colonies are counted, and the results are reported as the percentage (proportion) on the basis of comparison of the number of colony-forming units (CFU) on drug-containing and drug-free quadrants. The isolate is considered "resistant" if this proportion is 1 % or greater for all drugs except PZA. The criterion for PZA is 10 %; however, PZA is no longer tested on agar media. If growth is not sufficient at the 3-week reading, then the plates are reexamined at the 6-week reading, but in such a case only "susceptible" results are considered valid. This is because growth at 6 weeks in drug-containing quadrants may be related to drug degradation during the prolonged incubation period, rather than to the occurrence of true drug resistance.

6 Antimicrobial Susceptibility Testing (Indirect) in Liquid Medium

The need for expedited detection of drug resistance was first addressed in the 1980s by developing the antimicrobial susceptibility testing procedure [12–14] for the semiautomated BACTEC460 system introduced by Becton Dickinson (Sparks, MD). Non-radiometric, walkaway systems, all fully automated and computerized, are now commercially available: BACTEC960 MGIT (Becton Dickinson Microbiology Systems, Sparks, MD) and VersaTREK, formerly ESP-II Culture System, by Difco (Thermo Fisher Scientific, Cleveland, Ohio).

In the BACTEC960 MGIT system, the bacterial growth detection is based on consumption of oxygen, which causes the indicator embedded in the bottom of tubes to fluoresce, and the instrument continuously monitors the increase of fluorescence. Comparison of these patterns in drug-containing and drug-free tubes is analyzed by the instrument and automatically reported as "susceptible" or "resistant."

In the VersaTREK system, growth monitoring is based on reduction of pressure in the vials due to the consumption of oxygen by the growing bacteria. The conclusion is based on comparison between drug-free controls and drug-containing vials after positive readings have occurred for 3 consecutive days in the drug-free vial. "Susceptible" is reported if no growth is detected in drug-containing vials, and "resistant" is reported if growth is detected in a drug-containing vial at this time point.

For all of these systems, critical concentrations have been developed (Table 81.1). Two concentrations of INH are performed to distinguish between low and high levels of resistance to this drug [10, 11]. Critical concentrations of various second-line and newer compounds were suggested for the BACTEC960 MGIT systems [15] (Table 81.1).

A report simply stating "resistant" may not be enough for the clinician to provide adequate care for a patient with MDR-TB or even XDR-TB. There is a growing body of evidence that additional information is needed such as the specific genetic mutation and/or the determination of the minimal inhibitory concentration (MIC) for patients with drug-resistant TB [16]. Furthermore, strains with elevated MIC for RIF may be reported by systems using liquid media as susceptible, missing a potential red flag [17]. There are mainly two approaches to determine the MIC of a particular compound: macro-dilution and micro-dilution assays. In 2009, Springer et al. described a new method for quantitative AST by the use of BACTEC960 MGIT and EpiCenter instrumentation [18], and in 2012, Hall et al. published an evaluation of the Sensititre MycoTB Plate for AST of *M. tuberculosis* complex [19]. The Sensititre *Mycobacterium tuberculosis* MIC Plate (Thermo Fisher Scientific, Cleveland, Ohio) is CE/IVD marked and for research use only in the USA.

7 Molecular Phenotypic and Genotypic Methods

Traditional approaches based on solid and liquid media assays are hampered by long turnaround times due to the slow growth of *M. tuberculosis*. Molecular methods hold promise to speed up the process and to offer clinicians timely and accurate information that can be taken into account at the very beginning of therapy. With the advent of microfluidic "lab-on-a-chip" technologies, it may become possible to detect drug-resistant genotypes in point-of-care settings outside of the laboratory [20].

Genotypic methods detect the bacterial DNA or RNA sequences associated with drug resistance. A persistent challenge to the application of genotypic approaches is the complexity of the underlying genetics of drug resistance. High-level resistance to most tuberculosis drugs, including INH, can result from diverse mutations in multiple genetic loci [21, 22]. Adding to the complexity, some mutations in known drug resistance loci can confer low-level resistance, which may not be clinically significant. Although most drug resistance mechanisms are genetically complex, there is an exception to this rule with *M. tuberculosis:* More than 95 % of RIF-resistant isolates of *M. tuberculosis* carry point mutations or small deletions/insertions in an 81-base region of the *rpoB* gene coding for the β subunit of DNA-dependent RNA polymerase or in a smaller region near the 5′ end of the gene. This was established in numerous studies conducted throughout the world [22–25]. RIF is a first-line anti-tuberculosis drug, and it is considered to be a surrogate marker for MDR-TB [26, 27]. Accordingly, the development of genotypic antimicrobial susceptibility testing methods has mainly focused on this locus.

Most approaches use in vitro DNA amplification such as PCR in combination with diverse methods for detecting specific mutations known to result in drug resistance, most commonly line probe assays and molecular beacons.

Line probe assays allow identification of single base mutations that confer drug resistance. After DNA extraction from the clinical specimen, gene regions of interest are amplified via PCR and the single-strand amplicons bind to highly specific immobilized DNA probes. The bound DNA fragment becomes visible as a band by an enzymatic color reaction (e.g., biotin). Two currently commercially available line probe assays have received recommendation status by the WHO and related organizations, INNO-LiPA-Rif.TB (Fujirebio Europe, Ghent, Belgium) and GenoType MDR*plus* (Hain Lifescience, Nehren, Germany) which has been further developed to capture resistance markers of several drug classes.

The INNO-LiPA-Rif.TB was the first commercially available line probe assay. It uses a set of primers that allow PCR amplification of a region of interest in the *rpoB* gene where rifampin resistance mutations are located, and several oligonucleotide probes that span the resistance mutation region, as well as an upstream sequence that is specific for *M. tuberculosis* complex [28]. The assay proved very accurate when applied on culture specimens with sensitivities greater than 95 % and specificity of 100 %, with some loss in sensitivity (80–100 %) when directly applied to clinical specimens [29, 30].

The GenoType MTBDR assay and its successor, the GenoType MTBDR*plus*, are based on multiplex PCR and offer probes for both rifampin mutations (*rpoB*) and the most common INH resistance mutations (*katG* and, in the GenoType MTBDR*plus*, *inhA*) and thus allow detection of MDR-TB strains. In a 2008 meta-analysis on isolates, it was highly accurate for detection of RIF resistance with pooled sensitivity and specificity of 98 % and sensitivity for INH of 84 %, which improved to about 90 % when *inhA* testing was included (MTBDR*plus* assay) [29]. A further development is the GenoType MTBDR*sl* assay which allows detection for mutations that confer resistance to fluoroquinolones and the injectable drugs amikacin and capreomycin. Its performance against culture-based antimicrobial susceptibility testing was the subject of a recent Cochrane meta-analysis [31]. It was found to be highly specific with greater than 98 % for all classes. For quinolones, a pooled sensitivity of 83 % was found when applied on isolates, with similar results when applied to AFB smear-positive specimens. Pooled sensitivities for second-line injectable drugs amikacin, kanamycin, and capreomycin were 76.9 % for cultures and 94.4 % for AFB smear-positive specimens, respectively. The authors conclude that due to its modest sensitivity and excellent specificity, the test can be used as a rapid rule-in test on AFB smear-positive specimens (direct testing) for MDR-TB and

XDR-TB. However, it still would miss one in four cases of XDR-TB, which makes culture-based antimicrobial susceptibility testing necessary for specimens which were not identified as resistant.

In 2011, the Hain GenoType MTBDR*plus* V2 was introduced with improved sensitivity which may allow testing of AFB smear-negative sputum specimens [32, 33]).

Around the same time, the NTM+MDR-TB Detection Kit 2 (Nipro Corporation, Osaka, Japan) was developed which offers rifampin and INH resistance detection based on *rpoB*, *katG*, and *inhA* with excellent performance, as well as identification of various nontuberculous mycobacteria [34]. Both the Hain GenoType MTBDR*plus* V2 and the NTM+MDR-TB assay were compared to the GenoType MTBDR*plus* in an extensive non-inferiority analysis by FIND and, based on their excellent performance characteristics, received recommendation status for endorsement by the WHO for rapid detection of MDR-TB in AFB smear-positive specimens [35].

The Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) diagnostic system is a PCR-based platform which allows detection of *M. tuberculosis*-specific sequences as well as rifampin resistance mutations using **molecular beacons** [36]. Gene areas of interest are amplified via PCR. Molecular beacons are oligonucleotides that form hairpin-like loops. They carry the sequence of interest in the middle and short repetitive sequences at each end which close the loop by hybridization. A fluorophore is attached to the 5′ end and a quencher molecule to the 3′ end, respectively, which suppresses the fluorescent signal while to loop is closed. When the probe binds to its complementary DNA strand, the loop opens and fluorescence is restored which can be detected [37]. Specimens are simple to prepare and the single-use cartridge technology allows for safe and reliable handling with minimal training. Since its introduction, a large body of literature has accumulated. Its performance has been systematically reviewed by the Cochrane collaboration [38]: Pooled sensitivities from 27 studies were 89 % (95 %; CI 85–92 %) and pooled specificities 99 % (98–99 %). Performed on AFB smear-negative specimens, pooled sensitivities were still 67 % (60–74 %). For rifampin resistance, pooled sensitivity was 95 % with a specificity of 98 %. Based on these characteristics, Xpert MTB/RIF is now approved by the US Food and Drug Administration (FDA) for both AFB smear-positive and smear-negative sputum specimens.

It is increasingly recognized that the capabilities of currently available molecular platforms need to be expanded from being a screening tool alone toward the delivery of an individualized and detailed resistance profile, as recently outlined by *Somoskovi* and *Salfinger* [16]. The following are examples of potential limitations of currently used molecular testing platforms: Recent reports of rifampin mono-resistance rates as high as 11.6 % [39] highlight the problem of potential overdiagnosing MDR-TB when relying on detection of RIF resistance as a surrogate for MDR-TB. Similarly, INH mono-resistance has been described, which can remain undetected if RIF testing is not followed by conventional antimicrobial susceptibility testing and may lead to MDR-TB if inadequately treated with the standard regimen. INH is a prodrug that needs to be activated by a peroxidase encoded by *katG*. The most common *katG* mutation at codon 315 can be readily identified and is associated with high-level INH resistance. However, other less common *katG* mutations exist that lead to variable degree of resistance and would need to be identified by conventional INH testing, so that culture-based testing should not be foregone by a negative *katG* screen. In contrast, mutations in the promoter region of the target gene *inhA* also lead to low-level INH resistance, which can be overcome by increasing INH doses. On the other hand, these mutations have been associated with cross resistance to ethionamide.

The complex genetic background of most drug resistance mechanisms is currently being matched with the continuous technological progress to improve speed and quality of sequencing. Whole genomic scale analysis of individual isolates is currently being evaluated as a tool to predict the drug resistance phenotype [40].

For the foreseeable future however, molecular testing needs to be correlated with phenotypical antimicrobial susceptibility testing in order to capture novel resistance mechanism that are likely to arise. Similarly, as molecular tests are being expanded to different drug targets, there is a need for adaptation to prevailing resistance mutations at the local level [16, 41].

8 Pharmacokinetic and Pharmacodynamic Considerations

A growing body of evidence raises concerns that currently used standard dosing regimens for first-line agents do not reach adequate serum concentrations in a significant proportion of patients. This has been linked to treatment failure and development of drug resistance despite supervised directly observed therapy [42]. The concept of pharmacokinetic and pharmacodynamic (PK/PD) correlation aims at optimization of dosing regimens to attain drug exposure with maximal microbial killing effect (see [43] for review). The principal paradigm is that there is a quantifiable relation between drug concentration observed over time and microbiological killing, which can be evaluated in preclinical models (hollow fiber model, animal models) and translated into humans. The elements of this approach include: First is the determination of minimal inhibitory concentration (MIC) value that is associated with treatment success. Second is the determination of PK/PD parameter which

correlates best with bactericidal effect (Cmax/MIC, AUC/MIC, or T%/MIC). This is determined empirically and may be different for each drug. For example, in most of the first-line drugs, this is the ratio of total amount of drug in a given time interval over MIC (AUC/MIC), and efficacy is not affected by splitting doses. For aminoglycosides, in contrast, it is the maximal attained concentration over MIC (Cmax / MIC). This indicates that the aminoglycosides should be administered in large single doses to be most effective. It is noteworthy that this relation remains constant for different MIC, and an increase in drug exposure may be able to compensate for an elevated MIC. Third is the ability to reliably predict the serum concentration profile over time. On a population level, this is usually done by Monte Carlo simulations, which assume differences in drug absorption and clearance to be present in a certain distribution within the population. On an individual level, it is impractical to measure drug levels at multiple time points. Instead, therapeutic drug monitoring commonly relies on a single time point, usually drawn at 2 h post-drug administration to capture the serum peak. Limited sampling strategies aim to find a single time point specimen that correlates best with expected AUC [44].

It is clear that the ability to accurately determine the MIC is at the heart of this approach. Once the PK/PD parameter for optimal effect is determined, drug dosing can be optimized based on the MIC of the individual strain. Conversely, it has been argued that the currently employed MIC breakpoints are too optimistic and that using standard dosing regimens, treatment of drug-susceptible disease harbors the risk of treatment failure [45]. A further development of this approach is to determine PK/PD targets to prevent development of resistance. It has been shown that drug exposure that leads to effective killing may still be insufficient to prevent development of resistance [46].

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Fungal Drug Resistance Assays

82

Sevtap Arikan-Akdagli and John H. Rex

1 Introduction

The increase in opportunistic mycosis as well as the emergence of antifungal resistance and the development of novel antifungal drugs necessitated the development of standard phenotypic drug resistance assays for fungi. Microdilution methods for testing yeasts (CLSI M27-A3 and EUCAST E.Def 7.2 assay) and filamentous fungi (CLSI M38-A2 and EUCAST E.Def 9.2 assay) are available. Disk diffusion assays for testing *Candida* (CLSI M44-A2) and nondermatophytic molds (CLSI M51-A) have also been standardized. Despite their availability and increasing knowledge of epidemiological cutoff values, these assays still have limitations. Most notably, clinical MIC breakpoints are as yet undetermined for some important drug–genus combinations. In an effort to standardize methodologies that might solve these problems as well as approaches that would support assay automation, techniques based on gradient strip method, colorimetric microdilution, agar dilution, flow cytometry, sterol quantitation, and isothermal microcalorimetry are also being studied. MALDI-TOF MS and genotypic assays are other approaches currently explored for detection of antifungal resistance.

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2 The Need for Fungal Drug Resistance Assays

Fungal infections have drawn attention in the last three decades for several reasons. First, there has been a remarkable increase in the number of patients whose immune system is compromised due to various reasons. As invasive mycoses have emerged as significant causes of morbidity and mortality for this particular patient population, the term "fungal infection" no longer only means a "superficial infection." Second, the number and variety of antifungal agents increased. This is the outcome of the demand for more efficacious and less toxic antifungal drugs to treat serious infections and the developments in pharmaceutical industry. As a result, several possible therapies exist for some situations [1–5]. Third, fungal infections refractory to antifungal therapy because of primary or secondary resistance of the infecting strains to the antifungal agents used for treating these infections are observed [6–17].

In the era where we have more patients with serious fungal infections, more alternatives to treat these infections, and patients who become or remain resistant to therapy, the best way to optimize the antifungal therapeutic strategies and to predict clinical outcome is to determine the susceptibility profiles of the infecting fungal strains to the antifungal drugs. This great demand thus resulted in the standardization of fungal drug resistance assays and sustained efforts to define their utility.

2.1 Studies to Assess Correlation of In Vitro Susceptibility with Clinical Outcome

The ultimate goal of routine fungal resistance assays is to predict clinical outcome and permit monitoring and selection of antifungal therapy. The inquiry of to what extent this goal was achieved has been the key question. A meta-analysis of in vitro–in vivo correlation studies that included patients

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infected with *Candida*, *Cryptococcus*, or *Histoplasma* and treated with various azoles (fluconazole, itraconazole, or ketoconazole) found a clinical success rate of 91 % for infections due to isolates susceptible to the antifungal agent used for treatment and a 48 % response rate for those infections treated with agents predicted to be resistant [18]. Interestingly, these percentages approximated the clinical success rates reported for treatment of various bacterial infections due to susceptible/resistant strains. Based on these data, the concept referred to as "90–60 rule" has been proposed and states in summary that susceptible isolates respond about 90 % of the time and resistant isolates respond about 60 % of the time. This conceptual model reminds us that susceptibility assays are helpful in predicting clinical response but represent only one of many factors that influence response. Factors such as pharmacokinetic properties of the drug, immune status of the host, severity of the infection, presence (and removal) of prosthetic devices, and surgical management of the site of infection are all relevant, and each, in turn, may be the most powerful factor in a given situation.

Following this meta-analysis, available in vitro–in vivo correlation data expanded further to include those for various antifungal drugs against different fungal genera. Of specific note, some in vitro–in vivo correlation studies, particularly those for amphotericin B, have found limited correlations between in vitro susceptibility and clinical outcome. For amphotericin B and *Candida*, various in vitro susceptibility testing settings, including Clinical and Laboratory Standards Institute (CLSI) microdilution and Etest methods, antibiotic medium 3, and RPMI 1640 media, have failed to generate MICs that correlated with clinical outcome [19]. Similarly, in vitro susceptibility tests could not predict early clinical outcome in patients with cryptococcosis treated with amphotericin B-flucytosine or fluconazole [20]. As also discussed in Sect. 3.4.1, these observations of lack of correlation particularly for amphotericin B might be related to the technical difficulties in demonstration of amphotericin B resistance in vitro [21].

Correlation data for patients with candidiasis who are treated with echinocandins have recently expanded, particularly after the demonstration of isolates with secondary resistance to echinocandins, suggesting that clinical failure with echinocandin therapy correlates with FKS mutations and elevated echinocandin MIC values in patients with candidiasis due to FKS mutant *Candida glabrata* strains [22–27]. These data further strengthened the clinical significance of in vitro antifungal susceptibility testing and the correlation of in vitro susceptibility data with clinical outcome. Echinocandin resistance also appears to exist within *Aspergillus* spp. but is less well understood in part because susceptibility testing is not often performed and in part because susceptibility methods are suboptimal. Laboratory-engineered mutations in the target enzyme are associated with in vivo resistance and even hypervirulence in some cases [28]. Isolates from breakthrough clinical infections have not been exhaustively studied.

Finally, in vitro–in vivo correlation data for cases with *Aspergillus* infections and mucormycoses have also been reported but remain limited. For *Aspergillus*, the emergence of strains with well-defined point mutations in lanosterol 14-alpha demethylase produces increased azole MICs and is associated with clinical failure [14, 25, 29–33]. The demonstration of the presence of secondary azole resistance in *Aspergillus fumigatus* strains also further strengthened the use of antifungal susceptibility testing in routine practice. As previously discussed for yeast genera, correlations of MICs with outcome are less certain for amphotericin B against molds as well. Some of these data suggest no correlation of in vitro amphotericin B susceptibility profile with clinical response to aspergillosis [34], whereas other data appear to support an association between high amphotericin B MIC values and poor clinical outcome in cases of aspergillosis [35, 36] or those infected with Mucorales (*Apophysomyces elegans*, specifically) [37].

Overall and based on the available in vitro–in vivo correlation data, routine antifungal susceptibility testing is currently accepted as a useful adjunct for prediction of clinical outcome and guidance of antifungal therapy particularly in *Candida* and *Aspergillus* infections. However, it is not surprising that the prediction power of the in vitro antifungal susceptibility test results is incomplete given the influence of multiple factors on clinical response, including the immune system of the host in particular.

3 Reference Antifungal Susceptibility Testing Methods Interpretive Guidelines

3.1 CLSI Reference Antifungal Susceptibility Testing Methods

3.1 CLSI Reference Broth Dilution Methods (M27-A3 and M38-A2)

Based on multicenter studies that started at the beginning of the 1990s, the CLSI (Clinical and Laboratory Standards Institute, formerly National Committee for Clinical Laboratory Standards (NCCLS)) subcommittee on antifungal susceptibility testing standardized reference broth dilution methods for both yeasts (*Candida* spp. and *Cryptococcus neoformans*) and molds (*Aspergillus* spp., *Fusarium* spp., *Pseudallescheria boydii*, *Rhizopus* spp., mycelial form of *Sporothrix schenckii*, dermatophytes, and dematiaceous molds). The revised and currently available CLSI microdilution documents for testing yeasts and molds are CLSI M27-A3 [21] and M38-A2 [38], respectively. Of specific note, the standardization and inclusion of test parameters for dermato-

Table 82.1 Principal test parameters of CLSI M27-A3, EUCAST E.Def 7.2, CLSI M38-A2, and EUCAST E.Def 9.2 methods [21, 38, 80, 89]

Test parameters	Microdilution methods standardized for testing yeasts		Microdilution methods standardized for testing filamentous fungi	
	CLSI M27-A3	EUCAST E.Def 7.2	CLSI M38-A2	EUCAST E.Def 9.2
Test medium	RPMI 1640 medium (with L-glutamine and without bicarbonate), with phenol red as a pH indicator and buffered with MOPS [(3-*N*-morpholino) propanesulfonic acid] (pH = 7 at 25 °C), glucose, 0.2 %	RPMI 1640 medium (with L-glutamine and without bicarbonate), with phenol red as a pH indicator and buffered with MOPS [(3-*N*-morpholino) propanesulfonic acid] (pH = 7 at 25 °C), glucose, 2 %	RPMI 1640 medium (with L-glutamine and without bicarbonate), with phenol red as a pH indicator and buffered with MOPS [(3-*N*-morpholino) propanesulfonic acid] (pH = 7 at 25 °C), glucose, 0.2 %	RPMI 1640 medium (with L-glutamine and without bicarbonate), with phenol red as a pH indicator and buffered with MOPS [(3-*N*-morpholino) propanesulfonic acid] (pH = 7 at 25 °C), glucose, 2 %
Inoculum density	$0.5–2.5 \times 10^3$ cfu/mL adjusted by spectrophotometric measurement	$0.5–2.5 \times 10^5$ cfu/mL adjusted by spectrophotometric measurement	Molds other than dermatophytes: $0.4–5 \times 10^4$ cfu/mL adjusted by spectrophotometric measurement Dermatophytes: $1–3 \times 10^3$ cfu/mL adjusted by hemocytometric measurement	$1–2.5 \times 10^5$ cfu/mL adjusted by hemocytometric or spectrophotometric measurement
Microdilution plates	96 U-shaped wells	96 flat-bottom wells	96 U-shaped wells	96 flat-bottom wells
Incubation temperature	35 °C	35 °C	35 °C[a]	35 °C
Time of reading	24–72 h[b] (varies depending on the fungal genus, antifungal drug, and sufficiency of growth)	24–48 h[c] (varies depending on the fungal genus and sufficiency of growth)	24–72 h[d] (varies depending on the fungal genus and sufficiency of growth)	24–72 h[e] (varies depending on the fungal genus and sufficiency of growth)
MIC reading method	Visual	Spectrophotometric (530 nm)	Visual	Visual

[a]Incubation at 30 °C may be preferable for some strains of *Alternaria* spp. that do not grow well at 35 °C

[b]In case of adequate fungal growth, acceptable time of reading is 24 h for the echinocandins; 24 or 48 h for amphotericin B and fluconazole; 48 h for flucytosine, itraconazole, voriconazole, ravuconazole, and posaconazole; and 72 h for all referenced drugs against most *Cryptococcus neoformans* isolates

[c]MICs are read at 24 h for isolates that exhibit adequate growth. Further reincubation for 12–24 h is required for strains that grow poorly at 24 h. Of specific note, in case of poor growth of a *Cryptococcus* strain at 48 h, the next step should be repeat MIC testing by incubation at 30 °C

[d]MICs are read at 21–26 h for *Rhizopus* spp.; 46–50 h for *Aspergillus* spp., *Fusarium* spp., and *Sporothrix schenckii*; and 70–74 h for *Scedosporium* spp. The recommended echinocandin MEC reading time is 21–26 h for *Aspergillus* spp. and *Paecilomyces variotii*, 46–72 h for *Scedosporium* spp., or the first day when sufficient growth is observed in the growth control well. MIC readings for the dermatophyte isolates are performed after 4 days of incubation

[e]Specified recommendations for MIC reading time are 24 h for strains belonging to order Mucorales (in presence of sufficient growth) and 48 h for most of the remaining molds. A further extension of the incubation to 72 h (but not more) is acceptable (e.g., for *Scedosporium* strains) to achieve sufficient growth in the growth control well

phytes have been an addendum to CLSI M38-A2 methodology. These parameters for testing dermatophytes were standardized following multicenter analyses initiated in 2003 [39, 40] and summarized in Table 82.1.

The initial CLSI broth dilution method was a broth macrodilution performed in sterile tubes. Since the data obtained later provided a good correlation between macro- and microdilution assays [41, 42], broth microdilution method performed in sterile, disposable, microdilution plates with 96 U-shaped wells is now applied, based on its more practical and more cost-effective nature. The major test parameters proposed in CLSI M27-A3 and M38-A2 documents (as compared to those recommended in corresponding EUCAST documents) and the relevant MIC reading endpoints are summarized in Tables 82.1 and 82.2, respectively. In addition to the methodologic documents, CLSI also publishes informational supplements to provide updated information for quality control limits, interpretive guidelines, and test parameters. Among these is the CLSI M27-S4 document [43] which is the informational supplement for CLSI M27-A3 method. Interested readers are encouraged to review the cited CLSI documents for further details of these reference assays.

3.1.2 CLSI Reference Disk Diffusion Methods (M44-A2 and M51-A)

Following the successful development of standardized broth dilution methods for antifungal susceptibility testing of fungi, the next step was to simplify this approach and make it more attractive for small-volume testing. Disk diffusion has long been a popular and simple technique for susceptibility testing, and this methodology has been adapted by the CLSI

Table 82.2 CLSI and EUCAST recommendations for MIC reading endpoints for various fungal genera–antifungal drug combinations [38, 43, 80, 89]

Antifungal drug	Fungal genus or group	Recommended MIC reading endpoint	
		CLSI	EUCAST
Amphotericin B	All fungal genera relevant for testing amphotericin B	MIC-0	Yeasts: ≥90 % reduction in growth as compared to the growth control well in spectrophotometric[c] measurement Molds: The lowest concentration that yields complete inhibition of growth visually
Azoles relevant for testing against the specified fungal genus or group	Yeasts (*Candida* and *Cryptococcus*, specifically)	MIC-2	≥50 % reduction in growth as compared to the growth control well in spectrophotometric[c] measurement
	Aspergillus and most of the other opportunistic molds[a]	MIC-0	The lowest concentration that yields complete inhibition of growth visually
	Dermatophytes[b]	MIC-1	
Echinocandins	Candida	MIC-2	≥50 % reduction in growth as compared to the growth control well in spectrophotometric[c] measurement
	Aspergillus (and other opportunistic molds)[a]	MEC	The lowest concentration that yields the change from filamentous to granular growth pattern (as assessed macroscopically or in microscopic examination if macroscopic evaluation is not demonstrative). The microscopic reflection of this change is that from long, hyphal appearance (as observed in the growth control well) to short, stubby, branched hyphal clusters
Flucytosine	Yeasts (*Candida* and *Cryptococcus*, specifically)	MIC-2	≥50 % reduction in growth as compared to the growth control well in spectrophotometric[c] measurement
Ciclopirox	Dermatophytes[b]	MIC-1	–
Griseofulvin	Dermatophytes[b]	MIC-1	–
Terbinafine	Dermatophytes[b]	MIC-1	–

MIC-0: The lowest concentration that yields complete inhibition of growth visually
MIC-1: The lowest concentration that yields ~80 % reduction in growth as compared to the growth control well visually
MIC-2: The lowest concentration that yields prominent (~50 %) reduction in growth as compared to the growth control well visually
MEC: The lowest concentration that produces small, rounded growth pattern as compared to the filamentous growth observed in the growth control well macroscopically
[a]Referred to as "conidia-forming molds" in general in EUCAST EDef 9.2 document
[b]Includes species of *Microsporum*, *Epidermophyton*, and *Trichophyton*
[c]Recommended wavelength for measurement of the absorbance of the microplate is 530 nm. Alternatively, 405 or 450 nm may also be used

to antifungal agents. Based on studies of *Candida* vs. fluconazole and voriconazole [44–47], a standardized method (M44-A2) for antifungal disk diffusion susceptibility of yeasts, particularly for *Candida* vs. fluconazole, voriconazole, and caspofungin, is available and used for antifungal surveillance studies [9, 48, 49]. This method employs the basic rules of Kirby–Bauer disk diffusion method. Mueller–Hinton agar supplemented to 2 % glucose and 0.5 μg/mL methylene blue dye (pH = 7.2–7.4 at room temperature after gelling) is used as the test medium. While the supplementation with glucose provides favorable growth, addition of methylene blue enhances zone edge definition [50]. The inoculum density to be used in the test is adjusted in sterile saline to that of 0.5 McFarland standard either visually or spectrophotometrically at 530 nm, yielding a final concentration of $1–5 \times 10^6$ cells/mL. The results are read after incubation of the plates for 18–24 h at 35 °C at the inhibition zone diameter where a prominent reduction in growth is observed. Pinpoint colonies that are observed at the edge of the inhibition zone and large colonies within the zone should be ignored. The incubation period may be extended to 48 h only for isolates which grow insufficiently at 24 h. Disk diffusion interpretive criteria are available for fluconazole, voriconazole, caspofungin, and micafungin vs. *Candida* [51–54].

The disk diffusion method has also been investigated for other antifungal drugs and/or yeast genera, such as posaconazole vs. *Candida* [55–57]; micafungin vs. *Candida* [52]; amphotericin B, flucytosine, and azoles vs. *Saprochaete capitata* (formerly *Blastoschizomyces capitatus* and *Geotrichum capitatum*) [58]; and fluconazole and voriconazole vs. *Trichosporon* spp. [59].

Antifungal susceptibility testing by using disk diffusion method has been explored also for filamentous fungi causing invasive infections, particularly for testing of caspofungin and micafungin vs. *Aspergillus* and *Fusarium* [60, 61]; posaconazole vs. *Aspergillus*, *Rhizopus*, *Mucor*, *Scedosporium*, and *Fusarium* [62, 63]; and posaconazole, voriconazole, itraconazole, amphotericin B, and caspofungin vs. *Absidia*, *Aspergillus*, *Alternaria*, *Bipolaris*, *Fusarium*,

Mucor, *Paecilomyces*, *Rhizopus*, and *Scedosporium* [64]. The results of these studies in general suggested an acceptable degree of correlation between disk diffusion and CLSI reference dilution assays and were followed by documentation of a standardized disk diffusion assay by CLSI (M51-A) for testing filamentous fungi causing invasive infections [65]. The method is standardized for *Alternaria*, *Aspergillus*, *Bipolaris*, *Fusarium*, order Mucorales, *Paecilomyces*, *Pseudallescheria boydii*, and *Scedosporium prolificans*, in particular. Nonsupplemented Mueller–Hinton agar (without addition of calcium, magnesium, glucose, or methylene blue dye) (pH = 7.2–7.4 at room temperature) is the recommended test medium to be used in CLSI M51-A disk diffusion assay. Test inoculum containing conidia or sporangiospores is prepared spectrophotometrically and in accordance with the procedure outlined in CLSI M38-A2 document to yield an inoculum density of $0.4–5 \times 10^6$ cfu/mL. The inoculated agar media are incubated at 35 ± 2 °C for 16–24 h for order Mucorales; 24 h for *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*; 48 h for other *Aspergillus* spp.; and 48–72 h for *Alternaria*, *Bipolaris*, *Fusarium*, *Paecilomyces*, *P. boydii*, and *S. prolificans*. For mold-active triazoles, inhibition zone diameters are measured at the endpoint of prominent reduction in growth by ignoring the slight trailing around the zone edge as well as the filamentous growth extending into the inhibition zone. The recommended endpoint for reading the inhibition zone diameters for echinocandins is also similar to that of triazoles as specified for caspofungin in CLSI M51-A document. The inhibition zone diameters are measured by ignoring the trailing growth within a well-defined inhibition zone around an echinocandin disk. In contrary, trailing growth should be taken into consideration when interpreting the results for amphotericin B. Performance standards including the expected ranges for quality control isolates and the established epidemiological cutoff values (ECV) for CLSI M51-A methodology are documented in informational supplement CLSI M51-S1 [66]. Importantly, the ECVs documented in CLSI M51-S1 document remain yet tentative and have not been approved to be used for clinical testing.

CLSI M51-A method is standardized only for filamentous fungi causing invasive infections. While the results suggest an acceptable degree of correlation with CLSI reference dilution assay in general, disk diffusion susceptibility testing for dermatophytes remains yet investigational. It has been used for testing fluconazole, itraconazole, terbinafine, ravuconazole, and voriconazole against *Microsporum* and *Trichophyton* [67, 68] and ciclopirox olamine, fluconazole, griseofulvin, ketoconazole, itraconazole, miconazole, naftifine, posaconazole, ravuconazole, terbinafine, and voriconazole against *Microsporum*, *Epidermophyton*, and *Trichophyton* spp. [69–71].

Tablet diffusion assay is another agar-based susceptibility testing method which has also been studied in comparison with the CLSI reference disk diffusion assay. This method employs commercially available antifungal tablets (Neo-Sensitabs, Rosco Diagnostica, Denmark) instead of disks and has yielded acceptable percent agreement rates in general with reference disk diffusion and microdilution methods when testing amphotericin B, fluconazole, voriconazole, itraconazole, and caspofungin against *Candida* or *C. neoformans* [72–74]. Tablet diffusion assay has also been compared to CLSI microdilution and/or disk diffusion assays for testing filamentous fungi, including strains of *Absidia*, *Alternaria*, *Bipolaris*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Paecilomyces*, and *Scedosporium* spp. The categorical agreements of tablet and disk diffusion assays with microdilution assay were found to be similar for amphotericin B, caspofungin, itraconazole, and voriconazole but lower for posaconazole for tablet diffusion assay (84 % for tablet vs. 96 % for disk diffusion assay) [75].

Using the previous MIC breakpoints and interpretive zone diameters, reference disk diffusion assays occasionally yielding results that differ from those obtained by broth-based testing were reported [44, 50, 76–79]. Some isolates that were susceptible to fluconazole were categorized as fluconazole-resistant by disk diffusion assay. Disk diffusion assay also failed to differentiate some of the fluconazole-resistant isolates from the dose-dependent susceptible ones. Even more importantly, some fluconazole-resistant isolates were categorized as fluconazole-susceptible by disk diffusion assay using the previous breakpoints. Recent comparison of the fluconazole [51] and voriconazole [53] susceptibility categories obtained by broth-based testing to those generated by disk diffusion assay and by using the revised species-specific interpretive criteria yielded high agreement rates and very major error rates of 0—<1 %. For caspofungin and micafungin, on the other hand, disk diffusion breakpoints alternative to the revised ones have been proposed to improve the separation between wild-type and mutant isolates of *Candida* [52].

3.2 EUCAST Reference Broth Dilution Methods

A broth dilution assay for susceptibility testing of yeasts standardized by the European Committee on Antifungal Susceptibility Testing (EUCAST) Subcommittee on Antifungal Susceptibility Testing (AFST) is also available and has been revised as EUCAST definitive document EDef 7.2 [80]. The assay is similar to the CLSI broth dilution method, except that RPMI 1640 supplemented to 2 % glucose (CLSI uses 0.2 % glucose), an inoculum density of $1–5 \times 10^5$ cells/mL, and microdilution plates with 96 flat-bottom wells are used. The detailed comparison of the major test parameters proposed in EUCAST EDef. 7.2 and CLSI M27-A3 docu-

Table 82.3 Recommendations for application of *routine* fungal drug resistance assays for clinical yeast isolates [18, 92]

Indication	Antifungal drug(s) to be tested/recommendation(s)
Invasive infection due to *Candida* spp.[a]	Azoles, particularly fluconazole, and an echinocandin[c]
Invasive *Candida* infection (unexpectedly) refractory to initial therapy	Susceptibility testing for amphotericin B, flucytosine, fluconazole, voriconazole, and an echinocandin as an adjunct consultation with an experienced microbiologist
Refractory mucosal infection due to a *Candida* sp. that fails to respond to standard therapy at the standard dose	Azoles, particularly fluconazole
Refractory *C. neoformans* infection that fails to respond to standard therapy at the standard dose[b]	Fluconazole

[a]Susceptibility testing is particularly recommended for *C. glabrata*. Although susceptibility profiles may be predictable in general for other *Candida* spp., susceptibility testing appears still beneficial due to the possibility of strain-based secondary resistance to one or more antifungals
[b]Due to the lack of relevant MIC breakpoints for fluconazole vs. *C. neoformans*, susceptibility test results can provide general guidance only by comparison of the MIC results to those of the other strains of the same genus. If the MIC of that particular drug is relatively high for that strain when compared to those of the other strains of that genus, this may indicate microbiological resistance
[c]Due to the unresolved problems of interlaboratory variability observed when testing against *Candida* spp. by CLSI and EUCAST methods, in vitro susceptibility testing of caspofungin is currently not recommended. Anidulafungin or micafungin may be used as surrogate marker of echinocandin susceptibility until the problem is resolved [87]

ments and the recommended MIC reading endpoints are summarized in Tables 82.1 and 82.2, respectively.

Multicenter evaluation of the EUCAST assay showed that the method yields reproducible results [81], and comparative studies suggest a good correlation (92 % agreement rate) between CLSI and EUCAST antifungal susceptibility methods when testing amphotericin B, flucytosine, fluconazole, and itraconazole against *Candida* spp. [82].

Further studies that compared the fluconazole MICs generated by EUCAST and CLSI methods for *Candida* validated the very good correlation between the two methods. However and notably, the EUCAST MICs were found to be slightly lower than the CLSI MICs [83], especially for isolates with MICs above 2 μg/mL. Using the 24-h MICs and the ECVs, essential and categorical agreement rates suggested excellent correlation between EUCAST and CLSI methods when testing fluconazole, posaconazole, and voriconazole against various *Candida* spp. (categorical agreement rates varying from 91 to 99 %, 94 to 99 %, 94 to 99 % for fluconazole, posaconazole, voriconazole, respectively) [84]. Similarly, high categorical agreement rates of >90 % were observed for the EUCAST and CLSI methods in general when testing caspofungin, anidulafungin, and micafungin against *Candida* spp. except for lower agreement rates found for caspofungin against *C. glabrata* (85.3 %) and *C. krusei* (54.5 %). Importantly, both EUCAST and CLSI methods were able to distinguish FKS mutant strains of *Candida* spp. from the wild-type isolates at a high level [85]. A more recent study on susceptibility testing of ten systemically active antifungal agents against a collection of *Candida* strains including wild-type and non-wild-type collections for both azoles and echinocandins confirmed the high agreement rates for EUCAST and CLSI methods in general (overall categorical agreement rate 95.0 % with 2.5 % very major and major discrepancies) [86].

Importantly, lower (85 %) agreement rates were detected when testing caspofungin (10 % of the results categorized as non-wild-type by EUCAST and wild-type by the CLSI method). This study also revealed other specific problem points of lower agreement rates (amphotericin B, anidulafungin, and isavuconazole against strains of *C. glabrata*, itraconazole and posaconazole against most of the tested species, and caspofungin against *C. parapsilosis*, *C. tropicalis*, *and C. krusei*) that need to be addressed in future studies focused on further harmonization of the two methods [86]. Of specific note and as also discussed in Table 82.3, in vitro susceptibility testing for caspofungin using in the CLSI or EUCAST methodology is currently not recommended due to technical problems [87]. Anidulafungin or micafungin should instead be used for class-based testing. The currently accepted species-specific clinical MIC breakpoints to be used for EUCAST method and *Candida* are revised periodically and available on the EUCAST website [88].

Optimal parameters for antifungal susceptibility testing of conidia-forming molds have also been proposed and revised by EUCAST-AFST (EUCAST definitive document, EDef 9.2) [89]. These include the use of RPMI 1640 supplemented to 2 % glucose as the test medium and $1–2.5 \times 10^5$ conidia/mL (hemocytometric or spectrophotometric adjustment) as the inoculum density and are detailed in Table 82.1. An overall agreement rate of 92.5 % was achieved between the EUCAST standard and the CLSI method in previous studies when testing posaconazole and voriconazole against *Aspergillus*. Notably, the EUCAST method tended to generate higher MICs as compared to the CLSI method for *Aspergillus* isolates with discrepant results [90]. More recent studies also suggested very high essential agreement rates between the two methods within ±1 dilutions for posaconazole (87.7 %), voriconazole (96.3 %), and itraconazole (99.6 %) against *Aspergillus* [91]. Interpretive breakpoints have now been proposed by EUCAST for some *Aspergillus* spp. and antifungal drugs. These clinical breakpoints are available on the EUCAST website and periodically revised [88]. The detailed comparison of the major test parameters

proposed in EUCAST EDef. 9.2 and CLSI M38-A2 documents and the recommended MIC reading endpoints are summarized in Tables 82.1 and 82.2, respectively.

3.3 Clinical MIC Breakpoints and ECVs/ECOFFs for Interpretation of the Results Obtained by CLSI and EUCAST Microdilution Methods for *Candida* and *Aspergillus*

As also partly summarized in the previous subsection, species-specific clinical breakpoints for some antifungal agents and a number of *Candida* spp. determined by CLSI [43, 92] and EUCAST [25, 88] methods are available. ECVs have also been established for some species–antifungal drug combinations and may be useful particularly for cases where clinical breakpoints remain yet undetermined [92]. Current species-specific breakpoints are now commonly used in routine practice as well as in global surveillance studies to clarify temporal and geographic trends of resistance in *Candida* [93].

Clinical breakpoints for some *Aspergillus* spp. and antifungal drugs have also been established to be used for interpretation of results obtained by EUCAST methodology [25, 88, 94]. ECVs, on the other hand, are available for a number of *Aspergillus* spp. and antifungal drugs for CLSI [95–98] as well as EUCAST methodology [25, 99].

Due primarily to the difficulties of establishment of clinical breakpoints based solely on in vitro–in vivo correlation data, setting breakpoints based on pharmacokinetic and pharmacodynamic data and ECVs as well as clinical experience is now a common approach. To exemplify, EUCAST "rationale documents" [99] give an outline of the information on which the EUCAST clinical breakpoints are based [25]. The number of fungal genus–antifungal drug combinations where clinical breakpoints have been established is steadily expanding. Currently, the major goals of CLSI and EUCAST-AFST committees are determination of clinical breakpoints (and ECVs when determination of clinical breakpoints is yet not possible) for more and more fungal species and antifungal drugs and harmonization of CLSI and EUCAST broth microdilution methods [51].

3.4 Specific Points Under Discussion for Reference Antifungal Susceptibility Testing Methods

The development of standard assays for testing resistance to antifungal agents has been a remarkable progress. These assays are now widely used as a routine adjunct for prediction of clinical outcome and optimization of antifungal therapy. However, certain limitations of these methods have been under discussion.

3.4.1 Amphotericin B Susceptibility Tests

The distribution of amphotericin B MICs in a narrow range and the related difficulties in separation of resistant strains from the susceptible ones have long been a matter of discussion for both yeasts (*Candida*) and filamentous fungi. In efforts to overcome this drawback, various alternatives of the standard susceptibility testing method have been studied previously. Some investigators have found that the use of antibiotic medium 3 (AM3) instead of RPMI [100] and the application of Etest rather than broth dilution [101] enhanced the discrimination of amphotericin B-resistant *Candida*. However, the data obtained in other workers' hands did not support these findings [102] and the issue remained controversial. It was also observed that technical issues such as the lot of AM3 used for testing may also produce variation within the results [43, 103].

As an alternative approach, some studies using a combination of MIC and minimum fungicidal/lethal concentrations (MFC/MLC) suggested a meaningful correlation between in vitro and clinical resistance for some *Candida* [102] and *Aspergillus* [35] infections treated with amphotericin B. However, as with the MIC methods, further studies unfortunately failed to fully support these findings. An analysis of the amphotericin B MICs of strains isolated from candidemic patients showed that prediction of clinical amphotericin B resistance was not possible by any of the commonly used in vitro methods (CLSI microdilution, Etest) and test parameters (RPMI 1640 and AM3 as the test media, MIC and MLC as the interpretive criteria) [19]. On the other hand, the data published for the correlation of in vitro amphotericin B susceptibility with clinical outcome in mold infections (*Aspergillus* and Mucorales, in particular) also remained controversial. While some studies suggested the lack of potential of amphotericin B MIC testing in determination of resistance and prediction of clinical outcome [34], others supported its existence [35–37]. Overall, the technical problems in determination of in vitro amphotericin B resistance persist and await further approaches.

Amphotericin B remains as an important antifungal agent and patient care decisions must still be made with currently available data. EUCAST interpretive breakpoints have been established for amphotericin B vs. *Candida* and some *Aspergillus* spp. [88]. CLSI ECVs for amphotericin B vs. *Candida* [92] and some *Aspergillus* spp. [95] are also available.

3.4.2 Time Required to Finalize the Fungal Resistance Tests

The isolation of the infecting fungus, the growth of the fungus for the fungal resistance assay, and the interpretation of the assay take more than 48 h. This time is often even longer for molds. As a result of this drawback, fungal resistance

assays often give the clinician little early guidance in the choice of antifungal therapy for a particular infection. Fortunately, other clues can be applied during the early days of a given infection. Azole susceptibility profiles of *Candida* strains are generally predictable once the species of the isolate is known. For example, strains of *C. albicans* are mostly very susceptible to azoles, whereas those of *C. glabrata* may present with resistance at rates varying from one center to another [18, 93, 104–106]. Once antifungal therapy is initiated based on the knowledge on species-based primary resistance and the relevant epidemiological data for that specific center, the fungal resistance results of the particular infecting strain will then be available and can be used to guide therapy.

4 Other Methods for Antifungal Susceptibility Testing

4.1 Commercially Available Systems

4.1 Colorimetric Broth Dilution Methods

Determination of azole, flucytosine, and echinocandin MICs for yeasts demands grading of the amount of growth in comparison with that in growth control well. Making this assessment requires experience. In order to ease the challenge of grading the results, colorimetric indicators or fluorescent dyes may be used. Colorimetric methods have been employed by commercial assay systems, such as Sensititre YeastOne (Trek Diagnostic Systems Inc., Westlake, Ohio, which incorporates alamarBlue as the oxidation–reduction colorimetric indicator) and ASTY panels (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan).

For situations that do not permit use of commercially prepared systems, alamarBlue or other colorimetric indicators may also be added in-house to RPMI broth, followed by the application of conventional reference microdilution method. The endpoint determination in these methods is mostly based on the visual observation of the color change. Using alamarBlue, blue indicates no growth, purple indicates partial inhibition of growth, and red indicates growth.

The commercially available Sensititre YeastOne (Trek Diagnostic Systems Inc., Westlake, Ohio) [58, 107–119] and ASTY panels [120, 121] were compared to the CLSI or EUCAST method for *Candida*, *Aspergillus*, or other clinically significant filamentous fungi by various investigators. In a study that compared the Sensititre YeastOne panel with CLSI microdilution method for *Candida*, agreement rates of 93 %, 68 %, 78 %, and 80 % were attained for amphotericin B, fluconazole, itraconazole, and flucytosine, respectively. These results suggested relatively low rates of correlation, particularly for fluconazole and itraconazole [108]. While the agreement rate was found to be low (57 %) for itraconazole vs. *Candida* in some other studies as well [109], the results obtained by other investigators did not fully support this finding [110]. Some studies, on the other hand, have reported relatively lower agreement rates between Sensititre YeastOne and CLSI method when testing azoles and using the previous breakpoints (fluconazole, itraconazole, and voriconazole) against *C. glabrata*, specifically (categorical agreement rates of 34 %, 68 %, and 87 % for fluconazole, itraconazole, and voriconazole, respectively). These results emphasized the need for cautious interpretation of the Sensititre YeastOne results, particularly when testing azoles against *C. glabrata* [113]. Comparison of the Sensititre YeastOne with EUCAST reference method, on the other hand, when testing amphotericin B, flucytosine, fluconazole, and voriconazole against yeast isolates (that consisted of *Candida* strains, in major), yielded essential agreement rates of 98 %, 96 %, 97 %, and 96 %, respectively [118]. Given the revision of the breakpoints for *Candida*, comparison of the Sensititre YeastOne panel with the reference methods by using these current species-specific breakpoints is now the relevant approach. For caspofungin, micafungin, and anidulafungin and by using the species-specific breakpoints (or the ECVs when breakpoint remains undetermined for that species), Sensititre YeastOne was found to be in complete agreement with CLSI method (categorical agreement rates ranging from 93.6 % (caspofungin) to 99.6 % (micafungin) and less than 1 % very major or major errors) [122]. Of specific note, tentative wild-type populations and ECVs have been determined for Sensititre YeastOne method to be used when testing echinocandins, amphotericin B, and flucytosine against *Candida* spp. [123].

The utility of Sensititre panel/alamarBlue has been investigated also for susceptibility testing of *Aspergillus*. In one of these studies, the use of alamarBlue and reading the results spectrophotometrically yielded comparable results with CLSI method in 94 % of the cases for itraconazole and voriconazole, 25 % of the cases for flucytosine, and 64 % of the cases for amphotericin B [124]. In contrast to other results, agreement rates were found to be <66 % and >77 % for itraconazole and amphotericin B, respectively, at 24 h when the Sensititre method (endpoint: slight growth–MIC–purple for itraconazole, complete inhibition of growth–MIC–blue for amphotericin B) was compared to the CLSI method (endpoint: MIC-0 for both drugs) for *Aspergillus* strains. In the same study, the Sensititre method tended to produce lower MICs compared to the CLSI method, and agreement rates varied depending on the MIC endpoint used and the incubation period, yielding more comparable results at 48 h and by using MIC–blue endpoint [125]. Other studies also suggested higher agreement rates at 48 h [126]. In other studies that compared Sensititre YeastOne with CLSI M38-A method, overall agreement rates of 93 %, 90 %, and 97–99 % were obtained for amphotericin B, itraconazole, and voriconazole, respectively, for *Aspergillus* spp. [111, 127].

The agreement between Sensititre YeastOne panel and CLSI microdilution method has been explored in a few studies for filamentous fungi other than *Aspergillus* [114, 127, 128]. In one of these studies, posaconazole was tested by Sensititre YeastOne and CLSI methods against various filamentous fungi, including *Aspergillus*, *Fusarium*, *Rhizopus*, *Absidia*, and *Mucor*. By using the MIC–blue endpoint at 24 h, overall agreement rate within ±1 dilution range was found to be 94 % [114]. The comparison of these two methods for voriconazole against *Fusarium* spp., *S. apiospermum*, and *Rhizomucor pusillus*, on the other hand, showed percent agreement rates of 97–99 % at 48 or 72 h depending on the species [127].

Published data for ASTY panel are more limited. For *Candida*, ASTY panel provided high overall agreement rates of 93 (24 h) and 96 % (48 h) for amphotericin B, 5-fluorocytosine (5FC), fluconazole, and itraconazole against *Candida*. Agreement rates were found to range from 90 % with itraconazole and flucytosine to 96 % with amphotericin B at 24 h and from 92 % with itraconazole to 99 % with amphotericin B and flucytosine at 48 h [120]. For *Trichosporon asahii*, overall agreement rate between colorimetric microdilution panel and CLSI method was reported as 97.7 % when testing amphotericin B, flucytosine, fluconazole, miconazole, itraconazole, and voriconazole [121].

Conclusively, using the colorimetric assays, isolates with discordant results as compared to the reference method may be observed, and these need to be evaluated with the reference assays particularly for routine purposes. Now that the breakpoints have been revised and species-specific breakpoints are currently accepted, categorical agreement rates between the reference methods and Sensititre YeastOne are now being reexplored. One study investigating the correlation between CLSI method and Sensititre YeastOne for caspofungin and micafungin vs. *Candida* revealed promising results (very major and major errors, <1 %) [122]. Establishment of tentative method-specific ECVs for Sensititre YeastOne panel for testing echinocandins, amphotericin B, and flucytosine against *Candida* spp. is also noteworthy [123]. Further studies are required to determine the accuracy of the colorimetric assays particularly for strains with borderline MICs.

4.1.2 Fully Automated Broth Dilution Method

A fully automated commercially available system (VITEK-2 yeast susceptibility test, bioMerieux, Inc.) has been developed for antifungal susceptibility testing. VITEK-2 test evaluates the MIC results spectrophotometrically. Using the previous breakpoints, the system was found to be in very good agreement in general with the reference CLSI method (categorical agreement rates of 97.2 % and 88.3 % at 24 h and 48 h, respectively) when testing fluconazole against *Candida*, and very major errors were very seldom observed (0 % and 0.2 % at 24 h and 48 h, respectively) [129]. The VITEK-2 susceptibility results obtained for caspofungin, micafungin, and posaconazole against *Candida* were also found to be in very good agreement with those obtained by CLSI method by using the previously proposed breakpoints (categorical agreement rates of 99.8 %, 98.2 %, and 98.1 % for caspofungin, micafungin, and posaconazole, respectively) [130]. Using the current species-specific breakpoints, VITEK-2 system remained comparable to CLSI method when testing fluconazole and voriconazole against *Candida* [131] and fluconazole against *Candida* and *Cryptococcus* [132]. These comparative data using the current species-specific breakpoints are as yet limited, and further analyses using the new breakpoints and adequate numbers of resistant and intermediate strains are required.

4.1.3 MIC Strip (Gradient Strip) Method

MIC strip method is an agar-based diffusion assay that provides MIC values and quantitative measure of fungal resistance. Previously referred to as Etest® (AB BioDisk, Solna, Sweden) method, it has now been renamed due to the recent availability of multiple commercial products. It is being studied for both yeasts and molds although the available data are more extensive for yeast genera. The method uses plastic strips impregnated with a stable concentration gradient of the antifungal agent to be tested. MIC strips carrying amphotericin B, ketoconazole, fluconazole, itraconazole, flucytosine, voriconazole, posaconazole, caspofungin, anidulafungin, and micafungin are available.

Except for testing amphotericin B for which antibiotic medium 3 agar may be used [101, 133], the most relevant and commonly used test medium for MIC strip method is RPMI 1640 supplemented to 2 % glucose [134]. Casitone agar (azoles) [135, 136] and yeast nitrogen base (*C. neoformans*) [137] have been used by some investigators. In addition, similar to that in disk diffusion methodology, Mueller–Hinton agar supplemented with 2 % glucose and 0.5 μg/mL methylene blue is also being used [57, 74, 138–141] and may produce sharper edges of inhibition ellipse and less intra-elliptic growth when used particularly for testing azoles against heavy trailer *Candida* strains.

The inoculum density to be used in the test is adjusted in sterile saline to that of 0.5 McFarland standard either visually or spectrophotometrically at 530 nm, yielding a final concentration of $1–5 \times 10^6$ cells/mL. In accordance with the basic rules of disk diffusion assays, the adjusted inoculum is swabbed onto the agar plate and the Etest strip is placed onto the inoculated medium. The results are read as MICs after incubation of the plates at 35 °C for 18–24 h and 48 and 72 h (when needed, particularly for *C. neoformans*). The MIC of that particular drug is the concentration designated on the strip at the point where the inhibition ellipse intersects the strip. For azoles and other drugs such as flucytosine that tend

to produce partial inhibition, the growth inside the ellipse and the tiny colonies produced near the edge of the ellipse are neglected when reading the MIC value. This provides a reading endpoint that approximates MIC-2 of broth dilution assay and eases the precise determination of MICs particularly of isolates that tend to trail heavily.

The utility of MIC strip method for antifungal susceptibility testing of yeasts (predominantly *Candida* and *C. neoformans*) and filamentous fungi (mostly *Aspergillus* and less extensively *Rhizopus*, *Fusarium*, *Scedosporium*, *Paecilomyces*, and *Acremonium*) has been explored by several investigators. For *Candida*, percent agreement rates of the method (specifically Etest®) with CLSI reference method were found to be 90–98 % for amphotericin B [142], 82–100 % for fluconazole [113, 134, 143, 144], 80–95 % for itraconazole [113, 143], 91–100 % for voriconazole [113, 144, 145], 83–95 % for posaconazole [56, 57, 146], and 77–100 % for caspofungin [142, 147]. Of note and importantly, when the previous breakpoints were used and the correlation of the susceptibility categories was considered, Etest® tended to be less correlated with CLSI method (percent categorical agreement rates of 55 %, 74 %, and 76 % for fluconazole, itraconazole, and voriconazole, respectively), particularly when testing azoles against *C. glabrata* [113]. Correlation of Etest with EUCAST reference microdilution method has also been explored for yeasts (*Candida* strains, in major), yielding essential agreement rates of 98 %, 96 %, 97 %, and 95 % for amphotericin B, flucytosine, fluconazole, and voriconazole, respectively [118].

For *C. neoformans*, percent agreement rates of Etest® with CLSI reference method were found to be 99 % for amphotericin B [148] and 81 % for fluconazole [149]. Overall, Etest appeared in good agreement with the CLSI method when testing amphotericin B and fluconazole against *C. neoformans* [74]. The percent agreement rates for the other antifungal drugs were 54 % for itraconazole [149], 89 % for flucytosine [149], and 94 % for voriconazole [148]. Of specific note and in some previous studies, Etest appeared to ease the discrimination of amphotericin B resistance for both *Candida* and *C. neoformans* regardless of the test medium used (RPMI 1640 supplemented to 2 % glucose or antibiotic medium 3) [101, 133, 150].

For *Aspergillus*, percent agreement rates of Etest® with CLSI reference method were found to be 89–98 % for amphotericin B [111, 151], 67–100 % for itraconazole [111, 151, 152], 93–100 % for voriconazole [151–153], and 69–80 % for caspofungin [154]. Less data are available for Etest and other filamentous fungi (*Rhizopus*, *Fusarium*, *Scedosporium*, *Paecilomyces*, and *Acremonium*) [136, 155, 156]. For these genera, percent agreement rates were overall high (80 and 96 % on Casitone and RPMI, 2 % glucose agar, respectively) but tended to vary extensively (0–100 %) from one genus to another [136].

In some instances, as for testing *Trichosporon asahii*, Etest® was found to yield consistently lower MICs with a wider MIC range for amphotericin B and higher MICs for azoles (fluconazole and itraconazole) when compared to reference microdilution method [157]. Similar findings were recorded when testing *C. neoformans* as well; Etest® voriconazole MICs were higher than reference microdilution MICs for isolates that yielded discordant results with the two methods [148]. However, such a consistent trend of Etest to increase the MICs for all azoles was not always observed [152].

The performance of the Etest® for direct antifungal susceptibility testing of yeasts in positive blood cultures has also been investigated. The results of this study showed that correlation of direct Etest® with reference macrodilution method was ≥80 % for amphotericin B, flucytosine, and ketoconazole, while it was 64–70 % for itraconazole [158].

In summary, MIC strip method is a practical method that provides quantitative measure of fungal resistance. The agreement of the Etest® method with the reference assay has been found to be high in general [78, 113, 134, 145, 152, 159]. However, genus-, species-, and incubation period-dependent variations in Etest–CLSI reference method percent agreement rates may be observed [42, 113, 125, 136, 153, 160]. Standardization of the test parameters and the interpretive reading criteria as well as its correlation with clinical outcome should be addressed further. Most importantly and in relation to the recent revisions of the CLSI and EUCAST breakpoints, categorical agreement rates of MIC strip method with the reference microdilution methods by using these revised species-specific breakpoints need to be revisited.

4.2 Other Investigational Methods for Antifungal Susceptibility Testing

4.1 Flow Cytometry

The utility of flow cytometric susceptibility tests for rapid determination of fungal resistance of *Candida* (amphotericin B, fluconazole, caspofungin, and flucytosine), *Aspergillus* (amphotericin B, itraconazole, voriconazole), and *C. neoformans* (amphotericin B, fluconazole) has also been investigated [161]. The method employs various membrane potential-sensitive or DNA-binding vital dyes (3,3′-dipentyloxacarbocyanine iodide, propidium iodide, acridine orange, or FUN1) and is based on determination of alterations in fungal cell viability. The decrease or increase in fluorescence intensity of the cells stained with the dye following exposure to the drug and the "minimum fluorescence-enhancing concentration" (MFEC) of the drug are determined. The results are available in 3–8 h. The method appears to be well correlated in general with the reference

method and Etest [162–167], as well as clinical outcome [168, 169]. In contrary to the high agreement rates in general, essential agreement rate of 40 % with CLSI reference method has been reported for flow cytometry when testing caspofungin against *C. krusei* [170]. Flow cytometry provides rapid detection of resistance and has also been proposed as a useful and accurate method for identification of *Candida* strains that are resistant to amphotericin B [171]. Despite these advantages, its availability remains limited only to some centers due to the need for a flow cytometer, and the method is not used in routine practice of antifungal susceptibility testing [172].

4.2.2 Ergosterol Quantitation

Sterol quantitation method that measures cellular ergosterol content rather than growth inhibition has also been investigated as a fungal resistance assay. The method appeared to be useful particularly for *Candida* isolates that exhibit heavy trailing as these tend to produce unclear visual MIC endpoints for fluconazole and itraconazole at 48 h [173]. The method provides accurate results in general but is not practical for use in routine susceptibility testing. Also and importantly, since the currently accepted MIC reading time point is mostly 24 h in general for the reference methods when testing *Candida*, the problem of unclear visual endpoints particularly at 48 h is not much experienced in daily practice of antifungal susceptibility testing.

4.2.3 Metabolic (XTT) Assay

Tetrazolium salts may be used to detect in vitro antifungal susceptibility by determination of metabolic activity [174]. The eventual color change that reflects metabolic activity may be evaluated spectrophotometrically by measuring the optical density. The yellow tetrazolium salt turns purple when it is cleaved to its formazan derivative.

An antifungal susceptibility assay that uses the tetrazolium salt 2,3-bis{2-methoxy-4-nitro-5-{(sulfenylamino) carbonyl}-2H-tetrazolium-hydroxide} (XTT) was reported to yield high levels of agreement of >97 % for MIC-0 of amphotericin B and 83 % for MIC-0 of itraconazole vs. *Aspergillus*, suggesting potential reliability of this method [175]. The XTT assay has been also used for rapid susceptibility testing of fungi belonging to order Mucorales (*Rhizopus*, *Cunninghamella*, *Mucor*, and *Absidia* spp.). Percent agreement rates were found to be 93 %, 76 %, and 67 % for amphotericin B, posaconazole, and voriconazole, respectively. Importantly, the results were achievable as early as 6–12 h after inoculation [176]. Of specific note, XTT assay is commonly used for determination of antifungal susceptibilities of *Candida* biofilms [177, 178]. The method needs to be standardized for its potential use in routine antifungal susceptibility testing.

4.2.4 Agar Dilution and Agar Screening Methods

In accordance with its basic principles, agar dilution method employs agar medium plates containing twofold dilutions of the antifungal agent and inoculated with the suspensions of the fungal strains to be tested. The agar dilution method has been explored for amphotericin B, fluconazole, itraconazole, ketoconazole, and flucytosine vs. *Candida* [179, 180]; caspofungin, micafungin, and anidulafungin vs. *Candida* [181]; fluconazole vs. *C. neoformans* [182]; flucytosine vs. *C. neoformans* [183]; amphotericin B, itraconazole, and voriconazole vs. *A. fumigatus* [184]; ketoconazole, itraconazole, and terbinafine against *Malassezia* [185]; caspofungin vs. *Aspergillus* [186]; and terbinafine, naftifine, and itraconazole vs. *Microsporum*, *Epidermophyton*, and *Trichophyton* spp. [69, 187]. Agar dilution was also used for testing the antifungal activity of other compounds, such as boric acid [188] and *Melaleuca alternifolia* (tea tree) oil [189] against *Candida*.

While the agar dilution method remains unstandardized and labor-intensive for antifungal MIC determinations, an agar screening method recently appeared to gain significance for initial screening and rapid presumptive determination of secondary triazole resistance in *Aspergillus* (particularly, *A. fumigatus*) strains. The proposed method is based on determination of growth on a four-well RPMI 2 % glucose agar plate supplemented with itraconazole (4 μg/mL), voriconazole (1 μg/mL), and posaconazole (0.5 μg/mL) in each of the three wells and no antifungal drug in the fourth well for growth control assessment [30, 190, 191]. The agar screening plates are also commercially available (Balis Laboratorium V.O.F., Boven-Leeuwen, the Netherlands). In case of existence of growth in any of the triazole-containing wells, the strain may further be tested by a reference method for determination of corresponding MICs and definitive azole susceptibility categories. Further recommendations are awaited for the utility of the agar screening method in routine practice of azole susceptibility testing of *Aspergillus*.

4.2.5 Isothermal Microcalorimetry

Isothermal microcalorimetry is a novel method investigated for determination of in vitro antifungal resistance as well. Specifically, the method uses the growth-related heat production and the endpoint of "minimal heat inhibitory concentration" (MHIC, μg/mL) for determination of antifungal resistance [192]. It has so far been investigated for rapid detection of voriconazole resistance in *A. fumigatus* strains, where detection of voriconazole resistance was possible in 8 h [193]. The method has also been explored for antifungal susceptibility testing of Mucorales, *Fusarium*, and *Scedosporium*, yielding correlation rates of 67 %, 92 %, 75 %, and 83 % with CLSI MIC data generated for amphotericin B, voriconazole, posaconazole, and caspofungin, respectively

[194]. The applicability of isothermal microcalorimetry for determination of antifungal susceptibility of *Candida albicans* in artificial urine sample and determination of MIC values for amphotericin B, flucytosine, fluconazole, and tioconazole were also studied and provided promising results [195]. Isothermal microcalorimetry yet remains investigational as a fungal drug resistance assay. The limitations of the method are the availability of limited data for a few number of fungal genera, the requirement of special instrumentation, and the current lack of validation studies.

4.2.6 MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization–Time-of-Flight Mass Spectrometry)

Besides its utility for fungal identification, MALDI-TOF MS is now being explored for its potential use as a fungal resistance assay as well. MALDI-TOF MS was so far tested for determination of in vitro susceptibility to caspofungin using wild-type and FKS mutant *Candida* and *Aspergillus* strains and proved to yield accurate results when compared to CLSI reference method (complete essential agreement for all isolates and categorical agreement rates of 94 % for *Candida*) [196]. A MALDI-TOF MS-based method that relies on the proteome changes detectable after incubation of *C. albicans* strains with caspofungin for 3 h at a concentration of MIC "breakpoint" value has also been developed and reported for rapid detection of caspofungin resistance [197].

The available data for antifungal susceptibility testing using MALDI-TOF MS-based technology remain yet preliminary and limited in terms of fungal genera as well as tested antifungal drugs. Further studies are required for clarification of any potential utility and relevance of MALDI-TOF MS as a fungal drug resistance assay in the future of antifungal susceptibility testing.

5 Determination of Fungicidal Activity

For specific settings, determination of fungicidal activity may provide useful hints of likely clinical outcome. This may be achieved either by determination of the MICs initially, followed by MFC on solid media (mostly defined as the least concentration yielding growth of <3 colonies, approximating 99–99.5 % killing activity) or by time–kill experiments [198–203]. Animal models are also being used for assessment of fungicidal activity. Experimental models of disseminated candidiasis and aspergillosis have proven to be very useful for determination of fungicidal effect. Specifically, the assessment of residual fungal burden in animal models has been shown to be well correlated with the MFC measurements and time–kill results [204].

There is yet no standard procedure for determination of MFCs. A multicenter study investigated the reproducibility of MFC testing for itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B vs. *Aspergillus* spp. In this study, MFC was defined as the lowest drug concentration that yielded <3 colonies which approximated 99–99.5 % killing activity, and the reproducibility of using four different media (RPMI 1640, RPMI 1640 supplemented to 2 % glucose, antibiotic medium 3, and antibiotic medium 3 supplemented to 2 % glucose) was investigated. The highest reproducibility (96–100 %) was achieved with amphotericin B and the results were good across all four media. Reproducibility rates were still high but more medium-dependent for azoles (91–98 %) [205]. Similarly, optimal testing conditions for MFC determinations were investigated for filamentous fungi other than *Aspergillus* as well [206]. These studies remain significant as being the initial steps for standardization of MFC testing.

While investigations are being carried out for determination of fungicidal activities of various drug–fungal genus combinations, it seems likely that demonstrations of the utility of fungicidal measures will be limited. It may possibly be useful for specific clinical presentations, such as endocarditis, meningitis, septic arthritis, and osteomyelitis, or in existence of poor clinical response to standard, normally effective antifungal therapies in neutropenic patients [204].

For time–kill experiments, the test isolates are exposed to varying concentrations of the drug (e.g., ranging from 0.0625 to 16 times the MIC). Samples are then withdrawn at predetermined time points and plated. The viable colony counts on the plates are determined after incubation, and the results are plotted as time–kill curves. The method is labor-intensive but provides more detailed information about the pharmacodynamic properties of the drug and whether the killing activity of the antifungal agent for an individual strain is dependent on the concentration [198, 200, 207–213]. Similar to MFC testing, no reference method is available for time–kill experiments. Time–kill assay parameters that have been shown to yield reproducible results for *Candida* were proposed by some investigators. These parameters were specified as 10^5 cfu/mL as the inoculum size, RPMI 1640 medium as the test medium for antifungal drugs other than echinocandins (AM3 for echinocandins), 30 μL as the transfer volume, 35 °C with agitation as the incubation setting, and ≥99.9 % reduction in cfu/mL from the starting inoculum as the endpoint [214].

Overall and conclusively, determination of fungicidal activity by MFC measurements or time–kill assays is yet far from global standardization and awaits further investigations.

6 Indications for Use of Fungal Drug Resistance Assays

Fungal drug resistance assays, determination of MIC values in particular, are used (a) for routine purposes to predict the clinical outcome and optimize the antifungal therapy, (b) to provide epidemiological data for the susceptibility profiles and resis-

tance rates of the infecting strains to commonly used drugs at a particular center, and (c) to determine the in vitro antifungal activity of the novel compounds under investigation.

Unlike the application for bacteria and antibacterial agents, the use of routine fungal drug resistance assays is indicated only for some fungal strains isolated from clinical samples. These indications are currently more clearly defined for yeasts, particularly for *Candida*, and are listed in Table 82.3 [18, 92, 215]. Of specific note, susceptibility testing does not appear beneficial when intrinsic resistance or reduced susceptibility is known for an antifungal drug against all strains of a genus (e.g., fluconazole against *Candida krusei*). However and importantly, it may provide significant data for an infecting species with possibly high rates of acquired resistance for antifungal drugs (e.g., fluconazole against *Candida glabrata*; amphotericin B against *C. glabrata*, *Candida guilliermondii*, and *C. krusei*; amphotericin B, fluconazole, and echinocandins against *Candida rugosa*). In this latter case of possibility of high rates of acquired resistance, monitoring closely for signs of clinical failure and performing susceptibility tests are needed [92].

As the initial step in routine practice, identification of the infecting fungal strain to "species" level remains of uppermost significance not only for prediction of any possible existence of primary resistance to one or more antifungal agents but also for interpretation of the antifungal susceptibility test results which now are based on "species-specific" MIC breakpoints and ECVs [92].

For filamentous fungi, the relevance and benefit of routine application of these tests are less well-defined and require to be warranted by further in vitro–in vivo correlation studies. One important exception is *Aspergillus*. The existence and clinical significance of secondary triazole resistance in *A. fumigatus* strains, in particular, have recently been documented, and azole resistance in *Aspergillus* is now a concern in clinical practice. Based on this, testing mold-active triazoles against *Aspergillus* (particularly, against *A. fumigatus*) strains (by an initial agar screening test, followed by MIC determination where needed) now appears to be beneficial at least in a number of situations, including the existence of high prevalence of azole resistance in *A. fumigatus* in environmental or clinical isolates in that particular geographic area or center and/or the history of previous azole exposure for the particular case. Further recommendations are awaited for determination of definitive indications of routine azole susceptibility testing in clinical *Aspergillus* strains [7, 10, 28, 172, 190, 216, 217].

7 In Vitro Antifungal Combination Studies

Due to the low clinical response rates to monotherapy particularly in some opportunistic mycoses, such as aspergillosis, fusariosis, and zygomycosis, as well as availability of the new drugs, antifungal combination studies are now appealing. The best and most relevant method for testing in vitro interaction of antifungal agents is yet unknown. Most of the accumulated data on in vitro combination studies used checkerboard method (based on determination of fractional inhibitory concentration (FIC) indices) [218–226], and it still remains as the most commonly applied method for in vitro combination studies. However, there are significant problems about its performance, standardization, and interpretation [227]. Crossed Etest method [218, 228–231] and time–kill studies [218, 230–232] are the other methods used for testing in vitro antifungal interactions. Assessments of the antifungal interactions by a fully parametric response surface approach (Greco model) have also been undertaken [233–237] but appear equally difficult to interpret. Many questions yet remain to be resolved for rationale use of combination antifungal therapy, and standard in vitro methods and animal models followed by clinical trials appear to be the most relevant way of determination of the actual clinical efficacy of antifungal combinations [238–242].

8 Conclusions and Current Recommendations for Antifungal Susceptibility Testing

In the last two decades, there has been a great progress in standardization and application of fungal resistance assays. However, the issues that still remain to be resolved and clarified do exist though are getting less. Finally, while fungal resistance assays currently appear as a significant aid in prediction of clinical outcome and guiding therapy, the influence of the host factors is strong and limits the overall ability of susceptibility testing to completely predict response.

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Viral Phenotypic Resistance Assays

83

Jacqueline D. Reeves and Neil T. Parkin

1 Introduction

The emergence of the human immunodeficiency virus (HIV) pandemic in the early 1980s led to a marked escalation in virology research. A rapidly expanding knowledge base percolated not only within the HIV field but also in that of other viral diseases. The identification of drug targets in these viruses led to the development and approval of antiviral agents. However, especially for HIV, it quickly became apparent that the use of these agents could select for drug-resistant viruses. The need for assays to identify resistant strains and to guide physicians in treatment decisions was urgent. Today, the availability of numerous antiretroviral agents for HIV therapy, combined with assays to guide their use, allows the selection of combination regimens that can effectively suppress HIV replication for many years. The vast amount of experience gained over many years of HIV drug development and clinical research notably hastened more recent hepatitis C virus (HCV) drug development efforts. Combination drug regimens for HCV that include one or more direct-acting antiviral agents to different targets have been evaluated rapidly and optimized to minimize the emergence of resistance-associated variants and to promote viral clearance.

Phenotypic susceptibility assays are used for some viruses in a clinical setting. For HIV, they can help with the selection of the most active drug regimen for an individual's viral population. They are also employed in research studies, drug discovery, and preclinical and clinical stages of drug development, for example, to characterize resistance and cross-resistance patterns for new drugs and to establish correlations between discrete genotypic changes and drug susceptibility.

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Viral phenotypic susceptibility assays are designed to determine the observable susceptibility or resistance of a virus to an antiviral agent. Numerous types of assay have been described including classic plaque assays and more recent recombinant virus assays (RVAs). Susceptibility or resistance to an antiviral agent in cell culture is often reported as the concentration of antiviral agent that inhibits viral replication by 50 or 90% (IC_{50} or IC_{90}, respectively). The IC_{50} or IC_{90} is typically compared to that of a control or reference virus that is assumed to be drug sensitive, and the results are expressed as a ratio (often referred to as fold change or resistance index) of the experimental virus versus the control (e.g., IC_{50} experimental virus/IC_{50} control virus).

This chapter reviews the major phenotypic antiviral susceptibility assays, with a focus on HIV- and HCV-related assays. The use of intact virus assays, the development and clinical applications of recombinant virus assays for HIV drug resistance, replication capacity and coreceptor tropism determination, the use of HCV replicon assays for drug development, and the status of phenotypic assays for other viruses including HBV, CMV, HSV, and influenza virus are discussed.

2 Intact Virus Susceptibility Assays

2.1 Plaque Assays

Plaque assays were originally developed to study bacteriophages in the early twentieth century [1]. In the early 1950s, the assay was adapted for poliovirus by Dulbecco and Vogt [2–4] and catapulted animal virology forward. Plaque assays are based upon the principle that a single virus particle infecting a single cell in a monolayer culture will lead to a local area of cytopathology (a "plaque") after subsequent infection of adjacent cells when the culture is overlaid with a semisolid nutrient medium to prevent long-range secondary infection through diffusion. The amount of time required for plaque formation depends on the type of virus, cells, and growth conditions. Plaques are identified visually, often by staining the remaining viable cells. The plaques then appear

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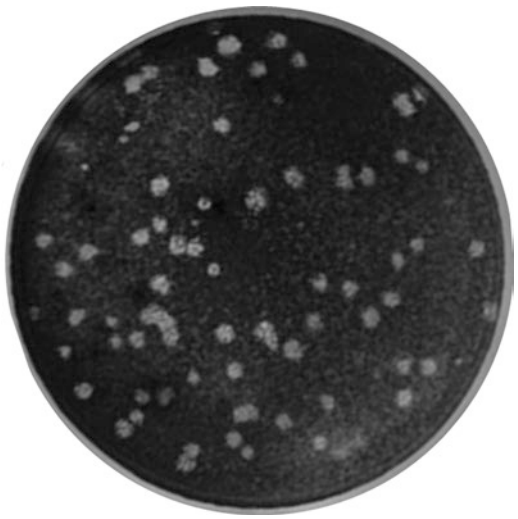

Fig. 83.1 Plaque assay. Crystal violet stained microtiter plate well showing HSV plaques in Vero cells (Image *source*: http://en.wikipedia.org/wiki/File:Plaque_assay_macro.jpg)

as clear circles in a stained monolayer of cells (Fig. 83.1). Alternatively, the monolayer can be stained with an antibody specific for viral antigens and the plaques (or foci) identified by colorimetric or fluorescence detection methods. The number of "plaque-forming units" (pfu) or "focus-forming units" (ffu) in a given volume is a measure of the infectious virus titer in a sample.

Plaque assays can be used to measure drug susceptibility. For example, serial dilutions of an antiviral agent can be added to the growth medium of both control and test virus infections. A dose-response curve (pfu/mL versus drug concentration) can then be generated, and the IC_{50} or IC_{90}, or change in IC_{50} or IC_{90} relative to control, can be determined. These types of "plaque reduction assays" have been utilized to measure drug susceptibility of many viruses, including influenza [5], herpes simplex (HSV) [6], cytomegalovirus (CMV) [7], varicella zoster virus (VZV) [8], and HIV-1 [9] (see below). One advantage of plaque assays over some other types of infectivity assays is that they can provide a visual assessment of viral fitness, as reflected by the size of the plaque. In addition, the presence of a low-level minority species of resistant virus can be detected by virtue of in vitro selection that can occur during a culture-based assay.

2.2 Virus Yield or Antigen Expression Assays

As an alternative to plaque reduction assays, virus released into the liquid medium of an infected cell culture in the absence and presence of antivirals can be measured by various techniques and used to quantitate antiviral susceptibility. The quantity of virus in the medium can be determined based on infectivity (e.g., by plaque assay or 50 % infectious dose ($TCID_{50}$) titration), viral antigen production (e.g., by ELISA), cytopathic effect (CPE), or viral nucleic acid production. Virus yield reduction assays have been used to measure drug susceptibility of several viruses including HIV [10], HSV [11–13], influenza virus [5, 14], and CMV [12, 15, 16], as detailed below.

2.3 Limitations of Intact Virus Assays

Plaque reduction and viral yield reduction assays are labor intensive, and some have limited precision, making them difficult to perform on a large scale for routine clinical use. The assays use replication-competent virus, which may undergo multiple rounds of infection during the assay. Thus for viruses that replicate with a high error rate, the virus tested in the assay could have acquired altered characteristics compared to those of the original virus sample. Additional limitations of intact virus assays include biosafety concerns that can make large-scale operations involving handling of infectious virus stocks a logistical obstacle. The ability to recover infectious virus from clinical specimens is not always reliable and is dependent on titer and fitness, which can vary considerably. Finally, some viruses do not form visible plaques, and others lack an in vitro cell culture system (or a system amenable to routine use) for clinical isolates and thus cannot be studied using plaque or other cell-based assays that rely on infection by intact viruses derived from clinical material.

3 Phenotypic Drug Susceptibility Assays for HIV-1

3.1 Plaque Reduction Assays

Initial measurements of HIV drug susceptibility, including the first description of zidovudine-resistant HIV-1 from infected individuals [9], were made using a plaque reduction assay in HeLa cells engineered to express the CD4 receptor [17]. Plaques, or foci, of infected cells could be identified and counted based on the propensity of the infected cells to fuse and form multinucleated syncytia; reduction in plaque/focus number in the presence of drug was used to derive IC_{50} values. Detection of infected cells was simplified by introduction of a β-galactosidase reporter gene under the control of the HIV-1 LTR [18]. Initially, these assays only generated plaques or foci with syncytium-inducing (SI) virus, since HeLa cells naturally express the CXCR4 coreceptor, but not CCR5 (see Sect. 3.5). Artificial expression of CCR5 in HeLa/CD4 cells, or other cell lines, overcame this obstacle [19–22].

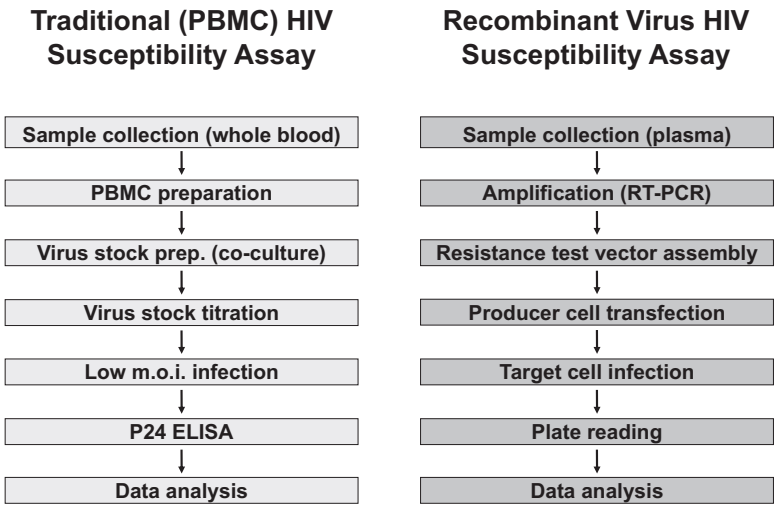

Fig. 83.2 Comparison of the process flow for intact virus (PBMC) and recombinant virus (PhenoSense HIV) assays

3.2 Peripheral Blood Mononuclear Cell-Based Assays

In the early 1990s, an alternative HIV phenotypic assay method was developed in which peripheral blood mononuclear cells (PBMCs) from an HIV-infected individual were co-cultured with phytohemagglutinin (PHA)-stimulated PBMCs from a seronegative donor [10] (Fig. 83.2). After approximately 7 days, the supernatant of the culture was collected as the viral stock and was subsequently titrated (based on p24 antigen production) on more PHA-stimulated donor PBMCs for an additional 7 days. An appropriate dilution of the viral stock was then added to PHA-stimulated donor PBMCs and grown for a further 7 days in the absence and presence of an antiretroviral agent. The supernatant was harvested and p24 antigen measured by an ELISA to quantitate virus production and generate susceptibility curves and IC_{50} or IC_{90} values. While this assay was standardized and provided useful phenotypic drug susceptibility/resistance data, it was cumbersome, imprecise, and slow. In addition, it is possible that the HIV stock derived from latent provirus in infected PBMCs does not reflect the strains circulating in the plasma.

3.3 Recombinant Virus Assays

The first recombinant virus assay for HIV generated viable virus by homologous recombination of a reverse transcriptase (RT)-deleted SI viral clone with a PCR-derived pool of RT sequences derived from proviral DNA samples [23]. Recombinant, replication-competent virus was amplified in a T-cell line and the virus harvested after 8–10 days, followed by virus titration and determination of drug susceptibility in a HeLa CD4+ cell foci reduction assay [23] or cell killing assay using a colorimetric readout [24, 25]. This assay represented a major step forward as it eliminated the need for donor PBMC cultures, thus standardizing viral stock production. Additionally it reduced the potential for the selection of virus stocks in culture that might differ from those represented in original sample due to the selective effects of different HIV gene products, particularly envelope. However, the use of proviral DNA may not fully reflect the circulating replication-competent virus, and the turnaround time for these assays (3–4 weeks) was still significant. This assay was later modified to measure HIV protease (PR) inhibitor susceptibility and to amplify sequences from plasma viral RNA instead of proviral DNA [26]. The assay was commercialized by Virco (Antivirogram®) in 1998 but discontinued for routine clinical use in 2010.

Significant advances that facilitated the use of phenotypic assays for routine clinical use occurred in the late 1990s. Both VIRalliance and ViroLogic (now Monogram Biosciences Inc.) developed and commercialized more rapid HIV phenotypic assays to measure resistance to antiviral drugs. The VIRalliance assay (Phenoscript™) [27] involves separate amplification of the gag-PR and the RT regions of HIV from RNA extracted from plasma samples. Each PCR product is then separately co-transfected into HeLa cells along with a proprietary plasmid vector. Infections are limited to a single cycle to ensure that the recombinant virus accurately reflects the amplified region from a clinical sample. Single-cycle infection is achieved by the deletion of the envelope region from the vector; recombinant virus is pseudotyped with the G-protein of the vesicular stomatitis virus (VSV-G). For testing of protease inhibitors, the transfected viral producer cells are incubated in the presence of serial dilutions of drug. The resulting recombinant virus is then used to infect indicator cells containing a *lacZ* gene under the control of the HIV-1 LTR. For testing of RT inhibitors, virus produced in the absence of drug is added to cells pretreated with serial dilutions of drug. β-Galactosidase in infected cells is quantitated using a CPRG-based colorimetric assay. This assay is no longer available for routine clinical use.

In the PhenoSense® phenotypic assay developed by Monogram Biosciences Inc., plasma-derived PR/RT sequences are amplified as one amplicon and inserted into a luciferase reporter resistance test vector (RTV) using restriction enzyme digestion and DNA ligation [28] (Fig. 83.2). Viral stocks are prepared by co-transfecting HEK293 cells with the test vector DNA and an expression vector that produces the amphotropic murine leukemia virus (aMLV) envelope protein. For the testing of protease inhibitor susceptibility, transfected producer cells are incubated in the presence of serial dilutions of drug. Pseudotyped viruses harvested from the transfected cells are then used to infect fresh HEK293 cells. For the assessment of RT inhibitors, virus produced in the absence of drug is added to cells pretreated with serial dilutions of drug. The production of luciferase is dependent on the completion of a single round of replication (infection, reverse transcription, and integration). Drugs that inhibit viral replication reduce luciferase activity in a dose-dependent manner, allowing the quantitative measurement of antiretroviral drug susceptibility (Fig. 83.3). The assay was subsequently adapted to allow the measurement of HIV integrase (IN) inhibitor susceptibility (PhenoSense® Integrase) [29, 30] and, more recently, the measurement of HIV PR/RT/IN inhibitor susceptibility, in conjunction with genotypic resistance analysis, from a single RTV (PhenoSense® GT plus Integrase) [31]. The assay was also adapted to allow assessment of maturation inhibitor susceptibility (Gag assay) for research and drug development purposes [32]. The distinguishing features of various HIV drug susceptibility assays are summarized in Table 83.1.

The recombinant virus assays described above share some drawbacks. Clinically relevant thresholds that define resistance are not known for all drugs (see below). The presence of a minority species of resistant virus(es) may be missed if their relative proportion and/or fitness is below that required for the IC_{50} to shift above the cutoff; the proportion required varies for each drug and mutation pattern. However, both of these limitations (interpretation and detection of minor species) also apply to standard population genotyping assays. Alternative approaches such as traditional clonal phenotypic or genotypic analysis are too expensive and cumbersome for routine clinical use. However, recent advances in "single genome sequencing" methodologies could allow the cost-effective genotypic analysis of minor species if deemed clinically relevant. Partly to minimize the potential for missing the presence of resistant virus, current recommendations emphasize the need to draw a blood sample while an individual is still taking a failing drug regimen to avoid the possibility of archived drug-sensitive virus from outgrowing the resistant variants [33].

Studies that have compared results from different HIV-1 phenotyping assays are limited. Qari et al. tested a panel of 38 samples, many of which were sensitive to all antiretrovirals, in the PhenoSense and Antivirogram assays [34]. Over 90 % of individual results were considered concordant, using a dichotomous scoring system based on susceptibility cutoffs in use at the time of the study. The majority of discordant results had a fold change in IC_{50} values close to the cutoff used. Miller et al. used a panel of 28 specimens, which included a greater proportion with drug resistance, and compared all three assays that were commercially available at that time [35]. Again, the results generally had a good concordance. The most comprehensive analysis comparing PhenoSense and Antivirogram was published by Zhang et al. and demonstrated an improved precision for PhenoSense with nucleoside RT inhibitors [36].

3.3.1 Phenotype Test Interpretation

The interpretation of phenotypic susceptibility assay results is enhanced by relevant thresholds, or "cutoffs", that are

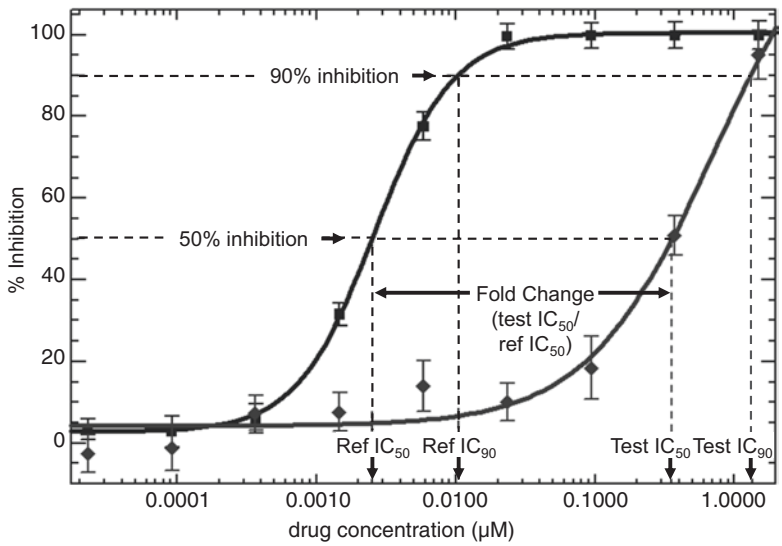

Fig. 83.3 Inhibitor susceptibility curve (PhenoSense HIV assay). Derivation of reference and test sample IC_{50}, IC_{90}, and IC_{50} fold-change values

Table 83.1 Phenotypic assays for HIV protease, reverse transcriptase, and integrase inhibitor susceptibility testing

	ACTG/DOD PBMC [10]	Antivirogram[a] [26]	Phenoscript[b] [27]	PhenoSense [28]	PhenoSense Integrase [29]	PhenoSense GT Plus Integrase [31]
Supplier	Various academic labs	Virco, Belgium	VIRalliance, France	Monogram Biosciences Inc., USA	Monogram Biosciences Inc., USA	Monogram Biosciences Inc., USA
Region of virus tested	All	PR 1–99, RT 1–400	PR 1–99, RT 1–503	PR 1–99, RT 1–305	IN 1–288	PR 1–99, RT 1–400, IN 1–288
		Gag variable	Gag variable	Gag 418–500	RNaseH	RNaseH
Readout	p24 antigen	MTT/cell viability (colorimetric)	β-Galactosidase (colorimetric)	Luciferase (luminescent)	Luciferase (luminescent)	Luciferase (luminescent)
Cells	Donor PBMCs	MT-4	P4 HeLa	HEK 293	HEK 293	HEK 293
Replication competency	Replication competent	Replication competent	Replication defective, single cycle	Replication defective, single cycle	Replication defective, single cycle	Replication defective, single cycle
Recombinant virus construction methodology	N/A	Homologous recombination	Homologous recombination	DNA ligation	DNA ligation	DNA ligation
Amplification sensitivity	N/A	>1000 copies/mL	>1000 copies/mL	>500 copies/mL	>500 copies/mL	>500 copies/mL
Envelope	HIV env from virus tested	HIV (HXB2) env	VSV-G	aMLV	aMLV	aMLV
Turnaround time (weeks)	4–6	3–4	2–3	2	2	2–2.5
Validated according to CLIA/local guidelines	No	Yes	Yes	Yes	Yes	Yes

[a]Discontinued
[b]No longer available for routine clinical use

intended to define the point above which the utility of a given drug begins to decline. "Clinical cutoffs" based on virologic response data from clinical trials provide the most clinically relevant threshold but are also the most difficult to define. To date, clinical cutoffs included in the PhenoSense, PhenoSense GT, PhenoSense Integrase, and PhenoSense GT Plus Integrase HIV assays have been defined for 14 drugs [31, 37–47]. The Phenoscript assay included clinical cutoffs for nine drugs [48, 49] and Antivirogram for four drugs [37, 40, 50–52] (Table 83.2). In the absence of clinical cutoffs, two alternative types of cutoffs have been used. The "assay" cutoff is defined by the intrinsic variability and technical limits of the assay during repeated testing of clinical samples. The "biological" cutoff is defined by an upper limit of the distribution of susceptibility exhibited by wild-type viruses, for example, the mean fold-change +2 standard deviations [53] or the 99th percentile [54]. The clinical relevance of biological cutoffs is limited, however, since the FC value that may be associated with declining virological responses can vary according to the drug. Importantly, the biological cutoff reflects both natural variation in viral susceptibility and inherent assay variability. Thus, such cutoffs may differ among assays that have different intrinsic variability.

3.3.2 Adaptation of Recombinant Virus Assays to Entry Inhibitors

HIV entry inhibitors include peptide inhibitors of virus-cell fusion and small molecules or antibodies that can target the viral envelope protein (Env) or cell-surface proteins (e.g., CD4, CCR5, or CXCR4) to prevent infection of cells [62, 63]. Enfuvirtide (ENF) is a synthetic peptide fusion inhibitor based upon the heptad repeat 2 (HR2) domain in the gp41 subunit of HIV-1 Env. ENF binds specifically to the HR1 domain in gp41 and resistance maps to this region [64–66]. To monitor the emergence of ENF resistance, two of the rapid phenotypic assays (Phenoscript and PhenoSense) that were originally developed for evaluating PR/RT resistance were modified [61, 67]. For Phenoscript, a fragment of the envelope gene (*env*) spanning gp120 and part of gp41 is amplified and co-transfected with an *env*-deleted proviral vector. Recombinant virus is used to infect cells containing an HIV LTR-β-gal reporter gene and expressing CD4 and one or both of the HIV coreceptors, CCR5 or CXCR4. In the PhenoSense Entry assay, the entire *env* gene (gp160) is transferred to an expression vector and co-transfected with a luciferase reporter viral vector. Resulting viral pseudotypes are used to infect cells expressing CD4 and CCR5 and/or

Table 83.2 Phenotypic susceptibility cutoffs

		PhenoSense			Phenoscript[a]			Antivirogram[b]		
Drug class	Drug	Cutoff (FC)	Type[c]	Ref.	Cutoff (FC)	Type	Ref.	Cutoff (FC)	Type	Ref.
NRTI	Abacavir	4.5	C	[39]	8	C	[49]	3.2	C	[52]
	Didanosine	1.3	C	[43]	2.5	C	[49]	2.3	B	[55]
	Lamivudine	3.5	C	[41]	5.5	B	[48]	2.1	B	[55]
	Emtricitabine	3.5	D	[56]				3.1	B	[50]
	Stavudine	1.7	A	[57]	3	C	[49]	2.2	B	[50]
	Tenofovir	1.4	C	[58]	4	C	[48]	2.2	B	[50]
	Zidovudine	1.9	B	[59]	4.5	B	[48]	2.5	B	[50]
NNRTI	Delavirdine	6.2	B	[59]	10	B	[48]	7.7	B	[55]
	Efavirenz	3	B	[59]	5	C	[49]	3.3	B	[50]
	Etravirine	2.9	C	[31]				3.2	B	[50]
	Rilpivirine	2.5	B	[31]				3.7	B	[60]
	Nevirapine	4.5	B	[59]	6.5	B	[48]	6	B	[50]
PI	Atazanavir	2.2	C	[46]				2.1	B	[50]
	Atazanavir/r	5.2	C	[46]	7	C	[49]			
	Amprenavir[a]	2	B	[59]	2.5	A	[49]	2.2	B	[50]
	Amprenavir/r[a]	4	C	[45]						
	Darunavir/r	10	C	[47]				10	C	[51]
	Fosamprenavir/r	4	C	[31]						
	Ritonavir	2.5	B	[31]						
	Indinavir	2.1	B	[59]	2.5	A	[49]	2.3	B	[50]
	Indinavir/r	10	C	[42]	20	C	[49]			
	Lopinavir/r	9	C	[40, 45]	10	C	[48]	10	C	[40]
	Nelfinavir	3.6	B	[59]	3	B	[48]	2.2	B	[50]
	Saquinavir	1.7	B	[59]	2.5	A	[49]	1.8	B	[50]
	Saquinavir/r	2.3	C	[45]	11	C	[49]			
	Tipranavir/r	2	C	[45]				3	C	[37]
INI	Dolutegravir	4	C	[31]						
	Elvitegravir	3.5	B	[31]						
	Raltegravir	2.2	B	[31]						
EI	Enfuvirtide	6.5	B	[61]						

FC: fold change from reference
[a]No longer available for routine clinical use
[b]Discontinued
[c]A, assay/reproducibility cutoff; B, biological cutoff; C, lower clinical cutoff; D, clinical cutoff derived by analogy to critical parameters of lamivudine

CXCR4. Both assays use inhibition of the reporter gene activity to generate IC_{50} or IC_{90} data. Studies using these assays, as well as others, revealed that natural variation in ENF susceptibility can be quite extensive [61, 67]. A clinical interpretation of these differences has been hindered by the lack of studies allowing for the derivation of a clinical cutoff for ENF; therefore, a biological cutoff is used to define a virus as having reduced susceptibility.

Recombinant virus entry assays can also be used to assess resistance to entry inhibitors that target Env interactions with CD4, CXCR4, or CCR5, including attachment inhibitors and chemokine receptor antagonists. For some inhibitors, including the CD4 antibody ibalizumab and the CCR5 antagonist maraviroc (MVC), resistance in a phenotypic assay can be observed as increases in IC_{50} and IC_{90} values and/or as a reduction in the maximum percent inhibition (MPI) obtained, visualized as a "plateau" at which infection can no longer be inhibited further with increasing drug concentrations [68–70].

3.4 Assays for HIV Fitness and Replication Capacity

Viral fitness is defined as the ability of a virus to reproduce within a defined environment. Mutations that confer drug resistance often reduce viral fitness in the absence of drug by interfering with one or more critical steps in the replication cycle. Replication capacity (RC) refers to the ability of a virus to replicate in the absence of drug as compared to that of a wild-type, drug-sensitive control virus. Several methodologies for determining viral fitness have been described, including replication-competent virus growth kinetic assays that

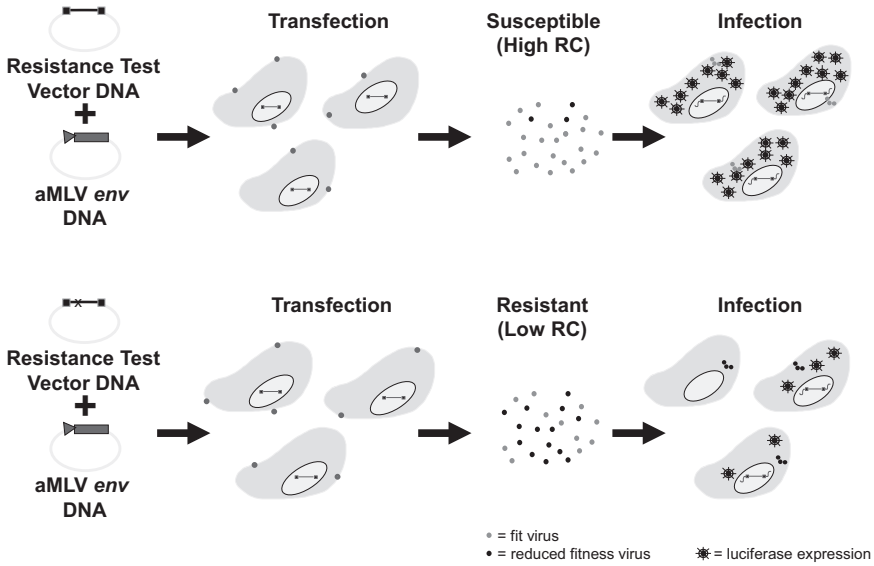

Fig. 83.4 Replication capacity assay (PhenoSense HIV). Drug-resistant viruses often exhibit reduced replication capacity (RC) compared to drug-susceptible viruses

compare the efficacy of viral replication of two or more variants in parallel or competitive cultures. Competitive culture assays measure the proportions of competing viruses over time using a variety of techniques including a recombinant marker virus assay [71] and a heteroduplex tracking assay [72]. A competition assay is regarded by many as the standard methodology to evaluate viral fitness because of its ability to measure the replicative abilities of two viral strains under identical conditions. However, the laborious nature and extended turnaround time of these assays make them impractical for routine clinical use. More rapid, single-cycle, phenotypic susceptibility assays have been adapted to measure RC (Fig. 83.4). In this case, the reported RC only relates to the portion of the amplified sequence transferred to the recombinant virus (i.e., PR and the partial gag and RT sequences included in the amplified fragment), and so the data must be interpreted carefully. Nonetheless there is evidence that if fitness differences are related to changes in PR/RT, the recombinant virus RC assay is a good surrogate of in vivo fitness [73].

Studies have shown that there is a wide distribution of RCs among wild-type HIV lacking phenotypic or genotypic resistance [54, 74, 75]. In general, drug-resistant HIV has been found to possess reduced RC and in vivo fitness, as demonstrated by the reappearance of less resistant virus in individuals whose antiretroviral therapy is interrupted, concomitant with an increase in viral load and decrease in CD4 cell count [73]. However, transmitted multidrug-resistant forms of HIV remain resistant for long periods of time even in the absence of drug pressure and with low viral fitness [75–77], presumably because the reversion rate is slower than that for outgrowth of archived drug-sensitive strains or due to unfavorable (unfit) intermediate forms on the pathway back to a drug-sensitive progenitor [78]. The availability of a convenient RC assay and accumulation of large amounts of data has enabled studies correlating the presence of specific resistance-associated mutations with low RC [79–86]. Such analyses may facilitate the formulation of treatment strategies designed to force the development of certain mutations which also reduce viral fitness [87, 88]. While the clinical utility of measurements of viral fitness or RC for a given individual is unclear, some reports have indicated a correlation between low RC and preservation of CD4 cell counts [74, 75, 89, 90].

3.5 Determining Coreceptor Tropism for HIV-1

HIV-1 infection requires interactions between the viral Env surface glycoprotein (gp120), the cellular receptor (CD4), and a coreceptor (e.g., CCR5 and/or CXCR4) [91]. CCR5 is expressed on primary T-cells and macrophages and is predominately used as a coreceptor by HIV transmitted between individuals and viruses present during early infection [92]. CXCR4 is expressed on many cell types, including primary T-cells, macrophages, thymocytes, and T-cell lines. CXCR4-using viruses are more commonly found in individuals with advanced disease [92]. However, it is not clear whether CXCR4 use precedes and causes more rapid disease progression or is merely the consequence of a change in target cell availability.

The discovery of HIV coreceptors enabled the development of HIV-1 entry inhibitors that target CCR5 in particular, including MVC (Pfizer, approved), vicriviroc (Schering-Plough, development halted), aplaviroc (GlaxoSmithKline, development halted), cenicriviroc (Takeda Pharmaceutical and Tobira Therapeutics, development for HIV on hold), and PRO 140 (CytoDyn Inc.) [62, 63]. The clinical development

of coreceptor inhibitors, and subsequent approval of MVC, necessitated the development of validated assays to determine coreceptor tropism [93, 94]. More recently, gene therapy-based approaches targeting CCR5 have further heightened interest in coreceptor usage and assays to measure it [95].

3.5.1 MT-2 Assays

CXCR4-using viruses can induce the formation of syncytia (syncytium-inducing (SI) virus) when cultured on the CXCR4-bearing MT-2 cell line. MT-2 cells lack CCR5 and are unable to be infected by CCR5-using HIV-1. Thus prior to the identification of coreceptors, CCR5-using HIV-1 isolates were classified as non-syncytium inducing (NSI). Two standardized MT-2 assay approaches have been described to evaluate coreceptor tropism. In one [96], there is a requirement to generate viral stocks from PBMC co-cultures, as described above. These stocks are titrated and can then be used to infect MT-2 cells. Since MT-2 cells express CXCR4 but not CCR5 [97], only SI (CXCR4-tropic) HIV-1 will be able to infect and induce the formation of syncytia. The assays are typically read 14 days or more after infection. Assessment requires microscopic inspection of individual cultures to determine the presence (SI) or absence (NSI) of syncytia. The second method utilizes direct cocultivation of MT-2 cells with an HIV-infected individual with PBMCs, followed by microscopic examination [98]. Prior to the identification of coreceptors, MT-2 assays were a common method of determining HIV phenotype in clinical research settings. Early studies utilizing an MT-2 assay established the SI phenotype as an important marker of disease progression [99]. Despite these findings, the MT-2 assay has not become a routine clinical monitoring test, owing to the time- and labor-dependent nature of the assay process, the lack of ability to directly alter this phenotype by previously available antiretrovirals, the potential drawback that the virus tested is derived from stimulated lymphocytes and not plasma virus and thus may not be representative of circulating virus, the nonquantitative nature of the assay readout (SI or NSI), the variable ability of CXCR4-tropic viruses to induce syncytia, and the potential for some non-CXCR4-tropic viruses to induce syncytia via an alternative coreceptor(s) [100].

3.5.2 Recombinant Viral Assays for Tropism

Entry susceptibility assays (see above) have been modified to enable the determination of HIV coreceptor tropism [93, 94, 101]. Recombinant viruses are used to infect mammalian cell lines expressing CD4 and either CXCR4 or CCR5. One such high-throughput assay (Trofile®, Monogram Biosciences Inc.) [93, 94] has been utilized in the clinical development of coreceptor inhibitors and is commercially available for selecting individuals suitable for MVC treatment. This single-cycle assay utilizes luciferase reporter pseudotype viruses and quantitates luciferase activity as relative light units (RLUs) to assess infection of U87 cells expressing CD4 and either CXCR4 or CCR5. As a confirmatory step, luciferase production must be inhibitable by an antagonist specific for the coreceptor being evaluated. This step is particularly relevant when infection levels are low and result in luciferase activity close to background levels. In June 2008, the original Trofile assay was superseded by an assay with enhanced sensitivity for the detection of minority variants [94]. This improved sensitivity allowed for the earlier detection of emergent CXCR4-using subpopulations in longitudinal samples and further optimized the selection of individuals for CCR5 antagonist therapy [94, 102–105]. The enhanced sensitivity Trofile assay is considered the current benchmark for coreceptor tropism evaluation. A version of this assay that utilizes cell-associated HIV-1 DNA as a template (Trofile® DNA), rather than plasma virus RNA, became available in 2010 to support treatment decisions in the context of virologic suppression [106].

The Tropism Recombinant Test (TRT; VIRalliance) is similar to the original Trofile assay except that a smaller region of the *env* gene (V1–V3) is amplified, and the readout is based on colorimetric assessment of β-galactosidase activity [101]. This assay was to be made available through Eurofins, but is not currently offered for routine clinical testing. The two recombinant tropism assays (TRT and the original Trofile assay) gave largely concordant tropism results (85 %) in a comparative study, with a few unresolved discordances and no evidence of differences in sensitivity [107]. While the V3 loop in the gp120 domain of Env is the major determinant of coreceptor use, regions outside of V3, and even outside of gp120, can also influence coreceptor tropism and thus may account for some discordant results between V3-based assays and those that utilize the entire Env [108].

A number of other recombinant virus-based tropism tests have been developed for research applications or exploratory clinical applications. These include:

(a) The Toulouse tropism test (TTT) which evaluates gp120 and the ectodomain of gp41 cloned from plasma virus or cell-associated DNA [109]. From a comparative analysis of tropism results for 24 samples, 92 % concordance to the enhanced sensitivity Trofile assay was obtained [109].
(b) A promoter-PCR (pPCR) assay in which overlapping PCR is used to assemble a CMV promoter to a population of full-length *env* genes which are then directly co-transfected with an Env-defective luciferase reporter HIV construct to generate pseudovirions, avoiding cloning/recombination steps [110]. Using this assay, results for 9/9 samples were concordant with the original Trofile assay [110].
(c) The VERITROP™ cell-to-cell fusion assay which utilizes a yeast-based homologous recombination approach to clone *env* genes into a HIV vector [111]. A comparative study to the original Trofile assay demonstrated 74 % (56/76) concordant results [111].

3.5.3 Comparison of MT-2 and Recombinant Virus Coreceptor Tropism Assays

There are important differences between MT-2 and recombinant virus assays. These assays typically evaluate HIV from distinct compartments: stimulated lymphocytes versus plasma. MT-2 assays utilize intact virus and recombinant assays evaluate the viral *env* gene. MT-2 assays permit multiple cycles of replication (and possible amplification of viral subpopulations and/or viral adaptation to culture conditions), while recombinant assays limit replication to a single cycle.

An SI result in an MT-2 assay is an established surrogate for HIV-1 CXCR4 utilization. This is supported by limited data examining the relationship between phenotypes determined by the MT-2 assay and the Trofile coreceptor tropism assay. In one study, 11 individuals with HIV determined to be SI in the MT-2 assay [112] had coreceptor typing performed retrospectively with the Trofile assay; virus from all 11 individuals was X4 or dual/mixed (DM (dual: CCR5 plus CXCR4. Mixed: populations of viruses with mixed tropisms that include CCR5- and CXCR4-using viruses)). Luciferase activity obtained on CXCR4-expressing cells infected with pseudovirions from these 11 samples was not uniform but rather varied over a very broad range of RLUs. Further studies will be required to determine whether this is clinically meaningful.

In a second study, the Trofile assay was utilized to determine the coreceptor tropism of virus from individuals prior to entry into a clinical trial of vicriviroc for the AIDS Clinical Trials Group 5211 study [113]. MT-2 assays were performed retrospectively among baseline isolates and revealed only limited discordance between the two assays [114]. Notably, the virus recovery rate among lymphocyte samples processed for the MT-2 assay was low (50 %) compared to the proportion of samples successfully phenotyped by the Trofile assay (>90 %). In a third study, the original and enhanced sensitivity Trofile assays were used to retrospectively evaluate sequential samples from individuals previously evaluated in an MT2 assay. Results were highly concordant and the evolution of coreceptor tropism from R5/NSI to DM/SI over time was noted in both assays [105].

4 Phenotypic Drug Susceptibility Assays for Hepatitis B Virus

Several specific antiviral drugs are now available for chronic HBV infection, including pyrimidine analogues (telbivudine, lamivudine) and purine analogues (tenofovir, entecavir, adefovir). As is the case for HIV, the use of these drugs can lead to the emergence of drug-resistant strains, associated with mutations within the polymerase gene [115] (see also chapter by Stephen Locarnini). With prolonged therapy and continued viral replication, mutations can accumulate and lead to significant cross-resistance between some polymerase inhibitors. Thus it may be important to detect and measure HBV drug resistance to manage the therapy of treatment-experienced HBV-infected individuals. To date, no detectable resistance has been observed following up to 7 years of treatment with tenofovir [116, 117]. However, preexisting adefovir resistance can decrease tenofovir activity [118].

While some HBV cell culture models have been described [119, 120], HBV presents unique challenges due to the fact that no routine robust cell culture system has been established to support the replication of HBV isolates (e.g., for viral spread assays). Therefore, phenotypic assays for the measurement of HBV antiviral drug susceptibility typically rely on several alternative methodologies and are limited to research/clinical research applications.

Phenotyping assays using full-length genomes from parental or mutant laboratory strains have been applied to study HBV resistance in transient assays [121, 122]. Cells able to support transient HBV replication (e.g., HepG2 or Huh7) are transfected with HBV plasmid vector constructs. Intracellular genome replication, dependent on the activity of the parental or altered HBV polymerase, is then compared in the presence and absence of the antiviral drug. Replication is traditionally monitored by Southern blotting; however this technique has limited clinical application due to the cumbersome nature of the readout. Additional concerns include questionable relevance of the behavior of individual mutations in a laboratory virus strain background.

Baculovirus vector-based HBV phenotyping assays to evaluate drug susceptibility have also been described [123, 124]. These approaches allow for efficient transduction of recombinant HBV baculoviruses into hepatoma cell lines. Most HBV drug-resistant variants have been found to replicate in such a system and to demonstrate the expected drug resistance phenotype. However, the procedure is still too cumbersome for routine use in the clinic.

A HBV phenotyping approach that employs PCR amplification of full-length HBV genomes from clinical samples may provide more relevant drug susceptibility information [125]. Clones or quasispecies populations of these genomes can be used instead of parental or mutant laboratory strains in transient transfection studies, using Southern blotting or real-time quantitative PCR approaches to monitor replication [126–129]. A modified version of one assay was commercialized by VIRalliance, but is no longer offered routinely [127]. A variant assay allows the phenotypic assessment of HBV polymerase/RT sequences from clinical specimens of genotypes A to H in the context of a recombinant genotype A HBV backbone [130, 131]. Polymerase/RT sequences are more easily amplified compared to full-length genomes; therefore, this approach facilitates the analysis of clinical samples with lower viral loads.

5 Phenotypic Drug Susceptibility Assays for Hepatitis C Virus

From 2001 through May 2011, HCV infection was treated with a combination of pegylated interferon alpha (peg-IFNα) and ribavirin (RBV) [132]. This entailed a long treatment course with significant side effects that was only approximately 50 % effective for individuals with genotype 1 HCV, the most common HCV genotype in North America [133–135]. Over the past few years, extensive antiviral drug discovery/development efforts have focused on direct-acting antiviral (DAA) agents that primarily target the NS3/4A protease, NS5B polymerase, or NS5A protein of HCV [132]. This has resulted in the approval of a number of different treatment regimens that variably incorporate protease inhibitors (boceprevir, telaprevir, simeprevir, asunaprevir, paritaprevir, grazoprevir), nucleoside (sofosbuvir) or non-nucleoside (dasabuvir) polymerase inhibitors, and NS5A inhibitors (daclatasvir, ledipasvir, ombitasvir, elbasvir, velpatasvir) [132]. Viral strains resistant to most of these compounds can rapidly emerge with suboptimal treatment regimens, given the error-prone nature of the HCV RNA-dependent RNA polymerase and high replication rate of HCV in vivo [136–142]. Thus, as for HIV, DAAs are utilized in combination/coformulated regimens, including with other DAAs with a different mechanism of action and with peg-IFN-α and/or RBV [132].

As for HBV, there is no cell culture system available for the routine culture of clinical isolates of HCV. To date, most in vitro HCV virology studies have been performed using genotype 1 or 2 subgenomic replicons [143–150] or a genotype 2a infectious cDNA clone [151–153]. Adaptive mutations can facilitate replication in cell culture. Replicons with resistance to virtually every compound tested so far can be selected in vitro. Such studies have been highly informative with respect to determination of the location of sites on the protease, polymerase, or NS5A protein that interact with the inhibitor and for the characterization of cross-resistance [139, 154–163]. For example, there appear to be four and possibly five distinct sites where allosteric inhibitors of the NS5B polymerase bind, as determined by the largely non-overlapping sets of mutations selected by the different classes of compound [164]. Variants associated with in vitro resistance to polymerase, NS3/4A protease, and NS5A inhibitors have also been detected in HCV from individuals treated with these inhibitors and largely overlap the in vitro findings [139, 165].

Recombinant replicon systems for assessing the drug susceptibility of plasma-derived HCV have been developed. These assays are currently utilized for research purposes and to support the phenotypic analysis of DAA susceptibility in preclinical and clinical drug development programs [166]. Plasma virus NS3 protease and NS5A or NS5B sequences can be transferred to a luciferase reporter-based replicon vector for susceptibility testing [161, 167–172], such as in the PhenoSense HCV NS3 protease and NS5A and NS5B assays (Monogram Biosciences Inc.; Fig. 83.5). Assay formats are similar to recombinant assays for HIV-1, in that target sequences are amplified from plasma by RT-PCR, transferred to a viral vector, introduced into cells, and cultured with serial dilutions of various inhibitors. Key differences include the requirement for in vitro RNA transcription (since the system relies on RNA, not DNA), typically an electroporation step, rather than transfection, and the use of limited number of cell types (derivatives of Huh-7 cells including those "cured" of HCV infection) which are able to support the high level of replication needed for the transient transfection assay format (Fig. 83.6).

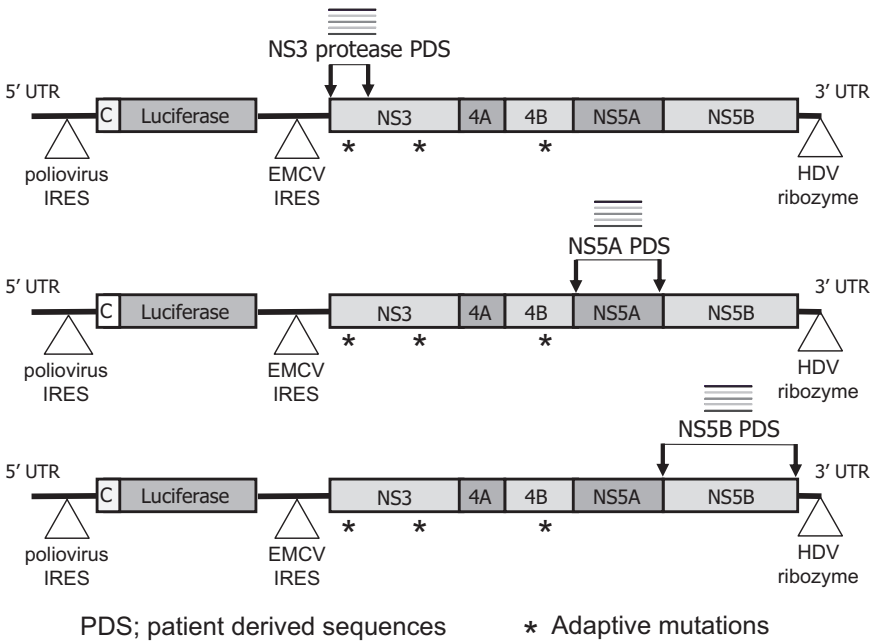

Fig. 83.5 Resistance test vectors for HCV replicon assays (PhenoSense HCV)

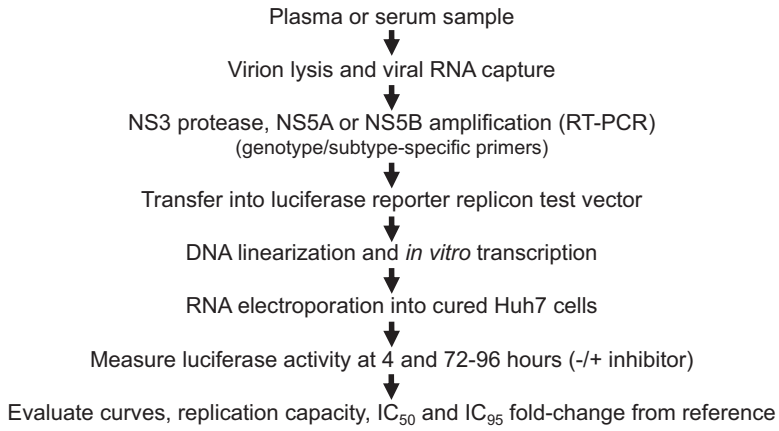

Fig. 83.6 Process flow for HCV replicon assays with clinical samples (PhenoSense HCV)

Challenges for phenotyping HCV clinical samples are related to the extensive diversity between HCV genotypes and subtypes and include (a) the design of primers and RT-PCR conditions that enable the amplification of a high percentage of samples at low viral loads; (b) the relatively low replication capacity of replicons containing some plasma-derived viral sequences, such as NS3 protease regions from protease inhibitor-resistant variants; (c) the lack of replication with some inter-genotypic recombinants, such as non-GT1 NS3 protease regions in a GT1 replicon backbone; and (d) the availability of a limited number of replicon backbones. HCV diversity has also proven challenging for drug development, with a number of inhibitors exhibiting variable potency within and between HCV genotypes. Natural variation in susceptibility to DAAs within a genotype can range from relatively narrow (e.g., within approximately 10-fold for some nucleoside inhibitors) to wide-ranging (e.g., over 1000-fold with some non-nucleoside polymerase inhibitors), in the absence or presence of known resistance-associated variants [167, 173]. However, as high sustained virologic response (SVR) rates can be obtained with combinations of potent antivirals, phenotypic viral resistance assays are not currently appropriate for routine clinical use as they are for HIV-1. Current guidelines do recommend the use of a genotypic viral resistance assay to select appropriate candidates for treatment with simeprevir in combination with peg-IFN-α/RBV or sofosbuvir [174,174b]. Clinical trials have shown that the efficacy of simeprevir/peg-IFN-α/RBV can be substantially reduced when the NS3 protease Q80K polymorphism is detected at baseline in HCV genotype 1a. Similar findings were observed following simeprevir/sofosbuvir treatment of individuals with cirrhosis. In phenotypic assays, Q80K confers an approximate 10-fold reduction in simeprevir susceptibility [175–177]. Guidelines also recommend genotypic viral resistance analysis of NS5A prior to the use of elbasvir/grazoprevir in HCV genotype 1a infected individuals [174b]. The presence of resistance-associated polymorphisms at amino acid positions 28, 30, 31 or 93, that confer at least a 5-fold reduction is elbasvir susceptibility in phenotypic assays, are associated with reduced efficacy in a 12 week treatment regimen. Treatment duration of 16 weeks with RBV intensification is recommended if variants at positions 28, 30, 31 or 93 are identified [174b].

6 Phenotypic Drug Susceptibility Assays for Herpesviruses (HSV, CMV, VZV)

While virus isolation and growth for the clinically important alpha herpesviruses, such as herpes simplex virus (HSV), cytomegalovirus (CMV), and varicella zoster virus (VZV), are technically possible, as with HIV it is wrought with practical obstacles including low reproducibility, long turnaround time, labor intensity, and biosafety concerns. Therefore, traditional plaque reduction assays for HSV [6], CMV [7] and VZV [8, 178] have been adapted for higher throughput [179] or are being replaced by recombinant virus systems [180–182], including some which rely on reporter gene readout such as secreted alkaline phosphatase (SEAP) [183]. Uncertainty about the clinically meaningful level of resistance is a major issue with the use of some of these assays [184, 185], as it is for HIV-1. Plaque reduction assays for the clinical evaluation of HSV-1/2 drug resistance are available from a limited number of reference or specialized laboratories.

7 Phenotypic Drug Susceptibility Assays for Influenza Virus

Phenotypic drug susceptibility assays for intact influenza virus have mainly been limited to plaque assays, often in Madrin-Darby canine or bovine kidney (MDCK or MDBK) cells. These assays have been successfully used to test the amantadine, rimantadine (adamantane derivative M2 ion channel inhibitors), and ribavirin (not approved for influenza

treatment) susceptibility of multiple strains of influenza [186]. Adamantanes are ineffective for the treatment of influenza B viruses, which lack the M2 protein, and widespread adamantine resistance among influenza A viruses has limited their utility this past decade [187].

In the mid-1990s, the advent of potent neuraminidase (NA) inhibitors such as zanamivir and oseltamivir provided new antiviral options for influenza treatment and created renewed interest in assays to assess influenza antiviral susceptibility. Phenotypic assays to measure NA activity were developed and are based on an enzymatic assay of virus particle-associated NA, using fluorescent or chemiluminescent NA substrates [188–191]. Commercial kits (Applied Biosystems), as well as in-house assays, are currently utilized routinely. In these assays, viral stocks are first titrated to select an assay input that is on the linear portion of the enzyme activity curve. An appropriate dilution of virus and drug are then mixed and incubated together, after which the fluorescent or chemiluminescent substrate is added. After incubation, the reaction is terminated and the amount of NA-released product is measured [192]. Fluorescent assays are more cost-effective, while chemiluminescent assays can have shortened incubation times and wider dynamic ranges, but both enzymatic assays are faster and more reliable than plaque assays. Alternative assays using virions pseudotyped with hemagglutinin and/or neuraminidase have also been described and can allow the biosafe evaluation of susceptibility to neuraminidase inhibitors [193–195]. However, for pseudotype as well as the traditional fluorescent or chemiluminescent assays, since some aspects of NA inhibitor resistance are associated with the hemagglutinin protein [196–199], NA enzyme or pseudovirion release assays may not completely reflect the inhibitor susceptibility of the intact native virus. An assay in which HA-expressing cell lines provide HA in trans to pseudotype HA-deleted, green fluorescent protein-expressing influenza viruses may facilitate analysis of influenza antivirals as well as neutralizing antibodies in a reconstituted virus system [199b].

Both fluorescent and chemiluminescent assays are rapid and reproducible and are used clinically as well as for surveillance [200, 201]. Phenotypic testing for neuraminidase inhibitor susceptibility is particularly useful when new viruses arise or new inhibitors become available, such as peramivir. Given the concern about spread of NA inhibitor-resistant influenza viruses, the Neuraminidase Inhibitor Susceptibility Network (NISN) was originally established to monitor resistance around the world using the chemiluminescent assay outlined above. In 2006, the NISN reported that at 3 years post the introduction of NA inhibitors, the detection of resistant viruses was limited (8 out of 2287 samples tested), but required continued surveillance as inhibitor use became more widespread [202]. Indeed, subsequent surveillance efforts by the NISN, the World Health Organization, as well as other groups, using fluorescent or chemiluminescent phenotypic assays, as well as sequence-based assays, identified widespread resistance to oseltamivir in circulating seasonal influenza from late 2007 to early 2008 and in the 2008–2009 season [187]. Viruses that arose late in the 2008–2009 season and that circulated/arose in following seasons through 2013–2014 had a low incidence of resistance on whole (2 % or less globally); however, clusters of resistant viruses identified in a number of communities in different countries warrant ongoing surveillance [187, 203, 204].

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Attributions

PhenoSense and Trofile are registered trademarks of Monogram Biosciences, Laboratory Corporation of America Holdings. Antivirogram is a registered trademark of Janssen. Phenoscript and VERITROP are trademarks of Eurofins VIRalliance Inc., and Diagnostic Hybrids, Inc., respectively.

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Drug Resistance Assays for Parasitic Diseases

84

Danielle Légaré and Marc Ouellette

1 Introduction

Protozoa and helminths have, by far, the greatest impact in terms of morbidity worldwide. The status of protozoa and helminths control, both in human and veterinary medicine, is challenged as the current medications against these parasites are losing their efficacy due to increasing and even further spreading drug resistance. Despite this alarming statement and the high burden imposed by parasites, research progress in parasitic diseases lags behind many other infectious diseases. Recent innovative technologies may significantly impact parasite diagnostics and their control in the near future, catalyzed by a better knowledge in drug resistance mechanisms. The present chapter review drug resistance assays in major protozoan and helminthic diseases, point-of-care tests and multiplexing assays for drug resistance testing, and opportunities for innovations in the field.

2 Drug Resistance

Parasitic diseases cause millions of human deaths every year with a major impact in terms of disability-adjusted life year (DALYs) [1]. Drug resistance is the ability of parasites to sustain growth and persist despite the presence of a drug. As long as drugs are used to treat parasitic infections, the chance of resistance developing to those drugs is present. All parasites covered in this chapter (Table 84.1) have developed resistance to nearly all available drugs used against them or will eventually develop resistance if drugs are not used appropriately. One useful strategy for reducing the appearance of resistance is drug combinations. This has been most useful against malaria [2] and is now advocated for other parasites, although the limited number of drugs often limits the possibility for effective combination. Apart from drug combination, the monitoring of drug resistance in parasitic pathogen collected from patients is not only helpful to guide initial treatment decisions (e.g., to avoid the use of nonoptimal medicine in case of drug resistance) but also helps in preserving the efficacy of existing molecules. Testing for resistance in parasites is labor intensive, however not well standardized, and hence not routinely available in clinical laboratories, especially in low-income countries.

2.1 Generalities: *Protozoa*

Drug resistance in protozoa can be inferred from clinical studies or from animal models, more frequently using in vitro tests and, with our increased understanding of the molecular mechanisms of resistance, using molecular DNA-based methods. Briefly, during clinical studies, a cohort of patients with clinical symptoms are treated and monitored over time for either failure to clear parasites or for relapse. Although relevant in terms of clinical context, one may not have the possibility of following closely the patients, and treatment failure or relapse may be due to several factors, including noncompliance, host immunity, variations of drug absorption and metabolism, reinfection, etc. A second form of in vivo tests deals with animal models and could be used (when available) when parasite isolates cannot be easily adapted to in vitro conditions. These animal model tests are expensive however and require special settings and qualified personnel and consequently, are not used frequently. In vitro tests are thus by far the most frequently used way to monitor drug resistance in protozoan parasites, but they require that the pathogenic organisms are grown in culture, in the presence of increasing concentrations of drugs and in either the presence or absence of in vitro cultured host cells. In most in vitro assays, the drug concentration that inhibits parasite growth (or its maturation or development) is used as an endpoint. Some in vitro methods call for adaptation of parasites to

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Table 84.1 Major protozoan and helminthic diseases in humans

Disease	Pathogen	Burden
Trypanomatid parasites		
Chagas disease	*Trypanosoma cruzi*	An estimated 25 million people are at risk in 21 countries in Central and South America (and more recently in the USA) with ~10 million people infected. There are 1 million new cases of chronic disease and more than 10,000 deaths annually. DALYs[a] burden is estimated at 0.55 million
Human African trypanosomiasis (sleeping sickness)	*Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense*	Of the 36 countries considered endemic for HAT, the Democratic Republic of the Congo alone accounts for 2/3 of reported cases. Other highly affected countries include Angola, Southern Sudan, and Uganda. The estimated number of actual cases is 20,000–50,000 with an estimated population at risk of 70 million people and a DALYs burden of 0.56 million. HAT has been targeted by WHO for elimination by 2020
Animal African trypanosomiasis (Nagana)	*T. vivax*, *T. congolense*, *T. brucei brucei*, *T. evansi, T. theileri*, and *T. equiperdum*	Economically, *Trypanosoma congolense* is the most important species and represents a major constraint to livestock productivity, particularly in the developing countries of Africa
Leishmaniasis	*Leishmania* spp.	The various forms of leishmaniasis affect populations in more than 95 countries with 350 million people living at risk, 12 million people infected, about 2 million of new cases per year, and an annual death rate of ~60,000 people. DALYs burden is estimated at 3.32 million
Apicomplexan parasites		
Malaria	*Plasmodium* spp.	Malaria occurs in over a hundred countries. Malaria encounters for 300–500 million annual cases with nearly a million deaths. DALYs burden is estimated at ~40 million
Toxoplasmosis	*Toxoplasma gondii*	Toxoplasmosis is present in every country. The global annual incidence of congenital toxoplasmosis alone has been estimated at 1.5 per 1000 live births, causing 1.2 million DALYs. High burdens were seen in South America and in some Middle Eastern and low-income countries
Cryptosporidiasis	*Cryptosporidium* spp.	*Cryptosporidium* spp. are recognized as major waterborne coccidian parasites worldwide. The disease may account for up to 25 % of childhood diarrhea cases in developing countries. Coccidian parasites are considered AIDS-defining opportunistic pathogens, but their screening is not done even in known HIV patients in most routine laboratories at the primary care level due to the lack of knowledge, expertise, and technique. DALYs burden for cryptosporidiosis is unknown
Coccidiosis	*Eimeria* spp. and *Isospora* spp.	*Eimeria* spp. is causing avian coccidiosis. Infections in human are rare. *Isospora belli* only infects human and some primates. DALYs burden for *Eimeria* and *Isospora* spp. in human is unknown
Anaerobe parasites		
Trichomoniasis	*Trichomonas vaginalis*	Trichomoniasis is the most common curable nonviral sexually transmitted infection worldwide, accounting for about 276 million cases annually. Among women, the prevalence of trichomoniasis has ranged from 3 % in adolescent and student to over 45 % in incarcerated women. Among men, the prevalence of trichomoniasis has ranged from 3 to 12 %
Giardiasis	*Giardia lamblia (or G. intestinalis or G. duodenalis)*	G*iardia duodenalis* (syn. *G. intestinalis*; *G. lamblia*) is the most frequently reported intestinal parasite in the world. According to estimations of WHO, giardiasis accounts for almost a billion cases worldwide with ~3 billion people living in areas in which the incidence of the infection is around 30 % (e.g., in developing countries). A prevalence range of between 2 and 8 % is estimated in developed countries. About 280 million people are infected each year worldwide. *Giardia* causes acute and chronic diarrhea, particularly among children in underprivileged communities
Amoebiasis	*Entamoeba histolytica*	Worldwide, approximately 50 million people develop colitis or extraintestinal diseases caused by amoebiasis. *E. histolytica* alone is responsible for 40,000–100,000 annual deaths worldwide. Amebic colitis and hepatic abscess are mostly responsible for the mortality, but the lung can be also infected which may also lead to death
Stramenopiles Parasites		
Blastocystosis	*Blastocystis hominis*	In some epidemiological surveys, *Blastocystis* is the most frequently isolated parasite; the prevalence varies between countries and between communities. In general, the estimated prevalence of *Blastocystis* spp. is higher in developing than developed countries (30–50 % and 1.5–10 %, respectively). This may be related to poor hygiene, animal exposure, and consumption of contaminated food or water

(continued)

Table 84.1 (continued)

Disease	Pathogen	Burden
Helminths		
Schistosomiasis (bilharziasis)	*Urogenital schistosomiasis is caused by S. haematobium and intestinal schistosomiasis by any of the organisms S. guineensis, S. intercalatum, S.mansoni, S. japonicum,* and *S. mekongi*	The schistosomes cause intestinal, hepatosplenic, pulmonary, urogenital, cerebral, and other forms of schistosomiasis. Schistosomiasis affects almost 240 million people worldwide and more than 700 million people live in endemic areas. DALYs burden is estimated at 3.31 million. The vast majority of the burden occurs in Africa
Lymphatic filariasis (also termed elephantiasis in extreme cases)	Filarial worms *Wuchereria bancrofti* and *Brugia malayi*	*W. bancrofti and B. malayi* are transmitted by mosquitos. Many patients are asymptomatic. Clinical features of *B. malayi* are similar to those of *W. bancrofti*. However in *B. malayi*, unlike *W. bancrofti*, genital involvement is rare. After recurring lymphangitis, a late complication resulting in thickening and verrucous changes in the skin known as elephantiasis may occur. An estimated 1.2 billion people are at risk in 83 countries throughout the tropics and subtropics of Asia, Africa, the western Pacific, and parts of the Caribbean and South America. An estimated 120 million people are infected worldwide with at least 40 million people severely affected. LF is marked by WHO for eradication by 2020. Lymphatic filariasis is responsible for 2.78 million DALYs
Onchocerciasis (river blindness)	Filarial worm *Onchocerca volvulus*	Onchocerciasis is one of the world's most distressing diseases of helminth origin, often resulting in blindness. The etiological agent *Onchocerca volvulus* is transmitted by the species *Simulium* or black flies whose breeding habitat is by fast flowing rivers or streams. The disease is prevalent in 30 countries of Africa, 13 countries in the Americas, and in Yemen. Worldwide, approximately 120 million people are at risk for the disease, over 35 million people are infected, 500,000 have visual impairment, and 270,000 people who have become blind as a consequence of long-term infection. DALYs burden is estimated at 0.49 million
Cysticercosis/ Taeniasis	*Taenia solium* (pork tapeworm) or *Taenia saginata* (beef tapeworm)	An infection due to an adult *Taenia,* in man or animals, is referred to as *taeniasis.* Only *T. solium* may cause cysticercosis in man. More than 50 million people are infected with *T. saginata* worldwide and about 60 million are infected with *T. solium*, causing ~50,000 deaths annually. Humans become infected by accidental ingestion of the embryonated ova (fecal-oral route) or by ingesting inadequately cooked beef or pork. Both humans and cattle or pigs are necessary to the complete life cycle of *Taenia* species. In the tropics, several other ruminants, e.g., goat, sheep, lama and giraffe, may serve as the intermediate hosts. DALYs burden is estimated at 0.5 million
Echinococcosis (Hydatid disease)	Caused by the larval stages of the *Echinococcus* spp. *(mainly **E. granulosus*** and *E. multilocularis but also **E. ortleppi**, **E. intermedius*** and **E. Canadensis**)[b]	Hydatid disease is most extensively found in East Africa, North Africa, South Africa, the Middle East, parts of South America, and Australia. The intermediate hosts are cattle, sheep, pigs, goats, or camels and the definitive host for this disease is the dog or other canids. Hydatid disease in humans is potentially dangerous depending on the location of the cyst (lung, liver, other tissues). Some 200,000 new cases of cystic echinococcosis are diagnosed annually. Echinococcosis are responsible for 0.14 million DALYs
Soil-transmitted helminthiases (intestinal worms)	*Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (Whipworm), and hookworms (*Ancylostoma duodenale* and *Necator americanus*)	Soil-transmitted helminths infect a large proportion of people worldwide with significant morbidity in more than 450 million people, primarily children and pregnant women, resulting in over 39 million DALYs. Worldwide, over 950 million people are infected with *Ascaris* with 60,000 deaths annually. *Necator americanus* and *Ancylostoma duodenale* are hookworms classed as one of the most destructive of human parasitic helminths. Hookworms affect more than 700 million people across the globe. *Trichuris trichiura* is less prevalent (estimates are nonetheless close to 700 million people infected). *T. trichiura* is responsible for ~10,000 deaths each year
Foodborne trematode (FBT) infections	Over 100 species of foodborne trematodes are known to infect humans	The word trematode is derived from the Greek word "hole" which references to their two attachment organs also called suckers, an anterior oral one and a posterior ventral sucker. At least 56 million people suffer from one or more foodborne trematode infections (clonorchiasis, opisthorchiasis, fascioliasis, paragonimiasis, and others) worldwide. FBT infections are responsible for 1.88 million DALYs
Strongyloidiasis	*Strongyloides stercoralis* (also caused rarely by *Strongyloides fülleborni*)	*Strongyloidiasis* is a soil-transmitted disease. *Strongyloides stercoralis* infects ~40–100 million people worldwide. *Strongyloides fülleborni* is found sporadically in Africa and Papua New Guinea. The true prevalence of strongyloidiasis is unknown, because infection is often subclinical.

[a]DALYs, disability-adjusted life years

[b]These *Echinococcus* spp. were, until recently, all considered to be strains of one species (e.g., *E. granulosus*), but following a taxonomic revision, a number of species are now proposed

culture first, while others used directly freshly isolated parasites from patients into the test. Although cheaper, faster, and most of the time easier to perform in comparison to in vivo tests, in vitro tests have their intrinsic limitations also, the first one being the low correlation of in vitro response with clinical response observed in patients in several parasitic infections. While a lack of significant correlation between in vitro sensitivity of artemisinins and clinical response was found in some studies [3, 4], good correlation was observed with most antimalarial studies [5–10]. The imperfect nature of non-standardized techniques for measuring resistance/susceptibility in a large number of protozoan parasites may be one of the key factors contributing to this discrepancy in correlation between various studies.

Current molecular techniques have allowed to pinpoint several genes associated with drug resistance in protozoan (Table 84.2), at least when induced under laboratory conditions. The discovery of molecular targets and molecular

Table 84.2 Major drug treatments for human parasitic diseases, mode of action, and known resistance mechanisms

Disease	Drug	Mode of action	Mechanisms of resistance
Trypanosomatid parasites			
Chagas disease	Nitroimidazole (benznidazole)	Reduction of BZ results in the generation of the cytotoxic metabolite glyoxal which covalently modify macromolecules (reductive stress)	• Gene deletion of TcOYE • Overexpression of TcFeSODA • SNPs and gene deletion of NTR • TcABCG1 overexpression • TcAAAP069 overexpression • TcPGP1 and TcPGP2 overexpression • Overexpression of DNA repair proteins
	Nitrofuran (nifurtimox)	NFX reduction leads to the production of an unsaturated open-chain nitrile (reactive species) that leads to parasite death	• SNPs and gene deletion of NTR • TcAAAP069 overexpression
Human African trypanosomiasis (sleeping sickness)	Organic arsenical (melarsoprol)	Melarsoprol is degraded to melarsen oxide, a metabolite which is highly toxic Melarsoprol also interacts with TSH forming a stable complex called MelT, which in turn is an inhibitor of TR	• Mutations or loss of P2/AT1 • MRPA overexpression • ODC and γ-GCS overexpression • Loss of the high-affinity transporter AQP2 (alias HAPT1) • Generation of a chimeric AQP2/AQP3 with concomitant loss of AQP3
	Ornithine analogue (eflornithine)	Suicide inhibitor of ODC	• Mutations or loss of TbAAT6
	Diamidines (pentamidine)	Binds to kDNA and inhibits topoisomerase thus interfering with kinetoplast replication May also cause inhibition of multiple cellular targets including SAMDC	• Mutations or loss of P2/AT1 • Changes in the low-affinity pentamidine transporter LAPT1 • Loss of the high-affinity transporter AQP2 (alias HAPT1) • Generation of a chimeric AQP2/AQP3 with concomitant loss of AQP3 • Changes in nucleobase transporters NT11.1 and NT12.1
	Naphthalene derivative (suramin)	Inhibition of LDL uptake, prohibiting the parasite's supply of cholesterol and phospholipids	• Overexpression of TbMRPE
	Nitrofuran (nifurtimox)	NFX reduction leads to the production of cytotoxic species that cause damage to DNA, lipids, and proteins leading to parasite death	• SNPs and gene deletion of NTR
Animal African trypanosomiasis (Nagana)	Diminazene aceturate (Berenil)	The main biochemical mechanism of Berenil's trypanocidal actions is thought to be by binding to kinetoplast DNA, thereby inducing complete and irreversible loss of kDNA in certain strains of trypanosomes. In addition, diminazene aceturate modulates the host cellular and inflammatory responses to Trypanosome infection	• Loss of HAPT1 function
	Isometamidium chloride (e.g. veridium, samorin)	Mode of action of ISMM is not fully understood but evidence is there that kinetoplastic topoisomerase type II of *trypanosoma* is selectively inhibited by the drug	• Not well defined. Modulation of mitochondrial electrical potential has been pinpointed as a candidate mechanism for drug resistance • SNPs in mitochondrial topoisomerase enzymes of *T. congolense* are not involved in isometamidium resistance

(continued)

Table 84.2 (continued)

Disease	Drug	Mode of action	Mechanisms of resistance
Leishmaniasis	Polyene antibiotic (amphotericin B)	Binding to ergosterol creates a transmembrane channel, allowing cytoplasmic content including K^+ to leak out Autoxidation forms free toxic radicals	• Loss of SCMT increases membrane fluidity in resistant parasites with changes in lipid composition • Decreased AMB uptake • Increased efflux due to MDR1 overexpression • Upregulation of the tryparedoxin cascade • Increase in the reduced intracellular thiol content • Upregulation of Sir2
	Pentavalent antimonials (SbV) (sodium stibogluconate/ meglumine antimoniate)	Reduced in trivalent form in vivo Generation of oxidative stress which leads to the disruption of the synthesis of macromolecules	• Decrease or absence or drug reduction to the trivalent form • Mutations or loss of AQP1 • Detoxification of the trivalent form by conjugation with thiols (whose levels are increased in resistant parasites due to an increased activity in ODC and γ-GCS) • Intracellular drug sequestration of thiol-conjugates through the overexpression of MRPA • Efflux pump whose regulation is probably MAPK1-dependent, able to pump out the drug-thiol conjugate • ARM58 overexpression • Overexpression of the host MDR1 transporter at the macrophage cell surface
	Diamidines (pentamidine)	Accumulation within parasites leads to disintegration of the network of kDNA and collapse in the mitochondrial membrane potential	• Efflux pumps
	Aminoglycosides (paromomycin)	Inhibition of protein synthesis and interference with vesicle-mediated trafficking	• Increase in PM vacuolar sequestration followed by exocytosis • Other potential resistance mechanisms may exit, see text
	Azoles (itraconazole/ ketoconazoles/ metronidazole)	Inhibitors of cytochrome P-450-dependent lanosterol C14 α-demethylase, a step in ergosterol biosynthesis	• Overexpression of squalene synthase confers itraconazole resistance
	Alkyllysophospholipids (miltefosine)	Perturbation of the metabolism of lipids, especially phospholipids Inhibition of cytochrome c oxidase which causes mitochondrial depolarization resulting to an apoptosis-like death	• Decrease in miltefosine uptake due to point mutations in the P-type ATPase transporter or in its specific beta subunit • Increase in efflux through MDR1 • Role of ABCG4 and ABCG6 in phospholipid trafficking at the plasma membrane • Possible role of other ABC transporters • Role of HSP83 and SKCRP1 that both could protect against programmed cell death induced by MIL • Role of a pyridoxal kinase and alpha-adaptin like protein as well as a large 299 KDa protein
	Pyrazolopyrimidine (allopurinol)	Mainly used in canine leishmaniasis. Its conversion to ribonucleoside triphosphate analogues and further incorporation into RNA disrupts biosynthesis of macromolecules Allopurinol is known to inhibit enzymes of the purine salvage pathway	• Differences in the affinity of enzymes of the purine salvage pathway

(continued)

Table 84.2 (continued)

Disease	Drug	Mode of action	Mechanisms of resistance
Apicomplexan parasites			
Malaria	Quinolone derivatives (chloroquine, quinine)	Inhibition of heme polymerase resulting in accumulation of cytotoxic-free heme	• SNPs in pfCRT and pfMDR1 • Changes in pfMRP1 and pfNHE-1 • SNPs in pfMRP2 • Changes in pfMDR2
	Antifolates (sulfadoxine-pyrimethamine)	DHPS is the target of sulfadoxine DHFR is the target of pyrimethamine	• SNPs in DHPS • SNPS in DHFR • CNVs in GCH1
	Mefloquine	Interference with hemoglobin digestion by the parasite	• CNVs in pfMDR1 • SNPs in pfMRP1 and pfMRP2 • Changes in pfMDR2
	Artemisinins	Artemisinins act via the generation of free radicals (ROS) that are initiated by iron bioactivation of endoperoxides and/or catalyzed by iron-dependent oxidative stress	• Changes in pfMDR5 • SNPs in the kelch domain of the K13 propeller protein
	Atovaquone	Inhibition of the cytochrome Bc1 complex in the mitochondrial electron transport chain Indirect inhibition of DHODH	• SNPs in cytochrome b • Changes in pfMDR2
	Proguanil	Antifolate metabolized into cycloguanil which targets the malarial enzyme DHFR	• SNPs in DHFR
Toxoplasmosis	Antifolates (pyrimethamine)	DHFR is the target of pyrimethamine	• No SNPs detected in the therapeutic targets DHFR, nor to the ABC transporters TgABC.B1, TgABC.B2, and TgABC.C1. The mechanisms of resistance are unknown
	Sulfonamide (sulfadiazine)	DHPS is the target of sulfadiazine	• No SNPs detected in the therapeutic targets DHPS nor to the ABC transporters TgABC.B1, TgABC.B2, and TgABC.C1 • Differentially expressed proteins detected in sulfadiazine-resistant strains of *T. gondii*, but their formal role in resistance requires further investigation
	Atovaquone	Inhibition of the mitochondrial electron transport process (binding to the cytochrome bc1 complex)	• No mutation was found on the cytochrome b gene so the resistance mechanisms are unknown
Cryptosporidium	Paromomycin	Targets the ribosomes, where it binds to the A-site and disrupts protein synthesis	• Upregulation of Cgd1_1350 (e.g., CpABC4) and Cgd7_4510 transcript levels encoding ABC transporters
	Azithromycin	Macrolide that is probably acting by inhibition of protein synthesis	ND
	Nitazoxanide	Nitazoxanide and its two metabolites, tizoxanide and tizoxanide-glucuronide, inhibit the growth of *C. parvum* sporozoites and oocysts	ND
Coccidiosis (*Eimeria* spp., *Isospora belli*)	For *Eimeria*: Ionophores (e.g., iasalocid, monensin, narasin, salinomycin, and semduramicin)	Disruption of ion gradients across the parasite cell membrane	ND
	For *Eimeria*: Synthetic drugs (e.g., decoquinate, clopidol, sulfonamides, amprolium, diclazuril, halofuginone, nicarbazin, and robenidine)	In many cases, the mode of action is unknown	ND
	For *Isospora*: Sulfonamide (trimethoprim-sulfamethoxazole)	Inhibition of the folic acid pathway	ND

(continued)

Table 84.2 (continued)

Disease	Drug	Mode of action	Mechanisms of resistance
Anaerobe parasites			
Trichomoniasis	Metronidazole	Metronidazole is reduced by the pyruvate to ferredoxin oxidoreductase system in obligate anaerobes. Reduction of metronidazole creates a concentration gradient that drives uptake of more drug and promotes formation of intermediate compounds and free radicals that are toxic to the cell	*In* in vitro *resistant parasites:* • Shrinking of the hydrogenosome in laboratory-generated *Tv*-resistant strains • Downregulation of PFOR • Ferredoxin with an exceptional redox potential • Reduced amount of intracellular ferrodoxin • Reduced thioredoxin reductase activity and free flavins • Decrease in the activity of FR1 *In clinical resistant isolates:* • Decrease in FR1 and ADH1 • SNPs in the nitroreductase genes *ntr4Tv* and *ntr6Tv*
	Tinidazole	The nitro group of tinidazole is reduced in *Trichomonas* by a ferredoxin-mediated electron transport system. The free nitro radical generated as a result of this reduction is believed to be responsible for the antiprotozoal activity. It is suggested that the toxic free radicals covalently bind to DNA, causing DNA damage and leading to cell death	• Downregulation of PFOR in in vitro generated resistant strains
Giardiasis	Metronidazole	Same as Trichomoniasis	• Epigenetic regulation • Resistance to MTZ is negatively correlated with the intracellular concentration of PFOR, leading to a concomitant decrease in the uptake of free MTZ into the cell • Nitroreductases GlNR1 and GlNR2
	Tinidazole	The mechanisms by which tinidazole exhibits activity against *Giardia* species is not known but are probably similar to the one described for Trichomoniasis	• Cross-resistance to TDZ has also demonstrated with MTZ-resistant *Giardia* strains
	Albendazole (in combination with mebendazole)	The benzimidazole drugs bind selectively to beta-tubulin and inhibit microtubule formation Albendazole-induced ROS accumulation in the albendazole susceptible *Giardia* parasites, but not in the resistant ones	• Cytoskeletal changes but not with a mutations at amino acid 200 in β-tubulin • Eight proteins involved in energy metabolism, cytoskeleton dynamics and antioxidant response • Antioxidant enzymes are upregulated in ABZ-resistant clones, leading to an increase in the R-SH pool
	Quinacrine	The exact mechanism of antiparasitic action is unknown; however, quinacrine binds to DNA in vitro, inhibiting transcription and translation. Quinacrine does not appear to localize to the nucleus of *Giardia* trophozoites however, suggesting that DNA binding may not be the primary mechanism of its antimicrobial action. Fluorescence studies using *Giardia* suggest that the outer membranes may be involved. In other organisms, quinacrine inhibits succinate oxidation and interferes with electron transport	• Resistant parasites accumulate less drug
	Nitazoxanide	Nitazoxanide and its two metabolites, tizoxanide and tizoxanide-glucuronide, inhibit the growth of *Giardia*	• Nitroreductases GlNR1 and GlNR2 • Recombinant PDI2 and PDI4 are inhibited by nitazoxanide

(continued)

Table 84.2 (continued)

Disease	Drug	Mode of action	Mechanisms of resistance
Amoebiasis	Metronidazole	kill the trophozoites by alterations in the protoplasmic organelles of the amoeba, but are ineffective in the treatment of cysts	• Increased expression of Fe-SOD and peroxiredoxin • Decreased expression of flavin reductase and ferredoxin 1 (FR1)
	Tinidazole	kill the trophozoites by alterations in the protoplasmic organelles of the amoeba, but are ineffective in the treatment of cysts	• Increased expression of Fe-SOD and peroxiredoxin • Decreased expression of flavin reductase and ferredoxin 1 (FR1)
Stramenopiles parasites			
Blastocystosis	Metronidazole	Metronidazole induces programmed cell death in *Blastocystis* and apoptosis-like features are observable in growing axenic cultures	• Drug resistance in certain *Blastocystis* strains might result in fitness cost that manifested as impairment of parasite adhesion and, consequently, virulence • Tolerance of nitrosative stress
Helminths			
Albendazole		The benzimidazole drugs bind selectively to β-tubulin and inhibit microtubule formation	• SNPs which cause amino acid substitutions in β-tubulin • SNPs present in a drug transport glycoprotein
mebendazole		The benzimidazole drugs bind selectively to β-tubulin and inhibit microtubule formation	Same as albendazole
Levamisole		Agonist at nicotinic acetylcholine receptors of nematode muscle which cause spastic paralysis	• Modulation or loss of the levamisole-sensitive acetylcholine receptor • Mutations in modulatory proteins of the acetylcholine receptor
Pyrantel		Agonist at nicotinic acetylcholine receptors of nematode muscle which cause spastic paralysis	• Modulation of nicotinic acetylcholine receptor subunits that form the pyrantel-sensitive receptors • Mutations in modulatory proteins of the acetylcholine receptor
Macrolides lactone (ivermectin)		Ivermectin increases the opening of glutamate-gated chloride (GluCl) channel and produce paralysis of pharyngeal pumping	• Changes in the frequencies of two alleles of the glutamate-gated chloride channel • Modulation in P-glycoproteins
Praziquantel		A rapid influx of calcium upon treatment probably causes death. Praziquantel may block adenosine receptors of the worms, causing calcium influx PZQ can specifically bind to the intersubunit cleft of glutathione S-transferase (GST) of schistosomes, but it was demonstrated that GST is not the molecular target of PZQ	• Cytochrome-c oxidase (SCOXl) expression was increased in PZQ-resistant *Schistosoma* strains compared to sensitive strains, but the levels of SCOXl enzymatic activity were reduced in resistant worms. This raises the possibility of an involvement of mitochondrial/respiratory processes in resistance to PZQ • Alteration in calcium regulation or membrane composition (modulation or loss of the putative calcium receptor?) • *S. mansoni* expresses higher levels of SmMRP1 in response to praziquantel • Modulation in SmMDR2 expression
Diethylcarbamazine		DEC blocks host, and possibly parasite enzymes involved in arachidonic acid metabolism, and enhances the innate, nonspecific immune system by altering the parasite surface structure, making them susceptible to destruction by host defense DEC interferes with parasite signaling pathways, including the nitric oxide pathway	ND

ND, not defined; NTR, nitroreductase; AAT6, amino acid transporter 6; AT1, adenosine transporter 1; MRP, multidrug resistance protein; HAPT, high-affinity pentamidine transporter; LAPT, low-affinity pentamidine transporter; SNPs, single-nucleotide polymorphism; MDR, multidrug resistance; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; MRP, multidrug resistance protein; GCH1, GTP-cyclohydrolase; NHE-1, sodium hydrogen exchanger 1; CRT, chloroquine resistance transporter; SIR2, silent information regulator 2; PGP, P-glycoprotein; ABC, ATP-binding cassette; ODC, ornithine decarboxylase; γ-GCS, γ-glutamylcysteine synthase; AQP, aquaporin; SCMT, S-adenosyl-L-methionine-C24-Δ-sterol methyltransferase; AMB, amphotericin B; BZ, benzimidazole; NFX, nifurtimox; SAMDC, S-adenosylmethionine decarboxylase; kDNA, kinetoplast DNA; LDL, low-density lipoprotein; MAPK1, mitogen-activated protein kinase 1; ARM58, hypothetical protein ARM58; MIL, miltefosine; TR, trypanothione reductase; DHODH, dihydroorotate dehydrogenase; ROS, reactive oxygen species; SOD, superoxide dismutase; FR1, flavin reductase 1; PDI, disulfide isomerase; PFOR, pyruvate to ferredoxin oxidoreductase; ADH1, alcohol dehydrogenase-1; ex, example

resistant determinants in clinical isolates has been more complicated, but progresses are being made. In the molecular method, genetic markers, already known to be linked to resistance, are assessed by polymerase chain reaction (e.g., PCR or its variants) or by gene sequencing, allowing the prediction to some degree of resistance to drugs. Clear advantages of molecular tests over the other assays are the need for only small amounts of genetic material as opposed to live parasites (that need to be obtained in sufficient quantity) and the ability to conduct large number of tests in a relatively short period of time. Disadvantages include the obvious need for sophisticated equipment and training and the fact that the whole set of gene mutations that confer drug resistance are not completely known for the majority of the antiprotozoal drugs. Thus, the predictive value of these molecular tests is not always perfect and caution in interpretation is needed. Sometimes, in vitro bioassays need to be performed in parallel to confirm molecular assays.

2.2 Generalities: *Helminthes*

Helminths are a diverse group of parasitic worms, encompassing nematodes, cestodes, and trematodes. Collectively, parasitic worms are among the most common causes of chronic human infections worldwide, particularly schistosomiasis, filariasis, onchocerciasis, and intestinal roundworm infections (Table 84.1). The WHO estimates that 2.9 billion people worldwide are currently infected with one or more helminthic species. Current efforts to control parasitic helminthes rely on the use of a few active anthelmintic drugs, namely, macrocyclic lactones (e.g., ivermectin), benzimidazoles (e.g., albendazole, mebendazole), praziquantel, and levamisole/pyrantel derivatives. The high rate of reinfection in helminth infection after drug therapy means that vaccines remain the best hope for worm control in human. Although promising avenues exist [11], no vaccines are yet available. Periodic mass administration of anthelmintic drugs to school-age children and other at-risk groups has proven to effectively limit the burden of helminthes, particularly for soil-transmitted helminths. Laboratory diagnosis of helminth infections is usually performed by microscopical detection of eggs and in some cases larvae in urine or stool or by immunological methods, e.g., through antibody or antigen detection (Table 84.3).

The development of parasitic helminthes with resistance to one or more anthelmintics is an increasing problem, especially in livestock. Indeed, reports of drug resistance have been made in every livestock host and to every anthelmintic class. Sheep and goats are the most affected by parasitic nematodes in which a high prevalence of multidrug resistance (MDR) has been reported. Resistance in nematodes of horses and cattle has not yet reached the levels seen in small ruminants, but evidence suggests that the problems of resistance, including MDR worms, are also increasing in these hosts. In human helminthes, resistance is rare although there are more and more signs of an emergence of drug resistance as demonstrated by some studies reporting low cure rates and fecal egg count reductions in stool/urine samples [12–18].

Helminthes in general are much more difficult to culture than protozoan. They pass through a complex life cycle and it is not always possible to grow and test them for all stages of the parasite outside their natural hosts. Thus, similar to protozoan parasites, testing for resistance in parasite nematodes is difficult and hence not routinely available in diagnostic laboratories. Nonetheless, a variety of in vitro tests and few in vivo assays have been developed for the detection of nematode populations resistant to the main anthelmintic groups, but most of them suffer in reliability, sensitivity, and reproductivity because most of them are not well standardized. The majority of the assays are currently based on coprological methods (e.g., stool samples). From these, the most widely used method for detecting and monitoring the presence of anthelmintic resistance in nematodes is the so-called fecal egg count reduction test (FECRT) which is suitable for all types of anthelmintic and is essentially a measure of changes in egg output before and after drug treatment. Alternatively, a series of phenotypic assays as well as some molecular assays have been developed (see helminth's section).

The next sections will discuss about the various susceptibility tests that are currently used for specific parasites, starting with protozoan and followed by helminths.

3 Drug Susceptibility Testing in Trypanosomatid Parasites

Trypanosomatid parasites infect over 21 million people worldwide with over 2 million new cases per year [1, 19, 20]. The highest burdens are reported for *Trypanosoma cruzi* causing Chagas disease, *Trypanosoma brucei* causing African trypanosomiasis, and *Leishmania* spp. responsible for a group of clinical manifestations collectively known as leishmaniasis. Jointly, these three diseases cause over 4 million DALYs (Table 84.1).

3.1 Chagas Disease

Chagas disease (CD), also known as American trypanosomiasis, is a zoonosis caused by the protozoan parasite *Trypanosoma cruzi* [21, 22], an important parasitic burden that thrives since ancient times, mostly in Latin America. *T. cruzi* is a heterogeneous species population circulating in human, insect vectors, and animals, categorized recently in

Table 84.3 Diagnostic tests and examples of drug resistance assays in the control of NTDs

Disease	Diagnostic tests[a]	Drug resistance assays[b]
Trypanomatid parasites		
Chagas disease	**Multiple serological tests using different platforms (e.g., ELISA, indirect immunofluorescence antibody test (IFAT), and indirect hemagglutination (IHA)) but cross reactions occur especially with visceral and cutaneous leishmaniasis and HAT which co-exist in the same geographic region as Chagas disease.** Ex: Chagas Stat-Pak® Rapid Assay (RDT) from Chembio Diagnostic Systems Inc.; Chagas Immunochromatographic Strip (ICS) Test developed by PATH; Hemagen Chagas kit (ELISA); Chagas instantest (Silanes); Ortho *T. cruzi* ELISA test system; SD Chagas Ab Rapid (Standard Diagnostic); Serodia Chagas (Fujirebio Inc; OligoC-test and the *T. cruzi* kDNA OligoC-test (Coris BioConcept); several others RDTs **Microscopy of thin or thick blood or buffy coat films stained with Giemsa or other appropriate stains. Hemoculture using special media (e.g., Novy-MacNeal-Nicolle agar or Evan's modified Tobies's medium) can also be done** Various PCR-based assays including LAMP assays Radioimmunoprecipitation assay (RIPA) Trypomastigote excreted-secreted antigen immunoblot assay (TESA-blot) Abbott PRISM Chagas test (automated chemiluminescence analyzer) Xenodiagnosis (uninfected laboratory kissing bugs are allowed to feed on patients and are then examined for trypanosomes) In new born: the microhematocrit concentration method in capillary tubes or the Strout concentration method in eppendorf tubes Peptide-based lineage-specific serology-based assay Microfluidic/lab-on-chip-based detection (e.g., VereTrop from Veredus Laboratories Ltd)	**Lack of methodology that allows monitoring of drug susceptibility of *T. cruzi* at public health laboratories** In vitro susceptibility assay with epimastigotes based on the tetrazolium dye [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] enzymatic micromethod (BZ resistance) In vitro epimastigote test combining hemoculture with quantification of BZ/NFX susceptibilities In vitro intracellular amastigote assay which involves the differentiation of hemoculture-derived epimastigotes into metacyclic trypomastigotes in order to establish infection in mammalian cell monolayers In vivo drug susceptibility assays in animals, mainly in the mouse model
Human African trypanosomiasis* (sleeping sickness)	CATT serological field tests for *T.b. gambiense* (e.g., the classical Card agglutination test on whole blood, the CATT-TP version and the CATT-D10 thermostable version). There is no comparable CATT test for *T.b. rhodesiense* The Immune trypanolysis tests LiTAT1.3 and LiTAT1.5 *for T. brucei gambiense* Coris BioConcept HAT Sero-strip (Dipstick) and HAT sero-*K*-SeT test (Lateral-flow device) for blood or plasma samples. These tests were developed by the NIDIAG consortium (http://www.nidiag.org/) and contain variant surface glycoproteins of the *T. brucei gambiense* variable antigen types LiTat1.3 and LiTat1.5 SD BIOLINE HAT test (immunochromatographic rapid serological test for *T.b. gambiense* detection in blood) IFA,ELISA and Dot-ELISA methods LATEX/*T.b. gambiense* serological test **Blood/lymph node/CSF microscopy and examination (e.g., thin or thick blood or buffy coat films stained with Giemsa or other appropriate stains. Fresh wet preparations of blood of buffy coat samples can also be used). Currently, stage determination in HAT relies on direct examination of CSF**; parasites in CSF are first centrifuged and examined by light microscopy in a mAECT viewing chamber or mAECT tubes. The HCT or QBC technique can also be used Various PCR and RT-PCR assays (conventional, TMA, NASBA, NASBA-OC, LAMP, RIME-LAMP, NEAR, HAD, RPA). However, molecular diagnostics are not yet developed to a level appropriate for widespread field use. A molecular diagnostic test for *T.b. rhodensiense* was developed based on the serum resistance associated (SRA) gene FISH test using peptide nucleic acid (PNA) probes Biomarkers in human plasma Light-emitting diode (LED)-based fluorescence microscope using acridine orange Nanobodies-based parasite antigen detection (co-developed by FIND and Standard Diagnostics) Single-chain variable fragment (scFv) engineered antibodies for trypanosome antigens detection in blood Microfluidic/lab-on-chip-based detection (e.g., VereTrop from Veredus Laboratories Ltd)	**No standardized methodology that allows monitoring of HAT drug effectiveness at public health laboratories** Molecular tests Assessment of drug sensitivity in mice Assay based on the metabolism of the dye Alamar blue (resazurin) by live cells to generate both a colorimetric and fluorescence signal Fluorimetric assay based on the interaction of propidium iodide with DNA The fluorescent diamidine DB99 test for melarsoprol resistance

Animal African trypanosomiasis (Nagana)	**Standard parasitological methods (Microscopy)** PCR-based assays (some based on 18S) Microsatellite loci analysis	Tests in ruminants **Tests in mice** in vitro assays (e.g., drug-ELISA technique) **Molecular detection** (e.g., the DpnII-PCR-RFLP assay or a similar BclI-PCR-RFLP assay specific for *T. congolense*. Several PCR-based assays are also available)
Leishmaniasis	Current rapid diagnostic tests for VL are antibody/antigen based. However, serological tests are positive in asymptomatic carriers and in past cases. Examples include the direct agglutination test (DAT), tests based on the K39 repeat antigen including the FDA-approved Kalazar Detect rapid test from InBios International Inc. and the urine latex agglutination test *KA*tex by Kalon Biological Ltd. RDTs based on the fusion synthetic protein rK28 (Chembio Diagnostics systems) **For VL: Microscopy (Giemsa or hematoxylineosin stained), culture of spleen, lymph node or bone marrow aspirates** **For CL and MCL: Microscopy (Giemsa or hematoxylineosin stained)/culture of biopsies/aspirate. Recently a Press-Imprint-Smear method was reported** Various PCR and RT-PCR assays including the Coris BioConcept *Leishmania* OligoC-test (e.g., PCR assay coupled to Oligochromatographic detection) and a LAMP assay The field-adaptable in-a-tube Liat/Cobas Analyzer (IQuum Inc. now Roche diagnostics) which is a simplified PCR assay that uses a colorimetric detection system and a portable instrument that can extract and amplify DNA as well as detect the PCR product PCR-ELISA assays Aptasensors Leishmanin skin test (LST); this assay measures delayed hypersensitivity response after 72 h Interferon-release assays (IGRAs); detect T-cells in peripheral blood reactive to *Leishmania* antigens IQuum Liat Analyzer, a four-color real-time PCR fluorescence detector For animal, biomarkers of leishmaniasis have been found in hair	**No standardized methodology that allows monitoring of antileishmanial drug effectiveness at public health laboratories** BALB/c mice infected with *Leishmania* strains and treated with antileishmanial drugs Amastigote/macrophage culture assays for SbV resistance Promastigote assay and the axenic amastigote culture assay Resazurin-based fluorimetric assay for promastigotes (see HAT section for details)
Apicomplexan parasites		

(continued)

Table 84.3 (continued)

Disease	Diagnostic tests[a]	Drug resistance assays[b]
Malaria	**Microscopy examination of thick and thin blood smears** Antigen detection (dipstick or ELISA formats) (antigens: HRP-II, Pan-malarial *Plasmodium* aldolase, pLDH) Antibody detection (ex: Pan malaria antibody CELISA from Cellabs) REED technology Micromagnetic resonance relaxometry Microarray-based techniques PCR and LAMP assays Aptasensors, immunosensors, and DNA biosensors Paper microfluidic Lab-on-chip-based detection (e.g., VereTrop from Veredus Laboratories Ltd) Flow cytometry and automated blood cell counting techniques to detect hemazoin or Plasmodium dsDNA in infected erythrocytes Focal Plane Array technology to detect the fatty acids in the parasite (infrared signature) Vapor nanobubbles technology (Rice University)	Rodent models for drug susceptibility testing Monitoring of the parasitological and/or clinical response over time in patients Spectroscopic analysis method In vitro assays based on [^{3}H]-hypoxanthine incorporation or other labeled precursors In vitro assays measuring parasite LDH activity, detecting antibodies against HRPII or LDH, or by staining parasite DNA with fluorescent dyes **The schizont maturation test/Mark III microtest** The "visual agglutination test" for detection of hemozoin production during parasite maturation Malaria Ag CELISA Double-site enzyme-linked pLDH immunodetection (DELI) assay In vitro and ex vivo phenotypic assays for artemisinin resistance PCR-based assays and pyrosequencing methods for detecting SNPs in target genes linked with resistance CDC is developing next-generation sequencing methods to detect resistant parasites rapidly Clamp-probe real-time PCR assay for detection of *Pfcrt* K76T SNP Gene Chip for drug resistance testing (can analyze ~7000 SNPs spaced throughout the parasite genome. Developed by Notre Dame's Eck Institute for Global Health)
Toxoplasmosis	**Isolation of *T. gondii* in stool, histological examination** Serological tests-immunoperoxidase stain toxoplasmin skin test antigen-specific lymphocyte transformation assay Inoculation of laboratory animals PCR-based and Lamp assays	**No standardized methodology that allows monitoring of anti-*Toxoplasma* drug effectiveness at public health laboratories** Sulfadiazine susceptibilities can be evaluated on Vero cells using an enzyme-linked immunosorbent assay (ELISA) Sulfadiazine susceptibilities can be evaluated on MRC-5 cells

Cryptosporidiasis	**Antigenic tests in stool specimens** (direct fluorescent antibody tests including the Meridian Merifluor kit for *Cryptosporidium*/*Giardia*, Crypto CELISA, PARA-TECT, Cryptosporidium Antigen 96 and ProSpecT from Remel, and several others) Modified acid-fast staining techniques and microscopy PCR assays	**No standardized methodology that allows monitoring of anti-*Cryptosporidium* drug effectiveness at public health laboratories** An in vitro model for *C. parvum* infection in human primary intestinal cells has been recently described but it has not been used yet for drug resistance testing Susceptibility testing for oocysts: inclusion or exclusion of fluorogenic vital dyes (DAPI/Propidium iodine, others) or by an excystation technique which reflects the metabolic potential of oocysts and their potential for infectivity Animal models available: *C. hominis* can be propagated in gnotobiotic piglets and immunosuppressed gerbils whereas *C. parvum* can be propagated in calves and lambs. In vivo drug screening/drug resistance testing has been done mostly in immunosuppressed rodents however
Coccidiosis	**Microscopic detection of oocysts in the feces** (acid-fast staining or use of fluorescence dyes, e.g., auramine-rhodamine) Examination of duodenal specimens by biopsy String test (Entero test) PCR assays	**No standardized methodology that allows monitoring of anti-coccidian drug effectiveness at public health laboratories** For *Eimeria*, in vivo assays in animal models (Chickens) Oocyst counts using a McMaster slide chamber or Neubauer counting chamber prior/post treatment Anticoccidial index (ACI), global index and optimum anticoccidial (OAA) index are commonly used to evaluate drug efficacies Experimental animal models (calves, Sinclair miniature pigs conventional pigs)
Anaerobe parasites		
Trichomoniasis	**Direct detection of trophozoites in vaginal secretions or urethral specimens by wet mount microscopic examination** Latex agglutination test for antigen detection in vaginal swab specimens OSOM Trichomonas Rapid Test (Genzyme Diagnostics, an immunochromatographic capillary flow dipstick) Aptima TV transcription-mediated amplification (TMA) test Direct fluorescent antibody staining culture (Pouch TV culture) PCR assays Rheonix CARD technology, an automated molecular diagnostic device	***T. vaginalis* susceptibility testing is not available routinely** In vitro susceptibility assays in aerobic conditions (initially developed by Meingassner and Thurne) In vitro susceptibility assays in anaerobic conditions in tubes or plates: • [^{3}H]-thymidine uptake assay • Anti-trichomonal activity colorimetric assays Anaerocult minisystems Mouse model and nonhuman primate animal models

(continued)

Table 84.3 (continued)

Disease	Diagnostic tests[a]	Drug resistance assays[b]
Giardiasis	**Fecal examinations in microscopy** Antigenic tests (Wampole, Antibodies Inc., Cellabs and Remel companies) Uni-Gold Rapid test (Trinity Biotech) Meridian Merifluor kit for *Cryptosporidium/Giardia* PCR-based and LAMP assays Wheatley's trichrome staining and microscopy in stool specimens Entero Test (e.g., the string test) Endoscopic aspiration and microscopy	**Susceptibility testing is not available routinely** Radiometric approaches to look for reduction in uptake of ^{3}H-thymidine (measuring parasite multiplication) or to look for reduction in parasite adherence with host cells Colorimetric assays for products released by killed trophozoites in the presence of drugs Colorimetric method of MTT (tetrazolium salts reduction to MTT-formazan) or on the fluorescent substrate resazurin Anaerocult assay Flow cytometry assay using propidium iodide Microfluidic devices In vivo Mongolian gerbil and mouse model assays Resistance to metronidazole and nitazoxanide has been detected using microarray technology and quantitative real-time PCR
Amoebiasis	**Immunoassay kits (antigen detection)** are commercially available that detect *E. histolytica.* Most tests detect the galactose-inhibitable adherence protein in the pathogenic *E. histolytica* in fresh stool specimens (ex: TechLab, Cellabs and Wampole are offering tests specific for *E. histolytica*). Currently, these tests require the use of fresh or frozen stool specimens and cannot be used with preserved specimens Trophozoites in trichrome stained smears and microscopy Rapid immunochromatographic cartridge assay: detects antigens of *E. histolytica*/*E. dispar*. However this assay does not distinguish between *E. histolytica* and *E. dispar*. This assay also detects antigens of *Giardia* and *Cryptosporidium.* Stool samples must be fresh or frozen	**Susceptibility testing is not available routinely** Nitro blue tetrazolium (NBT) reduction assay Experimental animal models (germfree guinea pig, hamsters)
Stramenopiles Parasites		
Blastocystosis	Microscopic observation (presence of vacuolar forms in feces and the amoeboid form in diarrheal stools) Direct smear, iodine stained smear, formalin-ether concentration techniques, and trichrome stained smear **In vitro cultivation using Jones' medium**	**Susceptibility testing is not available routinely** Caco-2 model of human intestinal epithelium Resazurin and XTT viability microassays
Helminths		
Schistosomiasis (bilharziasis)	**Microscopy of stool/urine/rectal biopsy (e.g., Kato-Katz fecal smear technique)** ELISA, Dot-ELISA, immunoblot tests Indirect hemagglutination test for *S. mansoni* Ether concentration technique Midi ParaSep SF solvent free fecal parasite concentrator PCR and RT-PCR assays including a OC-PCR dipstick assay for *S. mansoni* Immunological methods (antibody or antigen detection Urine-CCA and urine-CAA (circulating cathodic and anodic antigens) cassette test from rapid medical diagnostics; for *S. mansoni*, *S. haematobium*, and *S. japonicum.* Detection of antibodies directed against the soluble egg antigen (SEA) On-chip imaging of *S. haematobium* eggs in urine has been achieved by computer vision Thoracic imaging Luciferase immunoprecipitation system (LIPS) assay PCR-based assays On-chip imaging of eggs in urine by computer vision PCR-based assays	**Susceptibility testing is not available routinely** Animal models for praziquantel: worm count in treated infected rodents. A snail (*Biomphalaria glabrata*) model also exits Miracidial morphology and survival analysis in the presence of praziquantel Muscle contraction studies and $^{45}Ca^{+2}$ uptake FECRT (all anthelmintics) EHT (ivermectin) LFIA (ivermectin) assays xCeLLigence system (praziquantel)

Lymphatic filariasis* (also termed elephantiasis in extreme cases)	**Biopsy sample examination or microscopic examination of thick/thin blood film or buffy coat film stained with Giemsa or other appropriate blood stains** The ICT filariasis Antigen detection test (for *W. bancrofti* only) (BinaxNOW Filariasis; Binax) PCR and PCR-ELISA assays (Filariasis CELISA, Cellabs) TROPbio ELISA test for *W.bancrofti* *Brugia* rapid dipstick test Motile adult worms may be visualized by ultrasound exam of involved lymphatics	**Susceptibility testing is not available routinely** PCR assays for albendazole resistance
Onchocerciasis (river blindness)	**Microscopy of multiple Giemsa-stained Skin-snips** Observation of microfilaria in the eye by slit-lamp exam Ultrasonic detection Electromagnetic path (grant to Dr Manu Prakash, see: https://biox.stanford.edu/highlight/) Ov16 rapid test (POC prototype developed by PATH; detection of antibodies to the parasite antigen Ov16) SD BIOLINE Onchocerciasis IgG4 rapid POC test (developed by PATH and manufactured by Standard Diagnostics Inc.)	**Susceptibility testing is not available routinely** PCR assays detecting SNPs in β-tubulin selected in *O. volvulus* following repeated ivermectin treatment
Cysticercosis (taeniasis)	**Serology:** Various immunodiagnostic tests on serum or CSF (ex: RIDASCREEN *Taenia solium* IgG) PCR and LAMP assays for *Taenia solium* **Microscopy of tissue biopsies (recovery of the segments or scolex in the stool) or aspirate samples of cysts** CT or MRI scans of the brain or spinal cord for neurocysticercosis, plain radiographs of skeletal muscle	**Susceptibility testing is not available routinely** PCR, multiplex PCR Experimental encephalitis caused by *Taenia crassiceps* cysticerci in mice
Echinococcosis (Hydatid disease)	**Microscopy of tissue biopsies or aspirate samples of cysts** Serodiagnosis (ELISA-assays, indirect hemagglutination test, complement fixation test, western blot tests) Imaging—e.g., CXR, CT scan of the liver or abdomen, brain CT or MRI scan	**Susceptibility testing is not available routinely** PCR assays for BZ resistance testing Small laboratory animal models (mice and Mongolian jirds)
Soil-transmitted helminthiases (intestinal worms)	**Microscopy** The five most used techniques to detect eggs in feces are the FLOTAC method (hookworms), the Midi Parasep® SF Solvent Free Fecal Parasite Concentrator method, the McMaster Method, Kato-Katz technique (not very good for hookworms due to lysis of fragile hookworm eggs) and the formol-ether concentration technique A fully automated vision-based instrument called Ovaspec for *Trichuris* A Whipworm Antigen ELISA assay detects whipworm infections (in dogs)	**FECRT (all types of anthelmintics)** Egg hatch test (EHT) (BZ resistance in hookworms). EHT works well in hookworms but is not useful for *Ascaris* and *Trichuris* since they do not hatch externally to the host Larval development tests (BZ, levamisole, pyrantel and mebendazoles, ivermectin) Motility and the larval arrested morphology assay (LAMA) (Pyrantel, Levamisole and mebendazoles) PCR, real-time PCR and pyrosequencing assays (Benzimidazoles) In vitro assay using tritiated benzimidazole carbamates to tubulin extracts of third stage larvae
Foodborne trematode (FBT) infections	**Microscopy** ELISA and Dot-ELISA tests Immunoblot assays	**Susceptibility testing is not available routinely** In vivo animal model: dose and slaughter trial The murine model is the most commonly used animal model for fascioliasis

(continued)

Table 84.3 (continued)

Disease	Diagnostic tests[a]	Drug resistance assays[b]
Strongyloidiasis	Chest radiography and contrast enhanced scans **Parasitological diagnosis** (e.g., identification of larvae in stool using coproculture methods such as the Koga Agar culture method and the Baermann method, as well as the lesser known vermiculite stool culture. The sensitivity of these methods can be increased further by examining stool samples for several consecutive days. The direct saline smear method, and quantitative formalin ethyl acetate concentration technique can also be used Serological and molecular methods Luciferase immunoprecipitation system (LIPS) assay using a recombinant antigen (NIE)	**Susceptibility testing is not available routinely**

[a]Reference technique is given in bold

[b]Assays applied in field for treatment decisions are in bold. TMA, transcription-mediated amplification; NASBA, nucleic acid sequence-based amplification; LAMP, loop-isothermal amplification; NEAR, nicking enzyme amplification reaction; HAD, helicase-dependent amplification; RPA, recombinase polymerase amplification; mAECT, mini anion exchange centrifugation technique; HCT, microhematocrit centrifugation technique; NASBA-OC, nucleic acid sequence-based amplification coupled to an oligochromatographic dipstick detection. FISH, fluorescence in situ hybridization; QBC quantitative buffy coat; PATH, an international nonprofit organization; REED, rolling circle-enhanced enzyme activity detection; HRP-II, histidine-rich protein II; pLDH, parasite lactate dehydrogenase; CATT, card agglutination test for trypanosomiasis; CSF, cerebrospinal fluid; DAT, direct agglutination test; FECRT, fecal egg count reduction test; EHT, egg hatch test; LFIA, larval feeding inhibition assay; LDT, larval development test; LPT, larval paralysis test; MMT, micromotility test

six genetically defined groups, *T. cruzi* I to *T. cruzi* VI [23]. CD is usually a lifelong disease that is primarily transmitted by large blood-sucking insects (*Triatominae* spp.) widely known as "the kissing bugs" in endemic countries. Infection can alternatively results from blood transfusion, organ transplantation, vertical and congenital transmission, by ingestion of food or drinks contaminated by feces of insects or from domestic or wild mammal reservoirs of *T. cruzi*. CD presents three clinical stages in humans: the acute, indeterminate, and chronic phases. The acute disease is characterized by a number of symptoms which often spontaneously resolve in few weeks or months. In the asymptomatic "indeterminate" phase, patients can transmit the parasite to others while showing no signs of the disease. This asymptomatic phase can last for decades. An estimated 10–30 % of individuals infected with *T. cruzi* will develop the final phase known as the chronic symptomatic CD, which manifests as cardiac disease or pathological gut enlargement (e.g., megaesophagus and/or megacolon), with damage to the nervous system. If left untreated, patients infected with *T. cruzi* at this critical phase most often die precociously from heart (myocarditis) and gastrointestinal damages. The primary methods for diagnosing CD are serology and parasitology, but these tests have suboptimal sensitivity and low specificity (Table 84.3) [24]. Molecular techniques recently developed offer better sensitivity and specificity, although in the chronic phase, PCR seems less sensitive than serology. There is no effective vaccine for CD, and chemotherapy is restricted to two registered drugs, the nitroheterocyclics nifurtimox (NFX) and benznidazole (BZ). Considered as the gold standard treatment against *T. cruzi* for more than 40 years, NFX and BZ are nonetheless far from optimal; these treatments have low effectiveness (10–20 % of parasitological cure) in the chronic phase of the disease and limited effectiveness (60–80 % of parasitological cure) in the acute phase [25, 26]. In addition, *T. cruzi* treatment is often complicated by the natural variation in sensitivity in *T. cruzi* field strains, by differences in immune response among populations, and by the emergence of acquired drug-resistant strains which in fact are only suspected in the field since there are cases of treatment failure that are now documented in endemic areas [27–30]. Despite the urgent need for new CD therapies, only allopurinol and a few azoles (including fexinidazole currently in trials for African trypanosomiasis, posaconazole, and ravuconazole) have moved to clinical trials [31], but the results appear to be disappointing (80 % treatment failure) [32]. A number of preclinical promising agents are currently being evaluated (e.g., SCYX-6759, EPL-BS967, EPL-BS1246, SQ109 [33, 34], and few others [35–37]), but their potential clinical development is only expected in the medium- to long-term horizon [34].

3.1.1 Resistance and Diagnostic Assays

T. cruzi, in contrast to African trypanosomes and Leishmania, do not have a specific chapter on resistance and we will describe it here in greater details. Both NFX and BZ are prodrugs that are activated intracellularly in *T. cruzi* by mitochondrial NADPH-dependent type I nitroreductases (NTR) that are absent in human [38]. The reduction of BZ results in the generation of the cytotoxic metabolite glyoxal [39], while NFX reduction leads to the production of an unsaturated open-chain nitrile which has trypanocidal properties [40] (Table 84.2). Up to now, treatment failure in CD to these drugs has been largely attributed to variations in natural drug susceptibility of *T. cruzi* strains and not per se to drug-resistant isolates since no formal proof for true genetic-based resistance mechanisms in clinical isolates has been reported yet, despite the fact that resistance can be easily generated from in vitro selected clinical isolates [41–46] as well as in laboratory strains [38, 47] and animals [41, 48]. Several putative mechanisms of resistance to BZ have been however reported in in vitro generated cell lines (Table 84.2). Indeed, deletion of copies of the gene encoding the old yellow enzyme (TcOYE), a NAD(P)H flavin oxidoreductase associated with in vitro induced BZ resistance in *T. cruzi* was reported [49] as well as the overexpression of an iron-containing superoxide dismutase A (TcFeSODA) enzyme, possibly helping the detoxification of toxic metabolites [50] (Table 84.2). The tyrosine aminotransferase (TcTAT) was also overexpressed in strains of the parasite that were resistant to BZ [51]. Although not directly associated with the drug resistance phenotype, the overexpression of the TcTAT enzyme was considered as a general secondary compensatory mechanism or stress response factor in the parasite. Point mutations disrupting the flavin mononucleotide (FMN)-binding capacity of the NTR enzyme and gene deletion of NTR have been observed in BZ-resistant *T. cruzi* selected in vitro [52, 53] (Table 84.2). Interestingly, and although NTR genotypes in clinical isolates have never been tested yet, a stepwise BZ-induced resistance from a sensitive clinical isolate revealed that a resistant clone had lost a copy of NTR [46]. The participation of P-glycoprotein efflux pumps, TcPGP1 and TcPGP2, part of the ATP-binding cassette (ABC) transporter superfamily [54, 55] in *T. cruzi* BZ resistance has been also proposed recently in parasite lines submitted to in vitro induced resistance [56] (Table 84.2). Similarly, an AQBCG-like transporter gene, *TcABCG1*, was shown to be overexpressed in parasite strains naturally refractory to BZ. This gene in clinical BZ refractory strains exhibited several SNPs as compared to the CL Brener Bz-susceptible reference strain [57], but unfortunately the analysis revealed no direct correlation of any of these SNPs with the BZ resistance phenotype. It is thought however that

the overexpression of this ABC transporter in naturally *T. cruzi* BZ refractory strains (most likely favored by the high genome plasticity observed in *T. cruzi*) is a key determinant factor for the "natural drug resistance phenotype" observed in the field [57]. Recently, resistance to NFX and BZ in in vitro generated *T. cruzi* strains was also correlated with the overexpression of a D,L-proline transporter, the *T. cruzi* amino acid/auxin permease denoted TcAAAP069, located in a defined structure close to the flagellar pocket [58]. Augmented proline intracellular concentration in resistant parasites overexpressing this transporter not only improved resistance to trypanocidal drugs but also to reactive oxygen species (ROS), supporting the fact that proline is a free radical scavenger, radicals generated by the trypanocidal drug reduction [59] (Table 84.2). Interestingly in *T. brucei*, the etiological agent of African trypanosomiasis (see below), the orthologous protein TbAAT6 is not only capable of transporting proline but also the trypanocidal drug eflornithine and mutations in this gene are sufficient to generate resistance [60] (Table 84.2). Despite a clear involvement of aquaporin (AQP) homologs in drug response in several human parasites including African trypanosomes (see below), there is up to now no report of association between any of the four *T. cruzi* AQP homologs and drug response in *T. cruzi*, in neither in vitro drug-resistant selected strains nor clinical resistant isolates. Finally, although BZ is known to induce the formation of free radicals and electrophilic metabolites (e.g., glyoxal) in *T. cruzi* which potentially leads to cell death, its precise mechanism of action was still elusive until recently. Indeed, it was demonstrated that BZ preferentially oxidizes the nucleotide pool of *T. cruzi* during parasite growth and that the extensive incorporation of oxidized nucleotides during parasite DNA replication leads to potentially lethal double-stranded DNA breaks [61]. Moreover, it was elegantly shown that the overexpression of DNA repair proteins in BZ-treated cells increase resistance to BZ in vitro [61] (Table 84.2). It remains to be verified if the mechanisms of resistance detected in in vitro generated resistant *T. cruzi* strains are potentially operating in clinical isolates. Discrepancies may exit however between in vitro selected strains and clinical isolates as reported with the alcohol dehydrogenase enzyme TcADH that presented a decrease in expression in the in vitro induced BZ-resistant population, a situation that was not observed in naturally resistant strains [62].

Simple and rapid procedures to evaluate BZ and NFX "natural susceptibilities" of *T. cruzi* parasites isolated from humans have been difficult to standardize but are nonetheless possible in vitro on defined stages of the *T. cruzi* life cycle. Briefly, the life cycle of *T. cruzi* includes the non-replicative bloodstream trypomastigotes and the replicative intracellular amastigotes in mammalian hosts, and epimastigotes and mammalian infective metacyclic trypomastigotes in the triatomine vector. Currently, trypomastigotes are not widely used in drug resistance testing since their numbers are too low in the chronic phase of CD. A colorimetric method based on the tetrazolium dye MTT was developed more than 16 years ago to determine the susceptibility of *T. cruzi* epimastigotes in vitro and is still widely used [63] (Table 84.3). Briefly, the epimastigote forms obtained from in vitro culture in stationary phase are cultured in 96-well plates at 28 °C for 24 h, in the presence (or absence) of various concentrations of drugs. After this period, the MTT solution is added to each well, and the plate is incubated for 1 h. Then HCl and SDS are added to stop the MTT incorporation, and the plate is kept at room temperature for a further 30 min. The reading is performed on a spectrophotometer at 595_{nm}. The MTT assay should always include triplicate, and the results are normally expressed as IC_{50}, e.g., the concentration of the drug that kills 50 % of the parasites.

More recently, an in vitro procedure that couples the isolation of parasites by hemoculture with quantification of BZ/NFX susceptibilities in the resultant epimastigote form was reported [64, 65] (Table 84.3). This assay was also standardized with epimastigotes, as this developmental stage is predominant in hemocultures obtained from infected individuals. A reference strain, classified as resistant to BZ, is employed as positive control and incubated for 72 h with various BZ/NFX concentrations (serial dilutions 1:2) to determine IC_{50}. The assay is then applied to isolates from chronic patients prior to administration of BZ/NFX therapy and post-therapy. Suitable epimastigote density for the assay can be achieved after approximately 60 days in hemocultures derived from 30 mL blood. The IC_{50} of the isolates are determined as described above and values compared to the reference strain. It is important to mention however that this in vitro assay does not predict therapeutic outcome in CD [64]. The most obvious explanation of this disappointing conclusion would be that the epimastigote is not the infective stage in CD and its susceptibility to BZ/NFX does not reflect the susceptibility of other stages (e.g., trypomastigotes and amastigotes) encountered in the human host. Indeed, differences of several orders of magnitude of the IC_{50} values of epimastigotes and amastigotes were reported in *T. cruzi*. To alleviate this limitation, a robust intracellular amastigote model was developed (http://www.nature.com/protocolexchange/protocols/2240) (Table 84.3). The amastigote model involves the differentiation of hemoculture-derived epimastigotes into metacyclic trypomastigotes in order to establish infection in mammalian cell monolayers. Finally, drug susceptibility assays in animals exist in *T. cruzi*, and the majority uses the mouse model where the compounds are administered early in the acute phase of the infection [44, 66] (Table 84.3). More recently, an interesting real-time in vivo bioluminescence imaging method has been developed for drug screening [67, 68] which allows parasite burdens to be tracked

throughout the chronic stage of infection. This system is based on bioluminescent parasites expressing a red-shifted luciferase that emits light in the tissue-penetrating orange-red region of the spectrum. Although not suitable for drug resistance testing in clinical isolates (since no fluorescent reporter is present in clinical strains), this system may nonetheless accelerate drug discovery in CD.

3.2 Human African Trypanosomiasis

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a vector-borne parasitic disease caused by two subspecies of the protozoan parasite *Trypanosoma brucei*, e.g., *T.b. gambiense* in Western and Central Africa and *T.b. rhodesiense* in Eastern and Southern Africa (Table 84.1). Other species, not infective to humans, causes animal African trypanosomiasis (see next section) [69, 70]. In humans, *T.b. gambiense* accounts for more than 90 % of reported cases and manifests as a chronic condition that claims its victims after several months. In contrast, *T.b. rhodesiense* causes an acute infection that may kill patients within a few weeks. HAT occurs in two stages which are linked with the location where the parasites proliferate in the human body. Stage 1 is called the hemolymphatic phase which includes nonspecific symptoms like headaches, joint pain, and bouts of fever. At this stage, the parasites proliferate in lymph and blood peripheral organs. Stage 1 is generally undiagnosed without active HAT surveillance. Stage 2 is termed the neurologic or meningoencephalitic phase and occurs when the parasite crosses the blood-brain barrier and invade the central nervous system. This later stage can lead to serious sleep cycle disruptions (hence the name), paralysis, progressive mental deterioration, confusion, and coma and ultimately results in death without effective treatment.

Diagnosis of sleeping sickness involves case detection followed by staging (Table 84.3), which is crucial in the decision of the treatment to be given. HAT case identification relies primarily on the use of microscopy to confirm the presence of parasites in body fluids (blood, lymph, or cerebrospinal fluid (CSF)). A rapid blood test under the acronym CATT is available for field diagnosis, but this test only detects *T.b. gambiense* infections but not *T.b. rhodesiense* cases. Very good markers for *T.b. gambiense* HAT (e.g., neopterin and CXCL13) have been found recently in CSF of HAT patients, and a dual purpose rapid test for both staging and monitoring treatments is being developed http://www.finddiagnostics.org/. This innovative test should bypass the need for lumbar puncture necessary for CSF examination during follow-up [71]. Numerous diagnostic methods have been recently reviewed for HAT and readers are encouraged to consult references to get more information on this topic [72]. Whatever the method, the earlier the disease is identified, the better the prospect of a cure.

Although cases of healthy carriers have been reported [73, 74] which suggests that prophylaxis should be possible, no vaccination exits for HAT. The control of HAT thus largely relies on chemotherapy for which there are only a few drugs that are old and toxic (Table 84.2): pentamidine and suramin for stage 1 and melarsoprol, nifurtimox, and eflornithine for stage 2. The latest regimen, introduced in 2009, is NECT that combines nifurtimox that is normally indicated for Chagas disease in combination with eflornithine. Unfortunately, NECT is active only against *T.b. gambiense* since *T.b. rhodesiense* is naturally tolerant to eflornithine [75–77]. Presently, fexinidazole is in clinical trial [78] while two other compounds (a benzoxaborole called Scyx-7158 and compound DB829) are being tested for the treatment of stage 2 HAT [79, 80] (see also http://www.dndi.org/diseases-projects/portfolio/oxaborole-scyx-7158.html). The research community is thus actively trying to improve therapy against trypanosomes and novel compounds should enter soon the market [35, 81].

Treatment failures have been reported for all of the currently licensed HAT monotherapies, though in the case of pentamidine, these are rare [82]. Small numbers of relapses have been also reported for the NECT combined therapy [83, 84]. It is though that trypanosomes develop resistance to trypanocidal drugs during asexual multiplication in the animal or human host or during the passage through the tsetse fly, genetic exchange (sexual recombination) may occur, contributing further to the high degree of genetic diversity observed in these parasites. Current data derived mainly from experiments on drug-resistant laboratory strains of *T. brucei* made resistant to the various antitrypanosomal drugs in vitro has highlighted multiple mechanisms of drug resistance in this parasite (Table 84.2). These have been reviewed in this book in the chapter of Graf and Maser.

The mediator of both melarsoprol and pentamidine uptake in African trypanosomes is the P2 adenosine transporter AT1 [85, 86]. Mutations or loss of TbAT1 renders *T. brucei* less sensitive to both drugs [87]. However, not all resistant clinical isolates have this locus modified [88, 89], suggesting that other resistance mechanisms may operate in resistant cells (Table 84.2). Furthermore, AT1 gene deletion only confers a twofold decrease in melarsoprol sensitivity [90], supporting addition resistance mechanisms against this drug. Indeed, the overexpression of the thiol conjugate transporter TbMRPA was reported to cause melarsoprol resistance in laboratory human strains of *T. brucei* [91] although MRPA overexpression in clinical strains doesn't seem to be a frequent event [92]. The overexpression of the trypanothione biosynthetic enzymes ornithine decarboxylase (ODC) and gamma-glutamylcysteine synthetase (γ-GCS) alone gave two- to fourfold melarsoprol resistance, but the overproduction of these enzymes is not apparently contributing synergically to the resistance caused by MRPA [91]. More recently, the

T. brucei aquaglyceroporin 2 (AQP2) was pinpointed as the main genetic determinant of resistance for pentamidine and melarsoprol since it corresponds to the high-affinity uptake transporter previously known as HAPT1 [93]. The loss of TbAQP2 leads to melarsoprol-pentamidine cross-resistance [94] (Table 84.2). Interestingly in some melarsoprol- and pentamidine-resistant clinical isolates, a chimeric AQP2/AQP3 was found, with concomitant loss of AQP3 alleles from the genome [94, 95]. Finally, apart from the AT1 transporter [96–98] and AQP2/HAPT1, a low-affinity pentamidine transporter (LAPT) [99–101] has been also described as well as two AT1-related nucleobase transporters, NT11.1 and NT12.1 [102]. These transporters seem specific for pentamidine uptake (Table 84.2).

NECT is the treatment of choice for the Gambian form of sleeping sickness due to the toxic combined effects of nifurtimox and eflornithine [76]. Eflornithine is an analogue of ornithine that blocks spermidine synthesis and thus the formation of TSH, the redox regulator, through the inhibition of ODC [103, 104] (Table 84.2). Eflornithine-resistant trypanosomes were generated in the laboratory, and these cells exhibited significant reduced drug uptake and accumulate less drug than the susceptible parasites [60, 105]. The amino acid uptake transporter TbAAT6 was identified as a key determinant of eflornithine uptake in *T. brucei*, and it was demonstrated that mutations in TbAAT6 or loss of this transporter causes resistance to eflornithine [60] (Table 84.2). The second active ingredient in NECT is NFX which is apparently not acting in synergy with eflornithine [104], although it increases oxidant stress in trypanosome parasites. NFX is believed to function as a prodrug requiring enzyme-mediated reduction by nitroreductases (NTRs) to generate cytotoxic species that cause damage to macromolecules (e.g., DNA, lipids, and proteins), a process linked with ubiquinone availability [106] (Table 84.2). Modulation of NTR levels within trypanosomes directly affects their sensitivity to nitro compounds, with reduced levels of the enzyme leading to nitro drug resistance. Resistance to NFX can be generated relatively easily in bloodstream trypanosomes in vitro and NFX-resistant cell lines are cross-resistant to a number of other nitro drugs including the lead compound fexinidazole [107]. In clinical isolates of *T.b. gambiense* from Sudan and West and Central Africa, a tenfold range of sensitivities to NFX was reported, but the levels of NTR have not been evaluated in these field isolates [108].

The last drug part of the antitrypanosomal arsenal is suramin, a sulfonated naphthylamine, exhibiting binding affinities to many plasma proteins including low-density lipoprotein (LDL) [109]. In fact, this drug cannot cross lipid membranes by passive diffusion due to strong negative charge and must be taken up via a bloodstream stage-specific invariant surface glycoprotein called ISG75 [106]. Once internalized, suramin has been shown to inhibit various glycolytic enzymes among other activities [110, 111], but its main trypanocidal action is probably due to inhibition of LDL uptake via ISG75, prohibiting the parasite's supply of cholesterol and phospholipids (Table 84.2). Few resistance mechanisms to suramin were described up to now, but one of these is the overexpression of the ABC transporter TbMRPE that was shown to give two- to threefold resistance [91] (Table 84.2). More recently, a genome-scale RNA interference (RNAi) target sequencing (RIT-seq) screen has been performed in *T. brucei*, and a number of proteins involved in the mode of action of suramin have been revealed [106]. Some of these proteins might be also involved in drug resistance, but further investigation is required.

Few PCR tests are available for drug resistance testing in African trypanosomes, e.g., for the detection of the genetic status of particular resistant determinants or for parasite detection posttreatment, although the majority of these were developed for animal trypanosomes [44, 112–115] (Table 84.3). Apart from molecular assays, two techniques are commonly used to identify drug resistance in these parasites: tests in mice and in vitro assays (Table 84.3). The mice test is conducted as following: groups of at least six mice are inoculated intraperitoneally with 10^5 parasites of the isolates of interest. Twenty-four hours after inoculation or at the first peak of parasitemia, a range of trypanocidal drug doses are administered, also intraperitoneally. A control group, e.g., not treated (only buffer), is also required. After treatment, the parasitemia is monitored daily during the first week, three times a week during the second week, and twice a week thereafter in wet smears of tail blood. The treated groups are monitored until relapse occurred or until 60 days posttreatment, when the mice are euthanized. A trypanosome isolate is considered as drug sensitive if at least five out of six treated mice were cured. If fewer than five mice were cured, the isolate is considered resistant. The ED_{50} or ED_{95} (the effective dose that gives temporary clearance of the parasites in 50 or 95 % of the animals, respectively) can be calculated, as can the CD_{50} or CD_{95} (the curative dose that gives complete cure in 50 or 95 % of the animals, respectively). These values are then compared with those obtained using reference-sensitive trypanosome strains.

A fluorimetric/colorimetric assay based on the metabolism of the dye Alamar Blue by live cell cultures in vitro in the presence of various concentrations of drugs is available [116] (Table 84.3). Briefly, the nonfluorescent dye Alamar Blue (resazurin) is reduced intracellularly to resorufin in live cells, a pink and fluorescent molecule (excitation and emission at 544_{nm} and 590_{nm}, respectively). Although very useful for drug resistance testing or drug screening, this assay does not easily distinguish between cell death and growth arrest. An alternative fluorimetric assay, based on the interaction of propidium iodide with DNA, that allows either real-time monitoring of cell viability or the generation of EC_{50} values

at a predetermined time-point was thus developed [117] (Table 84.3). This assay is highly sensitive and fluorescence readings easily correlate to numbers of parasites or DNA content.

For melarsoprol drug resistance testing, a quick, simple, and sensitive test was reported [118] (Table 84.3). The assay is based on the fact that resistant parasites are defective in a plasma membrane transporter responsible not only for drug uptake but also for the specific uptake of the fluorescent diamidine DB99 (2,5-bis-(4-amidinophenyl)-3,4-dimethylfuran) into trypanosomes. The two DNA-containing structures in the trypanosome, the nucleus and the kinetoplast, begin to fluoresce within 1 min of introduction of DB99 into the medium, unless parasites are resistant. With the molecular determinants of resistance being discovered, it is likely that numerous PCR-based assays will be developed in trypanosomes.

3.3 Animal African Trypanosomes

Animal African trypanosomoses (AAT) or Nagana is a disease transmitted biologically by tsetse flies and mechanically by various hematophagous biting flies [119–121]. The disease is caused by some species of the *Trypanosoma* genus, e.g., *T. vivax*, *T. congolense*, *T. brucei brucei*, *T. evansi, T. theileri*, and *T. equiperdum*. Together, these species contribute to considerable losses in animal production in Africa, Latin America, and Southeast Asia. Economically, *Trypanosoma congolense* is considered the most important species. To maintain livestock in acceptable health condition, farmers relies on either curative or prophylactic treatment of animals with diminazene aceturate (DA) or isometamidium chloride (ISM), respectively. However, since these two trypanocides have been on the market for several decades, treatment failures and drug resistance are now reported [122–126]. Drug resistance to ISM is more widespread than to DA, but increasingly there are reports of resistance to both drugs [126–128]. Similar to what have been described for human trypanosomes, in animal trypanosomes adenosine permeases (P1 and P2 types) turned out to play an important role in the uptake of, and resistance to, trypanocides. Changes in mitochondrial electrical potential have been also demonstrated in ISM-resistant *T. congolense* [129].

At present, four types of technique are commonly used to identify drug resistance in animal trypanosomes: tests in ruminants; tests in mice, in vitro assays, and molecular detection (Table 84.3). None of these is, however, an ideal test and other tests are still in the phase of development or validation. The in vivo assessment of trypanocidal efficacy in experimentally infected animals is one of the most useful ways to detect drug resistance in AAT isolates [130] (Table 84.3). Briefly, animals of a group are experimentally infected with an isolate. After all animal of the group became parasitemic, they are treated with the recommended curative doses of DA or ISM. From the treatment date, animals are then monitored for parasitemia by the buffy coat technique [131] twice a week for 100 days. When relapse (e.g., detection of trypanosomes by microscopy after drug treatment) is confirmed in an animal, the animal is treated with a second different drug. If no relapse is detected 100 days after the first and the second trypanocidal drug administration, the treatment is considered successful and the trypanosomes sensitive to drug treatment. Relapse infections detected within 100 days of administration of a trypanocidal drug are taken as indicative of resistance. If relapse occurred in more than 20 % of the animal tested, the isolate is considered resistant to the dose of drug used [130]. Since microscopic methods have poor sensitivity, follow-up for up to 100 days after treatment is recommended to increase the chance of detecting recurrent parasitemia waves. An alternative to this limitation is PCR-based detection assays [132]. For example, a touchdown PCR assay targeting the internal transcribed spacer 1 of the ribosomal DNA (ITS1 TD PCR) was developed as a useful tool in assessment of drug efficacy against *T. congolense* infection in cattle [113]. As the assay bears the potential for detection of mixed infections of various trypanosomal species, it may be applicable for drug efficacy studies and diagnostic applications.

The mouse test is performed by expanding an isolate in a donor mouse, which is then inoculated in groups of five or six mice. Twenty-four hours later, or at the first peak of parasitemia, each group except the control group is treated with a range of drug doses. Thereafter, the mice should be monitored three times a week for 60 days. The effective dose ED_{50} or ED_{95} can be calculated, as can the curative dose CD_{50} or CD_{95}. The advantage of the mouse test over the test in ruminants is that it is cheaper and less cumbersome. However, most *T. vivax* isolates, and also some *T. congolense* isolates, do not grow in mice. Secondly, higher dose of drug (normally ~ ten times higher) must be used in mice in order to obtain results comparable to those from cattle because of the vast difference in metabolic activity, in spite of the fact that there is reasonable correlation between drug sensitivity data in mice and cattle. Therefore, results in mice cannot be directly extrapolated to calculate the curative dose to be used in animals. Thirdly, a large number of mice per isolate are required in order to obtain a precise assessment of the degree of resistance. Finally, the test takes as long as 60 days to evaluate the drug sensitivity of an isolate.

DA resistance might alternatively be monitored by the *Dpn*II-PCR-RFLP resistance test (Table 84.3). Briefly, all positive samples from *Trypanosoma*-infected animals (usually based on 18S-PCR-RFLP) are amplified using two primers targeting the P1-type purine transporter TcoNT10 gene. Then, the PCR products are digested by *Dpn*II restriction

enzyme and the digestion pattern is analyzed for DA resistance [133]. This test remains a reliable readout for DA resistance although Munday et al. [134] showed that the target gene in this test (e.g., the P1-type purine transporter) is not directly involved in DA transport. In fact, the transporter HAPT1 was shown to be responsible for most of the P2-independent diminazene uptake in animal trypanosome, and its absence seems generally to correlate with high levels of diamidine resistance [135].

Finally, in vitro assays in animal trypanosomes are expensive to perform and require good laboratory facilities and well-trained staff. A competitive ELISA which allowed the detection of small amounts of isometamidium in serum of cattle is available [136–138]. The test is both sensitive, detecting subnanogram concentrations, and specific. It allows the monitoring of drug levels over extended periods from the plasma. The presence of trypanosomes in animals with an ISM concentration of >0.4 ng/mL suggests resistance. A similar test for DA has been developed [139].

3.4 Leishmaniasis

Leishmaniasis is caused by over 20 different species of the protozoan parasite genus *Leishmania* and is spread by the bite of the female sand fly (*Phlebotomus* and *Lutzomyia* spp.) (Table 84.1). Leishmaniasis is a complex disease, with visceral, mucosal, and cutaneous presentations, each of which varies in incidence and severity. Early case detection and resistance testing followed by adequate treatment is central to control leishmaniasis, especially the VL form which can be fatal within months if not adequately treated. For decades the direct demonstration of parasites in tissue (lymph nodes, bone marrow, spleen, or skin) smears has been the gold standard in *Leishmania* diagnosis, a technique that is invasive and requires considerable expertise (Table 84.3). Alternatively, the high levels of serum antibody specific for parasite antigens allow serological diagnosis of VL using ELISA. PCR is usually highly sensitive for detection of leishmanial infections, but since there are carriers of the infection in endemic areas, these molecular assays are not useful to discriminate between acute infections from asymptomatic cases.

The treatment options for leishmaniasis are limited and far from satisfactory (Table 84.2). For more than 60 years, treatment of leishmaniasis has centered on pentavalent antimonial (Sb^{V}) formulations (i.e., sodium stibogluconate or meglumine antimoniate). Widespread misuse has led however to the emergence of Sb^{V} resistance, notably in the hyperendemic areas of North Bihar in India. Other antileishmanials including amphotericin B (AmB), miltefosine (MIL), and paromomycin (PM) could also face the same fate as there are increasing reports of relapses [140–143]. Another factor contributing to the rise of drug resistance in *Leishmania* is certainly HIV/VL-coinfected patients that are extremely difficult to treat and have been reported in at least 35 countries worldwide [144, 145].

Antimonials mediate their antileishmanial activity via generation of oxidative stress which leads to the disruption of the synthesis of macromolecules in the parasite cell. To survive, the parasite must control this oxidative assault and this is achieved by a complex molecular and multifactorial response. It is generally accepted that Sb^{v} are prodrugs that require biological reduction to their trivalent form (Sb^{III}) in order to acquire antileishmanial activity [146]. Antimony reduction apparently may occur in both the host cell or in parasites. In macrophages, the reduced trivalent form enters the parasite cell through the aquaglyceroporin AQP1 [147]. It has been shown that a lower activity of AQP1 by point mutations acquisition or a complete loss of function through a telomeric gene deletion resulted in Sb^{III} increased resistance in *Leishmania* [147–149]. In contrast to Sb^{III}, the Sb^{V} form is speculated to enter into the parasite via a protein that recognizes a sugar moiety-like structure shared with gluconate [150]. Increased intracellular levels of the antioxidant molecule trypanothione (TSH) have been observed in antimony-resistant parasites, an event usually related to the overexpression of rate-limiting enzymes involved in the synthesis of glutathione (γ-GCS) and polyamines (ODC), the two building blocks of TSH. The ABC transporter MRPA confers resistance by sequestering Sb^{III}-TSH conjugates within an intracellular organelle near the flagellar pocket, where the antimonial target(s) are probably absent [151]. In addition, a protein localized at the parasite cell surface was reported to be responsible for the active efflux of TSH-conjugated antimonial compounds outside the parasite [152], although the identity of this plasma membrane thiol-X-pump remains elusive [153]. Several other markers of resistance have been described and are reviewed in the chapters of this book authored by Mandal and collaborators and by Sundar and Chakravarty. Several of the markers found while studying in vitro antimonial resistance were confirmed in natural antimony-resistant *Leishmania* clinical isolates recovered from patients unresponsive to sodium antimony gluconate [154–158]. Nonetheless, since different paths lead to resistance in *Leishmania*, alternative in vitro mechanisms other than those described here may also operate in field isolates [159–161]. Recently, it has been discovered that *Leishmania* parasites influence cell functions of the mammalian host cell via glycans deployed at their cell surface [162]. Indeed, particular glycans at the parasite cell surface outwit the immune system of the host and permit to resist to the toxic effect of antimonial drugs by making the human host cell expelling antimony drugs through the ATP-binding cassette (ABC) transporter MDR1 localized at the macrophage cell surface.

AmB is the current secondary treatment of choice against leishmaniasis and the best treatment against antimonials

refractive leishmaniasis in highly endemic regions. The mechanism of action of AmB is complex and based on the binding of the AmB molecule to ergosterol, the predominant sterol in the membranes of *Leishmania* parasites (Table 84.2). AMB binding to ergosterol produces an aggregate that creates a transmembrane channel, allowing the cytoplasmic contents to leak out, probably accelerating cell death [163–165]. The level of sensitivity to AMB is species dependent and depends on the variation in the ergosterol content in membranes [166]. Resistance in in vitro generated *Leishmania* promastigotes was shown to be caused by a significant change in plasma membrane sterols, with ergosterol being replaced by a precursor, cholesta-5,7,24-trien-3βol [167] (Table 84.2). This change is apparently due to a loss of function of the S-adenosyl-L-methionine-C24-Δ-sterol methyltransferase (SCMT) that impaired C_{24} transmethylation. In addition, AmB uptake was decreased in in vitro resistant cells and efflux, most likely due to the overexpression of an ABC transporter (MDR1), was increased (Table 84.2). To date, only few cases of AmB clinical resistance were reported [168, 169], but the analysis of one clinical isolate of *L. donovani* has shown that similar resistance mechanisms previously observed in in vitro AmB-resistant mutants are also operating in clinical isolates [169]. Finally, an upregulation of the silent information regulator 2 (Sir2) was associated with AmB resistance in clinical isolates by regulating MDR1, ROS concentration, and the apoptosis-like phenomena upon AmB treatment [170] (Table 84.2).

The mode of action of paromomycin (PM) against *Leishmania* has been investigated by proteomics and, like other aminoglycosides, appears to act by inhibition of protein synthesis and interference with vesicle-mediated trafficking [171] (Table 84.2). In vitro generated resistant strains have a higher number of vesicular vacuoles and an increase in a number of proteins involved in vesicular trafficking compared to the parental sensitive strain. Several other products may also be involved but awaits further confirmation [172]. Interestingly in a recent study [173], experimental PM resistance could be readily selected in amastigote stage of several species and strains, although promastigotes remained fully PM susceptible. This study strongly suggests that the use of intracellular amastigotes, at least for PM susceptibility testing, is strongly recommended as promastigote resistance mechanisms may differ from amastigotes.

Although miltefosine (MIL) is the latest antileishmanial agent to reach the market, field reports note an increasing trend in treatment failures [174–176]. The mode of action of MIL includes perturbing the metabolism of lipids (especially phospholipids) [177], inhibition of cytochrome c oxidase activity and mitochondrial depolarization resulting to an apoptosis-like death [178] (Table 84.2). Drug uptake is a prerequisite for MIL activity against *Leishmania*, and a common feature in all MIL-resistant lines is a decreased drug accumulation. This is achieved by a decrease in uptake and/or an increase in efflux. The MIL uptake machinery is composed of two proteins, the miltefosine transporter LdMT (a member of the P4-ATPase subfamily) and its specific beta subunit LdROS3 (reviewed in [179]). Both are essential for MIL uptake at the parasite cell surface, and any mutations inactivating or decreasing the expression of any of these two components render the parasite cells highly resistant to MIL [180–182] (Table 84.2). The leishmanial MDR1, a P-glycoprotein-like transporter part of the *Leishmania* ABC family was the first molecule shown to be involved in in vitro MIL resistance [183]. Two members of the ABCG subfamily were also reported to be involved in MIL resistance in *Leishmania*, namely, ABCG4 and ABCG6, whose localization is mainly to the parasite plasma membrane and flagellar pocket [184–186]. Other proteins were involved in experimental MIL resistance in *Leishmania*, but their roles in clinical isolates remain to be elucidated (Table 84.2).

Drug resistance in *Leishmania* parasites can be assayed in two major stage forms of the life cycle of the parasite (Table 84.3). *Leishmania* spp. are digenetic organisms shuttling between a flagellated promastigote in the gut of the sand fly vector and an intracellular amastigote, mainly in phagolysosomes of macrophages of the mammalian host. Both forms can be cultivated in vitro in culture flasks, and both these stages have been exploited in drug discovery and drug resistance assays, but the consensus is that the only reliable method for monitoring resistance of *Leishmania* isolates is the technically demanding in vitro amastigote-macrophage model [187] (Table 84.3). In fact, various in vitro host cell models such as murine peritoneal macrophages [188], human monocytes (U-937) [189], THP-1 [190], and Chinese hamster ovary (CHO) cells [191] have been investigated to test the antileishmanial activities of promising candidate drugs or to monitor drug resistance levels in clinical isolates. In all these in vitro models, infection rates are usually measured by microscopic examination of adherent cells, although the technique in THP1 and mouse peritoneal macrophages can also be performed with free non-adherent cells. Once infection is established and confirmed, infected cells are treated with drugs for a certain period of time and pathogen growth/multiplication or inhibition is recorded. Flow cytometry can also be used as an alternative way to microscopy to measure the extent of drug action on various infected mammalian cells [192–195]. A potential substitute to the amastigote-macrophage model, although not presently as reliable as the former in its actual format, is based on axenic amastigotes in the absence of macrophages, thus corresponding to a "semi-in vivo" condition [196–198] (Table 84.3). Although axenic *Leishmania* parasites are more easily obtained in large numbers compared to intramacrophagic amastigotes, axenic amastigotes apparently only mimic the real intramacrophagic amastigote form and thus may not lead to reliable conclusions in terms of clinical drug resistance levels as the ones obtained with the

amastigote-macrophage model. More recently, a novel *Leishmania*-macrophage 3D model has been reported that might be an interesting model for initial drug screens or to study drug resistance mechanisms in a more close to in vivo context [199].

Alternative to in vitro tests, in vivo systems exist in *Leishmania* which are based on experimental animal models (mainly for VL) like rodents (mice, rat, hamsters), dogs, and monkeys [200] (Table 84.3). However, they are not widely used for drug resistance monitoring in clinical isolates. Finally, despite their intrinsic limitations, the use of promastigotes grown in flasks is often used as the first drug resistance test to evaluate the susceptibility level of isolates to current treatments since it is simple, relatively cheap, and easily applicable in several settings (Table 84.3). Briefly for drug resistance profiling using promastigotes, parasites are diluted to a concentration of $1–2 \times 10^6$ per mL of cultivation medium, and the drugs in appropriate concentrations are added to the experimental cultures. The inhibition of promastigote multiplication is assessed after approximately 3 days, depending of the species. Results derived from this in vitro promastigote test would always need to be counter verified in amastigote models, however.

4 Drug Susceptibility Testing in Apicomplexan Parasites

Apicomplexan parasites impose devastating impacts on much of the world's population. The phylum includes several pathogens of clinical and veterinary importance, such as *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, and *Eimeria*. In particular, apicomplexan diseases of domestic animals are associated mainly with farmed animals and are renowned for the large economic costs incurred by the agricultural industry. All apicomplexan parasites are characterized by the presence of a representative organelle, the apicoplast, a relic chloroplast-like organelle of uncertain function that contains hundreds of functional predicted proteins and gives the phylum its name. Most if not all apicomplexans are obligate intracellular parasites. They typically invade host cells by forming a ring-like junction with the host cell membrane through which the zoites will be internalized. It was estimated that out of a probable 1.2–10 million apicomplexan species, only about 0.1 % have been named and described to date [201].

4.1 Malaria

Each year an estimated 300–500 million clinical cases of malaria occur, resulting in nearly a million deaths, mostly young children under the age of five [202–204]. Malaria occurs in over a hundred countries (Table 84.1). The ever growing problem of drug resistance has hindered many malaria control programs. Transmitted from person to person by the bite of anopheline mosquitoes, malaria is caused by one of the five *Plasmodium* spp. *Plasmodium falciparum* is the main cause of severe clinical malaria and death, but the most common of all five human malarial species is *P. vivax*. Prompt diagnostic confirmation of malaria can be achieved through microscopy, serology, or rapid diagnostic tests (RDTs) with more than 200 malaria RDTs currently available on the market (representatives are listed in Table 84.2). Up to now, the most accurate tests to detect malaria parasites and for confirming the species are PCR-based tests but microscopy still remains the gold standard tool for malaria diagnosis although the accuracy and sensitivity of this method highly depends on well-trained and experienced technologists.

Drug resistance is now a major concern in the management of malaria. In addition to widespread resistance to chloroquine and sulfadoxine-pyrimethamine, parasite resistance to mefloquine, quinine, and other antimalarial drugs have been reported including to the last resort artemisinin-based combination therapies for which resistance has been reported in the Greater Mekong subregion of Cambodia, Laos, Myanmar, Thailand and Vietnam [205–208].

The mode of action and resistance mechanisms to several antimalarials have been extensively studied over the last decades and are reviewed in the chapters authored by Biagini and Ward and by Pradines (see also Table 84.2). Several molecular markers for chloroquine (CQ) resistance have been identified, including SNPs in the *pfCRT* (the digestive-vacuole transmembrane chloroquine resistance transporter) and *pfMDR1* (multidrug resistance 1) genes that are now well established [209–213] (Table 84.2). Other factors involved in modulating *P. falciparum* quinoline response include *pfMRP1* (multidrug resistance protein 1) [214] and *pfNHE-1* (sodium hydrogen exchanger) [215, 216], the latter specifically related to low levels of QN resistance. Mutations in other ABC transporters were associated to resistance [217, 218]. Mefloquine (MF) resistance was strongly associated with amplified pfMDR1 locus (e.g., copy number variations, CNVs). Both analyses of field isolates [219, 220] and cultured parasites [221] support a link between increased pfMDR1 CNVs and MF resistance. In fact, there is a complex relationship of CNVs and SNPs that contribute to MF resistance in the parasite. Some mutations in pfMRP1 as well as pfMRP2 were also found to be associated with reduced susceptibilities not only to MF but also to CQ [217, 222].

The sulfadoxine-pyrimethamine (SP) combination targets the folate pathway in malaria parasites. The primary determinants of resistance in *Plasmodium* against the SP combination are well-described point mutations in the enzymes dihydropteroate synthase (DHPS, the target of sulfadoxine) and dihydrofolate reductase (DHFR, the target of pyrimethamine)

(reviewed in [223]). Recent work has highlighted however the contributions of additional parasite adaptation to antifolate resistance (Table 84.2). Indeed, gene amplification (e.g., CNVs) of the first enzyme in the parasite folate synthesis pathway, GTP-cyclohydrolase (GCH1), was strongly associated with resistant parasites and potentially contributes to the development and even persistence of resistant parasites [223].

Atovaquone (ATQ) with proguanil is a component of Malarone that targets malaria respiration, more precisely inhibition of the cytochrome Bc1 complex in the mitochondrial electron transport chain. Resistance to ATQ in the field is associated with point mutations in cytochrome b, most notably near the conserved Pro(260)-Glu(261)-Trp(262)-Tyr(263) (PEWY) region in the ef loop [224] (Table 84.2). Even a single point mutation (at position Y268) in the active site of cytochrome b protein can rapidly render ATQ ineffective against *Plasmodium falciparum* parasites [225].

Artemisinins are the current cornerstone of effective therapy in malaria. These drugs are thought to act via the generation of free radicals (ROS) that are initiated by iron bioactivation of endoperoxides and/or catalyzed by iron-dependent oxidative stress [226, 227]. Recently, a molecular marker of artemisinin resistance, the K13 propeller protein in *P. falciparum* has been identified [228, 229] (Table 84.2). Mutations present in the kelch domain are now prevalent (>40 %) in parasite populations from the China-Myanmar border where artemisinin use has the longest history. In particular, a predominant mutation (F446I) and a prevalent microsatellite variation in the N-terminus were identified [230].

The three basic approaches routinely used to evaluate the antimalarial activity of compounds in malaria parasites are in vivo and in vitro assays along with molecular characterization. Of the available tests (Table 84.3), in vivo tests most closely reflect actual clinical or epidemiological situations, i.e., the therapeutic response of currently circulating parasites. Briefly, in vivo assessment of antiplasmodial activity, and thus resistance, can be achieved using rodent models in which assays measure mainly (a) the clearance of parasites as detected by optical microscopy or other more sensitive methods (e.g., PCR based), (b) the time that elapses between last drug dose and clearance of parasitemia, and (c) the drug dosage that clears parasites in a dose-response manner. In vivo studies can be conducted also in humans and usually represent the following of a selected group of symptomatic and parasitemic individuals that underwent carefully controlled treatment with subsequent monitoring of the parasitological and/or clinical response over time. Recently, a spectroscopic analysis method was proved to be sensitive for recognition of the effects of antimalarial treatment on the structure and composition of the parasites and infected red blood cells [231]. It is anticipated that this novel technology may aid and improve the accuracy and clinical relevance of laboratory or field testing for malaria drug resistance testing.

For *P. falciparum*, the in vitro assessment of parasite drug susceptibility, involving short-term culture of parasites in the presence of serial drug concentrations, has proved to be extremely useful in assessing intrinsic susceptibility to antimalarial drugs. Thus, in vitro tests in general consist by removing parasites from the host and placing them into a strictly controlled experimental environment. There are several in vitro tests that have been described over the last decades, but most of them are based on measuring parasite growth or growth inhibition under various drug concentrations either by counting parasites, measuring [^{3}H]-hypoxanthine incorporation [232] or other isotopic labeled precursors (e.g., palmitate, serine, choline, inositol, and isoleucine), measuring parasite lactate dehydrogenase (LDH) activity, detecting antibodies against histidine-rich protein II (HRPII) or LDH, or by staining parasite DNA with SYBR green, DAPI (4,6-diamidino-2-phenylindole), PicoGreen, or YOYO-1 dyes [233–238]. These malarial in vitro tests are demanding however and relatively expensive. A low-cost standardized in vitro assay called the schizont maturation test (SMT) also known as the Mark III microtest was developed by WHO more than 15 years ago (http://www.who.int/malaria/publications/atoz/ctd_mal_97_20_Rev_2_2001/en/) and is based on the maturation of parasites in a 24–36 h microculture (in the absence or in the presence of drugs) followed by microscopically counting the number of parasites that successfully develop from ring into schizonts (i.e., parasites with three or more chromatins) in Giemsa-stained thick films. The Mark III microtest (Table 84.3) was optimized for chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine, and artemisinin susceptibility testing. Experienced microscopists should carry out this test however as it is prone to individual variability. The "visual agglutination test" for detection of hemozoin production during parasite maturation is also commonly used. In general all in vitro methods involve direct exposure of malaria parasites to drugs in culture plates. These were extensively described elsewhere [239]. A commercial kit (Malaria Ag CELISA) which takes only about 2.5 h to perform was developed, where if parasite growth is inhibited by antimalarial drugs, the inhibition is reflected in the HPR2 levels and can therefore easily be quantified by antibody-mediated detection. The drawback of this assay is that some isolates from a number of regions including the Amazon region of Peru lack the *pfhrp2* gene [240, 241] and will produce a false-negative result in this test. A double-site enzyme-linked pLDH immunodetection (DELI) assay has been also used to access *P. falciparum* antimalarial drug susceptibility [242, 243]. This assay is equally as sensitive as PCR and much simpler to perform compared to isotopic assays.

In 2013, two novel tests were developed that can discern within 3 days whether the malaria parasites in a given patient

will be resistant or susceptible to artemisinin, the key drug used to treat malaria [205] (Table 84.3). In both tests, young parasites are briefly exposed to a high dose of artemisinin, mimicking the way parasites are exposed to the drug in people being treated for malaria, and their survival is measured 72 h later. One test quickly determines how a malaria parasite from a specific patient responds to artemisinin. It involves taking a blood sample and treating it with artemisinin in a test tube for 6 h. The drug is washed and the treated parasites are incubated for another 66 h, and counted. The second test is designed to isolate the malaria parasite at an early stage of its life cycle, the so-called ring stage. At this stage, malaria parasites are uniquely susceptible to artemisinin so the test can determine how these immature forms are becoming resistant to the drug.

Finally in malaria, the presence of distinct point mutations in established molecular markers (e.g., chloroquine resistance transporter (*pfCRT*), dihydrofolate reductase (*dhfr*), dihydropteroate synthase (*dhps*), and cytochrome b (*cytb*)) is highly correlated with drug resistance, and PCR-based assays are now available for detecting drug resistance to most antimalarial drugs in clinical isolates, including artemisinin [244–246] (Table 84.3). However, when designing a molecular assay for drug resistance testing in malaria, one should always keep in mind that minor DNA alleles linked with resistance that are present in a parasite population at ≤10 % (e.g., in mixed *Plasmodium* genotype infections which are prevalent in endemic malaria areas) are hardly being detected by genotyping methods like RT-qPCR, pyrosequencing, or microsatellite typing. Thus, even if PCR is highly sensitive, it may present some difficulties to detect low-level resistance allele that may nonetheless influence the outcome of the treatment.

4.2 Toxoplasmosis

Toxoplasmosis is a widespread zoonotic coccidian disease that occurs in both animals and humans. Approximately one third of the global human population is infected with *Toxoplasma gondii* [247] (Table 84.1). Clinically, the lifelong presence of the parasite in tissues of a majority of infected individuals is usually considered asymptomatic. The definitive hosts are representatives of the felid family. There are three infective stages of *T. gondii*: a) a rapidly dividing invasive tachyzoite; b) a slowly dividing bradyzoite in tissue cysts; and c) an environmental stage, the sporozoite, which are protected inside an oocyst and are the product of a sexual cycle operating in the intestine of the cat. In human, *T. gondii* is usually transmitted by consumption of lightly cooked meat. It can also be acquired by inadvertent ingestion of oocysts containing sporozoites, which are remarkably stable environmentally. The diagnosis of toxoplasmosis may be established by serologic tests, amplification of specific nucleic acid sequences (i.e., PCR), histologic demonstration of the parasite and/or its antigens (i.e., immunoperoxidase stain), or by isolation of the organism [248] (Table 84.3). Other rarely used methods include demonstration of antigenemia and antigen in serum and body fluids, a toxoplasmin skin test, and antigen-specific lymphocyte transformation. To diagnose toxoplasmosis during pregnancy, a sample of amniotic fluid may be used to detect the parasite. Ocular disease is diagnosed based on the appearance of the lesions in the eye, symptoms, course of disease, and often serologic testing. The current treatment options for toxoplasmosis are limited and include only few compounds such as pyrimethamine and sulfadiazine, which act synergically to block the folate biosynthesis pathway by inhibiting DHPS and DHFR, and atovaquone which binds to the cytochrome bc1 complex and therefore inhibits the mitochondrial electron transport process (Table 84.2). Treatment failures have been reported for these drugs however [249–251] and "natural resistance" to sulfadiazine have been observed [252].

T. gondii strains are genetically highly diverse, but only a few lineages are widely spread. The three different genotypes of *T. gondii* show great diversity in pathogenicity and "natural" drug sensitivity. As mentioned previously, *T. gondii* strains "naturally resistant" to sulfadiazine have been reported [252], but sulfadiazine resistance, either "natural" or "acquired," doesn't seem to be related to changes in the expression levels or SNPs in none of the targets (e.g., DHPS and DHFR) [252, 253]. For atovaquone resistance, mutations (M129L and I254L) were found within the cytochrome b gene [254]. The mechanism of pyrimethamine resistance in *T. gondii* is currently unknown (Table 84.2).

Very few assays are available for drug susceptibility testing in *T. gondii* (Table 84.3). Sulfadiazine susceptibilities can be evaluated on Vero cells infected with tachyzoites in 96-well plates using an enzyme-linked immunosorbent assay (ELISA) [255]. Alternatively, pyrimethamine, sulfadiazine, and atovaquone can be evaluated on MRC-5 fibroblast-infected cells [256].

4.3 Cryptosporidiosis

Cryptosporidiosis is commonly a self-limiting disease in healthy hosts but represents a life-threatening disease in immunocompromised and young individuals (Table 84.1). The disease is caused by *Cryptosporidium* spp. which is recognized as major waterborne coccidian parasites worldwide [257–259]. Most infections worldwide have been attributed to *C. hominis* and *C. parvum*. Diagnostic tests for *Cryptosporidium* infection are suboptimum however, necessitating specialized tests that are often insensitive (Table 84.3). Antigen detection and PCR improve sensitivity.

There is no effective treatment against *Cryptosporidium* spp., although limited efficacies have been reported for paromomycin, azithromycin, and nitazoxanide which is the first FDA-approved drug for treating cryptosporidiosis in non-immunodeficient children and adults [259]. Resistance to paromomycin seems attributed to the modulation of ABC transporters [260]. No mechanism of resistance for azithromycin and nitazoxanide has been described in *Cryptosporidium* spp. (Table 84.2).

Many obstacles exist to the development of novel drugs for cryptosporidiosis, including difficulty in propagation of the organisms in vitro. Recently, a novel in vitro model for *C. parvum* infection in human primary intestinal cells has been reported [261]. Animal models for drug assessment and drug resistance testing are poorly standardized, however (Table 84.3). Gnotobiotic piglets and immunosuppressed gerbils are the only animal models available for *C. hominis* [259] whereas *C. parvum* can be propagated in calves and lambs. In vivo drug screening has been done mostly in immunosuppressed rodents. An assay that could be converted eventually in an in vitro drug resistance assay for current anti-cryptosporidial drugs is the enzyme-linked immunosorbent assay (ELISA) that has been initially described to examine the effects of 13 antivirals on the development of *C. parvum* in human ileocecal adenocarcinoma (HCT-8) cells [262]. Finally, the viability of purified *C. parvum* oocysts exposed for different period of time to different concentrations of drugs (or disinfectants) can be also evaluated by inclusion or exclusion of various fluorogenic vital dyes and by an excystation technique [263, 264].

4.4 Eimeria

Eimeria is the cause of important livestock diseases with a high impact in the poultry industry where parasite transmission is favored by high-density housing of large numbers of susceptible birds. Indeed, *Eimeria* spp. are responsible for a $1.5 billion loss to the poultry broiler industry each year worldwide [265]. Transmission of the disease to human is rare and proceeds via the fecal-oral route. Coccidiosis is usually diagnosed by demonstrating oocysts in the feces (Table 84.3). Because the oocysts may be passed in small amounts and intermittently, repeated stool examinations and concentration procedures are recommended. Acid-fast staining is the preferred method for *Coccidia* (Table 84.3). If stool examinations are negative, examination of duodenal specimens by biopsy or string test (entero test) may be needed. Alternatively, molecular tools have been developed for the diagnosis of *Eimeria* [266, 267].

In livestock, *Eimeria* has developed drug resistance against all the dozen or so drugs approved for use in avian, and varying levels of resistance are present for those currently employed [268, 269]. Relatively little is known about the mode of action of anticoccidial drugs and even less about the mechanisms of resistance (Table 84.2). Anticoccidial drugs used in avian can be broadly divided into two categories: the ionophores and the synthetic drugs and these are described in the chapter authored by Aubert et al. in this series.

The development of resistance in chickens can be detected by means of different indices and criteria, but the assays should include at least a group of medicated infected birds, a group of unmedicated infected birds, and a third group comprising unmedicated uninfected birds [270] (Table 84.3). The most useful criterion for evaluating the effects of anticoccidial drugs is body weight gain during the acute phase of infection. Gain can be measured from the day of inoculation until the sixth or seventh day postinoculation or during the period of maximum growth depression (3–7 or 4–8 days postinoculation). Weight gains in medicated infected birds may be compared directly with unmedicated infected and unmedicated uninfected controls. An isolate is considered resistant if the weight gain of medicated infected birds is not significantly different from that of unmediated infected birds. Apart from weight gain, a lesion score system is also available [271]. This procedure is inherently subjective since it requires visual assessment of the condition of different regions of the intestine of infected birds, medicated or not. One can also rely on oocyst counts from intestinal contents which estimate the magnitude of infection in terms of parasite numbers, although there may be considerable variation in the number of oocysts produced by individual birds (Table 84.3). An anticoccidial index (ACI) is also used to evaluate drug resistance in birds. A 50 % or greater reduction in the ACI for medicated infected birds compared with that of unmedicated uninfected birds is ascribed to resistance where as a 25–50 % reduction is considered to indicate reduced sensitivity. A global index and an optimum anticoccidial (OAA) index are two other usual anticoccidial efficacy indices useful to monitor drug efficacy in avian [272].

4.5 *Isospora belli*

Isospora belli is believed to be a species which only infects human and some primates. It has a worldwide distribution but is more common in tropical regions and areas with poor sanitation. Infections are often asymptomatic and those with symptoms tend to be self-limiting with a duration of a few weeks. Infections are more common and the symptoms more severe in AIDS patients. In general, symptoms are similar to those of cryptosporidiosis. The infection is acquired through the ingestion of sporulated oocysts contaminating the external environment, including food and water supplies. There is no accepted gold standard method for diagnosing isosporiasis, but infections are usually diagnosed

by the coprological examination of host feces for coccidial oocysts (concentrated using various sedimentation-flotation techniques) (Table 84.3). Feces from carnivores can also be pretreated with ether/chloroform to remove fatty material. Unstained oocysts are best observed by light microscopy using suboptimal transmitted illumination (condenser wound down to introduce diffraction), phase-contrast or interference-contrast optics. Alternatively, oocysts can be stained with Giemsa or acid-fast stains of dried smears or with fluorescence dyes (auramine-rhodamine) in wet preparations (Table 84.3). Fresh fecal samples may only contain unsporulated oocysts, so differential specific diagnosis may sometimes require short-term storage to facilitate sporulation (2 % potassium dichromate is often used to suppress microflora during storage, and refrigeration can slow the process down if so required for field samples). The recommended treatment for *Isospora* is the combination of trimethoprim-sulfamethoxazole [273]. Few failure treatment cases in human have been reported [274, 275]. Drug resistance in these cases is only suspected but not proven since parasite sequestration in immune privileged sites may also play a role in recurrent cases [276]. Monitoring susceptibility in *Isospora* is not well standardized and thus not performed routinely (Table 84.3).

5 Drug Susceptibility Testing in Anaerobe Parasites

Drugs and resistance mechanisms in anaerobic parasites have been reviewed in details in this series by Smith et al. and Orozco et al., and we will concentrate on salient points and on diagnostics.

5.1 Trichomoniasis

Trichomoniasis is the most common curable nonviral sexually transmitted infections worldwide, accounting for about 276 million cases annually [277] (Table 84.1). Diagnostic tools for *Tv* have improved significantly in the last decade and various laboratory methods are now employed routinely (Table 84.3). Trichomoniasis can be treated with metronidazole (MTZ) or tinidazole (TDZ), two 5-nitroimidazole compounds that are taken up by the parasite as a prodrug by passive diffusion and activated by reduction in the hydrogenosome, the *Tv* equivalent of a mitochondrion [278] (Table 84.2). Electrons required for the drug reduction are generated by the key hydrogenosomal enzyme pyruvate to ferredoxin oxidoreductase (PFOR). Electrons released in the PFOR reaction are accepted by ferredoxin that is subsequently reoxidized by a hydrogenase. This hydrogenosomal model for MTZ activation in *Tv* has been recently challenged however, since a flavin-based mechanism of MTZ activation has been suggested as an alternative mechanisms [279]. Whatever the activation pathway, drug activation always results in the production of toxic nitro-radical molecules that likely interfere with proteins and protein trafficking, leading to cell damage and ultimately parasite death [280] (Table 84.2).

Resistance to MTZ and TDZ has been demonstrated both in field isolates of *Tv* from patients refractory to treatment and in laboratory-developed strains obtained by exposing trichomonads to sublethal pressure of the drug in vitro. Clinical MTZ resistance in *Tv* is currently found in 2.5–10 % of isolates tested [281–285]. Fortunately, the prevalence of resistance to TDZ is lower but cross-resistance between MTZ and TDZ is of great concern as the two drugs are similar in modes of action. In laboratory-generated *Tv*-resistant strains, an altered conformation (shrinking) of the hydrogenosome was observed [286, 287], as well as a downregulation of enzymes required for drug activation including the enzyme PFOR [288], a ferredoxin with an exceptional redox potential [287] and a reduced amount of intracellular ferredoxin [289] (Table 84.2). However, resistant clinical isolates do not harbor downsized hydrogenosomes and do not exhibit reduced transcription of the PFOR or ferredoxin genes [290]. Laboratory-generated resistance was also associated with reduced thioredoxin reductase activity and free flavins, both of which are proposed to reduce MTZ as well [279, 291]. In clinical isolates a decreased flavin reductase activity (FR1, formerly known as NADPH oxidase) has been similarly observed [279, 292, 293] as well as a downregulation of alcohol dehydrogenase 1 (ADH1) (Table 84.2). MTZ resistance in clinical isolates was clearly associated with single-nucleotide polymorphisms in the nitroreductase genes *ntr4Tv* and *ntr6Tv* [294], although their formal role in resistance needs further investigation. Once confirmed, these SNPs may have clinical utility in identifying MTZ-resistant *Tv* in a rapid PCR-based assay. It is salient to point out however that MTZ resistance does occur in *Tv* isolates with intact *ntr4Tv* and *ntr6Tv* genes, indicating that alternative resistance mechanisms may also operate in clinical strains.

Resistance to nitroimidazole drugs has been studied by growing *Tv* strains in the presence of different drug concentrations under aerobic and anaerobic conditions in vitro (Table 84.3). Aerobic versus anaerobic resistance in *Trichomonas* isolates is an important consideration since most reports indicate significant levels of aerobic resistance and few cases of anaerobic resistance [295], the latest being characterized by very high minimum lethal concentration (MLC) values in vitro (over 1000 µg/mL MTZ) and has been demonstrated only in laboratory-developed strains up to now [296, 297]. Low-level resistance in *Tv* to MTZ is usually defined as aerobic MLC of 50–100 µg/mL, moderate-level resistance as 200 µg/mL, and high-level resistance as ≥400 µg/mL [298]. In general, elevated aerobic MLCs are associated with a greater likelihood of treatment failure, but inconsistency does exist [298].

The standard procedure to determine the in vitro aerobic MTZ (or TDZ) resistance among *Tv* isolates is performed as follows: swab specimens are obtained from infected person and parasites are cultured at 35–37 °C in the InPouch Tv (BioMed Diagnostics) culture media for 24–96 h. The cultures are examined at the microscope to visualize trichomonad parasites. Positive cultures are then incubated in multi-well plates (in triplicate) in TYM medium at 37 °C until axenic cultures are obtained. Axenic parasites are then assayed for MTZ and TDZ susceptibility under aerobic conditions, using serial dilutions of drug concentrations from 0.2 to 400 μg/mL [299, 300]. Briefly, stock solutions of MTZ or NTZ are prepared in dimethyl sulfoxide (DMSO) and diluted with TYM medium to obtain a stock solution, and then further serially diluted with the same medium in a multi-well plate. DMSO (0.05 %) in TYM is used as vehicle in control wells. Parasites (5×10^3 trophozoites/well) are added to the wells as well as control *Tv* strains (resistant and sensitive) and the plates are incubated at 37 °C. The MLC is the lowest dilution at which no motile trichomonads could be observed by microscopic observation, in at least two independent experiments.

For anaerobic condition, traditionally two major susceptibility assays have been optimized, e.g., tube assays and microtiter plate assays, and both have their own limitations. Microtiter plates are problematic due to the need to remove the plates from the anaerobic or low-oxygen environment to monitor the progress of the assay. On the other side, tube assays are much more cumbersome and time-consuming. Optimized in tubes or in plate format, a number of anaerobic susceptibility assays have been reported for *Tv* over the last decades, for example, [^{3}H]-thymidine uptake derived from a *Giardial* test [301]; colorimetric assays [302] based on the giardiasis colorimetric test [303] which employ synthetic substrates of purine salvage pathway enzymes [302, 303] are available (Table 84.3). Even a commercial system does exist for anaerobe parasites, the Anaerocult minisystems, which has been used for the determination of chemosensitivity in several anaerobic protozoan species including *Tv* [284] (Table 84.3). This system allows the use of multi-well plates in sealed bags or airtight jars for parasite culturing in low-oxygen environment. Finally, experimental animals employed in studies of *Tv* infection are available including a mouse model [304] and nonhuman primate animal models [305–307] (Table 84.3), but they are not routinely used for in vivo drug susceptibility testing.

5.2 Giardiasis

The anaerobe diplomonad parasite *Giardia lamblia* (also known as *G. duodenalis or G. intestinalis*) is a common enteric parasite spread all over the world in contaminated food and water (Table 84.1). Chronic infections of months to years can occur, but asymptomatic carriage of *Giardia* is common in human [308]. The fecal-oral route is regarded as the major source of infection. As few as ten cysts may establish infection in human [309]. The control of this infection requires both the inactivation of the infectious cysts disseminated in the environment and the elimination of pathogenic trophozoites attached to the small intestinal epithelium. The diagnosis of giardiasis is generally made by the identification of cysts or trophozoites in a total of three fecal samples over a period of several days. Enzyme immunoassays and fluorescent antibody assays of fecal specimens are available as well as the Entero Test (e.g., the string test) [310] (Table 84.3). Endoscopic aspiration from the small intestine is also possible in some patients with chronic diarrhea with repeatedly negative fecal examinations.

Giardiasis is treatable with metronidazole (MTZ) or tinidazole (TDZ), but alternatives such as albendazole (in combination with mebendazole), quinacrine, and nitazoxanide are available (Table 84.2). Treatment failures with MTZ occur in ~ 20 % of cases and resistance has been confirmed among clinical isolates of *Giardia* in in vivo Mongolian gerbils and mouse model assays [311] (Table 84.3). In in vitro generated MTZ strains, resistance was associated with DNA changes (epigenetic regulation) (Table 84.2). DNA probes which hybridize with specific chromosomes and repetitive sequences indicated that rearrangements both at the chromosome and repetitive DNA level occurred concurrently with the development of MTZ resistance [312]. Resistance to MTZ also is negatively correlated with the intracellular concentration of pyruvate to ferredoxin oxidoreductase (PFOR, which replaces pyruvate dehydrogenase in aerobic organisms) leading to a concomitant decrease in the uptake of free MTZ into the cell [313, 314] (Table 84.2). Similar to *Tv*, nitroreductases play a role in activating MTZ (and other nitronidazole drugs) and *Giardia* parasites encode two nitroreductases, GlNR1 and GlNR2, that were shown to be involved in this process [315]. Trophozoites overexpressing GlNR1 presented a higher susceptibility to MTZ and nitazoxanide [316] whereas trophozoites overexpressing GlNR2 were less susceptible to both nitro drugs as compared with control trophozoites [315]. Thus, susceptibility to nitro drugs in *Giardia* may depend not only on activation, but also on inactivation of the drugs by the two giardial nitroreductases (Table 84.2). Finally, it was shown that recombinant protein disulfide isomerases 2 and 4 (PDI2 and PDI4) are inhibited by nitazoxanide [317] (Table 84.2). In *Giardia*, resistance against nitazoxanide and MTZ is thus linked. Cross-resistance to tinidazole has also been demonstrated with MTZ-resistant *Giardia* strains [318, 319].

The emergence of albendazole resistance in giardiasis is also an issue of growing concern for public health. Albendazole resistance can be generated in vitro in Giardia

[320], and it was shown that resistance was correlated with cytoskeletal changes but not with mutations at amino acid 200 in β-tubulin, a common mutation found in almost every albendazole-resistant helminthic species (see below) [321]. Albendazole induces ROS accumulation in albendazole susceptible *Giardia* parasites, but not in resistant ones, and the accumulation of albendazole oxidant metabolites (e.g., sulfoxide/ABZ-SO and sulfone/ABZ-SOO) is lower in albendazole-resistant cultures compared to susceptible strains [322]. The NAD(P)H- and flavin-generating pathways, and possibly redox-sensitive epigenetic regulation are also probably involved in the complex albendazole resistance mechanism operating in *Giardia* [323]. It was thus suggested that the strong antioxidant response in resistant parasites may contribute to overcome the pro-oxidant cytotoxicity of albendazole observed in susceptible *Giardia* parasites, and thus may contribute to the resistance phenotype in this parasite (Table 84.2).

In vitro susceptibility assays are not easy to perform in *Giardia* and these difficulties result from the requirement for anaerobic growth and the difficulties in adapting the parasite strains to in vitro cultivation, e.g., excystation and axenization of trophozoites [324–326]. Nonetheless, a number of different methods have been developed to assay drug susceptibility in *Giardia,* which are similar to the ones developed for *Tv* (Table 84.3). In general, the axenized trophozoites are cultured in "large volume" airtight tubes [327], in vials [328], or in microtiter plates [329–332]. Some methods rely on a radiometric approach to look for a 50 % reduction in uptake of ^{3}H-thymidine measuring parasite multiplication [301, 333, 334], a 50 % reduction in parasite adherence [335–337], colorimetric assays for products released by killed trophozoites in the presence of drugs [303], assays based on soluble formazan production [338, 339], or the fluorescent substrate resazurin [330] (Table 84.3). In cases were anaerobic parasites are co-cultured with intestinal cells, *Giardia* trophozoites are normally quantified using real-time PCR with parasite-specific targets [340]. An Anaerocult assay is also available for drug susceptibility testing in *Giardia* [284], as well as a novel flow cytometry assay using propidium iodide [341] (Table 84.3). Recently, an integrated microfluidic device for culturing *Giardia* was reported, and this system also enables dose-response experiments for drug resistance monitoring [342]. A gerbil model [343] and mice model [311, 344] also exit for in vivo drug susceptibility testing.

5.3 Amoebiasis

Amoebiasis is caused by *Entamoeba* spp., anaerobes of worldwide high prevalence (Table 84.1). There are two distinct, but morphologically identical species of *Entamoeba*: *Entamoeba histolytica*, which is pathogenic and *Entamoeba dispar* which is non-pathogenic to human. Hepatic disease (amoebic liver abscess) is the most common symptomatic manifestation and occurs in 4–10 % of cases when organisms penetrate the bowel mucosa and enter the portal circulation. Antigen detection is the preferred tool in amoebiasis diagnosis (Table 84.3). Emetine, a plant alkaloid, was initially used to treat infections by *E. histolytica*, but MTZ/TDZ have become the drugs of choice following recognition of their amoebicidal properties in the mid-1960s (Table 84.2).

Emetine kills the trophozoites of *E. histolytica* mainly by inhibiting protein synthesis whereas MTZ and TDZ, two 5-nitroimidazoles, kill the trophozoites by alterations in the protoplasmic organelles of the amoeba. Both are ineffective however in the treatment of cysts [345]. Indiscriminate use of drugs has led to an increase in the minimum inhibitory concentration (MIC) of these therapeutic agents. In fact, MTZ/TDZ resistance in *E. histolytica* does not appear to be a serious problem since there are only occasional reports of failure with MTZ [346, 347]. Nonetheless, using stepwise incremental increases in drug dose, MTZ resistance can be induced in axenic lines of *E. histolytica* [348, 349]. In contrast to other anaerobes, resistant amoebae do not substantially downregulate pyruvate to ferredoxin oxidoreductase (PFOR) or upregulate P-glycoproteins, but exhibit increased expression of iron-containing superoxide dismutase (Fe-SOD) and peroxiredoxin and decreased expression of flavin reductase and ferredoxin 1 [348] (Table 84.2).

Because the organism is difficult to culture axenically from patients, there are very few assays to monitor drug resistance levels in clinical isolates (Table 84.3). Clinical isolates can be maintained in polyxenic cultures followed by monoxenic cultures. In vitro drug sensitivity of clinical isolates is usually assessed by nitro blue tetrazolium (NBT) reduction assay after exposure to various concentrations of each drug, in parallel with and standard reference strains [345]. Experimental animal models in the germfree guinea pig [350] and in hamsters [351] are also available using axenic or monoxenic cultures of *E. histolytica*.

6 Drug Susceptibility Testing in Stramenopiles (Heterokonts) Parasites

6.1 Blastocystosis

Blastocystosis is caused by a microscopic parasite, *Blastocystis hominis,* and the only stramenopile known to cause infections in humans [352] (Table 84.1). Several animals (e.g., cats, dogs, pigs, horses, cattle) can be also infected and infection in human often results from ingestion of contaminated food or water (fecal-oral route). In vitro cultivation is the most sensitive in detection of *B. hominis* than simple smear and concentration technique (Table 84.3) but is

not used routinely [353]. The taxonomy of *Blastocystis* remained elusive for many years, but there has been a sequence information on the complete SSU rRNA gene; *B. hominis* has been placed within an informal group, the stramenopiles, a branch of the *Chromalveolata* [354]. Once a person or animal has been infected with *B. hominis*, the parasite lives in the intestine and is passed in feces. Because the parasite is protected by an outer shell, it can survive outside the body and in the environment for long periods in some cases. Indeed, asymptomatic individuals with few cysts are usually not treated. Although MTZ is standard therapy for *Blastocystis* infections (Table 84.2), there have been accumulating reports of treatment failure, suggesting the existence of drug-resistant isolates [355]. One of the many reasons could be that the cyst forms, in addition to being genetically heterogeneous, are resistant to the cytotoxic effect of the drug [356] (Table 84.2). Alternatives include NTZ, trimethoprim-sulfamethoxazole, paromomycin, iodoquinol, ketoconazole, secnidazole, emetine, TDZ, and the probiotic *Saccharomyces boulardii* [355].

MTZ induces programmed cell death in *Blastocystis* and apoptosis-like features [357]. Reduction of ferredoxins in the mitochondrion-like organelle seems to play a role in the conversion of MTZ into its active state [357, 358]. A Caco-2 model of human intestinal epithelium also has been also developed for infection studies that may probably serve also in drug resistance testing [359] (Table 84.3). Rapid, in vitro high-throughput viability assays for *Blastocystis* spp. were optimized for MTZ resistance testing and extensive subtype-dependent variations in drug susceptibilities [360]. These are based on resazurin and XTT viability microassays (Table 84.3).

7 Drug Susceptibility Testing in Helminthes

There are a limited number of anthelmintic that can be used in medicine to treat helminthic infections, and most of them are also used since decades in animals. As a consequence of this long-term usage, resistance in livestock has been described for every anthelmintic available on the market. Resistance in helminthic infections in human, while not widespread, is now an emerging concern worldwide. There are only few broad-spectrum anthelmintic groups available for treatment and for the control of nematodes in human (Table 84.2). The first one is the benzimidazole class of drugs (e.g., albendazole and mebendazole), the second group includes imidazothiazoles (e.g., levamisole) and hydropyrimidines (e.g., pyrantel), and a third group corresponds to the macrocyclic lactones (MLs, e.g., ivermectin). In addition in the 1970s, the pyrazinoisoquinoline derivative praziquantel was successfully developed as a new broad-spectrum anthelmintic and is now used against most parasitic trematodes and cestodes on a large scale [361]. Praziquantel causes rapid contraction of the worm musculature of trematodes, which leads to a loss of worm movement, rapid bleb formation and vacuolization of the tegument, followed by rupture of the blebs and vacuoles [361]. Rare occurrence of allergic and hypersensitivity reactions after praziquantel administration may limit its use however [362]. The exact mechanism of action remains unclear [377]. Similarly, a synthetic derivative of piperazine, diethylcarbamazine (DEC), was discovered during the 1940s [363] and is now used as an antifilarial drug along with other drugs in mass drug administration programs (Table 84.2). DEC is part of the Global Program for the Elimination of Lymphatic Filariasis (GPELF) in human and is yearly administrated along with albendazole (or with ivermectin). DEC blocks host, and possibly parasite enzymes involved in arachidonic acid metabolism, and enhances the innate, nonspecific immune system by altering the parasite surface structure, making them susceptible to destruction by host defense. Each of these anthelmintic classes of drugs have their own mechanisms of action and resistance mechanisms (Table 84.2) that will be briefly described here.

The class of anthelmintic drugs that has been the most extensively studied up to now is certainly benzimidazoles (BZs). Members of this class bind selectively to β-tubulin and inhibit microtubule formation in parasites (Table 84.2). Parasites are thus immobilized and die slowly upon treatment. BZ resistance is characterized by single-nucleotide polymorphisms (SNPs), which cause amino acid substitutions in β-tubulin [364–366]. Mutations in β-tubulin inhibit drug binding and thus confer resistance (Table 84.2). More recently, SNPs present in a drug transport glycoprotein have been similarly involved in BZ resistance [367, 368]. Biologically, BZs prevent embryonation and hatching of nematode eggs. Therefore, a number of egg hatch/embryonation assays have been developed (see below) for the detection of resistance to this group of anthelmintics.

Levamisole and pyrantel are agonists at nicotinic acetylcholine receptors of nematode muscle and cause spastic paralysis (Table 84.2). Both drugs are used against nematodes of medical and veterinary importance, in particular soil-transmitted helminths. The molecular mechanism of resistance to such drugs is poorly understood however. In animals, reduced transcription of the mRNA coding for nicotinic acetylcholine receptor subunits that form the pyrantel-sensitive receptors was described as a component of the pyrantel resistance mechanism [369] (Table 84.2). A number of changes in several genes, which together encode the acetylcholine receptor, result in loss of the levamisole-sensitive acetylcholine receptor and thereby may cause resistance [370]. In *Haemonchus contortus*, a nematode infecting mainly sheep and goats, a potential marker for levamisole resistance has been discovered [371]. Indeed, the presence or absence of an indel of 63 bp located just downstream from

the splice acceptor site for the alternative third exon of a specific locus, Hco-acr-8b, was correlating with levamisole resistance status. Using this knowledge, a DNA-based assay for the detection and monitoring of levamisole resistance in parasitic nematodes of animals has been developed [371]. This test has not been validated for human nematodes yet.

Macrolide lactones (MLs) are primarily used against onchocerciasis, lymphatic filariasis, and strongyloidiasis. Most studies on the mode of action of MLs were based on the avermectins class of drug which includes ivermectin. Ivermectin resistance is now a serious problem for parasite control in livestock. Avermectins inhibits larval motility and the functioning of the pharyngeal pump which can inhibit feeding of nematodes. More specifically, ivermectin at certain concentrations increases the opening of glutamate-gated chloride (GluCl) channels and produces paralysis of pharyngeal pumping in nematodes through hyperpolarization of the target neuromuscular cell (Table 84.2). Changes in the frequencies of two alleles of this glutamate-gated chloride channel gene confer resistance [372] (Table 84.2). Interestingly in *Onchocerca volvulus*, changes in genotype frequencies in β-tubulin gene were associated with ivermectin treatments [373]. Finally, P-glycoproteins may be also involved in resistance to ivermectin [374–376] (Table 84.2).

7.1 Diagnostic Assays

A range of in vitro tests for both veterinary and human applications as well as few in vivo assays in animals have been developed for the detection of nematode populations resistant to the main anthelmintic groups (Table 84.3). Each suffers to some degree from reliability, reproducibility, sensitivity, and ease of interpretation however.

7.1.1 In Vitro Methods

The in vitro tests available to monitor drug resistance in helminths fall into three broad groups: (1) the Fecal Egg Count Reduction Test (FECRT) in which the fecal egg counts in pre- and post-drug treatment human samples are compared to indicate the percentage reduction in egg count as a result of the drug treatment [378]; (2) phenotypic assays in which the effects of drugs on free-living life cycle stages are examined with in vitro bioassays [378–380]; and (3) molecular tests in which the genotypic changes associated with drug resistance are monitored using polymerase chain reaction (PCR)-based methods [380, 381].

The FECRT assay measures changes in fecal parasite egg counts following chemotherapy and is currently the standard method for determining the therapeutic efficacy of all anthelmintic chemotherapy in human or animal use. The assay is valid however only when resistance becomes common place in the nematode population, e.g., when at least 20–25 % of the population developed resistance [382]. All FECRT protocols (e.g., FLOTAC, McMaster, Kato-Katz) start with fecal samples collected just before treatment with an antiparasitic drug and again after treatment. Parasites' eggs are counted in both the pre- and posttreatment fecal samples. If the egg numbers in the posttreatment sample are not reduced by at least 90 %, resistance may be suspected, although the proposed cut-off values have been set to 70 % and 50 % in the case of *Ascaris lumbricoides* and *Trichuris trichiura,* respectively [383]. The FECRT protocols can be used in human applications [384–386]. The Kato-Katz protocol is most widely used to detect eggs of *Schistosoma* spp., but less so for the detection of eggs of STHs and particularly for quantification of egg burden of hookworms (e.g., *Ancylostoma duodenale* and *Necator americanus)* due to lysis of fragile hookworm eggs during processing. The McMaster technique is the most common technique used in veterinary parasitology. Both the FLOTAC and McMaster techniques are designed to be quantitative, in comparison to the Kato-Katz method which can nonetheless be used for drug susceptibilities screening in helminths. Although very useful, one disadvantage of the FECRT is that some drugs, like ivermectin, may temporarily suppress egg laying so resistant worms appear to be susceptible in routine testing [387]. Furthermore, density-dependent fecundity effect among different worm populations is also a concern with this method. Indeed, egg output by females varies between species and changes under certain conditions which may complicate interpretation of results for this assay [388].

One of the phenotypic methods commonly used for drug susceptibility testing in parasitic helminths is known as the egg hatch test (EHT). This test assesses the ability of benzimidazole drugs (BZ), at given drug concentrations, to inhibit the embryonation and hatching of freshly collected nematode eggs, expressed as the dose required to inhibit 50 % of the eggs (ED_{50}). The EHT has been widely used with nematodes of livestock to detect resistance [389, 390], but several studies have tested human hookworm populations using this type of assay [391, 392]. The assay can be performed using the "agar-based protocol" or the "water-based" format. For the agar-based method, a stock solution of BZ is prepared in dimethyl sulfoxide (DMSO) and serially diluted twofold in the same solvent. Aliquots from a series of dilutions are added to 96-well microtiter plates, such that each row of the plate comprised a gradient of ten dilutions. The first two wells of each row are used as control wells (e.g., receive DMSO only). Each drug concentration is present in at least triplicate wells on each plate. Aliquots of a 2 % agar solution are dispensed into each well of the plate and allowed to set. Plates are placed into plastic press-seal bags and stored at 4 °C for no more than 3 months. Prior to use, plates are equilibrated to room temperature for 2 h before an aliquot of egg suspension in H_2O is dispensed onto the surface of the agar in each well.

Depending on the species, the number of eggs distributed per well may vary. For example, for *N. americanus*, it corresponds to ~30–35 eggs per well. Plates are then returned to a bag and incubated for 48 h at 26 °C. Lugol's iodine is then added to each well. The numbers of larvae present in each drug well are counted using an inverted microscope, and numbers of larvae and unhatched eggs are also counted in 12 control wells for each experiment. For some species, the amount of fecal material in each well prevents direct counting within the well. In those cases, the contents of each well are pipetted onto a slide for counting of larvae. Experiments are repeated three times. In the water-based assay, each drug well contains aliquots of the series of BZ solutions and control wells contain DMSO only. Egg solutions are added, the plates are sealed in plastic bags, agitated briefly and incubated at 26 °C for 48 h. Numbers of larvae and/or eggs are then counted as described previously for the agar assay. The EHT is the most appropriate test for use with human hookworms, since their eggs hatch rapidly. EHT is not useful for *Ascaris* and *Trichuris* which develop to the infective stage within the egg in the external environment but do not hatch externally to the host. As FECRT, low levels of resistance (below 25 %) are being hardly detected by EHT [382].

Another commonly used in vitro test for monitoring anthelmintic resistance is the larval development test (LDT) which allows the detection of resistance irrespective of the mode of action of drugs. Several different methods have been published, measuring the effect of different anthelmintic drug classes in various parasite species. There are currently two larval development tests, the liquid-based test described [393] and the agar-based test [394]. LDTs are mainly used to detect resistance to benzimidazoles (BZs), pyrantel/levamisole, and some macrocyclic lactones. The use of agar was reported to eliminate solubility problems with avermectins, including ivermectin [395]. Essentially LDTs measure either the morphology (or motility) changes upon treatment on a worm population. Briefly, these assays are performed with larvae in 96-well microtiter plates and LDT assays are performed as the EHT assays, but L1 stage larvae are used instead of eggs. Their development is followed up to the third larval stage (L3). LDTs are mainly useful to monitor drug resistance in *Necator americanus* and *Ancylostoma* spp., two soil-transmitted helminths.

A larval paralysis test (LPT) has been developed for the detection of levamisole (and morantel in animals) resistance [396]. In the assay, infective third stage larvae are incubated for 24 h in serial dilutions of the anthelmintic. After this time the percentage of paralyzed larvae is determined at each concentration and a dose-response line plotted and compared to known reference strains. Sutherland and Lee [397] described a modification of the larval paralysis assay, suitable for detecting thiabendazole resistance, a macrolide lactone.

A micromotility meter test (MMT) has been developed in which a micromotility meter is used [398]. On the base of the micromotility meter, a light is located, projecting upward through the test tube and its contents. It refracts horizontally from the meniscus to the outside of the tube, where the light signals are measured by a photodetector. Movement of the worms causes a variation of the reflected light rays and therefore a variation in the signals received. The average deviation of the signals from its mean value is determined by means of an amplifier, followed by a converter and a computer. The numerical representation of this signal is termed the motility index. Software is then used to record the readings which give a good measure of the motility of tested helminths in the presence or absence of drugs at various concentrations. Dead helminths generated a reading comparable to those obtained from pure liquid; active helminths caused higher indices than less active worms. MMTs have been proved useful for the detection of resistance to macrolide lactones (ivermectin) and levamisole, but not to BZ drugs [399].

Larval motility and larval migration (LMT) in the presence of drugs can alternatively be measured by migration through a sieve or by direct observation [400–405]. Most of these methods require visual scoring by skilled operators. To alleviate this limitation, an automated objective assay for drug screening and resistance diagnosis has been optimized [406]. A system called the xCeLLigence system (Roche) is currently available commercially which monitors cellular events in real-time without the incorporation of labels by measuring electrical impedance across interdigitated microelectrodes integrated at the bottom of tissue culture E-plates. Each time a parasite hit the electrode, it is monitored. As the action of many anthelmintics is reflected by their ability to affect the motility of the target parasites, the magnitude of the decrease in parasite motility for a particular sensitive strain is an indicator of compounds presenting therapeutic activities. In contrast, in the presence of a resistant strain, the motility will not be affected. Thus, the motility index generated by the statistical analysis of data using control strains clearly differentiates between resistant and sensitive strains of parasites.

Larval feeding inhibition assays (LFIAs) for detection of nematode anthelmintic resistance to macrocyclic lactones (ivermectin and imidazothiazoles) are available [407]. These assays consist in the study of the reduction of food ingestion (e.g., labeled bacteria) by first stage larvae (called L1) incubated in serial dilutions of an anthelmintic. The percentage of larvae fed is determined for each dilution by examination of the larvae's intestine, and the dose of larval feeding inhibition 50 (IC_{50}) (i.e., the concentration of anthelmintic required to inhibit the ingestion in 50 % of the L1) is calculated. Resistant strains tend to have higher IC_{50} values since they continue feeding at higher concentrations of the drugs being assayed. Control wells provided a qualitative measure of

larvae viability. Fed larval counts (observation of intestinal fluorescence) are carried out through a fluorescence inverted microscope. Feeding assays with adult worms are also possible [408].

A chemiluminescent assay for measuring avermectin binding sites has been described [409]. A biologically active chemiluminescent compound (ivermectin-luminol) provides an extremely sensitive nonradioactive probe to study avermectin binding sites.

The tubulin binding assay was specifically optimized for benzimidazole resistance testing [410]. The assay is based on the mode of action of BZ drugs that is associated with a reduced affinity of tubulins for the anthelmintics observed in resistant parasites. Briefly, the assay involves the incubation of a crude tubulin extract from adult parasites, infective larvae or eggs, with a tritiated benzimidazole for a certain period of time. The free, unbound drug still in suspension is removed using charcoal and the tubulin-bound label is counted by liquid scintillation spectrophotometry. Tubulin extracts from resistant parasites bind significantly less strongly than do those from susceptible parasites. Some drawbacks of this test are, besides requiring radiolabeled drug and access to expensive laboratory apparatus, that it requires relatively large numbers of starting material (worms, larvae, or eggs) making it nonoptimal for routine field assays.

Microfluidic chips for live whole worm sorting are also showing great promise for drug screening and resistance testing, as recently demonstrated for the model worm *C. elegans* [411–413]. With microchannels to direct worms and microsuction valves that trap individual worms, the microchip device can sort whole worms depending on drug sensitivity phenotype. The sorting chip is combined with fluorescence and digital imaging and permits screening down to a single cell worm resolution. Although very attractive, the limit of this system is that some adult parasites of many species are too large to be screened by this device.

The role of molecular diagnosis for resistance to antihelminthic drugs is currently considered as the way of the future. Indeed, several tests to detect antiparasitic resistance in worms are PCR or pyrosequencing-based tests, mainly for benzimidazoles resistance testing. Indeed, a number of tests have been applied in veterinary parasitology, especially for soil-transmitted helminths [376, 414–417] and some have been adapted to humans [418–420] (Table 84.3). However, with the exception of the benzimidazole class of drugs, the molecular basis of anthelmintic resistance is poorly understood at present for the other class of drugs, which limit the development of accurate and sensitive PCR/pyrosequencing-based assays. This may change through the leadership of a consortium carrying genomic characterization of resistant strains in a number of species [421]. Since the mechanism of benzimidazole resistance appears to be mainly associated with a reduced affinity of tubulin for the anthelmintic [422–424], a diagnostic assay for the detection of benzimidazole resistant nematodes in animals using the binding of tritiated benzimidazole carbamates to tubulin extracts of third stage larvae has been developed [410]. The assay is claimed to be rapid (~2 h), robust, highly reproducible, and sensitive to minor changes in the resistance status of parasite populations, but it requires relatively large numbers of larvae making it unsuitable for routine field assays. This assay has not been standardized yet for human nematodes.

7.1.2 In Vivo Methods

An in vivo test for monitoring suspected anthelmintic resistance in trematode- and cestode-infected animals is available which is called the controlled efficacy test (CET). The CET can assess anthelmintic resistance against any type of anthelmintic. After artificial infection followed by treatment with a flukicide, the animals are killed and the number of flukes in the liver (or in specific gastrointestinal regions) are counted [389]. By using various control groups (e.g., untreated and susceptible worm isolates), dose-response curves can be generated and then the ED_{50} calculated. There is, however, currently no agreed view on how to determine the occurrence of resistance on basis of these counts so this test is not routinely used.

The next sections will describe the main helminthic infections in human and discuss about the various susceptibility tests that are currently used for specific parasitic helminths (Table 84.3). It is important to mention here that very few in vitro assays to detect anthelmintic resistance in human nematodes have been validated so far, and most were adapted from assays previously developed for nematodes of veterinary importance. A major problem which limits the validation process is obviously the lack of human reference-resistant strains.

7.2 Schistosomiasis

Schistosomiasis is a parasitic worm disease carried by freshwater snails infected with one of the six species of the parasite *Schistosoma*, e.g., *S. mansoni, S. haematobium, S. japonicum, S. intercalatum, S. guineensis*, and *S. mekongi,* of which *S. haematobium* and *S. mansoni* are the predominant causes of disease (Table 84.1). Only *S. haematobium* causes vesical (urinary) human schistosomiasis that can lead to bladder cancer. The others are responsible for intestinal diseases (abdominal bleeding) but may also attack the liver, lungs, and spleen with concomitant damage to the intestines. Roughly 240 million people in ~80 countries suffer from schistosomiasis [425]. Transmission occurs by contact with contaminated freshwater (lakes and ponds, rivers, dams) inhabited by snails carrying the parasite. Parasites penetrate the skin during contact with freshwater or soil containing

contaminated snails. The quantitative Kato-Katz fecal smear technique is considered as the golden standard method for diagnosing schistosomiasis (Table 84.3). Praziquantel is the primary form of treatment against all schistosome species, and it can be safely coadministrated with albendazole or ivermectin. The expression of the schistosome P-glycoprotein SmMDR2 was found to be altered in worms exposed to praziquantel (PZQ) and was expressed at higher levels in worms from isolates with reduced PZQ susceptibility [426] (Table 84.2). A second ABC transporter, SmMRP1, was associated with PZQ resistance [426]. Drug resistance testing in schistosomes is usually performed using the FECRT (all anthelmintics), EHT (ivermectin), and LFIA (ivermectin) assays. The use of the xCeLLigence system has been also reported (praziquantel) [406], and animal models (snails [427, 428] and mice [429]) are available, although these models remain difficult to handle and interpret.

7.3 Lymphatic Filariasis

Lymphatic filariasis affects more than 120 million people in 80 countries worldwide and is a painful and debilitating disease (Table 84.1). The disease is caused by the threadlike parasitic filarial worms *Wuchereria bancrofti* and *Brugia malayi*, which live in the lymphatic system and can cause extreme swelling of the extremities and genitals. The disease is transmitted to humans by mosquitoes. The standard laboratory test for this infection is detection of the filarial worm in biopsy samples (Table 84.3). Lymphatic filariasis is treated with a combination of albendazole and diethylcarbamazine (DEC). Alternatively, ivermectin has proven to be effective. Although ivermectin is the only macrocyclic lactone approved for treating filarioid infections of humans, moxidectin appears to be effective against *B. malayi,* although resistance can be acquired in vitro by the modulation of ABC transporters [430] (Table 84.2). Few evidence of nonsusceptibility to DEC in *W. bancrofti* has been reported yet [431]. Drug resistance testing in filarial worms is usually performed using the FECRT and LMT assays. PCR and pyrosequencing assays for screening for albendazole resistance among *W. bancrofti* populations have been reported [15, 432–434].

7.4 Other Helminth Diseases

7.4.1 Onchocerciasis

Onchocerciasis, also known as river blindness, is caused by the filarial nematode *Onchocerca volvulus* which infects 26 million people living near the rivers and fast-moving streams of sub-Saharan Africa (Table 84.1). The disease is transmitted from person to person by infected *Simulium* black flies. Approximately 37 million people are estimated to be infected with onchocerciasis. Larvae enter the skin at the bite site and form nodules in the subcutaneous tissue where they mature. Adult females release millions of microscopic larvae—called microfilariae—into the surrounding tissue, that can lead to visual impairment and blindness. Onchocerciasis is treated with a biannual dose of ivermectin. In recent years there have been reports of persistent microfilaridermias despite multiple treatments with ivermectin suggesting that *O. volvulus* is becoming resistant to the anti-fecundity effects of the drug [18, 435–437]. Drug resistance testing in this parasite can be performed using the FECRT, LDT, LMT, MMT, and sometimes LFIA assays. Single-nucleotide polymorphisms in β-tubulin selected in *O. volvulus* following repeated ivermectin treatment can also be monitored though PCR assays [373].

7.4.2 Cysticercosis/Taeniasis

Two species of *Taenia* are frequent human intestinal parasites. *T. saginata*, the most frequent, is found in almost all countries where beef is eaten. *T. solium,* also called the pork tapeworm, is endemic in Latin America, Africa, and some Asian countries (Table 84.1). Infection occurs when one eats infected beef or pork. Eggs are passed in the human feces [438]. More than 60–70 million people worldwide are infected with a *Tania* spp. Taeniasis/cysticercosis are treatable with albendazole or praziquantel. Only few cases of treatment failure have been reported [439, 440]. Drug resistance testing in parasites causing cysticercosis/taeniasis is usually performed using the FECRT assay. An experimental encephalitis caused by *Taenia crassiceps* cysticerci in mice is also available [441] (Table 84.3).

7.4.3 Cystic Echinococcosis

Cystic echinococcosis (CE) or hydatid disease is a disease that affects both humans and animals (Table 84.1). CE is caused by the larval stage of *Echinococcus granulosus*, a tapeworm cestode that is common in Asia, Australia, East Africa, southern regions of Spain, South America, and North America. The primary carriers are dogs and wolves, and humans are accidental hosts. The liver is the most commonly involved organ in the body. The lungs are involved in approximately 10–30 % of adult cases. Pulmonary echinococcosis may remain asymptomatic for years and symptoms often develop only after cyst rupture or superinfection, most commonly by *Aspergillus fumigatus* [442, 443]. In humans, the disease is treated by surgery with a supplementary option of chemotherapy. Small (<5 cm) stage cysts (CE1 and CE3a) may be primarily treated with benzimidazoles, the first-choice drug being albendazole. In some situations the combination of albendazole and praziquantel may be preferred [444]. Out of three β-tubulin gene isoforms of *E. granulosus*, β-tubulin gene isoform 2 showed a conserved point mutation indicative of BZ resistance [445]. A second species, *E. mul-*

tilocularis, may also infect humans. The presentation is similar to *E. granulosus*, but the cysts are multilocular. *E. multilocularis* is resistant to praziquantel although high doses of albendazole or mebendazole may be effective. Drug resistance testing in CE parasites is usually performed using the FECRT and LDT assays, and PCR assays were also developed for BZ resistance testing. Small laboratory animals such as mice and Mongolian jirds are also used for in vivo drug susceptibility assays.

7.4.4 Soil-Transmitted Helminths

The major soil-transmitted helminths (STHs) are *Ascaris lumbricoides*/*Strongyloides stercoralis* (roundworms), *Necator americanus/Ancylostoma duodenale* (hookworms) and *Trichuris trichiura* (whipworm) (Table 84.1). The main intervention available for controlling STH infections is the periodic administration of one of the four anthelmintics recommended by WHO, mebendazole, albendazole, levamisole, or pyrantel. Of these, benzimidazoles (BZs) (e.g., mebendazole, albendazole) are the most frequently used anthelmintic for treatment of SHTs. Ivermectin is not recommended for STHs, except for strongyloidiasis (see below). The FECRT is by far the most commonly used test for diagnosis of resistance in human STHs. Phenotypic tests are also used in the livestock industries with some drug groups, most notably egg hatch assays with BZ [378, 380]. Phenotypic assays have been also developed to measure drug sensitivity in human hookworms [446]. Molecular tests are also available for detecting resistance to BZ drugs in some livestock nematode species [421]. For the nAChR antagonist drug group (pyrantel and levamisole), phenotypic tests have been described for livestock and companion animal gastrointestinal species [447, 448].

Strongyloidiasis is caused by *Strongyloides stercoralis*, a roundworm present mainly in tropical and subtropical regions but also in temperate climates (Table 84.1). Some 40–100 million people are estimated to be infected worldwide with more than 50 % of *S. stercoralis* infections being asymptomatic and most of them being chronic [449]. The two aggressive forms of the disease are hyperinfection syndrome or disseminated strongyloidiasis. In the first case, the infection occurs with a very heavy worm burden, while in the latter the larvae penetrate the intestine wall and reach the bloodstream, causing meningitis and septic shock. Severe strongyloidiasis carries a high mortality rate (up to 80 %) because the diagnosis is often delayed. Parasitological diagnosis of *S. stercoralis* infection relies on identifying larvae in stool. Alternatively, serological and molecular methods as well as chest radiography can also be used (Table 84.2). Today, ivermectin is the standard treatment for *S. stercoralis* infections, although many reports revealed an incomplete cure [450, 451]. Other drugs have been used for treatment, including mebendazole and albendazole [452, 453]. An in vitro larval motility assay is usually used to determine anthelmintic sensitivity for *Strongyloides* species [454]. A modified filter paper culture technique has been described for in vitro screening of *Strongyloides stercoralis* ivermectin sensitivity in clinical specimens [455]. This procedure does not require parasite isolation and is based on the principle of the drug effect on the motility of the infective-stage larvae, combined with a modified coproculture using the filter paper culture technique. Briefly, stool samples are collected from *S. stercoralis*-infected subjects and feces are smeared in the center of narrow filter paper strips. The strips are then placed in glass test tubes and various concentrations of ivermectin or of the control (distilled water) are added to the bottom of each tube at 25 °C. On the third and fifth days of culture at 25 °C, the tubes are examined for the effect of the drug on worm viability based on the motility of the infective-stage larvae at × 40 magnification. All control (no drug) tubes should show motile worms. The criterion for drug resistance is the demonstration of at least one motile worm in duplicate tubes either after 3 or 5 days of culture. Drug resistance testing in *S. stercoralis* can be alternatively performed using the FECRT, LFIA, LDT, and through the xCeLLigence platform.

There are two common species of hookworms, *Necator americanus* and *Ancylostoma duodenale*. Collectively, hookworms affect more than 700 million people across the globe, particularly in Africa and Latin America. Left untreated, hookworms cause internal blood loss leading to iron-deficiency anemia and protein malnutrition, particularly in pregnant women and children. Moreover, chronic hookworm infection in children contributes to physical and intellectual impairment, learning difficulties and poor school performance. Hookworm transmission is a complex, repetitive cycle. Larvae are found in human feces and transmitted to humans from contaminated soil through the skin or by accidentally ingesting contaminated soil. The worms mature in the small intestine of the host. A vaccine is currently in clinical trials [456, 457]. Hookworm infections are treated with anthelminthic drugs albendazole or mebendazole on an annual basis. Alternative treatment may include levamisole or pyrantel. Drug resistance testing in *Necator americanus* and *Ancylostoma duodenale* is usually performed using the FECRT and EHT assays, but PCR assays exist for the detection of SNPs in β-tubulin gene conferring BZ resistance.

Trichuriasis in human is caused by the species *Trichuris trichiura*. The disease affects close to 700 million people worldwide, particularly in warm, humid, tropical climates (Table 84.1). Symptoms include abdominal upset, diarrhea, and, depending on the number of worms, malnutrition, dehydration, and anemia. Adult worms live in the colon. Treatment is based on anthelminthic drugs albendazole or mebendazole on an annual basis. Alternative treatments include levamisole, pyrantel, and ivermectin. None of these drugs are opti-

mal however since they display only moderate cure rates when administered as single doses. Although this has not arisen as a problem yet, potential emergence of drug resistance in *Trichuris* is of concern [458]. The identification of potential new drug candidates currently relies on the adult *Trichuris* motility assay originally developed for the mouse whipworm *T. muris* [459]. A variant of this assay has been adapted for drug testing purposes based on L1 stage larvae, but the assay is also suitable for detecting resistance to levamisole and other drugs [460]. Alternatively, drug resistance in *Trichuris* may be detected using the FECRT (all anthelmintic) and LDT (BZs) assays. The xCELLigence system has been also used for levamisole, nitazoxanide, and ivermectin drug resistance testing in *Trichuris* [459] and molecular assays are available to detect BZ resistance [461]. The EHT is not useful for *Trichuris* which develop to the infective stage within the egg in the external environment but do not hatch externally to the host.

7.4.5 Foodborne Trematodes

Foodborne trematodes may cause fascioliasis, clonorchiasis, opisthorchiasis, and paragonimiasis (Table 84.1). These infections are treatable with praziquantel. The most notorious trematode is *Fasciola hepatica*, an hermaphroditic helminth of cattle and sheep. Human infection occurs occasionally, mainly in certain areas of Europe, Africa, and Latin America. The adult worm lives in the liver or bile ducts of the host, passing eggs in feces. The eggs hatch in fresh water, and the parasite completes its life cycle in a snail. Infection occurs by ingestion of metacercaria in vegetation or in water. Praziquantel treatment failure in human patients infected with *F. hepatica* has been reported [462, 463]. In sheep in cattle, *Fasciola* hepatica drug susceptibility testing can be performed by a coproantigen reduction test (CRT). The coproantigen assay (ELISA format) measures the levels of antigenic gut enzymes released from the fluke into the feces of the animal host. Coproantigen assays have been also tested in human [464, 465]. Alternative drug resistance assays include FECRT and EHT. The murine model is the most commonly used animal model for fascioliasis [466] (Table 84.3).

8 Point-of-Care (POC) Drug Resistance Testing

POC assays are rapid detection devices capable of determining whether a patient (clinically ill or asymptomatic) is infected with a particular pathogen. This type of assays either detects the pathogen itself or an alternative biomarker that is highly specific for the pathogen. With the threat of parasites developing resistance to the currently available drugs, the importance of POC assays for detecting parasites and simultaneously their resistance determinants is obvious. New or improved diagnostics including POC assays for parasite identification and drug resistance testing are needed. To facilitate the implementation of novel POC assays in resource limited settings, a series of criteria under the acronym "ASSURED" have been proposed [467, 468]. According to these criteria, an ideal POC assay should generate results within minutes; should not require a laboratory, electricity, or any special piece of equipment; should use heat-stable reagents with no special storage requirements; and should be easily performed by individuals with minimal training. The assay should also be highly sensitive and specific for the particular agent, inexpensive, and portable. In addition to speed up pathogen detection with high accuracy and sensitivity, the assay should also identify cases of drug resistance in order to ensure a better management of patients. This is no small challenge. The few POC assays currently available for infectious parasitic diseases commonly use the lateral-flow immunochromatographic format which is similar to the pregnancy strip test. Notorious examples in malaria diagnosis based on this format include the histidine-rich protein 2 (HRP2) of *P. falciparum*, the parasite-specific lactate dehydrogenase (pLDH), and a pan-malarial *Plasmodium* aldolase. Other POC assays use agglutination or solid phase processes. Currently, very few POC tests identify cases of drug resistance.

A particularly challenging issue when developing a POC assay for low-income resource settings is how to achieve high sensitivity or quantitative detection without dedicated instrumentation. Isothermal DNA amplification (e.g., LAMP) assays are gaining interest for empowering developing countries as they do not require sophisticated equipment and prove to be cost-effective. LAMP is unique among all isothermal amplification techniques as it is robust, rapid (can amplify a low copy DNA to more than 10^9 copies within an hour), and easy to perform. Moreover, LAMP is able to discriminate SNPs so drug resistance testing using this technology (when mutations are already known) appears very promising.

The LAMP assay is characterized by the use of a DNA polymerase (e.g., *Bst* or *Bsm* polymerases) that has low sensitivity to inhibitors and a set of primers specially designed to recognize different sequences on the target gene. Amplification occurs only when all primers bind, thus forming a product. The reaction allows the release of pyrophosphate that causes turbidity due to precipitations, which can be interpreted with naked eyes or by others means like agarose gel electrophoresis as well as by real-time monitoring in a relatively inexpensive turbidimeter. A DNA-binding dye can also be used to increase further the sensitivity of the assay or one can alternatively use a metal ion indicator like calcein or the coloring dye hydroxynaphthol blue (HNB) for product colorimetric detection without the need to run a gel.

LAMP assays can not only be used for detection of DNA but also can be used for detection of RNA which is known as RT-LAMP. LAMP assays can be carried on a microfluidic chip (microLAMP) or combined with lateral flow array (LFA-LAMP). Typical LAMP assays have been developed for a number of parasites including *Trypanosoma* species [71, 469, 470], Brugian filariasis [471], malaria [472–476]), *Wuchereria bancrofti* [477], and *Leishmania* [478–482]. Regarding resistance genotyping in parasites, few "home-made" LAMP assays have been developed [483], and some ready-to-use LAMP kits are available on the market, although none yet for parasite drug resistance genotyping.

Nanofluidics are being more and more used in POC assays as these technologies are compatible with inexpensive materials and fabrication methods. A European Union-funded public-private consortium, Nanomal (http://www.nanomal.org/), has developed a DNA testing microfluidic smartphone-like prototype device, the nanomal DNA analyzer. The first prototype is intended to quickly test a sample of blood (from a pinprick) for genetic markers of malaria. The assay is based on microfluidic PCR and nanowire sensing technology. The assay is able to identify in 10–15 min which species of malaria parasite is responsible for the infection and whether the parasite is resistant to antimalarial medications. To use it, a health professional would put a sample from a patient into a credit card-sized disposable cartridge and pop the cartridge into the device for analysis. Following a rapid mechanical lysis, the sample flows through a special filter that removes all non-nucleic acid blood constituents under 3 min. The purified DNA eluate then rehydrates lyophilized PCR reagents and is flowed through different temperature zones for rapid thermal cycling (30 cycles in less than 4 min). The amplicons then simply flow into the nanowire array channel. Detection happens when fragments of parasite DNA from the sample bind to complementary strands, or probes, in the cartridge. These probes are associated with nanowires. The binding produces an electrical change in the wires, which the device interprets as a positive result. The prototype device is currently known as Q-POC™ but is still not used in the clinic. The platform is battery powered and do not need special grade water.

A third interesting field of research for POC applications is paperfluidics also known as lab-on-paper. Lab-on-paper was introduced in 2007 [484, 485] and since the field has exploded as it represents an attractive platform for pathogen identification and drug resistance genotyping at very low cost. A number of elegant prototypes have been developed in the last few years [486]. Most of them were inspired from the convergence of fields such as nanotechnology, microfluidics, proteomics, and genomics. Several techniques can be used to create microchannels on paper chips, namely, photolithography, inkjet printing with polydimethylsiloxane (PDMS), the wax printing method, and hot roller embossing technique. These new paperfluidic assays would be valuable tools for routine screening of individuals for the presence of parasites and their resistance profiles in the near future.

9 Multiplex Resistance Testing in Parasite Coinfections

There are close to 150 countries where parasitic diseases are endemic, at least 100 of which are endemic for 2 or more diseases, and 30 countries that are endemic for 6 or more [487]. In areas where coinfections commonly occur, diagnostics should allow for testing related or multiple infections as well as multiple drug resistance profiles. Few assays have been optimized and commercialized to simultaneously detect several parasites and none of them offer drug resistance testing. One example of multiplex assays for pathogen identification is the VereTrop™, a molecular lab-on-chip device able to identify 13 different major tropical diseases, including the five pathogenic *Plasmodium* species, sleeping sickness, and Chagas disease from a single blood sample in less than 3 h (http://vereduslabs.com/products/clinical/veretrop/). The VereTrop™ chip is a PCR-microarray-based diagnostic test that needs to be processed on the portable VerePLEX™ Biosystem. The company claims that the Chip is customizable so there is a possibility to include SNPs linked with resistance, which are not included in the present format. Other multiplex assays include a multiplex real-time PCR assay for the detection in stool of eight gastrointestinal parasites [488], an assay allowing simultaneous detection of eight pathogenic parasites, and a number of bacteria causing gastroenteritis (Savyon Diagnostics and its proprietary NC400 NanoChip molecular electronic microarray, see http://www.savyondiagnostics.com/).

10 Promising Technologies in Parasite Diagnosis and Resistance Testing

The molecular diagnostics market is unquestionably the most rapidly growing segment of the in vitro diagnostics industry. The next 5 years should witness significant developments in nucleic acid amplification systems, automation, and miniaturization, as well as introduction of a wide range of new products facilitating product detection and drug resistance testing with an increased sensitivity. These promising technologies certainly represent the next generation of platforms for parasite diagnostics and drug resistance testing, and these will be briefly overviewed here.

10.1 Next-Generation Sequencing (NGS)

The DNA sequencing field has seen dramatic advances in recent years with companies reporting ever faster and cheaper sequencing methods collectively known as *next-generation sequencing (NGS)* methods. These NGS methods have different underlying biochemistries and different sequencing protocols, throughput, and sequence length output (reviewed in [489]). At present, sequencing-based approaches are the more expensive for drug resistance testing but represent one of the most powerful ways to screen a full genome (or transcriptome by RNAseq) for the detection of resistance genes and resistance determinants, even from unprocessed clinical specimens. Providing that a good genome coverage is obtained, mixed parasite genotypes and coinfections can also be easily detected in patient isolates. Current obstacles to a routine use of NSG technologies in diagnostic parasitology and resistance testing comprise the acquisition cost of the sequencer, scarcely available user-friendly bioinformatics platforms [490] and adequate computing resources (supercomputers). Indeed, the data analysis step is very time-consuming and requires a competent amount of manpower and expertise in bioinformatics. Nonetheless, NGS may revolutionize drug resistance testing on the long term, eventually replacing traditional culture-based approaches. Currently, NGS is successfully used in detection of antiviral drug resistance in clinical specimens [491, 492] but only rarely used in parasitology [493, 494]. In addition, the power and speed of NGS technologies in SNPs detection has recently prompted the CDC (Centers for Disease Control and Prevention) to develop NGS methods applied for the control and surveillance of the potential spread of artemisinin-resistant malaria parasites recently emerged in Southeast Asia (http://www.cdc.gov/amd/project-summaries/next-gen-malaria-methods.html). Their premise is that early identification of low levels of resistant parasites by NGS methods will make it easier and faster to choose the right drugs for treatment than the current practices allow, thus limiting the risk of spreading parasites resistant to artemisinin-based combination therapies to other regions. It is expected that the decrease of costs and improvement of turnaround time will lead to increase use of NGS for parasitological routine diagnostics.

10.2 DNA Pyrosequencing-Based Assays

Pyrosequencing provides a fast, inexpensive, and sensitive alternative to conventional resistance detection methods and can be easily adapted in a high-throughput format for molecular surveillance of drug resistance in human pathogens. Pyrosequencing [495] is a technology ideal for detecting SNPs and short-read sequencing. It is a flexible bioluminometric method which does not need labeled nucleotides or gel electrophoresis. In this technique an enzymatic cascade reaction is used to convert the inorganic pyrophosphate (PPi) released during the incorporation of deoxynucleotide triphosphate, into proportional amounts of visible light, which can then be measured. Interestingly, it has been reported that pyrosequencing assays can detect minor alleles down to a concentration as low as 5 % [496]. Recently, DNA pyrosequencing of PCR amplicons has been successfully applied for genotyping and species level identification of protozoan parasites [497–501] and nematodes [415, 502–504].

10.3 Luminex xMAP Technology

Co-occurring SNP mutations in resistance genes is challenging when using qPCR, multiplex PCR, or even pyrosequencing. In response to this challenge, the Luminex xMAP technology was developed which allows for simultaneous detection of multiple targets in a single reaction, e.g., up to 500 unique analytes within a single sample. In essence, the Luminex technology is a color-coded bead-based multiplex flow cytometric assay (http://www.luminexcorp.com/). Luminex beads, come into 500 distinct sets, each emitting unique fluorescent signals when excited by lasers. Each bead set can be coated with a reagent specific to a particular bioassay (e.g., antigens, antibodies, or oligonucleotides in the case of SNP detection), allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, allowing target identification. Adapted to the study of parasites, the Luminex assay could either identify one particular organism, multiple organisms, or different genotypes (including drug resistance alleles) during the same reaction. The technology has been applied for parasitic diseases including malaria diagnosis [505] and intestinal parasites [506–508].

10.4 Oligonucleotide-Based DNA Microarrays

This methodology permits to rapidly and simultaneously identify a causative pathogen and generate its antimicrobial resistance profile based on its genome or transcriptome. Microarrays involve few up to several thousands of specific DNA sequences (probes) spotted in picomole amounts on any suitable surfaces. Hybridization of probes and target (e.g., the microbial DNA target) is usually detected and quantified via fluorophore labeling of the target sample. Most microarray imaging results are obtained by scanning of laser excitation coupled with photomultiplier tube light

detectors. At present, the DNA microarray technology is mostly used in the routine detection of antimicrobial resistance of TB, HIV, and influenza viruses [509–513] and has been used for *Leishmania* [514–516].

10.5 Minisequencing by Primer Extension Followed by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (PEX-MALDI-TOF) and PCR-Electrospray Ionization Mass Spectrometry (PCR/ESI-MS)

PEX/MALDI-TOF is a method used to rapidly detect resistance, but for each reaction, a single-nucleotide polymorphism (SNP) can be detected. The technique requires a primer whose 3′-end is located directly at the site of the mutation to be detected. The extension reaction catalyzed by a polymerase is terminated in the case of a WT allele just after one nucleotide complementary to the mutated nucleotide and, in the case of a mutant, after two nucleotides by a dideoxynucleotide (ddNTP). Because of the molecular weight difference, mutant and WT alleles can be easily discriminated using MALDI-TOF. In contrast to *PEX-MALDI-TOF*, PCR/ESI-MS allows multiplexing that enables the parallel detection of a wide panel of resistant alleles or genes, as well as pathogen identification. As its name states, the technology combines broad-range PCR amplification with ESI-MS for the sensitive detection of amplicons by mass spectrometry. ESI-MS is used to determine the molecular mass of each amplicon, which is then used to calculate the base composition of each amplicon, and compared to an extensive database for pathogen and resistance SNPs identification. Another feature of the PCR/ESI-MS technology is that it allows a relative quantification of the microbe (and its resistance gene) present in the specimen. One current limitation of this technology is that there are still many gaps in our knowledge of the mechanisms and evolution of resistance in most parasites and the full panel of genes that control resistance to many drugs are not yet identified. The PCR/ESI-MS technology has been commercialized under the trade name PLEX-ID (www.abbott.com/).

10.6 Single Cell Mass Cytometry-Based Techniques

Mass cytometry is a recently developed technology platform that allows for high-content multiparametric analysis of single cells in complex biological systems [517]. Applications may include drug screening and drug susceptibility profiling studies, biomarker discovery, and time-course treatment analysis to name a few. Two important mass cytometric-based techniques were developed, namely, the fluorescent cellular barcoding (FCB) [518, 519] and mass-tag cellular barcoding (MCB) [520] methods. Both methods have the potential to impact the way drug resistance mechanisms are studied and assayed in cells. The first technique, FCB, uses fluorescent tags whereas the second one, MCB, uses metal isotope reporters. These techniques allow high throughput, minimize inter-sample variation, and reduce reagent consumption. Briefly in MCB, individual cell samples are labeled with a unique combination of mass tags before being combined into a single sample. The pooled samples is then stained with a single antibody mix and analyzed in one run on the mass cytometer. Roughly the same methodology stands for FCB. Measured cells are thereafter assigned to the corresponding source sample based on their unique "mass barcode" signatures. These two methods have been used in cancer cells studies but could be applied to a number of infectious diseases including parasites.

10.7 Aptamer-Based Multiplex Proteomics Assays

Aptamers are oligonucleic acids (ssDNA or RNA) that bind to a specific target. Oligonucleic acid aptamers are usually selected from a large random sequence pool through an in vitro iterative selection-amplification process called SELEX (standing for systematic evolution of ligands by exponential enrichment) [521, 522]. Similarly, peptide aptamers can be selected from combinatorial peptide libraries constructed by phage display and other surface display technologies such as mRNA display, ribosome display, bacterial display, and yeast display. Aptamers are attractive tools for multiple analytical and diagnostic applications. Recently, an emerging diagnostic technique based on aptamers called aptamer-based multiplex proteomics was reported [523, 524], commercialized by SomaLogic (http://www.somalogic.com/). This technology enables multi-biomarker protein measurements that can aid diagnostic distinction of disease versus healthy states or to predict treatment outcome [523]. For the moment, the technique has not been optimized to detect signatures from resistant versus sensitive pathogens, but the high specificity of aptamers emphasizes the feasibility of this type of assay, as recently demonstrated for drug-resistant HIV-1 [525] and MRSA [526]. In this context, the company Operational Technologies (OPTech) is working on a handheld aptamer-based-magnetic bead-quantum dot sensor for active and latent leishmaniasis [527] (see also http://www.sbir.gov/sbirsearch/detail/383773).

11 Conclusion

Drug resistance in parasites is inevitable, but the rate at which it develops is not. Increasing levels of pathogen resistance against available drugs aggravate the state of health worldwide, particularly in developing countries, where parasitic diseases are responsible for a high level of mortality and morbidity. Fighting parasites, especially resistant parasites, will require new and low-cost diagnostic tools as well as more sensitive and specific drug resistance assays, ideally POC assays. More research is needed into the mechanisms and genetics of resistance. Knowing the key players involved in resistance will certainly be the basis for more sensitive and accurate diagnostic tests, for the early detection of resistance, and will certainly accelerate the discovery of novel therapeutic targets to develop safer medicines.

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Bacterial Genotypic Drug Resistance Assays

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A. Huletsky and Michel G. Bergeron

1 Introduction

The rise in antimicrobial resistance has significantly reduced the effectiveness of current antibiotics in treating common infections. However, there have been no successful discoveries of novel antibiotics since 1987. During that period, hundreds of mechanisms of resistance have evolved to such an extreme level that the World Health Organization (WHO) has declared antibiotic resistance as a worldwide threat to human health [1]. In 2015, President Obama has released the *National Action Plan for Combating Antibiotic-Resistant Bacteria* (https://www.whitehouse.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf) and has offered a $20 million prize to facilitate the development of rapid, point-of-care diagnostic tests for healthcare providers to identify highly resistant bacterial infections.

These are dangerous times but also exciting as genomic technologies have evolved rapidly and will facilitate the development of tools to better diagnose antibiotic-resistant bacterial infections, evaluate the spread of resistance, and study its epidemiology. Practically speaking, hospital-acquired infections (HAIs), 70 % of which are caused by multidrug-resistant (MDR) organisms [2], are spreading rapidly so that our hospitals are no longer safe, killing more than 75,000 people each year in the United States [3] (http://www.cdc.gov/HAI/surveillance/). Some hospital-acquired MDR pathogens—the so-called superbugs—were recently named as the "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) to emphasize that they "escape" the effects of antibacterial agents [4–6]. Some strains of the ESKAPE pathogens group are not only MDR but also extensively drug resistant (XDR) or pandrug resistant (PDR) [5]. In the community, the prevalence of multidrug resistance in *Streptococcus pneumoniae* is increasing and includes resistance to ß-lactams (intermediate- and high-level resistance to penicillin and cross-resistance to cephalosporins), the macrolides, and, more recently, the fluoroquinolones [7–9]. Furthermore, virulent strains of MRSA that differ from the hospital strains, the so-called community-associated MRSA (CA-MRSA), have emerged in the communities and have now entered healthcare facilities causing HAIs [10, 11]. Another major public health problem is the increasing incidence of MDR *Mycobacterium tuberculosis* (MDR-TB) and the emergence of XDR *M. tuberculosis* (XDR-TB) [12–14].

Physicians practicing in both hospitals and the community must treat infections caused by multiresistant organisms, and new emerging antimicrobial resistances are becoming more complex to detect [15–17]. With the limited number of antimicrobial agents available to treat the infections caused by multidrug-resistant organisms, the need for rapid and reliable susceptibility testing methods or alternative resistance testing methods for detection of antimicrobial resistance becomes increasingly important. Conventional phenotypic culture-based susceptibility test results are usually obtained in 24–48 h or more after a bacterial culture has been isolated. Moreover, susceptibility tests are not always accurate to detect difficult-to-detect emerging antimicrobial resistance, and often more than one method is needed to obtain an accurate susceptibility profile. The lack of accurate and timely susceptibility data by the microbiology laboratory has consequences on antibiotic usage and prescription. Patients have to be treated empirically and often with broad-spectrum antibiotics which results in increased resistance rates and healthcare costs [18]. The advances in our understanding of the genetic mechanisms of antimicrobial resistance and the progress in sample preparation, nucleic acid-based amplification, and sensitive nucleic acid detection have allowed the development of genotypic methods for rapid detection of antimicrobial resistance. It is now possible

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to identify a microorganism and its resistance to antimicrobial agents directly from clinical specimens in about 1 h [19, 20]. Some genotypic drug resistance assays are increasingly used in the clinical settings providing more accurate and rapid resistance testing.

This review describes the mechanism and the importance of this new plague and summarizes new rapid molecular diagnostic tests for the detection of antimicrobial resistance, some of which are having a great impact by allowing clinicians to intervene in real time (<1 h) not having to wait 2–3 days for the results of culture and susceptibility testing, this avoiding empirical treatment and the overuse of broad-spectrum antibiotics which is responsible, by disturbing the microbiota of patients, for the development of *C. difficile* infection and the spread of resistance. Microbes which have survived 4.2 billion years double their population in 20 min and are able to develop resistance very rapidly. Moreover, this resistance can be passed over to other pathogens through mobile genetic elements (plasmids, transposons, etc). The molecular diagnostic revolution has started in 2002 when our group has developed the first real-time PCR assay cleared by the FDA (IDI-Strep B, now BD GeneOhm StrepB from BD Diagnostics), a test that can be used to detect Group B *Streptococcus* in pregnant women during delivery [21]. In 2004, our second real-time PCR test (IDI-MRSA, now BD GeneOhm MRSA from BD Diagnostics) was the first molecular MRSA test cleared by the FDA for use directly on clinical specimens [19, 22].

Today, 10 years later, there are many molecular tests available and "the change in culture without culture" (using nucleic acid-based tests) [23] is occurring slowly but surely and hopefully will insure a better use of antibiotics and less empirical treatment. Rapid molecular tests at point of care are now also appearing and within the next 5–10 years will have great impact on clinical practices.

2 Mechanisms of Resistance to Antimicrobial Agents

Different strategies have been developed by bacteria to evade the action of the antimicrobial agents. In general, antimicrobial resistance results from:

(a) Production of enzymes that inactivate or destroy the antimicrobial agent or the target gene
(b) Acquisition of exogenous resistance genes that are not inhibited by the antimicrobial agent
(c) Reduced uptake of the antimicrobial agent
(d) Active efflux of the antimicrobial agent
(e) Overproduction, loss, or mutation of cellular target genes reducing the binding of the antimicrobial agent

Here are described the major resistance mechanisms for the important antimicrobial classes.

2.1 Resistance to Aminoglycosides

The aminoglycosides constitute a large family of antimicrobials that inhibit the translation process by binding to the bacterial 16S rRNA of the 30S ribosomal subunit. Five mechanisms of resistance to aminoglycosides have been described [24–26] including:

(a) Enzymatic inactivation by aminoglycoside-modifying enzymes (AMEs)
(b) Mutations in the ribosomal target site (*rrs* gene encoding 16S rRNA and *rpsL* gene encoding the S12 protein) that prevents binding
(c) Decreased cell membrane permeability
(d) Expulsion by efflux pumps
(e) Methylation of 16S rRNA target site

Inactivation by AMEs is the most important in terms of frequency and level of resistance [26]. Aminoglycosides are modified by three types of enzymes classified as: aminoglycoside phosphotransferases (APHs), aminoglycoside adenylases (ANTs), and aminoglycoside acetyltransferases (AACs). These enzymes covalently modify specific amino or hydroxyl groups, resulting in aminoglycosides that bind poorly to the target ribosomes. Within each class, there are enzymes with different specific sites of modification. More than 100 aminoglycoside-modifying enzymes have been described [26].

2.2 Resistance to β-Lactams

The β-lactams are a structurally diverse group of antimicrobials that interfere with the synthesis of the bacterial cell wall as a result of their interaction with penicillin-binding proteins (PBPs). Resistance to β-lactam antibiotics can be caused by four different mechanisms: (a) acquisition or hyperexpression of β-lactamases which is considered the most common resistance mechanism; (b) alteration, overexpression, or acquisition of PBPs; (c) permeability change in the outer membrane; and (d) active efflux of the antimicrobial [27, 28]. More than 1300 β-lactamases have been described to date [29]. β-Lactamases can be grouped on the basis of either their molecular structure or function. Four different molecular classes of β-lactamases have been defined based on the similarities in amino acid sequences. Class A, B, and C are serine β-lactamases, whereas class B are metallo-β-lactamases [30, 31].

2.3 Resistance to Glycopeptides

Glycopeptide antibiotics such as vancomycin and teicoplanin inhibit cell wall synthesis by binding to the terminal D-alanyl-D-alanine of the pentapeptide peptidoglycan precursor molecule. This binding prevents the cross-linking of peptidoglycan precursors necessary for the formation of cell wall. Acquired resistance to vancomycin in Gram-positive bacteria differs depending on the bacterial species where they have been described: (a) altered precursor formation in enterococci and staphylococci, (b) mutational cell wall changes in staphylococci, and (c) tolerance in *S. pneumoniae* [32–35]. To date, nine gene clusters conferring different glycopeptide resistance phenotypes have been described in enterococci; eight are acquired (*vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*), while the ninth (*vanC*) is usually intrinsic to *Enterococcus gallinarum* and *E. casseliflavus/flavescens* [32]. However, recent studies reported the presence of *vanC* genes in *E. faecalis* and *E. faecium* showing the ability of these genes to be transferred between bacteria [36–38]. The *vanA*, *vanB*, *vanD*, and *vanM* genes encode D-alanine-D-lactate ligases whereas the *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* genes encode D-alanine-D-serine ligases. Bacterial species other than enterococci have been described to contain *van* genes [39–55]. The world's first isolate of *S. aureus* containing *vanA* (vancomycin-resistant *S. aureus* (VRSA)) was reported in June 2002 in the United States [39]. Nowadays, the number of VRSA isolates worldwide is still low [34, 56–58]. Recently, *S. aureus* isolates containing *vanB* and both *vanA* and *vanB* were described in India, Iran, and Sudan [57–59].

2.4 Resistance to Macrolides, Lincosamides, and Streptogramins

Macrolides, lincosamides, and streptogramins (A and B) inhibit protein synthesis by reversibly binding to the peptidyl-tRNA binding region of the 50S ribosomal subunit, stimulating dissociation of the peptidyl-tRNA molecule from the ribosome during elongation [60, 61]. Three different mechanisms of macrolide, lincosamide, and streptogramin resistance have been described:

(a) Alterations in the ribosomal target site by several different acquired erythromycin ribosomal methylases (*erm*) that methylate the same adenine residue in 23S rRNA or by mutations in chromosomal genes (e.g., *rrl* gene encoding 23S rRNA), resulting in resistance against macrolides, lincosamides, and streptogramin B antibiotics (MLS_B) (alteration in the target site has not been described for streptogramin A resistance)
(b) Active efflux of the antimicrobial (e.g., *mef*(A) conferring macrolide resistance, *vga*(A) conferring streptogramin A resistance, and *msr*(A) conferring both macrolide and streptogramin B resistance)
(c) Drug inactivation by several different enzymes including esterases (*ere*), phosphorylases (*mph*), lyases (*vgb*), and transferases (*vat*) [62, 63]

2.5 Resistance to Quinolones

Quinolones interact with two type II topoisomerases, DNA gyrase, and topoisomerase IV, both of which are essential for bacterial DNA replication. Inhibition appears to occur by interaction of the drug with complex composed of DNA and either of these two target enzymes. The GyrA and GyrB subunits of DNA gyrase are respectively homologues with ParC and ParE subunits of topoisomerase IV. Quinolone resistance results mostly from chromosomal mutations in the drug target and alterations of drug access to target enzymes, either by altered permeation mechanism or increased drug efflux [64, 65]. Low-level resistance may also be mediated by (a) plasmid-encoded Qnr proteins which protect DNA gyrase from quinolone action, (b) an aminoglycoside acetyltransferase encoded by the *aac(6′)-Ib-cr* that acetylates quinolones, and (c) plasmid-mediated quinolone efflux pumps encoded by *qepA* [65, 66].

2.6 Resistance to Trimethoprim and Sulfonamides

Trimethoprim and sulfonamides are inhibitors of two enzymes (dihydrofolate reductase [DHFR] and dihydropteroic acid synthase [DHPS], respectively) that act sequentially in the formation of tetrahydrofolate (THF). The most common mechanism of trimethoprim resistance is the acquisition of low-affinity *dhfr* genes, of which approximately 20 have been described [67]. Resistance to trimethoprim can also be conferred by promoter mutations, leading to overproduction of DHFR, point mutations within the *dhfr* genes, or both mechanisms. Resistance to sulfonamides can be caused by acquisition of different low-affinity *dhps* genes and by point mutations in chromosomal *dhps* genes [67].

2.7 Resistance to Tetracyclines

The tetracyclines are a group of bacteriostatic antibiotics that act by binding reversibly to the 16S rRNA near the ribosomal acceptor A site, inhibiting the attachment of aminoacyl-tRNA to this site, thereby preventing the elongation step of protein

synthesis [68]. Tetracycline resistance is caused by four mechanisms: (a) active efflux which keeps tetracycline out of the cytoplasm, (b) ribosomal protection which prevents tetracycline from binding to the ribosome, (c) inactivation of the tetracycline molecules, and (d) rRNA mutations which prevents tetracycline from binding to the ribosome [68, 69].

2.8 Resistance to Chloramphenicol

Chloramphenicol binds to the 50S ribosomal subunit and inhibits prokaryotic peptidyl transferase. The most common mechanism of resistance to chloramphenicol is the production of chloramphenicol acetyltransferases (CATs) which inactivate the antibiotic. A large number of CAT genes have been reported, and these determinants generally confer high levels of resistance to chloramphenicol [70]. Resistance to chloramphenicol can also be caused by target site mutations, permeability barriers, phosphotransferase inactivation, and active efflux [71].

2.9 Resistance to Linezolid

Linezolid, an oxazolidinone antimicrobial, inhibits bacterial protein synthesis by binding to the domain V region of the 23S rRNA [72]. Resistance to linezolid is principally mediated by mutations in the central region of domain V of one or more alleles of the *rrl* gene encoding 23S rRNA [73]. Mutations in the *rplC* and *rplD* genes encoding the riboproteins L3 and L4, respectively, as well as methylation of the 23S rRNA base A2503, have also been described [73].

2.10 Resistance to Rifampin

Rifampin acts by binding to the beta subunit of the bacterial DNA-dependent RNA polymerase encoded by the *rpoB* gene resulting in transcription inhibition [74]. Resistance to rifampin is conferred by chromosomal mutations or short deletions and insertions in the central region of the *rpoB* gene [75].

2.11 Resistance to Isoniazid

Isoniazid is a synthetic antimicrobial agent used for the treatment of infections caused by *M. tuberculosis* complex. The precise mechanism of action is still unclear but the target seems to be inhibition of mycolic acid synthesis [76, 77]. Resistance to isoniazid may result from mutations in six different genes:

(a) The *katG* gene encoding a catalase-peroxidase
(b) The *inhA* gene encoding an enoyl reductase and its promoter
(c) The *ahpC* gene encoding an alkyl hydroperoxide reductase subunit and its promoter
(d) The *kasA* gene encoding a β-ketoacyl-acyl-carrier-protein synthase
(e) The *ndh* gene encoding a NAD dehydrogenase
(f) The *nat* gene encoding an arylamine N-acetyltransferase [75, 77–79]

2.12 Resistance to Ethambutol

Ethambutol is a synthetic antituberculosis agent. This compound alters outer mycobacterial membrane formation by inhibiting the synthesis of arabinogalactan [74, 80]. Resistance to ethambutol often results from mutations in the *embB* encoding the arabinosyltransferase [81].

2.13 Resistance to Pyrazinamide

Pyrazinamide, the pyrazine analog of nicotinamide, is a prodrug for *M. tuberculosis*, which requires conversion to the active pyrazinoic acid by the bacterial pyrazinamidase. Resistance to pyrazinamide is usually caused by mutations in the *pncA* gene encoding the pyrazinamidase or in the putative regulatory region upstream of the gene [82].

3 Methods to Detect Resistance

The clinical microbiology laboratory has the responsibility to provide reliable, accurate, and susceptibility data of significant bacterial isolates in a time frame that is useful to the clinicians to prescribe the most appropriate antimicrobial agent (least expensive and/or narrower spectrum) for a particular infection and, when possible, to reduce the development of resistance. Determining the antimicrobial susceptibility profile of a pathogen is considered as important as the identification of the pathogen involved in the infection. This is becoming even more essential with the increasing antimicrobial resistance in which treatment options are more limited. The antimicrobial susceptibility of a clinical isolate measured by conventional phenotypic susceptibility methods is presently the parameter provided to clinicians. However, an isolate which is defined as sensitive to an antimicrobial agent is not always treated with success, but most resistant isolates result in treatment failure. Therefore, it is important to develop methods that will allow detection of resistance mechanisms and to better understand which resistance mechanisms are the most difficult to detect

[83]. During the past years, optimization of conventional susceptibility tests and development of novel phenotypic methods for resistance mechanisms that are difficult to detect have been carried out [84, 85]. Moreover, several innovative phenotypic methods for antimicrobial susceptibility testing, such as matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), could provide valuable novel options in the near future [86, 87]. As alternative or complement to these phenotypic methods, several genotypic drug resistance assays have been developed and are increasingly used in the clinical microbiology laboratory offering rapid, accurate, and sensitive methods to detect the presence of antimicrobial resistance [16, 86, 88, 89].

3.1 Phenotypic Assays/Susceptibility Tests (Culture)

Conventional culture-based susceptibility methods measure the in vitro phenotypic expression of resistance which can be interpreted quantitatively as the minimum inhibitory concentration (MIC) or interpreted qualitatively as sensitive, intermediate, or resistant. MIC is defined as the lowest concentration of an antimicrobial agent that inhibits the visible growth of an organism over a defined interval. Several methods for routine antimicrobial susceptibility testing are used in the clinical microbiology laboratory [90]. The phenotypic resistance can be quantitatively reported as the MIC for dilution methods (broth and agar dilution) and antibiotic gradient diffusion (e.g., Etest) or may be expressed qualitatively with disk diffusion method (e.g., Bauer-Kirby disk diffusion). Broth microdilution methods have been adapted to automated instrument-based systems facilitating the reading and interpretation of results. These instruments can provide species identification and/or antibiotic susceptibility results within 24–48 h. The most common systems currently available include the Microscan WalkAway system (Siemens Healthcare Diagnostics), the Vitek 2 system (bioMérieux), the BD Phoenix system (BD Diagnostics), and the Sensititre system (TREK Diagnostic Systems). Detailed descriptions as well as advantages and limitations of these systems can be found in recent reviews [91, 92].

Regardless of the microorganism and antibiotic tested as well as the method used, the results obtained by in vitro antibiotic susceptibility testing can vary greatly depending on the culture medium, inoculum concentration of the organism tested, and the conditions of incubation (duration, temperature, and atmosphere). The Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [93–96] (clsi.org) and the European Committee on Antimicrobial Susceptibility (EUCAST) [97] (eucast.org) are the major bodies which contribute to antimicrobial susceptibility testing providing up-to-date guidelines on methodologies and standardized control procedures to ensure accuracy and reproducibility within and between laboratories [98]. Different national standardized methods may also be used including those published by the British Society for Antimicrobial Chemotherapy (BSAC) in the United Kingdom and equivalents in other countries [98, 99].

3.1.1 Clinical Significance of Breakpoints

The goal of antimicrobial susceptibility testing is to predict the clinical outcome by classifying a bacterial strain into clinically relevant categories (i.e., susceptible, intermediate, or resistant) on the basis of established breakpoints based on MIC. A breakpoint for an antibiotic is usually selected as the therapeutic concentration in blood that can be readily achieved with usual dosing regimens, but this is not easily determined. Several factors must be taken in consideration in establishing MIC breakpoints such as MIC distributions, pharmacokinetics and pharmacodynamics, clinical and bacteriological response rates, and zone diameter distributions for disk diffusion methods [100]. Breakpoints must be periodically reevaluated following change in bacterial resistance, susceptibility test methods, or antibiotic regimens. Determination of MIC is influenced in vitro by a number of variables such as the composition of the test medium, the inoculum size, the incubation time, and the presence of resistant subpopulations of the organisms. Moreover, the condition tested in vitro for determining MIC cannot mimic other factors that can influence the in vivo antimicrobial activity including sub-MIC effects, postantibiotic effects, protein binding, variations in redox potential at sites of infection, and differences in drug levels in blood and at the site of infection [100]. Nevertheless, when the MIC is determined under standardized condition, it provides a convenient reference point for the setting of breakpoints to predict the efficacy in vivo.

The interpretative breakpoints assigned to an antimicrobial agent can have a significant impact on the prescribing of that drug for empiric therapy by influencing the resistance rates measured at the local, regional, national, or international level [101]. In North America, the CLSI has the responsibility to establish breakpoints. However, different countries have defined different breakpoints to define resistance [98] (see Sect. 3.1). This difference may be related to different dosages or administration intervals or can result from technical aspects such as different susceptibility test media and test conditions [102]. Moreover, some countries may be more or less conservative in determining susceptibility. Therefore, it is sometimes confusing to compare resistance rates among countries if different methods have been used. In Europe, MIC breakpoints have been harmonized by EUCAST. However, there is an urgent need that susceptibility methods and MIC breakpoints be harmonized at the international level [98, 103].

3.2 Special Phenotypic Susceptibility Methods

Conventional susceptibility testing methods or automated systems are not reliable for certain fastidious organisms or organisms with difficult-to-detect resistance mechanisms. Fastidious organisms (e.g., *Mycobacterium* species, *Streptococcus* species including *S. pneumoniae*, *Haemophilus* species, *N. gonorrhoeae*, and anaerobic bacteria) require special growth media and conditions, and certain organisms with inducible resistance or subtle change in MICs (at or near the breakpoint) (e.g., methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE), vancomycin-intermediate *S. aureus* (VISA), as well as carbapenemase-producing *Enterobacteriaceae* (CPE)) require special phenotypic testing methods for detection of antimicrobial resistance. A complete description of susceptibility test methods used for these fastidious organisms and difficult-to-detect resistance mechanisms can be found in recent reviews [77, 84, 104, 105]. Some examples of phenotypic methods used for difficult-to-detect resistance are further described.

3.2.1 Detection of Oxacillin Resistance in Staphylococci

Oxacillin resistance in *S. aureus* results from at least three different resistance mechanisms:

(a) Acquisition of a mobile genetic element, the staphylococcal cassette chromosome (SCC*mec*) carrying the *mecA* gene which encodes an altered PBP—PBP2a/PBP2′—which has reduced affinity for β-lactam antibiotics (a new *mecA* homologue named *mecC* (originally $mecA_{LGA251}$), has been recently described [106–108].
(b) Inactivation of the drug by increased production of ß-lactamase which results in low-level or borderline resistance (BORSA).
(c) Production of modified intrinsic PBPs (MOD-SA) with altered affinity for the drug which also results in borderline resistance [84, 109, 110].

It is important to differentiate isolates that have *mecA*- and *mecC*-positive resistance from isolates that have the two other types of resistance because *mecA* and *mecC* genes confer resistance to all ß-lactams. Moreover, isolates carrying *mecA* are also usually multidrug resistant which is not the case for the two other types of resistance and for *mecC* isolates [108]. Although the majority of MRSA isolates are susceptible to the novel anti-MRSA extended-spectrum cephalosporin ceftaroline that has been approved recently in the United States and elsewhere in the world [111], some *S. aureus* isolates resistant to this new drug were recently described [112–114]. Moreover, some new emerging community-associated *mecA*-positive MRSA strains (named CA-MRSA) are usually susceptible to non-β-lactam antibiotics [115].

Some isolates carrying *mecA* are either homogeneous or heterogeneous in their expression of resistance. Contrary to isolates with homogeneous resistance, heterogeneous resistance results in MICs that appear to be borderline and can be confused with BORSA or MOD-SA isolates for which MICs are also borderline. To detect heterogeneous subpopulation, conventional susceptibility test with oxacillin requires special media, incubation temperature, and time. Moreover, some rare MRSA strains are inducibly resistant and need specific procedures to be detected [111]. BORSA resistance can usually be distinguished from *mecA* resistance by the addition of a ß-lactamase inhibitor (e.g., clavulanic acid) to the oxacillin MIC test, which lowers the MIC by two dilutions or more [84]. During the past years, cefoxitin has been shown to be more sensitive than oxacillin for detection of isolates containing *mecA* and is now widely used to predict *mecA*-mediated oxacillin resistance as a surrogate for oxacillin in MIC and disk diffusion methods [84, 93]. Moreover, cefoxitin is more reliable than oxacillin to detect *mecC* MRSA [116]. However, this test does not detect BORSA and MOD-SA isolates [84]. Therefore, both CLSI and EUCAST recommend testing both oxacillin and cefoxitin to detect all possible oxacillin resistance mechanisms [111].

Other commercially available methods are widely used in the clinical microbiology laboratory for rapid confirmation of oxacillin resistance in *S. aureus* isolates such as latex agglutination tests that detect the presence of PBP 2a in about 15–20 min including the MRSA-Screen test (Denka Seiken Co., Ltd.), the PBP 2′ latex agglutination test (Oxoid Limited), the Mastalex-MRSA test (Mast Diagnostics), and the Slidex MRSA detection test (bioMérieux) [84]. However, these methods do not detect isolates containing *mecC* [106]. The automated instruments described in Sect. 3.1 for bacterial identification and susceptibility testing have been improved during the past years and can now identify MRSA isolates reliably.

Rapid and accurate identification of MRSA isolates is essential not only for patient care but also for effective infection control programs to limit the transmission of MRSA [117–122]. Active surveillance for identification of MRSA carriers, either targeted or universal, is one of the most common and effective measure to prevent the spread of MRSA in healthcare facilities [2, 118, 123, 124]. However, culture-based methods for identification of *S. aureus* combined with susceptibility testing, as described above, usually requires at least 48 h. In recent years, several commercial chromogenic media containing a selective antibiotic (e.g., cefoxitin) have been developed (e.g., BBL-CHROMagar MRSA from BD Diagnostics, CHROMagar MRSA from CHROMagar Microbiology, Brilliance MRSA agar from Oxoid,

MRSA*Select* from Bio-Rad, and ChromID MRSA from bioMérieux) allowing direct colony identification of MRSA from the primary screening culture, obviating the need for subculture, and reducing the time to results to 20–26 h. The performance of these different media has been evaluated in several studies and is described in recent reviews [111, 121, 125, 126]. Sensitivity and specificity of these media are highly variable. A prolonged incubation time of 48 h or a selective enrichment broth prior to inoculation of the sample to these chromogenic media has been shown to improve sensitivity but increasing time for MRSA detection.

3.2.2 Detection of Vancomycin Resistance in Enterococci and Staphylococci

Among the nine glycopeptide resistance phenotypes described to date in enterococci [32] (see Sect. 2.3), three are most commonly found in the clinical settings: (a) high-level vancomycin resistance with teicoplanin resistance (VanA phenotype); (b) moderate- to high-level vancomycin resistance, usually without teicoplanin resistance (VanB phenotype); and (c) intrinsic low-level resistance associated with *E. gallinarum* and *E. casseliflavus/flavescens* (VanC phenotype) [84]. In the past, standard culture-based methods (especially disk diffusion) and automated systems commonly used in clinical laboratories sometimes failed to detect low-level vancomycin resistance (VanB and VanC phenotypes) in certain enterococcal strains. However, some automated systems such as the Vitek 2 (bioMérieux) and BD Phoenix (BD Diagnostics) systems have now been enhanced, and new recommendations have been added by the CLSI to improve vancomycin disk diffusion method [35, 84]. The use of the vancomycin agar screening test is recommended by the CLSI to detect low-level vancomycin resistance in enterococci [93]. However for an infection control perspective, it is important to distinguish the *vanC*-containing enterococcal species that can grow on the agar screening plate from the clinically important vancomycin-resistant enterococci (VRE), which include *E. faecalis* and *E. faecium* [93] (see Sect. 3.3.3). Special phenotypic tests are needed to differentiate these enterococcal species [84].

Active screening of VRE using rectal/perirectal specimen or stool specimen to detect carriage in at-risk patients has been shown to contribute to reducing VRE colonization, infections, and healthcare costs [127, 128]. Several selective agar media are commercially available (e.g., *Brilliance* VRE agar from Oxoid, chromID VRE from bioMérieux, CHROMagar VRE from CHROMagar Microbiology, and VRE*Select* from Bio-Rad) for VRE screening and have been shown to reduce time to identify VRE carriage. The performance of these selective media for detecting VRE has been evaluated in several studies [129–133]. While most agar media demonstrated excellent specificity, the sensitivity of these media (especially when assessed at 24 h versus 48 h) was suboptimal for detecting *vanB*-VRE with low vancomycin MICs.

Three types of vancomycin resistance phenotypes have been described in *S. aureus*: (1) vancomycin-intermediate resistant (VISA), (2) heterogeneous VISA, and (3) vancomycin resistant (VRSA) [33]. Whereas resistance to vancomycin in VRSA usually results from acquisition of the *vanA* gene (see Sect. 2.3), diverse mutations in a small number of staphylococcal chromosomal regulatory genes have been associated with VISA and hVISA, resulting in changes in cell wall volume and composition. The cell wall of these strains is usually thickened and is thought to prevent the diffusion of vancomycin to its active site in the cytoplasmic membrane in the division septum [33]. VRSA strains can be detected accurately with standard disk diffusion and broth microdilution methods as well as with the vancomycin agar screening test used to detect VRE and most commercial methods. However, detection of VISA strains is reliably detected only with the broth microdilution method [84]. Recently, some *S. aureus* isolates containing *vanA* or *vanB* with vancomycin-intermediate resistance or vancomycin-susceptible phenotype have been reported [57, 134].

3.2.3 Detection of Carbapenemase Producers in Gram-Negative Bacteria

Resistance to carbapenems in Gram-negative bacteria mainly results from the acquisition of carbapenemase-encoding genes, although other resistance mechanisms may be involved such as decreased permeability of the outer membrane due to porin alteration or high efflux pump activity [135]. Carbapenemases are β-lactamases being able to hydrolyze carbapenems (imipenem, ertapenem, meropenem, and doripenem) and most other β-lactams and are located on mobile genetic elements carrying many other resistance determinants, thus giving rise to transmissible multidrug resistance and even pandrug resistance and limiting the choice of antibiotic treatment [136]. Carbapenemases belong to three classes of β-lactamase, namely, class A (e.g., KPC type), class B or metallo-β-lactamases (MBLs) (e.g., IMP, NDM, and VIM types), and class D (e.g., OXA-48 type), each class harboring specific hydrolytic properties against β-lactams [135, 137]. Carbapenemase-producing Gram-negative bacteria (CPGN), mainly *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii*, are associated with increased mortality and have become a major concern worldwide [138–141]. The high rate of transmissibility of plasmid-encoded carbapenemase genes and their association with multiple antibiotic resistance determinants explain the need for detection of carbapenemase producers both for selecting the appropriate therapy and implementing effective infection control measures [142].

Detection of carbapenemases is usually performed on isolates which are resistant to carbapenem based on MIC

breakpoints [143]. However, carbapenem resistance in Gram-negative bacteria can usually be detected reliably only based on susceptibility testing results obtained with standard broth microdilution and disk diffusion methods since automated antimicrobial testing systems or gradient diffusion technologies such as the Etest have shown poor performance [144]. Nevertheless, some carbapenemase producers with MICs for carbapenems lower than the established CLSI breakpoints may not be detected, especially OXA-48-type producers [136]. It has recently been proposed that a search for carbapenemase production should be performed on any enterobacterial isolate that exhibits even a slight decrease in susceptibility to carbapenems compared with a wild-type phenotype [136].

Several phenotypic methods can be used to confirm carbapenemase activity. The CLSI recommends the modified Hodge test (MHT) to screen for production of carbapenemases in *Enterobacteriaceae* [93]. However, some isolates producing AmpC-type β-lactamases or ESBL coupled to porin loss can give false positive, and a lack of sensitivity has been reported for detection of class B carbapenemases [136, 144]. Other tests can also be performed to identify carbapenemase producers with molecules inhibiting carbapenemases and/or other types of β-lactamases using double-disk synergy and combined-disk methods [136, 144, 145]. Combination-disk tests based on the specific inhibition properties of the different carbapenemases are commercially available (e.g., KPC/MBL and OXA-48 Confirm Kit from Rosco Diagnostica and Carbapenemase Detection Kit from Mast Diagnostics). The sensitivity and specificity of these methods are highly variable depending on the carbapenemases detected [136, 143–147].

Recently, Nordmann et al. described a new rapid (<2 h) chromogenic carbapenemase detection assay based on hydrolysis of the β-lactam ring of imipenem, the Carba NP test [148–150]. This assay has been validated in several studies for detection of carbapenemase producers among *Enterobacteriaceae*, *Pseudomonas* spp., and *Acinetobacter* spp. Reported sensitivities varied between 78.9 and 100 %, whereas specificity was generally reported to be 100 % [148–155]. Two commercial versions of this test are now available (RAPIDEC CARBA NP from bioMérieux and Rapid CARB Screen Kit from Rosco Diagnostica). The performance of the Rapid CARB Screen Kit has been recently evaluated for detection of carbapenemase producers in *Enterobacteriaceae* and *P. aeruginosa* and was shown to perform less well than the Carba NP test [156].

Recently, several studies have reported methods to identify carbapenemase activity with Gram-negative bacterial isolates using MALDI-TOF-MS which detect hydrolysis of the carbapenem ring [145]. MALDI-TOF-MS has also been evaluated to detect cabapenemase activity directly from positive blood cultures [157]. These methods appear to be simple, rapid, and reliable to identify carbapenemases.

To cope with the increasing emergence and high transmission potential of carbapenemase producers, screening of stools or rectal swabs is recommended by the Centers for Disease Control and Prevention to identify carriers of carbapenemase-producing *Enterobacteriaceae* (CPE) and initiate appropriate infection control measures [158]. To screen for CPE carriers, the stools or rectal swabs are usually plated on selective media containing carbapenems [159, 160]. Selective chromogenic culture media are commercially available (e.g., Brilliance CRE from Oxoid, CHROMagar KPC from CHROMagar, and chromID Carba from bioMérieux) for detection of carbapenemase producers. The performance of these culture media has been evaluated in several studies which are described in recent reviews [136, 144, 145, 161]. These media showed variable specificity, and their sensitivity depends on the MICs of the carbapenemase-producing isolates. Carbapenemase producers growing on selective culture media should be confirmed by susceptibility testing and carbapenemase activity by the methods described above.

3.3 Genotypic Assays

Phenotypic methods for susceptibility testing are usually simple, and automated systems have greatly facilitated the susceptibility testing procedures and data analysis [91, 92]. Even though incubation time to obtain susceptibility data is reduced to 3–5 h with some automated systems or shorter (15–20 min) with special susceptibility tests (e.g., MRSA-Screen test) (see Sect. 3.2.1), all the phenotypic susceptibility methods require bacterial isolation, and hence the results are not available until 24 h or more after a treatment is started. Several of the presently performed susceptibility tests are highly dependent on experimental conditions, and special phenotypic tests must often be performed to obtain an accurate susceptibility profile. Moreover, there is presently no international agreement on susceptibility methods and interpretation of breakpoints in antimicrobial susceptibility testing (see Sects. 3.1 and 3.1.1).

There are several advantages of using genotypic methods for resistance testing compared to conventional susceptibility methods [86, 88, 162]:

(a) Detection of resistance genes is more accurate for detection of isolates with difficult-to-detect resistance profiles (MICs at or near the breakpoint or inducible resistance) since it does not depend on the variable gene expression under laboratory conditions [85].
(b) Genotypic tests can provide resistance profiles rapidly (less than 1 h with some molecular methods) since they

can be performed directly from clinical specimens; this is particularly important not only for organisms that cannot be cultured and are not easily cultured or for slow-growing organisms but also to choose the most appropriate therapy early in the course of disease before cultures are positive.

(c) Genotypic tests may diminish the biohazard risk associated with the propagation of a microorganism by culture.

(d) Genotypic tests are a powerful tool for epidemiological study of antimicrobial resistance in a hospital or the community by providing an immediate insight into the genetic mechanism underlying the resistance phenotype.

(e) Genotypic tests can be used as a gold standard for evaluating new improved susceptibility methods for testing clinical isolates with difficult-to-detect resistance profiles.

With the progress in the understanding of antimicrobial resistance mechanisms and the increasing number of resistance gene sequences available in public databases [163], several genotypic assays have been developed for detection of bacterial genes and mutations associated with resistance. Some reviews provided more details on several genotypic resistance assays [16, 86, 88, 89, 164–166]. Table 85.1 shows the most common antibiotic resistance genes for which genotypic resistance assays have been developed. For the majority of these genes, the genotypic resistance assays developed to date are in-house tests that have not been necessary validated through a thorough process by clinical microbiology laboratories. However, guidelines which described validation of laboratory-developed tests have been published by the CLSI [167]. Moreover, the number of commercially available kits which are Food and Drug Administration (FDA)-cleared and/or European Community (CE)-marked (Table 85.2) as well as analyte-specific reagents (ASRs) has considerably increased in recent years and has facilitated the use of these technologies. Nevertheless, ASRs still need to be optimized and validated by the users in the clinical microbiology laboratories under the Clinical Laboratory improvement Amendments (CLIA) [168], making it more and more difficult for laboratories to use ASRs [88].

Genotypic resistance assays usually target a nucleic acid sequence containing a part of, or the entire resistance gene, or the mutations associated with resistance which can be detected by DNA probe techniques or amplification technologies. However, non-amplified probe technologies are not sensitive enough to detect the small number of bacterial cells found in most clinical specimens and are therefore limited for direct detection of pathogens in specimens in which the number of organisms is large. For example, the *mecA Xpress*FISH (AdvanDX), a fluorescence in situ hybridization (FISH) assay using PNA probes, detects *mecA* from positive blood cultures [169]. Amplification techniques used for genotypic testing comprise:

1. Nucleic acid target amplification [e.g., polymerase chain reaction (PCR) including reverse transcriptase PCR (RT-PCR), nested PCR, multiplex PCR, real-time PCR, and digital PCR, as well as several isothermal amplification methods such as strand displacement amplification (SDA), self-sustaining sequence replication, transcription-mediated amplification (TMA), nucleic acid sequence-based amplification (NASBA), helicase-dependent amplification (HAD), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA)]
2. Probe amplification (e.g., ligase chain reaction, cycling probe technology, and cleavase-invader technology)
3. Signal amplification (e.g., signal-mediated amplification of RNA technology (SMART) and branched DNA (bDNA) assay) [165, 170, 171]

The amplification product or amplicon can be detected following amplification by different methods; the most common are hybridization arrays, line probe assays, gel and capillary electrophoresis methods, colorimetric microtiter plate systems, restriction fragment length polymorphism (RFLP) analysis, DNA sequencing, or mass spectrometry. A comprehensive description of these technologies is provided in recent reviews [170–172].

PCR amplification is the most commonly used nucleic acid amplification technique for the detection of antimicrobial resistance genes. However, the combination of PCR amplification with post-PCR amplicon detection have found limited acceptance for diagnostic laboratory testing due to the time-consuming nature of these post-PCR detection approaches and the problem of carry-over contaminations [173]. Real-time PCR is the most widely used technology for genotypic resistance testing in routine microbiology laboratories because this closed-tube amplification process, which is monitored in real time by using fluorescence techniques, is fast due to ultrarapid thermal cycling and easy to perform, while the risk of carry-over is minimized [174]. A variety of in-house or commercial real-time PCR assays have been developed for detection of antibiotic resistance genes and mutations (Tables 85.1 and 85.2). Real-time multiplex PCR can be used when there is a need to detect several bacterial species and/or antimicrobial resistance genes. However, real-time multiplex PCR is limited by the number of genetic targets that can be simultaneously detected because of the restricted number of fluorophores that can be discriminated by the optical detection systems. To detect a broader range of microbes and antimicrobial resistance genes present in some

Table 85.1 Common bacterial drug resistance genes detected by genotypic methods

Antimicrobial agent	Gene (reference)
Aminoglycosides	***aac (2 ′)- Ia*** [337], ***aac (3)- Ia*** [182, 201, 203, 337–344], ***aac (3)- Ib*** [337], ***aac (3)- IIa*** [203, 337, 340–343], ***aac (3)- IIc*** [345], ***aac (6 ′)- aph (2 ″)*** [179, 187, 298, 346–356], ***aac (6 ′)- Ia*** [337, 357, 358], ***aac (6 ′)- Ib*** [179, 182, 201, 203, 338, 343, 357–361], ***aac (6 ′)- Ic*** [337], ***aac (6 ′)- II*** [344, 362], ***aac (6 ′)- Ih*** [343, 358], ***aac (6 ′)- IIa*** [339], ***aac (6 ′)- IIb*** [339], ***ant (2 ″)- Ia*** [182, 201, 203, 337–343, 363], ***ant (3 ″)- Ia*** [203, 205, 337, 340, 341, 344, 363–366], ***ant (4 ′)- Ia*** [187, 341, 347, 349–352, 355, 356], ***ant (4 ′)- IIa*** [203, 337, 342], ***ant (4 ')- IIb*** [360], ***ant (6)- Ia*** [182, 187, 203, 355], ***ant (6)- Ib*** [205], ***ant (6 ′)- Ie*** [187], ***ant (6 ′)- Ii*** [187], ***ant (6 ′)- Im*** [187], ***ant (9)- Ia*** [187], ***ant (9)- Ib*** [205], ***aph (2 ″)- Ia*** [187], ***aph (2″)- Ib*** [187, 203, 348, 356], ***aph (2″)- Ic*** [187, 203, 355, 356], ***aph (2″)- Id*** [187, 203, 348, 356], ***aph (2″)- Ie*** [205], ***aph (3 ′)- Ia*** [203, 337, 343], ***aph (3 ′)- Ib*** [203], ***aph (3 ′)- IIa*** [203, 341, 367], ***aph (3 ′)- IIb*** [339, 360], ***aph (3 ′)- III*** [187], ***aph (3 ′)- IIIa*** [187, 203, 347, 349–351, 355, 356, 367], ***aph (3 ′)- IVa*** [187, 203], ***aph (3 ′)- VIa*** [340, 343, 368], ***aph (6)- Id*** [182, 337, 366], ***armA*** [359, 369–372], ***rmtA*** [369, 370, 372], ***rmtB*** [359, 369–372], ***rmtC*** [179, 369–372], ***rmtD*** [369, 370], ***rmtE*** [373], ***rmtF*** [374], ***npmA*** [369, 375], ***rpsL***[a] [376–383], ***rrs***[a] [209, 378, 379, 381–390], ***eis* / *eis* promoter**[a] [385–387, 389, 390], ***tlyA***[a] [385, 389, 390]
β-Lactams	***bla*SHV-type**[b] [201–204, 291, 359, 391–399], ***bla*TEM-type**[b] [188, 189, 201–204, 291, 359, 363, 391, 392, 394, 395, 398–403], ***bla*CTX-M-type** [201–204, 291, 359, 392, 393, 398, 399, 403–408], ***bla*PER** [179, 203, 392, 399], ***bla*GES-type** [179, 203, 399, 409, 410], ***bla*VEB** [179, 203, 399], ***bla*SFO** [398, 411], ***bla*KPC** [179, 203, 286, 289, 290, 359, 399, 409, 410, 412], ***bla*OXA-48-like** [179, 287, 291, 399, 410, 412], ***bla*NDM** [288, 291, 359, 410, 412, 413], ***bla*VIM** [203, 291, 344, 359, 399, 408, 410, 412–417], ***bla*IMP** [179, 189, 203, 291, 344, 359, 361, 399, 407, 408, 410, 412, 413, 415–419], ***bla*SME** [203, 359, 409, 410], ***bla*IMI/NMC-A** [179, 409, 410], ***bla*SPM** [179, 203, 291, 408, 410, 412, 413, 417, 419], ***bla*GIM** [179, 203, 410, 412, 413], ***bla*AIM** [291, 408, 412], ***bla*SIM** [203, 408, 410, 412, 413], ***bla*DIM** [291, 412], ***bla*BIC** [291, 412], ***bla*MOX** group [179, 201, 203, 399, 410, 420, 421], ***bla*DHA** group [201, 203, 399, 420, 421], ***bla*FOX** group [201, 203, 399, 420, 421], ***bla*CIT** group [179, 182, 201, 203, 399, 420, 421], ***bla*EBC** group [399, 420, 421], ***bla*ACC** group [201, 203, 399, 420, 421], ***bla*OXA-23-like** [179, 291, 408, 410, 413], ***bla*OXA-24/40-like** [179, 408, 410, 413], ***bla*OXA-58-like** [179, 291, 408, 410, 413], ***blaZ*** [187, 203, 297, 352, 353, 422, 423], ***mecA*** [184, 187, 203, 225–244, 297, 346, 349–352, 424–442], ***mecC*** [205, 225, 226, 243, 443], **SCC *mec* / *orfX*** [22, 441, 442, 444], ***pbp1a***[a] [203, 445–448], ***pbp2b***[a] [445, 446, 448–452], ***pbp2x***[a] [445, 446, 448]
Macrolides, lincosamides, streptogramin B	***erm* (A)** [180, 187, 203, 298, 346, 352, 353, 453–456], ***erm* (B)** [180, 187, 203, 298, 445, 453–458], ***erm* (C)** [180, 187, 203, 298, 346, 352, 353, 454–456]
Macrolides, streptogramin B	***msr* (A)** [180, 187, 203, 298, 352, 353, 454, 456, 459]
Macrolides	***mef* (A)** [187, 445, 453, 455, 457, 460], ***rrl***[a] [454, 456, 458, 461–466]
Streptogramin A	***vga* (A)** [203, 205, 352, 467–469], ***vga* (B)** [187, 203], ***vga* (C)** [205, 454] ***vat* (A)** [187, 203, 346, 352, 469], ***vat* (B)** [187, 203, 346, 352, 469], ***vat* (C)** [187, 346], ***vat* (D)** [187, 203, 205, 467, 469], ***vat* (E)** [179, 187, 203, 205, 467, 470]
Streptogramin B	***vgb* (A)** [187, 203, 352], ***vgb* (B)** [187, 203]
Linezolid	***rrl***[a] [471–476], ***rplC***[a] [477], ***rplD***[a] [476, 478], ***rplV***[a] [477], ***cfr*** [476, 479]
Quinolones	***gyrA***[a] [181, 184, 186, 190, 200, 209, 377, 378, 381, 383, 385, 386, 388, 389, 480–507], ***gyrB***[a] [184, 190, 378, 388, 389, 480, 482, 483, 489–491, 494, 495, 500, 503, 507, 508], ***parC* (*grlA*)**[a] [181, 184, 190, 200, 480, 483, 486, 487, 489, 490, 493–496, 498, 500, 503, 505, 509–511], ***parE* (*grlB*)**[a] [184, 190, 200, 480, 483, 487, 489, 490, 493–495, 500, 503], ***qnrA*** [32, 201, 359, 503, 512, 513], ***qnrB*** [32, 359, 503, 512], ***qnrC*** [512, 513], ***qnrD*** [513, 514], ***qnrS*** [32, 503, 513], ***aac*[6]- *Ib - cr*** [359, 503, 513, 515, 516], ***qepA*** [32, 503, 513, 515], ***oqxAB*** [512, 515, 517]
Chloramphenicol	***cat1*** [341, 363], ***cat2*** [341, 363], ***catP*** [187, 518], ***catQ*** [187, 518], ***catpC194*** [187, 203, 205, 518, 519], ***catpC221*** [187, 205, 518], ***florR*** [189, 201, 203, 341, 363–365, 520], ***cmlA*** [201, 341, 521, 522], ***cmlB*** [341]
Ethambutol	***embB***[a] [377, 379–383, 385, 502, 523–528]
Pyrazinamide	***pncA***[a] [199, 377, 380, 381, 385, 499, 501, 526, 529–534]
Rifampin	***rpoB***[a] [183, 191–198, 209, 377–380, 382, 383, 385, 386, 389, 502, 507, 524, 526, 528, 531, 535–560]
Isoniazid	***katG***[a] [183, 196, 197, 209, 377, 378, 380, 382, 383, 386, 389, 502, 507, 508, 524, 526, 528, 536, 545–550, 555, 561], ***inhA* / *inhA promoter***[a] [183, 197, 209, 378, 379, 382, 385, 386, 389, 502, 508, 526, 536, 545–547, 549, 556, 558, 562], ***ahpC* / *aphC* promoter**[a] [183, 209, 380, 526, 546, 549, 563–565], ***kasA***[a] [564], ***ndh***[a] [564], ***nat***[a] [78]
Vancomycin	***vanA*** [52, 187, 203, 257, 260, 264, 266–268, 354, 443, 566–575], ***vanB*** [52, 187, 203, 257, 264, 266–268, 354, 443, 566–568, 570–573, 575], ***vanC*** [187, 203, 257, 354, 566, 567, 571–573], ***vanD*** [52, 187, 203, 257, 354, 443, 576], ***vanE*** [52, 187, 203, 577], ***vanG*** [52, 187, 203, 578]
Tetracycline	***tet* (B)** [189, 201, 203, 341, 579–582], ***tet* (K)** [187, 203, 346, 352, 443, 458, 579, 580, 582–584], ***tet* (L)** [203, 443, 458, 580, 582–584], ***tet* (M)** [187, 203, 205, 346, 352, 443, 504, 580–585], ***tet* (O)** [205, 579–585], ***rrs***[a] [586, 587]
Sulfonamides	***sul1*** [189, 201, 203, 308, 341, 363, 401, 588–590], ***sul2*** [189, 201, 203, 308, 341, 363, 588, 590], ***sul3*** [201, 203, 308, 341, 521, 588, 590, 591]
Trimethoprim	***dhfrIa*** [67, 189, 363, 591–593], ***dhfrIb*** [67, 189, 592, 593], ***dhfrV*** [67, 203, 591–593], ***dhfrVI*** [67, 203, 592, 593], ***dhfrVII*** [67, 201, 203, 591–593], ***dhfrVIII*** [67, 203, 592–594], ***dhfrXII*** [201, 203, 591–593], ***dhfrXV*** [203, 595], ***dhfrXVII*** [201, 203, 591]

[a]Nucleotide mutations conferring resistance are usually detected in these genes
[b]Nucleotide mutations conferring resistance to extended-spectrum β-lactams can be detected in these genes

Table 85.2 Commercial bacterial genotypic drug resistance assays [CE IVD[a] or FDA 510(k)[b]]

Antimicrobial agent and resistance gene target	Organism	Molecular method	Manufacturer
Glycopeptides			
vanA + *vanB*	Enterococci	Real-time PCR	BD GeneOhm VanR Assay (BD Diagnostics) Xpert *vanA*/*vanB* (Cepheid)
23S rDNA + *vanA* + *vanB* + *vanC1* + *vanC2*/*C3*	Enterococci	PCR/DNA strip hybridization	GenoType Enterococcus (Hain Lifescience)
β-lactams			
mecA	Staphylococci	Real-time PCR	LightCycler SeptiFast MecA Test (Roche)
mecA	*S. aureus*	DNA probe	*mecA Xpress*FISH (AdvanDx)
23S rDNA + mecA + *mecC* + *lukS-lukF*	*S. aureus* + *S. epidermidis*	PCR/DNA strip Hybridization	GenoType MRSA (Hain Lifescience)
23S rDNA + *mecA* + *lukS-lukF*	Staphylococci	PCR/DNA strip hybridization	GenoType Staphylococcus (Hain Lifescience)
ldh1 + *mecA*	*S. aureus*	Real-time PCR	MRSA/SA ELITe MGB (ELITechGroup)
orfX-SCC*mec*	*S. aureus*	PCR/DNA strip hybridization	GenoType MRSA Direct (Hain Lifescience) GenoQuick MRSA (Hain Lifescience)
orfX-SCC*mec*	*S. aureus*	Real-time PCR	BD GeneOhm MRSA Assay (BD Diagnostics) Xpert MRSA Assay (Cepheid) LightCycler MRSA Advanced Test (Roche) FluoroType MRSA (Hain Lifescience)
Capsular polysaccharide enzyme-encoding gene + *orfX*-SCC*mec*	*S. aureus*	Real-time PCR	Cobas MRSA/SA Test (Roche)
nuc + *orfX*-SCC*mec*	*S. aureus*	Real-time PCR	BD GeneOhm StaphSR Assay (BD Diagnostics)
spa + *orfX*-SCC*mec* + *mecA*	*S. aureus*	Real-time PCR	Xpert MRSA/SA Nasal Complete Assay (Cepheid) Xpert MRSA/SA SSTI Assay (Cepheid) Xpert MRSA/SA Blood Culture Assay (Cepheid)
orfX-SCC*mec* + *mecA*	*S. aureus*	NASBA[c]	NucliSENS EasyQ MRSA Assay (bioMérieux)
orfX-SCC*mec* + *mecA* + *mecC*	*S. aureus*	Real-time PCR	BD MAX MRSA XT (BD Diagnostics) BD MAX StaphSR Assay (BD Diagnostics)
$bla_{\text{CTX-M-1, CTX-M-2, CTX-M-9}}$ groups + bla_{SHV} ESBL	*Enterobacteriaceae*	Real-time PCR	Check-*Direct* ESBL for BD MAX (Check-Points) Check-*Direct* ESBL Screen for BD MAX (Check-Points)
$bla_{\text{CTX-M-1, CTX-M-2, CTX-M-9}}$ groups + bla_{TEM} ESBL + bla_{SHV} ESBL	*Enterobacteriaceae*	Ligation-mediated real-time PCR	Check-MDR ESBL

(continued)

Table 85.2 (continued)

Antimicrobial agent and resistance gene target	Organism	Molecular method	Manufacturer
bla_{KPC} + $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM} + bla_{VIM} + bla_{IMP+} bla_{GES} + bla_{GIM} + bla_{SPM+} $bla_{OXA\text{-}23\text{-}like,\ \text{-}24\text{-}like,\ \text{-}58\text{-}like}$ + $bla_{CTX\text{-}M\text{-}1\text{-}like,\ CTX\text{-}M\text{-}2,\ CTX\text{-}M\text{-}3\text{-}like,\ CTX\text{-}M\text{-}15\text{-}like,\ CTX\text{-}M\text{-}32\text{-}like,\ CTX\text{-}M\text{-}8\ \&\ \text{-}25,\ CTX\text{-}M\text{-}9}$ groups + bla_{TEM} wt[d] + bla_{TEM} ESBL[e] + bla_{SHV} wt[d] + bla_{SHV} ESBL[e] + bla_{VEB} + bla_{PER} + bla_{BEL} + bla_{GES} + $bla_{CMY\text{-}1/MOX}$ + bla_{ACC} bla_{DHA} + $bla_{ACT/MIR}$ + $bla_{CMY\ II}$ + bla_{FOX}	Gram-negative bacteria	PCR/hybridization	Check-MDR CT103XL (Check-Points)
Bacterial specific genes + bla_{KPC} + bla_{VIM} + bla_{NDM} + bla_{IMP} + $bla_{OXA\text{-}23,\ \text{-}40,\ \text{-}48,\ \text{-}58}$ + $bla_{CTX\text{-}M}$	Gram-negative bacteria	Microarray gold nanoparticle probe assay	Verigene Gram-Negative Blood Culture (BC-GN) Nucleic Acid Test (Luminex)
bla_{KPC} + $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM} + bla_{VIM} + bla_{IMP}	Gram-negative bacteria	Real-time PCR	Xpert Carba-R (Cepheid)
bla_{KPC} + $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM} + bla_{VIM}	*Enterobacteriaceae*	Real-time PCR	Check-*Direct* CPE (Check-Points) Check-*Direct* CPE for BD MAX (Check-Points) Check-*Direct* CPE Screen for BD MAX (Check-Points)
bla_{KPC} + $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM} + bla_{VIM} + bla_{IMP}	Gram-negative bacteria	Ligation-mediated real-time PCR	Check-MDR Carba (Check-Points)
bla_{KPC} + $bla_{OXA\text{-}48\text{-}like}$ + bla_{NDM} + bla_{VIM} + $bla_{CTX\text{-}M\text{-}1\ \&\ CTX\text{-}M\text{-}9\ group}$	Gram-negative bacteria	Real-time LAMP[f]	eazyplex SuperBug CRE (Amplex BioSystems)
β-Lactams + glycopeptides			
Bacterial specific genes +*vanA* + *vanB* + *mecA*	Several Gram-positive bacteria Several Gram-positive and Gram-negative bacteria	Microarray gold nanoparticle Low density PCR arrays	Verigene Gram-Positive Blood Culture (BC-GP) Nucleic Acid Test (Luminex) FilmArray Blood Culture Identification Panel (bioMérieux)
Bacterial specific genes + *mecA* + *vanA* + *vanB* + *vanC1* + *vanC2*/*C3*	Streptococci + Staphylococci + Enterococci	PCR/ DNA strip hybridization	GenoType BC grampositive (Hain Lifescience)
Bacterial and fungal specific genes + *mecA* + *vanA* + *vanB*	Gram-positive and Gram-negative bacteria + fungi	Real-time PCR	Magicplex Sepsis Real-time Test (Seegene)
Bacterial and fungal specific genes + *vanA* + *vanB* + *mecA*	Gram-positive and Gram-negative bacteria + fungi	PCR/Tube or strip hybridization array	Prove-it Bone/Joint (MOBIDIAG)
Bacterial and fungal specific genes + *vanA* + *vanB* + *mecA* + bla_{KPC}	Gram-positive and Gram-negative bacteria + Fungi	PCR/ESI-TOF-MS[g]	IRIDICA BAC BSI Assay (Abbott Diagnostics)
Rifampin			
rpoB	*M. tuberculosis* complex	Real-time PCR	Xpert MTB/RIF Assay (Cepheid)
Rifampin + *isoniazid*			
23S rDNA + *rpoB* + *inhA* promoter + *katG*	*M. tuberculosis* complex	PCR/DNA strip hybridization	GenoType MTBDR*plus* (Hain Lifescience)

Table 85.2 (continued)

Antimicrobial agent and resistance gene target	Organism	Molecular method	Manufacturer
Mycobacteria specific genes +*rpoB* + *inhA* promoter + *katG*	*M. tuberculosis* and non-tuberculosis mycobacteria	Real-time based on DPO[h] and TOCE[i]	Anyplex plus MTB/NTM/ MDR-TB Detection (Seegene)
Rifampin + *isoniazid* + *fluoroquinolones* + *aminoglycosides/cyclic peptides* Mycobacteria specific gene + *rpoB* + *inhA* promoter + *katG* +*gyrA* + *rrs* + *eis* promoter	*M. tuberculosis* complex	Real-time based on DPO[h] and TOCE[i]	Anyplex II MTB/MDR/XDR Detection (Seegene)
Fluoroquinolones + *aminoglycosides/cyclic peptides* + *ethambutol* 23S rDNA + *gyrA* + *rrs* + *embB*	*M. tuberculosis* complex	PCR/DNA strip hybridization	GenoType MTBDR*sl* (Hain Lifescience)

[a]CE IVD, Conformité Européenne marking for in vitro diagnostics
[b]FDA 510(k), Cleared by U.S. Food and Drug Administration for in vitro diagnostics
[c]NASBA, Nucleic acid sequence-based amplification
[d]WT, Wild type
[e]ESBL, Extended-spectrum β-lactamases
[g]ESI-TOF-MS, Electrospray ionization time-of-flight mass spectrometry
[h]DPO, Dual priming oligonucleotide
[i]TOCE, Tagging oligonucleotide cleavage extension

clinical samples, multiparametric technologies are needed. Comprehensive reviews of such technologies have recently been published [16, 175, 176].

Array technologies represent one of the most powerful multidetection technologies, having the capacity to identify multiple targets, up to thousands, depending on the system [176–178]. However, because of the poor analytical sensitivities of array technologies, most of them include PCR amplification prior to array detection. Several in-house DNA probe arrays combined or not with PCR amplification have been developed to detect multiple antibiotic resistance genes and mutations [179–205] (Table 85.1). Different types of array technologies are also commercially available and increasingly used in the clinical microbiology laboratories including low-density PCR arrays such as the FilmArray technology (bioMérieux), liquid bead arrays such as the xTAG technology (Luminex), DNA microarrays such as the Check MDR technology (Check-Points) and the Verigene technology (Nanosphere), and line probe arrays such as the DNA strip technology (Hain Lifescience), some of which detect both antibiotic resistance genes and bacterial species whereas others also detect virulence genes (Table 85.2). Most commercial array technologies used PCR amplification prior to array detection, especially for detection of bacterial and drug resistance targets directly from clinical specimens. However, the potential of amplicon carry-over contamination of two-step non-integrated PCR/array technologies has pushed the development of new systems in which PCR amplification and multidetection on arrays are performed in a closed system (e.g., FilmArray Blood Culture Identification Panel from bioMérieux). The Verigene Gram-Positive (BC-GP) and Gram-Negative (BC-GN) Blood Culture Nucleic Acid tests from Nanosphere allow direct detection on DNA microarrays of bacterial and drug resistance targets from positive blood cultures without amplification by using a sensitive gold nanoparticle probe-based hybridization technology [206]. Though, the lack of prior amplification is possible because of the large bacterial load in positive blood cultures.

In the next section, some genotypic resistance assays that are increasingly used in the clinical microbiology laboratories are further described.

3.3.1 Genotypic Detection of Drug-Resistant *Mycobacterium tuberculosis*

The increasing rates of MDR-TB (resistance to rifampin and isoniazid) and the emergence of XDR-TB (MDR plus additional resistance to fluoroquinolones and at least one of the injectable drugs amikacin, kanamycin, or capreomycin) is a serious global health problem causing an important rise in morbidity and mortality [12–14]. The rapid identification of MDR-TB and XDR-TB is essential to improve TB treatment, prevention, and control [207]. However, because *M. tuberculosis* is slow growing, identification and determination of the susceptibility profile of this organism can take several weeks. In the last few years, broth-based methods,

either manual or fully automated, have allowed to accelerate the culture of mycobacteria and the availability of antibiogram. Nevertheless, culture-based drug susceptibility testing (CDST) methods may still take few weeks after the primary culture results are available. Reviews of susceptibility methods for mycobacteria were recently published [77, 208].

Advances in our understanding of the genetic mechanisms of drug resistance in *M. tuberculosis* have made possible the development of several different rapid genotypic drug resistance assays. Recent reviews describing these assays have been published [25, 89, 209, 210] (see Sect. 2). Most molecular methods for detecting resistance are based on determining the presence/absence of the mutations associated with resistance (Table 85.1). Comprehensive databases of mutations associated with antibiotic resistance in *M. tuberculosis* are available providing access to up-to-date information on mutations [211–213]. A few years ago, genotypic assays were mostly developed for detection of rifampin resistance because the genetic basis of rifampin resistance in *M. tuberculosis* is simple and well characterized, being caused by specific mutations in the *rpoB* gene in more than 95 % of rifampin-resistant TB [75] (see Sect. 2). Moreover, resistance to rifampin can often be used as a marker of MDR-TB since more than 90 % of rifampin-resistant TB are also resistant to isoniazid [75]. However, in the past few years, in-house molecular methods have been developed to detect mutations in most known target genes conferring resistance to rifampin (*rpoB*), isoniazid (*katG*, *inhA*, *ndh*, *kasA*, as well as *inhA* and *ahpC* promoters), ethambutol (*embB*), pyrazinamide (*pncA*), fluoroquinolones (*gyrA* and *gyrB*), streptomycin (*rrs* and *rpsL*), amikacin/kanamycin (*rrs*), kanamycin (*eis* and *eis* promoter), and capreomycin (*rrs and tlyA*) (Table 85.1) (see Sect. 2). Several molecular methods have been used to detect these mutations including Sanger sequencing, pyrosequencing, whole-genome sequencing (WGS), PCR-single-strand conformation polymorphism (PCR-SSCP), PCR-restriction fragment length polymorphism (PCR-RFLP), PCR hetero-duplex formation (PCR HDF), multiplex allele-specific PCR (MAS-PCR), real-time PCR using different types of fluorescent probes, LAMP, and hybridization on strips and on microarrays; and most of these methods have been described in recent reviews [25, 172, 209, 214–216].

Three molecular assays, namely, GenoType MTBDR*plus* and GenoType MTBDR*sl* (Hain Lifescience) and GeneXpert MTB/Rif (Cepheid) are commercially available for detection of TB drug resistance directly from specimens, concentrated specimens, and cultures (Table 85.2). The INNO-LIPA rif. TB assay (Fujirebio) that has been widely used in the past is now discontinued. GenoType MTBDR*plus* and GenoType MTBDR*sl* are line probe assays based on a multiplex amplification in combination with reverse hybridization to identify either wild-type sequence or specific mutations, whereas the GeneXpert MTB/Rif assay is a nested real-time PCR assay which uses molecular beacon probes to detect mutations. GeneXpert MTB/Rif assay detects *M. tuberculosis* complex (MTBC) and rifampicin resistance (*rpoB* mutations), whereas GenoType MTBDR*plus* detects MTBC as well as rifampin (*rpoB* mutations) and isoniazid resistance (*katG* and *inhA* promoter mutations). GenoType MTBDR*sl* assay detects resistance to fluoroquinolone (*gyrA* mutations), aminoglycosides/cyclic peptides (*rrs* mutations), and ethambutol (*embB* mutations). The advantages and limitations as well as the performance of these tests have been recently reviewed [25, 209, 214–218].

Noncommercial sequencing methods such as pyrosequencing and Sanger sequencing are increasingly used as they can provide, directly from specimens or from cultures, gene sequence information for specific targeted loci to identify known mutations as well as new unknown potential mutations that must be confirmed by CDST [209]. In recent years, WGS has emerged as a promising technology for characterizing antibiotic resistance in *M. tuberculosis* isolates [216]. Several studies have used WGS on hundreds of clinical isolates for identifying known and novel mutations conferring antibiotic resistance [219–223]. In a recent study, WGS was also used for TB diagnosis and drug-resistance screening (for 39 antibiotics), as well as strain typing on a sputum sample that became positive after 3 days [224]. This study showed that in well-resourced countries, rapid whole-genome sequencing may replace current methods of identifying and typing TB as well as rapidly identifying resistance when mutation known to confer resistance is detected.

The use of molecular methods to detect resistance markers in mycobacteria is an area of great potential benefit to the clinical mycobacteriology laboratory allowing diagnosis of MDR-TB and XDR-TB in the same day or a few days after sample collection. However, the correlation between phenotypic and genotypic resistance testing is not always accurate because the genetic mechanism of resistance for certain antituberculous drugs is still not fully known [209]. Therefore the "gold standard" for identification of resistant TB remains CDST.

3.3.2 Genotypic Detection of Oxacillin Resistance in Staphylococci

Despite improvement and development of phenotypic methods to detect oxacillin resistance (see Sect. 3.2.1), molecular detection of the *mecA* gene is considered the "gold standard" for detection of oxacillin resistance in *S. aureus* as it does not depend on the variable expression of the PBP 2a [109]. Unfortunately, the newly described *mecC* homologue of *mecA* (see Sect. 3.2.1) is usually not detected with *mecA*-specific primers since these two genes share <70 % identity [106]. Numerous molecular-based tests have been developed to increase the sensitivity, the specificity, and the speed for

MRSA detection. Most of these methods are in-house tests that detect an *S. aureus*-specific gene and/or *mecA* (Table 85.1). In-house molecular assays detecting *mecC* have also been developed recently [225, 226] (Table 85.1). Several commercial assays are also available for detection of MRSA, but only a few also detect *mecC* (Table 85.2). Molecular methods detecting *S. aureus* and *mecA*/*mecC* can be used to detect MRSA from pure cultures or directly from sterile specimens such as cerebrospinal and peritoneal fluids, endotracheal aspirates, blood, and blood cultures [169, 227–244] but can hardly be applied for detection of MRSA from nonsterile specimens such as nasal screening specimens containing a mixed flora of CoNS and *S. aureus*, because both can carry *mecA* [245–247]. During the past years, novel strategies, mainly PCR-based, have been developed to rapidly identify MRSA from nonsterile screening specimens. One of the most common PCR strategy used was pioneered by our group [22] and is based on our increasing knowledge of the genetic element containing *mecA* (see Sect. 3.2.1). PCR assays, which are based on this approach, generally include a primer specific to the *S. aureus* chromosomal *orfX* gene combined with primers specific to the right extremity sequences of the different staphylococcal cassette chromosome *mec* (SCC*mec*) containing *mecA* in proximity to the *orfX* junction, thus providing a link between *mecA* and *S. aureus*. By linking *mecA* to *S. aureus*, these PCR tests allow detection of MRSA directly from clinical specimens containing a mixture of staphylococci without previous isolation or enrichment of the bacteria, thereby reducing the number of sample preparation steps and time to results. Our original assay [22] led to the first real-time PCR test (IDI MRSA, now BD GeneOhm MRSA) cleared by the FDA for rapid detection of MRSA from nasal swabs (~2 h) and was initially commercialized by Infectio Diagnostic Inc. (IDI) (now BD Diagnostics). Several PCR assays based on this strategy are now commercially available on different system platforms to meet different clinical needs (batch processing or on demand) (Table 85.2). These assays reduce time for identification of MRSA from clinical samples from <1 to 3 h. The description and clinical performance of these different assays can be found in recent reviews [111, 121]. Some system platforms are fully automated including the sample preparation steps, thus requiring minimal hands on time (e.g., BD MAX MRSA, BD Diagnostics and Xpert MRSA, Cepheid) (Table 85.2). Some PCR assays also contained primers for identification of *S. aureus* and *mecA* in addition to SCC*mec*/*orfX* primers and can be used for detection of both MSSA and MRSA. Addition of *mecA* primers to the *S. aureus* SCC*mec*/*orfX* primers could reduce the number of false positive resulting from the presence of some *S. aureus* isolates harboring SCC*mec* but lacking *mecA* [22, 248–250]. Some assays have been adapted for detection of these microorganisms either in nasal swabs, wound swabs, or blood cultures (Table 85.2). False negative has also been described with some assays due to sequence variants of SCC*mec* not detected by the primers used [251, 252]. With the recent description of new SCC*mec* sequences and the new *mecC* gene in some MRSA isolates [108, 253], new versions of assays which now include primers specific to new SCC*mec* and *mecC* sequences have recently been cleared by the FDA (e.g., BD MAX MRSA XT, BD Diagnostics).

A new FDA-cleared PCR assay (MRSA/SA ELITe MGB, Epoch Biosciences) used another strategy to detect MRSA in nasal swabs. This PCR assay contains primers specific to *S. aureus* and *mecA*/*mecC*. MRSA identification is based on the presence of both markers at the same relative quantity measured by a difference in cycle threshold (Ct) value. This new test has not been evaluated in several studies, but the company claims clinical sensitivity and specificity of 92.3 and 95.2 % for detection of MRSA in nasal swab specimens when compared to conventional broth culture methods (Food and Drug Administration. Medical Device. MASA/SA ELITe MGB. 510 (k) Summary. http://www.accessdata.fda.gov/cdrh_docs/pdf11/K112937.pdf).

By providing an immediate detection of MRSA carriers (1–3 h), many studies have shown the positive impact of rapid molecular tests for reducing the transmission of MRSA [117, 254–256].

3.3.3 Genotypic Detection of Glycopeptide Resistance in Enterococci and Staphylococci

Most conventional phenotypic susceptibility methods can detect accurately high-level vancomycin resistance in enterococci (VanA and VanD phenotypes); however, detection of low-level vancomycin resistance (VanB, VanC, VanE, VanG, VanL, VanM, and VanN phenotypes) and differentiation between different Van types are difficult by phenotypic methods (see Sect. 3.2.2). Numerous in-house or commercial amplification or probe hybridization assays have been developed to detect the various *van* genes conferring glycopeptide resistance in pure cultures of enterococci (Tables 85.1 and 85.2). A multiplex PCR detects six types of glycopeptide resistance genes (*vanA*, *vanB*, *VanC*, *vanD*, *vanE*, and *vanG*) [257]. This multiplex PCR also contains primers specific to *E. faecium*, *E. faecalis*, *S. aureus*, and *S. epidermidis* allowing detection of both glycopeptide-resistant enterococci and *vanA*-containing *S. aureus* (VRSA). Other groups have used PCR assays for detection of *vanA* and/or *vanB* in *S. aureus* isolates [57, 58, 258–260]. However, the description of new *van* genes in recent years (e.g., *vanL*, *vanM*, and *vanN*) in enterococci emphasizes the need for more universal *van* gene primers in the future [261–263].

Despite development of various selective culture media and novel susceptibility testing methods for detecting VRE from screening specimens, it still takes at least 72 h to iden-

tify VRE by culture methods (see Sect. 3.2.2). Moreover, the sensitivity of selective culture media for detecting VanB-type VRE with low-level vancomycin resistance remains low [130, 133] (see Sect. 3.2.2). From the nine different glycopeptide resistance genes described in VRE, *vanA* and *vanB* are the most prevalent and clinically important from an infection control perspective because of the transmissibility of these genes. Since *vanA* and *vanB* are generally associated with *E. faecalis* and *E. faecium*, different in-house PCR assays, including gel-based PCR assays and real-time PCR assays, have been developed to detect these two resistance genes (some assays also include *vanC*) directly from fecal specimens or following enrichment in broth culture (for increased sensitivity) without the need to include PCR primers specific to these two bacterial species [264–269] (Table 85.1). Real-time PCR assays detecting *vanA* and *vanB* directly from fecal specimens are also commercially available (e.g., BD GeneOhm VanR, BD Diagnostics and Xpert *vanA*/*vanB*, Cepheid) (Table 85.2). The clinical performance of these commercial tests has been evaluated in several studies for detection of VRE in fecal specimens [270–276]. While the sensitivity of these assays is usually good, the specificity was limited in some studies largely due to false-positive results obtained for *vanB* [51, 271, 274–276]. These can be explained by the presence of *vanB*-containing anaerobic bacterial species in fecal specimens [50, 52, 53]. In this case, the presence of VRE must be confirmed by culture in stools that are positive for *vanB* [274–276]. A recent study has shown that rapid real-time PCR assays for VRE detection contributed to rapid decision about the best infection control measures and resulted in substantial cost savings [278].

3.3.4 Genotypic Detection of Carbapenemases in Gram-Negative Bacteria

The high mortality rate observed in infections caused by CPGN urges for rapid detection of these microorganisms [139–141] (see Sect. 3.2.3). However, culture-based methods for detection of carbapenemases in Gram-negative bacteria are time-consuming having a time to result of at least 24–48 h, often lack sensitivity and specificity, and cannot identify the type of carbapenemases [136, 144, 279–282] (see Sect. 3.2.3). Currently, the most prevalent carbapenemases include KPC, NDM, OXA-48-like, VIM, and IMP. The prevalence of each gene varies depending on countries or regions [135, 138, 283, 284]. Each carbapenemase type includes several variants, VIM and IMP harboring the greatest number of variants (http://www.lahey.org/Studies/access). To overcome limitations of the phenotypic culture-based methods, several in-house or commercial molecular tests (e.g., real-time and conventional simplex and multiplex PCR, DNA microarrays, and LAMP assays) which allow detection of a single or several carbapenemase genes in carbapenemase producers have been developed (Tables 85.1 and 85.2). These molecular assays are described in recent reviews [136, 144, 145, 161, 285]. Some in-house assays have been used to detect carbapenemase genes directly from clinical specimens such as urine, sputum, fecal, blood, or surgical site samples [286–291]. For example, the sensitivity and specificity of a real-time PCR used to detect KPC in 187 perianal/rectal swabs were shown to be 97.9–100 % and 96.4–95 %, respectively, depending on the nucleic acid extraction method used [286]. This study showed that the time to detect KPC carriers was reduced from 24 h to 4 h.

The different commercial molecular technologies available for detecting carbapenemase genes (Table 85.2) include real-time PCR assays such as the Xpert Carba-R kit (Cepheid) and the Check-*Direct* CPE kit (Check-Points), LAMP assays such as the eazyplex SuperBug CRE kit (Amplex BioSystems), and capture probe hybridization on microarrays (combined with multiplex PCR) such as the different Check-MDR kits (Check-Points). The type of carbapenemase genes and the variants detected depend on the different kits. These tests can be used for detection of carbapenemase genes from bacterial cultures, but some can be used directly from clinical specimens such as the Xpert Carba-R kit, the Check-*Direct* CPE kit, and eazyplex SuperBug CRE kit. The performance of some of these commercial assays has been assessed for detecting the major families of carbapenemase in pure cultures [292–294]. Most assays detect all isolates containing the carbapenemases included in their assays. However, the first version of the Xpert Carba-R kit which includes OXA-48 did not detect the common OXA-48 variant named OXA-181 [292, 294]. The new version of this kit now detects OXA-181 and OXA-232. The Xpert Carba-R kit is the only test detecting IMP carbapenemases, but it only detects IMP-1 subgroup [293]. The Check-*Direct* CPE kit combined with the NucliSENS easyMAG Extraction kit (bioMérieux) has been evaluated for detection of carbapenemase genes using spiked rectal samples and was shown to be comparable to the ChromID CARBA agar (bioMérieux) [295]. The use of molecular tests for rapid detection of carbapenemase producers directly in clinical samples should help to rapidly detect infected or colonized patients, improve treatment caused by these resistant microorganisms, and control their dissemination.

3.3.5 Potential Artifacts of Genotypic Resistance Testing

There are some potential artifacts of genotypic resistance testing to determine the resistance profile of a microorganism. For example, the presence of a resistance gene may not be always indicative of a resistant bacterium and does not necessarily lead to treatment failure, because the level of expression may be low. For example, the development of resistance by ß-lactamase production among members of

Enterobacteriaceae depends on the mode and level of expression [296]. However, the presence of a gene can be indicative of the potential to develop resistance. For example, in a study of antimicrobial resistance in *S. aureus*, it has been shown that the presence of *mecA* did not necessarily result in oxacillin resistance phenotype; however oxacillin-susceptible *S. aureus* isolates carrying this gene were easily selected for resistance expression by exposure to increasing antibiotic concentrations suggesting that, at least for certain resistance genes, the presence of a gene is sufficient for a bacterium to eventually become resistant to the drug [297, 298]. Another limitation of the resistance testing is that the absence of a gene coding for a resistance to a drug does not always mean that the bacterium is susceptible to that drug because resistance testing only identifies genes or mutations that have been characterized and other unknown resistance mechanisms may exist. Therefore, continuously updated epidemiological studies of resistant bacteria based on susceptibility testing and study of novel resistance mechanisms would help to develop genotypic tests for detection of the new types of resistance that undoubtedly will arise in bacteria in the future.

4 The Future of Genotypic Drug Resistance Detection

During the past decade, there has been enormous progress in the development of genotypic drug resistance assays that provide more accurate and rapid antimicrobial resistance testing. Genotypic drug resistance assays are increasingly used in the clinical microbiology laboratories, especially for detection of antimicrobial resistance in microorganisms that grow slowly such as MDR and XDR *M. tuberculosis* or for rapid detection of difficult-to-detect resistance mechanisms such as those found in MRSA, VRE, and carbapenemase-producing microorganisms. With the increasing prevalence of MDR and XDR pathogens, there is an urgent need for novel rapid genotypic diagnostic tests for detection of resistance without the need of the time-consuming culture-based systems. For infection control programs, the use of specific genotypic tests that can be used directly from screening clinical specimens to detect rapidly patients colonized with antimicrobial-resistant pathogens (e.g., MRSA, VRE, and carbapenemase-producing microorganisms) will help to prevent or reduce transmission [117, 139–141, 254–256, 278]. However, for diagnostic purpose, genotypic tests for detection of antimicrobial-resistant pathogens will have to detect and identify rapidly (in less than 1 h) all possible causative pathogens for a specific infection or syndrome (e.g., meningitis, nosocomial pneumonia, septicemia, etc.) as well as the associated genes or mutations conferring resistance to potentially effective therapeutic agents [175, 299, 300].

Several genome sequences as well as sequences of many conserved genetic targets for bacterial identification and antimicrobial resistance genes and mutations are available in public databases [163]. Though, the development of genotypic tests that will allow sensitive detection of multiple pathogens as well as multiple antimicrobial resistance genes and mutations directly from clinical specimens will pose major challenges. PCR-based techniques, especially multiplex real-time PCR, remain today the most common molecular methods when there is a need to detect a limited number of bacterial species and/or antimicrobial resistance genes and mutations. However, array technologies represent the most powerful tools for multiple target detection [176, 178]. To overcome the problem of amplicon carryover and the lack of sensitivity of array technologies, new array platforms are now available combining arrays to prior PCR amplification in closed systems (e.g., FilmArray technology from bioMérieux and eSensor technology from GenMark) [206, 301]. Other platforms enabling PCR amplification and array detection in a single closed chamber or closed systems combining amplification and innovative detection technologies are under development and should help to solve the challenge of sensitive multidetection [302–309]. Moreover, the development of ultrasensitive biosensors for nucleic acid analysis is another promising tool that could obviate the need for multiple target amplification in the future [310–313].

In recent years, the decreasing cost of next-generation sequencing (NGS) and the development of various rapid desktop sequencers have allowed these technologies to make significant impact in infectious diseases [176, 314–317]. It is now possible to rapidly sequence a whole bacterial genome in less than 1 day directly from a single colony on a primary isolation plate [318]. Whole genome sequencing (WGS) has found numerous applications in the clinical field of antimicrobial resistance [317] such as characterizing the genetic determinants of antibiotic resistance in clinical isolates and predicting antimicrobial susceptibilities [319, 320], measuring the rate at which resistance emerges [321], improving genotypic resistance tests [108], typing multidrug-resistant isolates for epidemiological surveillance [322, 323], and tracking outbreaks [324–326]. However, despite the high concordance between whole genome-based resistance genotypes and phenotypes observed in some studies [319], WGS will not be implemented for routine susceptibility testing of pure bacterial culture in the near future, considering the turnaround time, the lack of automated sequence analysis system, and still elevated cost of these technologies compared to traditional phenotypic methods and new molecular tests. Unbiased metagenomic NGS can also be performed directly from clinical specimens to identify antibiotic-resistant pathogens [327, 328], but this approach is very expensive, is less sensitive than phenotypic and molecular methods, and can-

not ascertain which pathogen harbors a plasmid-borne resistance gene in mixed cultures [176, 318, 328]. Nevertheless, the continuous technical advances (ultimately entirely automated) and the falling costs and turnaround time of NGS technologies as well as automation of sequence data analysis to generate data relevant for clinical use should push forward implementation of these diagnostic platforms in clinical and public health microbiology laboratories by providing unprecedented information on clinical isolates on a single platform [317, 329].

Finally, the next generation of genotypic tests for detection of antimicrobial-resistant pathogens should be fully automated with integrated sample preparation and nucleic acid detection (sample-to-answer). Indeed, automation of standard culture-based methods is steadily spreading throughout clinical microbiology laboratories [330], and genotypic tests should follow this trend while offering new faster technologies. Eventually, some molecular tests should be used at the point of care (POC) (i.e., close to a healthcare user) in low-complexity settings, such as the emergency rooms, for example, when rapid results (ideally less than 1 h) matter for patient outcomes or for infection control practices [331]. Although fully automated or integrated systems are commercially available for detection of bacteria and antibiotic resistance genes (e.g., BD MAX technology from BD Diagnostics, the Xpert technology from Cepheid, and the FilmArray technology from bioMérieux), there are no POC molecular tests available for detection of antibiotic resistance. Indeed, in January 2015, the 15-min molecular Alere I Influenza A & B test on the Alere I platform (Alere) was the first nucleic acid-based test ever to receive FDA CLIA waiver. In May 2015, a second nucleic acid-based POC test, the Strep A assay on the Liat system (Roche) was approved by the FDA. Other tests from these two companies have now received FDA CLIA waiver as well as tests for other companies (e.g., Xpert Xpress Flu/RSV from Cepheid and FilmArray Respiratory Panel EZ from bioMérieux). Several POC diagnostics devices are currently in development that can identify a variety of nucleic acid targets from multiple types of samples in under an hour [175, 332–334]. GenePOC, a Canadian company, has developed a simple microfluidic centripetal platform which enables a fully automated nucleic acid-based testing for infectious microorganisms within 1 h and less than 1 min of hands-on time. This system has the ability to process a wide range of clinical samples with up to 12 genetic targets [331]. Recent advances in nanotechnology and microfluidic "or lab-on-a-chip" systems should revolutionize the detection antimicrobial-resistant pathogens in the future [305–307, 331–336].

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Genotypic Assays for Monitoring Drug Resistance in HIV-1 Infection and for Other Chronic Viral Diseases

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1 Introduction

The routine introduction of genotypic drug resistance assays in the clinical microbiology setting represents a significant milestone in the treatment of HIV-1 infection; it has had practical utility in guiding antiviral therapy. These laboratory methods permit characterization of specific changes in the genomic nucleotide sequence of viral isolates in comparison to a HIV-1 reference strain to monitor the development of resistance to antiretroviral therapy [1, 2]. With genotypic testing, mutations that emerge spontaneously as a result of error-prone viral replication and/or that are selected by drug pressure in the HIV-1 polymerase (*pol*) or envelope (*env*) genes are commonly detected by automated techniques based on the Sanger method for dideoxy-terminator nucleotide sequencing [1] or, alternatively, with hybridization tests such as the line probe assay (LiPA) that monitor point mutations at codons known to be important for resistance to specific antiretroviral agents (ARVs) [1, 3, 4]. The effective utilization of genotypic drug resistance assays for HIV-1 infection also requires expert clinical interpretation of often complex mutational patterns. This task has been greatly facilitated by the use of several computerized algorithms that have been specifically designed for HIV-1 genotypic analysis [5–7].

Resistance-conferring mutations that encode single or multiple amino acid substitutions in the reverse transcriptase (RT) or protease (PR) enzymes or the heptad repeat 1 (HR-1) domain of gp41 in the HIV-1 envelope have been shown to be directly responsible for diminished susceptibility to the inhibitors of these viral targets and may, therefore, be viewed as important molecular markers that are predictive of drug resistance [8]. The prognostic value of genotypic resistance testing in improving virological outcomes to antiretroviral therapy for HIV-1 infection has been documented in several prospective and retrospective clinical studies, including comparisons against standard of care [9–12]. In addition, health economics analyses from the CPCRA 046 [13] and VIRADAPT [14] studies have confirmed the benefit conferred by genotypic resistance testing when used for guiding therapy choice decisions in patients who experienced virological failure on an initial antiretroviral regimen. In CPCRA 046, patients receiving standard antiretroviral therapy regimens were randomly assigned to one of two study groups in which therapeutic decisions were determined by clinical judgment alone or, alternatively, using genotypic antiretroviral resistance testing (GART) as an adjunct to clinical judgment [9]. With GART, 34 % of patients were reported to achieve a successful virological response compared to 22 % of patients in which therapy choice decisions were based entirely on physician clinical judgment [9–12]. Similar results have also been reported from the VIRADAPT study [10], in which 32 % of patients assigned to the drug resistance genotyping (DRG) group responded satisfactorily to antiretroviral therapy compared to a response rate of 14 % in patients without DRG [10, 13].

The benefit conferred by HIV-1 genotyping in treatment-experienced patients has also been further corroborated by the Havana trial [15]. In this study, a significantly greater proportion of patients in whom genotyping was used to guide therapy choice decisions achieved undetectable plasma viremia (i.e., HIV-1 RNA <400 copies/mL) after 24 weeks of therapy as compared to patients managed in accordance with the standard of care alone [15]. Additionally, the use of expert advice to assist with treatment decisions was also shown to be associated with improved virological response, especially in patients who had experienced a second virological failure [15]. Thus, the results from CPCRA 046, VIRADAPT, and Havana, as well as those from other related studies conducted in settings that more closely reflect current

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clinical practice [16], support the use of genotypic drug resistance assays and expert advice as important interventions to improve the sustained effectiveness of antiretroviral therapy in patients with HIV-1 infection.

The scope of utilization of genotypic drug resistance assays is increasing, and this technology is also being used, albeit on a more limited basis, to monitor resistance to antiviral drugs used for the treatment of hepatitis B virus (HBV) infection as well as a limited number of other chronic viral diseases associated with certain herpes viruses, e.g., cytomegalovirus (CMV) [1, 2, 17]. Although antiviral drugs have also recently become available for some other viral infections such as influenza, the routine use of genotypic drug resistance tests may not be equally practical or feasible in all situations [1]. Interestingly, molecular genotyping of validated tumor molecular markers, akin to drug resistance testing for HIV-1 infection, may also be of value in the future to help predict the development of resistance to novel targeted anticancer drugs [18]. For example, genotypic surveillance of the Bcr-Abl/c-kit tumor marker in chronic myelogenous leukemia (CML) may be used to detect resistance to targeted anticancer drugs such as Gleevec (imatinib mesylate, STI571) that are now used to treat this disease [19]. This therapeutic strategy could be advantageous with respect to the selection of alternate courses of therapy in patients with CML that has become refractory to treatment with Gleevec and, therefore, may also result in improved therapeutic outcomes compared to patients in whom genotypic drug resistance testing was not used for guidance of chemotherapy [18].

Although many of the techniques associated with genotyping were first developed for use in HIV-1 disease, these methods will also prove useful for other viral diseases including those associated with human cytomegalovirus (CMV) and herpes simplex viruses (HSV) among others; there is also likelihood that these techniques may prove useful in the management of certain types of cancer. This chapter reviews the methods available for detection of drug resistance by genotyping and the clinical utility of the testing procedures available at this time.

2 Genotypic Drug Resistance in HIV-1 Infection

The development of resistance to antiretroviral agents (ARVs) is largely thought to be a consequence of incompletely suppressive regimens and, moreover, constitutes a serious limitation in regard to the sustained effectiveness of these drugs for the treatment of HIV-1 infection [20–23]. HIV-1 variants that harbor resistance mutations to drugs from any of the currently approved classes of antiretroviral agents including the recently introduced fusion inhibitor, enfuvirtide (T-20), may precede the initiation of therapy because of spontaneous mutagenesis or transmission of drug-resistant viruses and are subsequently selected by antiretroviral therapy [8, 24]. Genotypic analysis has shown that prolonged exposure to combination therapy is associated with complex and often overlapping patterns of resistance-conferring mutations commensurate with increasing levels of resistance and, for that matter, cross-resistance, to some of the antiretroviral drugs comprising the therapeutic regimen. In general, multiple drug mutations to any single or combination of ARVs need to be selected in order to produce clinical resistance to most ARVs. However, this is not the case for a limited number of nucleoside reverse transcriptase inhibitor (NRTIs) such as lamivudine (3TC) and a closely related compound, emtricitabine (FTC), and also for most non-nucleoside reverse transcriptase inhibitors (NNRTIs) of HIV-1 reverse transcriptase (RT). These compounds possess relatively low genetic barriers for the development of drug resistance compared to the protease inhibitors (PIs) and can often experience substantial loss of antiviral activity following the appearance of a single primary drug resistance mutation in RT [25–29]. Table 86.1 shows most HIV-1 drug resistance mutations that are usually associated with antiretroviral therapy.

3 Nucleoside Reverse Transcriptase Inhibitor (NRTIs)

Unlike ARVs from other drug classes, NRTIs are administered to patients as precursor compounds that are phosphorylated to their active triphosphate form by host cellular kinases [30, 31]. NRTIs mimic the naturally occurring deoxynucleotide triphosphates (dNTPs) and can effectively compete with these intracellular substrates for binding to RT and incorporation into proviral DNA. However, NRTIs lack a 3′ hydroxyl group that is necessary for DNA polymerization, and, therefore, the antiviral activity of these compounds results from their ability to cause chain termination of nascent viral DNA strands [30, 32–34].

Mutations associated with drug resistance have been reported in response to the use of any single NRTI [8]. However, not all drugs elicit the same mutagenic response, and, consequently, resistance patterns and sensitivity must be considered on an individual drug basis. For example, resistance to 3TC develops quickly both in vitro [27, 29] and in patients treated with 3TC-containing regimens [35, 36]. High-level resistance to this nucleoside analogue (i.e., 500–1000-fold increase in IC_{50}) is mediated by a single mutation that encodes substitution of a methionine amino acid residue for either isoleucine (M184I) or, more commonly, valine (M184V) at position 184 in HIV-1 RT [29, 37–39]. Moreover, a novel mutational pattern in RT consisting of V118I alone or in association with E44A/D has also been shown to confer

Table 86.1 Common antiretroviral drug resistance mutations

Nucleoside (tide) reverse transcriptase inhibitor (NRTI) mutations associated with HIV drug resistance				
NRTI				
Abacavir	K65R, L74V, Y115F, M184V			
Didanosine	K65R, L74V			
Lamivudine/emtricitabine	K65R, M184V			
Stavudine	M41L, D67N, K70R, L210W, T215YF, K219KE			
Tenofovir	K65R, K70E			
Zidovudine	M41L, D67N, K70R, L210W, T215YF, K219KE			
TAMs	M41L, D67N, K70R, L210W, T215YF, K219KE		Affect all NRTIs	
69 insertion	TAMs plus T69X + X or XX		Affect all NRTIs	
151 complex	A62V, V75I, F77L, F116Y and Q151M		Affect all NRTIs except tenofovir	
Non-nucleoside (tide) reverse transcriptase inhibitor (NNRTI) mutations associated with HIV drug resistance				
NNRTI				
Delavirdine	K103N, V106AM, Y181C, Y188L, P236L			
Efavirenz	K103N, V106AM, V108I, Y181C, Y188L, G190SA, P225H			
Nevirapine	K103N, V106AM, V108I, Y181CI, Y188CLH, G190A			
Protease inhibitor (PI) resistance mutations according to the IAS-USA panel for antiretroviral drug resistance				
PI	Cross-resistance mutation		Unique mutations	
	Major	Minor	Major	Minor
Saquinavir	L90M, G48V	10IRV,24I,54VL,62 V,71VT,73S,77I,82AFTS,84 V		
Indinavir/RTV	46IL,82AFT,84 V	10IRV,20MR,24I,32I,36I,54 V,71VT,73SA,77I,90 M		
Nelfinavir	90 M	10FIRV,L24I, M36I, M46IL, A71VT,G73S, V77I,V82AFTS,I84V,N88DS	30 N	
Fosamprenavir/RTV	I50V	L10FIRV,V32I, M46IL, I47V, I54LVM, G73S,V82AFST,L90M		
Lopinavir/RTV	V32I, I47VA, V82AFTS	L10FIRV,K20MR, L24I,L33F, M46IL, I50V, F53L, I54VLAMTS, A71VT,G73S, I84V,I90M		L63P
Atazanavir	I84V, N88S,	L10IFVC,K20RMITV, L24I, V32I, L33IFV, M36ILV, M46IL, G48V, F53LY, I54,LVMTA, I62V, A71VITL, G73CSTA,V82ATFI, L90M	I50L	G16E, E34Q, D60E, I64LMV, I93LM
Tipranavir	L33F,V82LT, I84V	L10V,K20MR, E35G, M36I, K43T, M46L, I47V, I54AMV, L90M		I13V, Q58E, H69K,T74P,N83D
Darunavir	I50V, I54ML, I84V	V11I,V32I, L33F, I47V, G73S,	L76V	V11I, L89V
Integrase strand transfer inhibitors (INSTIs) mutations associated with HIV drug resistance				
Raltegravir	Y143R/C, Q148R/H/K, N155H			
Elvitegravir	E92Q, Y143R/C, Q148R/H/K, N155H			
Dolutegravir	G118R, R263K			

moderate phenotypic resistance (i.e., 3- to 4-fold increase in IC_{50}) to 3TC in the absence of M184V [40, 41]. Increased prevalence of both V118I and E44A/D is associated with long-term ZDV/d4T usage.

In contrast to these findings with 3TC, resistance to zidovudine (ZDV) and other NRTIs may become clinically important only about 6 months after initiation of therapy [42, 43]. Furthermore, prolonged exposure to ZDV is characterized by a stepwise accumulation of resistance mutations referred to as thymidine analogue mutations (TAMs) that can result in progressive loss of antiviral activity to this compound. The TAMs comprise a group of six drug resistance mutations (i.e., M41L, D67N, K70R, L210W, T215Y/F, and K219Q) in RT that were initially described in connection with ZDV resistance and have also been implicated in reduced sensitivity to stavudine (d4T) [8, 44–47]. In addition, TAMs can also confer moderate levels of resistance to other NRTIs such as didanosine (ddI) and zalcitabine (ddC), depending on the mutational pattern that is present. However, L74V is the primary resistance-conferring mutation that is selected by ddI that is responsible for the greatest loss of antiviral activity with this drug [8, 42, 44, 46]. Similarly, the selection of various TAMs is also associated with decreased susceptibility to ddC, although, as with ddI, other resistance-conferring mutations (i.e., K65R and T69N) are also important in this regard [8, 42, 45, 46]. It is also noteworthy that whereas discriminatory mutations in RT such as M184V confer resistance primarily against the drugs that select them, TAMs, on the other hand, can mediate diminished drug susceptibility against an extended array of unrelated NRTIs [8, 44–47].

Genotypic analysis of viral isolates from patients treated with antiretroviral regimens that included d4T or ZDV has pointed to the existence of two major genetic pathways in regard to the development of resistance to thymidine analogues as evidenced through the detection of differential patterns of TAMs over time [48–51]. Initially, each of the M41L and T215Y/F mutations is commonly present in both pathways (50), and they are followed by the stepwise accumulation of other TAMs at positions 210 and 215 (i.e., 41 L-210 W-215Y pattern) or, alternatively, positions 67, 70, and 219 (i.e., 67 N-70R-219Q/E pattern) [48, 49, 51]. Furthermore, the specific sequence of TAM accumulation observed may be dependent on whether ZDV monotherapy or dual-NRTI combinations were used for initiation of antiretroviral therapy. Monotherapy with ZDV has been shown to be more commonly associated with the K70R mutation appearing first, leading predominantly to selection of the 67 N-70R-219Q/E pattern [48], whereas patients who started treatment with either ZDV/ddI or ZDV/ddC usually initially developed 215Y/F followed by 41 L and 210 W [48]. Moreover, the 41 L-210 W-215Y pattern appears to be more prevalent than 67 N-70R-219Q/E [52]. The V118I and E44A/D mutations frequently cluster jointly with the 41 L-210 W-215Y pathway but have only been observed individually in association with the 67 N-70R-219Q/E pattern [52]. TAMs from the 41 L-210 W-215Y pathway generally yield higher levels of cross-resistance to other NRTIs when present together with other mutations than do the same number of TAMs from the 67 N-70R-219Q/E pathway [52].

Another less frequently observed resistance mutation, K65R, has been shown to be associated with prior treatment with abacavir (ABC)-containing regimens and results in reduced antiviral susceptibility to both ABC and the nucleotide analogue reverse transcriptase inhibitor tenofovir (TDF). Hence, resistance to these ARVs can develop independently via genetic pathways involving either the TAMs or K65R as the signature drug resistance mutations [53]. K65R is also selected by TDF in vitro [54] and has been observed with low frequency (i.e., 3 % of cases) in clinical trials of patients with HIV-1 infection who were treated with a TDF-containing regimen for up to 96 weeks [55].

The simultaneous presence of K65R together with TAMs is very rare in clinical samples. One study found only a negative association of K65R and TAMs (except for Q151M [positive association] and K70R [no association]) [56]. Site-directed mutagenesis experiments that introduced both TAMs and K65R into clinical isolates determined a reciprocal antagonistic phenotypic effect. TAMs reduced the resistance conferred by K65R to TDF, ABC, and ddC, and K65R decreased the resistance conferred by TAMs to AZT. TAMs had no effect on the resistance conferred by K65R against 3TC or FTC, but enhanced the resistance of M184V against each of ABC, ddI, and TDF [56]. This finding adds support to the sequential use of AZT- and TDF-based NRTI backbones.

Mutational patterns that are associated with broad cross-resistance to multiple NRTIs have also been identified. The Q151 multidrug resistance (MDR) complex is encoded by five mutations in RT: A62V, V75I, F77L, F116Y, and Q151M. These mutations were initially observed in viral isolates from patients with HIV-1 infection who received combination therapy with ZDV plus either ddC or ddI for over 1 year [57, 58]. Primary resistance mutations that are usually associated with resistance to ZDV, ddI, or ddC in monotherapy were not present in these isolates. Q151M is the first of these five mutations to develop in vivo and compared to the other Q151M MDR substitutions also produces the most resistance to additional NRTIs [57]. In addition, it has been shown that a family of insertion mutations between codons 67 and 70 in RT can cause resistance to a variety of NRTIs including ZDV, 3TC, ddI, ddC, and d4T. Usually, these mutations confer resistance to multiple NRTIs when present in a ZDV-resistant background [59, 60]. The development of these mutations is also correlated with prior treatment with ZDV/ddI and ZDV/ddC combination therapy regimens. However, the prevalence of the insertion mutations has been reported to be lower than that for the substitutions comprising the Q151M MDR complex [61].

4 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

Non-nucleoside RT inhibitors (NNRTIs) act as noncompetitive antagonists of enzyme activity by binding to a hydrophobic pocket that is located adjacent to the catalytic site of RT [62, 63]. NNRTIs reduce the catalytic rate of polymerization without affecting nucleotide binding or nucleotide-induced conformational change [64]. These drugs are particularly active at template positions at which the RT enzyme naturally pauses and, moreover, do not appear to influence the competition between dideoxynucleotide triphosphates (ddNTPs) and the naturally occurring dNTPs for insertion into the growing proviral DNA chain [65].

Diminished sensitivity to NNRTIs appears quickly both in tissue culture selection protocols and in patients [25, 62, 63]. NNRTIs share a common binding site, and mutations that encode NNRTI resistance are located within the binding pocket that makes drug contact [62–69]. This explains the finding that extensive cross-resistance is observed among all currently approved NNRTIs [25, 70, 71]. A substitution at codon 181 (i.e., Y181C) is a common mutation that encodes cross-resistance among many NNRTIs [25, 68, 70, 72]. Replacement of Y181 by a serine or histidine also conferred HIV resistance to NNRTIs [73]. A mutation at amino acid 236 (i.e., P236L), conferring resistance to a particular class of NNRTIs that include delavirdine, can also diminish resistance to nevirapine and other NNRTIs, particularly if a Y181C mutation is also present in the same virus [74]. Y188C and Y188H are other important mutations that can also confer resistance to NNRTIs.

Another drug resistance mutation, namely, K103N, is also commonly observed and is responsible for reduced susceptibility to all approved NNRTIs [25, 68, 70, 72]. Substitution of K103N results in alteration of interactions between NNRTIs and RT. The K103N mutation shows synergy with Y181C in regard to resistance to NNRTIs, unlike antagonistic interactions involving Y181C and P236L [75].

5 Protease Inhibitors (PIs)

Drug-resistant viruses have been observed in the case of all protease inhibitors (PIs) developed to date [76–78]. In addition, some strains of HIV have displayed cross-resistance to a variety of PIs after either clinical use or in vitro drug exposure [76–78]. In general, the patterns of mutations observed with PIs are more complex and extensive than those observed with RT antagonists [8]. This involves greater variability, as well, in temporal patterns of appearance of different mutations and the manner in which different combinations of mutations can give rise to phenotypic resistance. These data suggest that the viral protease (PR) enzyme can adapt more easily than RT to pressures exerted by antiviral drugs. At least 70 mutations in PR have been identified as responsible for resistance to PIs [8, 76–79].

In general, several mutations are necessary in order for PIs to lose activity against HIV-1. Certain of the mutations within the HIV-1 PR affect the enzyme more than others and can on their own confer resistance to certain PIs [76–78]. In particular, D30N and D50L are unique to nelfinavir and atazanavir, respectively. Saquinavir, an early PI, predominantly selects for the mutations L90M and G48V. Amprenavir and fosamprenavir can select for D50V, which can confer some degree of cross-resistance to darunavir (DRV) [80]. Regarding lopinavir, the accumulation of at least five mutations in PR is required for high-level resistance to develop this drug [81, 82]. Recently, the presence of mutation I47A, although uncommon, was shown to result in very high levels of resistance to lopinavir (>100-fold increase in IC50) and hypersusceptibility to saquinavir [83, 84]. Unique signature mutations have not been well defined for either tipranavir or darunavir.

A variety of mutations may confer cross-resistance among multiple drugs within the PI family. Cross-resistance mutations can lower the affinities of PIs, but the specific effects of these mutations vary according to each individual PI. As a classical example, the mutations V82F/I84V can contribute to resistance against almost all PIs currently available for therapy. These two positions are located in the β-sheet of the active site cavity of the PR, a structure to which all PIs must bind to inactivate the enzyme. Interestingly, the IAS-USA Drug Resistance Mutation Panel considers that mutations at position 82 can affect all PIs in clinical use to date, except DRV (although the resistance profile of DRV is not yet completely determined) [79]. Similarly, the I84V mutation affects all PIs in clinical use and is a major mutation for five of them. Despite being located outside the active site, the L90M mutation also affects all PIs except DRV and, on its own, does not contribute to tipranavir (TPV) resistance [79]. Extensive reviews on the effect of each resistance mutation on each particular PI goes beyond the scope of this review and can be found elsewhere [85].

On the other hand, wide arrays of secondary mutations have been observed that, when combined with primary mutations, can cause increased levels of resistance. Mutations such as L90M and L63P (a common polymorphism) have no discernible effect on binding affinity, but can partially restore PR catalytic activity and hence viral fitness [86]. It should be noted that resistance to PIs can also result from mutations within the substrates of the PR enzyme. The gag and gag-pol precursor proteins of HIV can acquire mutations at or close to their cleavage sites that render them more susceptible to hydrolysis by PR [87–90]. Thus, cleavage occurs more efficiently and viral fitness can be restored to some degree. Some of the gag and gag-pol mutations that have been

reported in treatment-experienced patients include the p7/p1mutations A431V, K436R, and I437V and p1-/p6-gag mutations L449F/V and P452S-P453L/A [91]. At least one of these mutations was detected in 60 % of therapy-experienced patients compared to 10 % in treatment-naïve patients [91]. Nevertheless, the full clinical significance of these cleavage site mutations in regard to PI resistance remains to be elucidated.

6 Fusion Inhibitors (Enfuvirtide, T-20)

Enfuvirtide (T-20) is the first entry in a novel class of antiretroviral agents known as HIV-1 entry inhibitors and has recently been approved for the treatment of HIV-1 infection [92]. This compound is a synthetic peptide consisting of 36 amino acids that are homologous to the residues located at positions 127–162 from the C-terminus of the heptad repeat 2 (HR-2) domain in the gp41 transmembrane glycoprotein of the viral envelope. T-20 binds competitively to the HR-1 domain within gp41, thus preventing interaction with HR-2 and formation of the hairpin-like structure that is required for fusion of the viral and host cell membranes [92, 93].

In the TORO-1 and TORO-2 studies, the addition of T-20 to optimized background therapy consisting of three to five active antiretroviral drugs, which were selected using genotypic drug resistance testing, was shown to result in significant reduction of plasma HIV-1 RNA and CD4 cell count increases compared to optimized background therapy alone in heavily treatment-experienced patients with HIV-1 infection that was resistant to NRTIs, NNRTIs, and PIs [94, 95]. The results from additional open-label and controlled clinical trials with this drug have similarly demonstrated improved treatment outcomes for up to 48 weeks in HIV-1 patients that were experiencing virological failure on previous regimens [96, 97]. In phase 1 clinical testing, resistance to T-20 developed rapidly and shown by rebounding plasma HIV-1 RNA after 14 days of monotherapy in four patients receiving an intermediate dose (i.e., 30 mg twice daily) of T-20 [98]. Genotypic analysis of cloned virus from these patients showed that resistance to T-20 was produced by substitutions in the highly conserved GIV motif which comprises a three-amino-acid sequence between residues 36–38 within the HR-1 domain that is essential for fusion of viral and cellular membranes to occur. Mutants that harbored a single amino acid substitution in GIV (i.e., G36D, I37V, and V38A/M) were frequently detected [98] (84). G36D and, in particular, V38A both exhibited significant fold increases in the IC_{50} for T-20 compared to HIV-1 strains with wild-type envelope sequences. In addition, dual mutants that contained G36D together with substitutions at other amino acid residues within HR-1 (i.e., Q32H/R and Q39R) were also observed and were shown to confer reduced susceptibility to T-20 to an extent similar to that produced with G36D by itself [98]. Interestingly, variability in the HR-1 domain at positions that are associated with resistance to T-20 has been demonstrated in both subtype B (i.e., residues 37, 39, and 42) and in non-B (i.e., residue 42) HIV-1 strains isolated from T-20-naïve patients [99]. However, the major GIV mutants commonly associated with T-20-resistant isolates were not observed in the absence of drug treatment, suggesting that primary genotypic resistance to this drug is uncommon [99]. Further study is needed to better understand the long-term implications of these uncommon resistance mutations in HIV-1 patients undergoing therapy with fusion inhibitors.

7 Integrase Inhibitors

HIV integrase inhibitors are the latest class of antiretroviral agents and inhibit the strand transfer reaction leading to the insertion of viral DNA into target host DNA and are therefore called integrase strand transfer inhibitors (INSTIs). INSTIs specifically and tightly bind to the active site of integrase and chelate the divalent metal ions located in the catalytic triad of integrase. Currently approved INSTIs include raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) [100, 101]. All approved INSTIs are well tolerated and highly efficacious against variants resistant to other classes of drugs with well-tolerant safety.

The development of resistance to INSTIs to varying extent has been reported both in vitro and in patients [102–104]. Genotypic studies showed that resistance to RAL and EVG develops rapidly both in cell culture selection and in the clinic. Resistance to RAL (i.e., FC 10–100) often occurs due to three primary mutations at positions N155H, Q148H/K/R, and less frequently Y143R/C/H in the active site of integrase, resulting in reduced integrase activities and virological failure. The primary mutations in combination with one or more secondary mutations are often observed after virological failure involving raltegravir, e.g., G140S/Q148H and G140S/Q148R conferring high-level resistance to RAL. The mutation at N155 commonly appears early in RAL therapy and is often replaced by other primary mutations (e.g., Q148H, K, or R) with secondary mutations. Cross-resistance between RAL and EVG has been observed. Major primary mutations that confer resistance to EVG have been found at positions T66I, E92Q, N155H, and Q148H/K/R. The addition of G140S to Q148H/R/K resulted in increased levels if resistance to EVG (i.e., FC $\geq$100). However, DTG, the second-generation INSTI, can still be either partially or completely active against RAL- and EVG-resistant viruses in vitro, and it is the only anti-HIV drug against which HIV has not developed resistance mutations in clinical practice. In treatment-naïve patients, only

low-level resistance to DTG (i.e., FC <10) has been observed on the basis of a primary mutation at R263K, accompanied by impaired viral replication fitness. However, so far no virological failure resulting from R263K or any other mutation has been reported since the approval of this drug in 2013.

8 Limitations of Genotypic Resistance Testing for Treatment of Infection with HIV-1

Although genotypic resistance testing represents an important advance in HIV therapy, it adds complexity to the management of HIV infection, since interpretations of results are not straightforward and clinical correlates do not yet exist for all resistance mutations. The limitations for genotypic resistance tests include inability to detect virus archived in viral reservoirs, insensitivity to viral minority populations (populations that are less than 20 % of the total viral mixture), and the requirement of a minimum viral load (500–1000 plasma HIV RNA copies/mL) for detection to be achieved. However, it is generally accepted that genotype is more sensitive for minority populations than phenotype testing. For instance, genotyping can detect sentinel mutations (e.g., M184V) before changes in phenotypic resistance become evident. Importantly, several studies have clearly demonstrated that expert advice adds benefit to results from resistance testing [12, 15, 16, 105–107].

On the other hand, large databases of paired genotype-phenotype assays have allowed the construction of "virtual phenotype" estimators that quantify HIV-1 resistance to ARV drugs based on a statistical prediction of the phenotype for a given genetic sequence. The accuracy of such estimations depends on the frequency of genotypes in the database that match the problem genotype and the variability in drug susceptibility of the phenotypes used to create the predicting pool. Uncommon sequences and those with suboptimal matches will have less accurate predictive value than those that are more frequent and better matched. Although a good correlation of virtual phenotypes with "real phenotypes" has been reported [108, 109], it should be kept in mind that "virtual phenotype" is a probability estimation. Further research is advancing in order for informatic aids to be able to display options of antiretroviral regimens starting from the computerized evaluation of a viral nucleic sequence.

Finally, there is subrepresentation of non-B subtype genetic sequences in current HIV resistance databases that have been used to generated resistance algorithms. Therefore, resistance pathways and mutations may be limited in the interpretation of resistance of non-B subtype clinical isolates. A classical example in subtype C HIV-1 exposed to efavirenz in tissue culture is the emergence of the V106M mutation which was observed to arise in the place of the V106A substitution, more commonly seen with subtype B viruses [110]. It is not yet known to which extent natural polymorphisms of different non-B subtypes can lead to different mutation patterns of frequency of individual mutations. For instance, a rapid emergence of K65R has been reported in tissue culture of subtype C HIV-1 in the presence of TDF. A high prevalence of this mutation has also been described in Botswana patients taking ddI [111, 112].

9 Hepatitis B Virus (HBV) Infection

Antiviral treatment of chronic hepatitis B has regained importance since only a small proportion of actively infected patients achieve the desirable outcomes with interferon-based therapy. Also new data suggests that higher viral loads are associated, at least in Asian populations with increased risk of developing cirrhosis and hepatocellular carcinoma [113, 114]. Hence, suppression of viral load with the use of antiviral drugs in chronic HBV infection emerges as a promising option for reduction of patient morbidity.

Four nucleoside reverse transcriptase inhibitors are currently licensed for treatment of chronic HBV infection: lamivudine (3TC), adefovir and entecavir, and telbivudine. They are used either alone or in association with immunotherapy, i.e., interferon-α (regular or pegylated) in treatment of HIV/HBV coinfection. All are highly active against HBV and are frequently used together with antiretroviral therapy directed against chronic HIV-1 infection. Other drugs, not yet specifically licensed for HBV treatment but that have excellent anti-HBV activity, are famciclovir, tenofovir, emtricitabine, clevudine, pradefovir, ANA 380, myrcludex, and valtorcitabine.

Lamivudine is a widely utilized antiviral drug often used in initiation of therapy in patients with hepatitis B virus (HBV) infection. In both immunocompetent and HIV/AIDS patients with HBV coinfection, the prevalence of lamivudine (3TC)-resistant hepatitis B virus (HBV) variants has been reported to be approximately 16–43 % after 1 year and up to 70 % at 4 years of treatment [115, 116]. The rtM204V (previously position M552V) mutation induces a 1000-fold decrease in susceptibility to lamivudine in vitro in comparison to wt HBV [117]. Drug-resistant virus can be selected after 6 months of lamivudine therapy in these patients, and its presence has been shown to increase with the duration of exposure to this drug [115, 118]. As is also the case with HIV-1, genotypic analysis has shown that resistance to lamivudine results principally from either isoleucine (I) or valine (V) amino acid substitutions in place of methionine (M) at position rt204 within the C domain of the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif of the HBV DNA polymerase [1, 115]. Compensatory mutations associated with lamivudine resistance (rtV173L, rtL180M) are found in the B domain [119, 120]. This mutation and similarly the M184V/I substitution in HIV-1 RT are responsible for high-level resistance to lamivudine.

In addition to rtM204I/V, several other mutations in the HBV polymerase gene have been shown to emerge following prolonged exposure to lamivudine and are associated with diminished susceptibility to this drug. Specific patterns of these mutations are used to assign lamivudine-resistant HBV variants to one of two genotypic groups. HBV group I mutants contain lamivudine resistance mutations that are located in both the polymerase B and C domains that include predominantly the rtL180M (previously L528M) and rtM204I/V (previously M552I/) substitutions, respectively. Group II viruses on the other hand are characterized by the presence of rtM204I in the C domain as the main lamivudine resistance-conferring mutation and have been shown to occur less frequently than their group I counterparts [115].

Resistance to other nucleoside analogues used for the treatment of HBV infection has also been documented. Compounds such as ganciclovir (GCV) and famciclovir (FCV) are potent inhibitors of the HBV polymerase both in vitro and in vivo, and, although they appear less effective than lamivudine [1, 118], both of these antiviral agents have been used on an investigational basis to treat HBV infection. Genotypic analysis has revealed that the most important resistance-conferring mutations to these drugs are selected outside of the YMDD motif and include the rtV173L (previously V521L), rtP177L (previously P525L), rtL180M (previously L528M/V), T184S (previously T532S), and rtV207I (previously V555I) substitutions in the B domain of the HBV polymerase gene. Furthermore, it has been shown that the rtV207I substitution produces the highest attenuation of antiviral susceptibility to FCV and that both this mutation and rtP177L are associated with cross-resistance to lamivudine [1].

Adefovir dipivoxil (PMEA), a novel antiviral drug from a class of compounds known as nucleotide analogue reverse transcriptase inhibitors (NtRTIs) [121], has been licensed for the treatment of chronic hepatitis B [122–124]. Resistance to adefovir appears to develop infrequently in vivo, and, in two large placebo-controlled trials that included 700 patients with HBV infection [122, 123], treatment with adefovir for 48 weeks did not select for mutations associated with resistance to this compound [125]. Substitutions in the conserved domains of HBV polymerase (i.e., rtS119A, rtH133L, rtV214A, and rtH234Q) were infrequently detected as minority species in the clinical isolates of four adefovir-treated patients from these studies. Moreover, these secondary mutations did not confer phenotypic resistance to adefovir and were not associated with diminished virological response to this drug during the treatment period [125]. However, continued exposure to therapeutic levels of adefovir for up to 96 weeks resulted in selection of an adefovir resistance mutation within the HBV polymerase D domain (i.e., rtN236T) in one patient [126]. Other reports from patients receiving 10 mg/d as monotherapy registered 2, 5.9, 18, and 29 % of resistant mutants after 2–5 years of treatment [126–128]. Clinical isolates that harbored this substitution were shown to have reduced antiviral activity to adefovir in vitro but remained susceptible to lamivudine and entecavir [126]. The mutation rtA181V in the B domain of the polymerase has been more recently described and can confer some loss of susceptibility to lamivudine [128, 129]. Also, adefovir resistance has been seen to emerge more frequently in lamivudine-resistant patients than in those without previous lamivudine resistance (10 % vs. 0 %) [130]. However, adefovir resistance is less likely to occur when adefovir is given in addition, rather than as a substitute, for lamivudine [131]. Therefore, the addition of adefovir to lamivudine-failing patients has become widely accepted. Importantly, a virus variant carrying the mutation rtI233V which occurs naturally appears to have lower susceptibility to adefovir [132]. This mutation has not been selected in vitro nor seen in patients experiencing virological breakthrough. In general, these data point to the essentiality of always initiating the therapy of HBV disease with combination therapy.

The nucleoside analogue entecavir was approved for treatment of HBV infection in 2005. Virologic breakthrough confirmed by genotypic analysis has been seen during phase II and III clinical trials in 5.8 % of patients treated by entecavir after lamivudine failure for 1 year, 10 % for 2 years, and 25 % for 3 years [133–136]. The patients reported with resistance to entecavir had two signature lamivudine resistance mutations in the HBV polymerase, the rtL180M and rtM204V, along with the novel mutations rtM250V or rtS202I and rtT184G. The mutation more closely linked to entecavir resistance appears to be rtM250V within a background of lamivudine resistance mutations [133]. To date, primary resistance to entecavir in the absence of previously existing lamivudine resistance has not been reported [133]. Also, recent studies indicate that entecavir possesses activity against HIV-1 as well as against HBV and can select for the M184V mutation in HIV-1 [137]. This finding my lead to revision of current guidelines of treatment in HIV/HBV coinfected patients.

Emtricitabine (FTC) is an L nucleoside very similar to lamivudine. When administered as monotherapy for HBV infection, it selects for the rtM204I/V (YIDD/YMDD) mutations in the C domain of the HBV polymerase. The rate of YMDD mutations emerging in patients receiving 200 mg of FTC per day has been reported to be 9–13 % at week 48 of treatment and 19 % at week 96 of treatment [138]

Telbivudine is a potent L-analogue and the latest antiviral drug to be approved for treatment of chronic hepatitis B. Resistance was seen in about 5 % of patients after 1 year of treatment and is attributable to a rtM204I mutation in the HBV polymerase "YIDD," but this does not seem to be linked to the rtM204V mutation in the "YMDD" motif of the HBV polymerase [139]. Telbivudine resistance mutations do not overlap with the entecavir resistance mutations, leaving a

full option for patients failing therapy with either of these agents. Finally, the emergence of resistance to all drugs used to date for the treatment of HBV provides testimony to the need for combination therapy in clinical practice.

10 Herpes Virus Infections: Cytomegalovirus (CMV) and Herpes Simplex Virus (HSV)

The incidence of opportunistic infections associated with HIV/AIDS has significantly declined as a result of the introduction of highly active antiretroviral therapy (HAART). However, in certain clinical settings, such as in patients with severe primary combined immunodeficiency (SCID) and patients requiring organ transplantation, the development of cytomegalovirus (CMV) infection remains a serious complication that generally requires the use of antiviral therapy [1, 140]. In the pre-HAART era, the rate of resistance to each of the anti-CMV drugs was estimated to be approximately 25 % per person-year [141–144].

Regarding human CMV therapy, a diverse array of drugs that includes acyclovir, ganciclovir (GCV), oral prodrug of valganciclovir (a prodrug which is transformed into GCV first-pass metabolism), foscarnet (FOS), nucleotide analogue, cidofovir (CDV), and fomivirsen are used to treat CMV disease [1, 121, 140]. These compounds have been shown to suppress viral replication through inhibition of the viral DNA polymerase which is encoded by the CMV UL54 gene. Sequence analysis has demonstrated that mutations in this gene can confer resistance to each of these three drugs. In addition, GCV, like all other nucleoside analogues, needs to be activated to its virologically competent form, GCV-triphosphate. This process initially involves the phosphorylation of GCV to its monophosphate moiety by a viral-encoded phosphotransferase (see Fig. 86.1). This enzyme is expressed by the UL97 gene. Studies have shown that resistance to GCV can manifest as early as 10 days following initiation of therapy with this drug and that numerous mutations, many of which are located between amino acid residues 590–606 or at position 460 or 520 [145] in UL97, may contribute to reduced susceptibility to GCV in some immunocompromised patients [140]. Similarly, a novel deletion mutant involving an eleven amino acid sequence between positions 590–600 in UL97 has also been identified in GCV-resistant isolates from a patient with SCID [146]. In other studies, GCV-associated mutations in UL97 were found to be highly prevalent in viral isolates that displayed varying degrees of resistance to GCV [147]. However, sequence analysis of UL97 alone cannot be used to predict the level of resistance to GCV without knowledge of additional genotypic and/or phenotypic information obtained in regard to UL54 [1]. During prolonged GCV therapy, UL97 mutations appear early and result in lower-level resistance, whereas UL54 mutations appear later and confer higher-level resistance [147–150].

Recent studies of clinical isolates from AIDS and solid organ transplant patients have documented that the most frequent UL97 mutations present in GCV-resistant mutants were A594V, L595S, M460V, and H520Q [150–152]. Other common UL97 mutations related to resistance include C592G and C603W. The mutations associated with the highest rate of increase in GCV resistance have been M460V, C603W, deletion of codons 595–603, H520Q, L595S, A594V, C607Y, and deletion of codon 595 with a fold change in resistance from 4.9 to 13.3 depending on the mutation [145, 153–156]. In contrast, mutations C592G, A594T, and E596G and deletion of codon 600 confer a lower decrease in drug susceptibility [145].

The CMV DNA polymerase, which is encoded by the gene pUL54, can also mutate in response to drug pressure, and such mutations can potentially affect all currently approved antivirals. Some of the most frequent DNA polymerase mutations

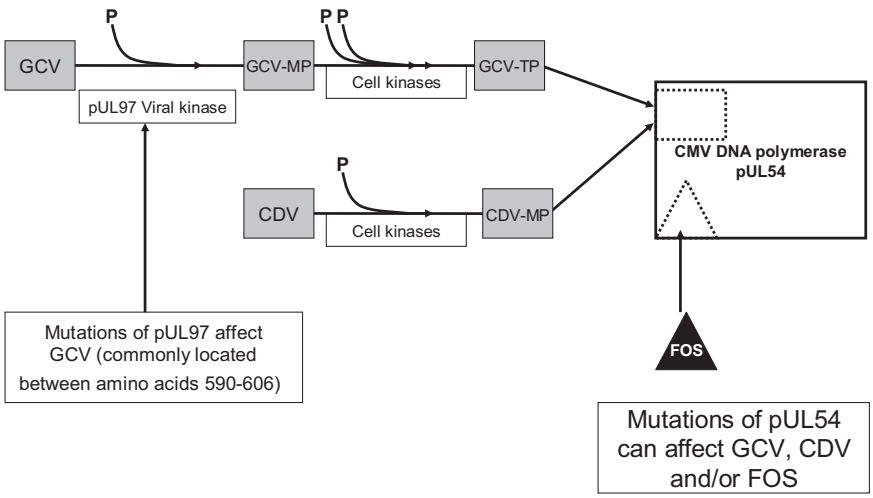

Fig. 86.1 Anti-CMV drugs: mechanisms of action and resistance. (Ganciclovir) GCV needs to be first phosphorylated by a viral kinase encoded by the gene UL97. The CMV DNA polymerase, encoded by the gene UL54, can also mutate and potentially cause resistance to CGV, cidofovir (CDV), and foscarnet (FOS)

causing drug resistance are V715M, V781I, and L802M, which confer resistance to FOS, and F412C, L501I/F, and P522S, which result in resistance to GCV and CDV. The mutation A809V, which confers resistance to GCV and FOS, the mutation N408K which confers resistance to GCV and CDV, and the mutation A834P which causes resistance to GCV, FOS, and CDV have also been reported [157, 158]. Some mutations (A834P, E756K, and V812L and the deletion of codons 981 and 982) can cause resistance to all three of those antivirals [157–159]. The effect of combined mutations can be synergistic, as it is the case with mutations N408K and A834P. N408K and A834P cause a 4.2-fold and a 5.4-fold increase in resistance against GCV, respectively. When present together, the resulting fold increase in resistance is 22.7, rendering the virus highly resistant to this drug [158]. The mutations L501I and K513N and deletion of codons 981–982 result in six- to eightfold decrease in GCV susceptibility, and mutations F412C/V, K513N, and A987G have been associated with a 10- to 18-fold decrease in CDV susceptibility [157, 159, 160]. In addition, the substitutions D588N, V715M, E756K, L802M, and T821I can reduce the susceptibility to FOS from 5.5- to 21-fold [157, 159–162].

Fomivirsen (ISIS 2922) is a 21-base oligonucleotide with phosphorothioate linkages that are complementary to human CMV immediate-early 2 (IE2) mRNA. Hence, fomivirsen binds to this complementary CMV mRNA sequence and inhibits translation of several CMV immediate-early proteins [32, 163]. Although a resistant virus has been isolated in vitro, the mechanism of resistance was not due to loss of encoded complementarity with the oligonucleotide. To date, no report has been published on fomivirsen resistance in patients [164].

As is also the case with CMV disease, the prevalence of drug-resistant herpes simplex virus (HSV) variants is both highest and of greatest concern in immunocompromised hosts [165]. It has been reported that up to 30 % of allogeneic bone marrow transplant patients may be infected with acyclovir (ACV)-resistant HSV [166] (97). These mutants arise spontaneously and are selected by exposure to antiviral agents. Resistance to ACV and also to related drugs such as penciclovir that are used for the treatment of HSV infection arises predominantly from mutations in the virally encoded thymidine kinase (TK) gene. The TK gene product is responsible for the phosphorylation of ACV to ACV-monophosphate, an important initial step that is essential for the activation of nucleoside analogues such as ACV in HSV-infected cells. ACV resistance mutations in TK involve nucleotide additions, deletions, or substitutions that often occur in regions that contain a high density of guanine-cytosine (G-C) sequences and which are thought to be more prone to mutagenesis. Examples of common ACV resistance mutations in TK include repeated nucleotides at codon 92; a frameshift mutation at codon 146 that is detected in the majority of ACV-resistant clinical isolates; an arginine substitution at codon 176 in HSV type 1 or, alternatively, at codon 177 in HSV type 2; and an amino acid substitution at codon 336 that is observed in both clinical and laboratory HSV strains with reduced sensitivity to ACV [166]. Furthermore, genotypic studies have shown that TK possesses an unusually high propensity for the development of mutations associated with polymorphisms that do not confer resistance to ACV. These polymorphisms are located throughout the TK gene but do not involve conserved domains or the nucleotide sequences that encode the ATP and nucleoside binding sites within TK. Lastly, mutations in the conserved domains of the HSV DNA polymerase gene have also been shown to be involved in resistance to ACV [166, 167]. For example, the L774V substitution in the polymerase conserved region VI has been shown to be associated with diminished susceptibility to both ACV and the pyrophosphate analogue foscarnet [168].

11 Future Perspectives

As reviewed herein, the application of genotypic resistance testing has proven to be instrumental for the clinical monitoring of antiretroviral drug resistance and now constitutes an important component of the standard of care for patients with HIV-1 infection in industrialized nations. Furthermore, the results of several studies including CPCRA 046 and VIRADAPT and the Havana trial have confirmed the prognostic value and cost-effectiveness of genotypic resistance testing for guidance of therapy in patients who experience virological failure during the second or later regimens. In particular, HIV-1 genotyping is an essential strategy for the optimization of combination therapy used in salvage regimens that include the fusion inhibitor T-20.

However, despite these significant advances, the high cost and complexity associated with genotypic drug resistance assays remain important economic and technological barriers in regard to their wider implementation, especially in resource-poor countries [169]. Careful planning and prioritized use of genotyping are essential in order to achieve the best cost-benefit in these circumstances. There are also several other potential applications for genotyping that may represent opportunities for further improvements in therapeutic outcomes for HIV-1 infection. An example concerns the use of genotypic analysis for monitoring polymorphisms in non-B HIV-1 subtypes that may be important in regard to differential patterns of antiretroviral susceptibility compared to HIV-1 subtype B viruses. For instance, some subtype C HIV-1 variants are known to possess naturally occurring polymorphisms at several RT and PR codons that are implicated in drug resistance [170, 171]. Studies have showed that the presence of these polymorphisms did not significantly reduce susceptibility to ARVs nor diminish the effectiveness of an initial antiretroviral therapy regimen for a period of up

to 18 months [170, 172]. However, it has also been suggested that polymorphisms at resistance positions may facilitate selection of novel pathways in some cases, leading to drug resistance especially with incompletely suppressive antiretroviral regimens [170]. This, in turn, may have important clinical implications with respect to the choice and long-term benefit of antiretroviral therapy that may indeed warrant increased genotypic surveillance, particularly as the worldwide prevalence of non-B HIV-1 infection is increasing rapidly [173]. HIV-1-infected pregnant women who have previously received antiretroviral therapy represent another important situation in whom genotypic drug resistance testing may be a perinatal strategy for guidance of therapy to prevent HIV transmission to infants [174, 175].

Genotypic monitoring may also be of prognostic value in the clinical management of patients with primary HIV-1 drug resistance. The prevalence of primary HIV-1 drug resistance (RT and PR resistance-associated mutations) in recently infected individuals in Europe [176] and North America [177, 178] has been estimated to be approximately 7 and 20 %, respectively. In addition, recent reports suggest that a trend exists toward worldwide transmission of drug-resistant HIV-1 variants in antiretroviral therapy-naïve individuals [179]. Of particular interest and concern is the transmission in primary HIV-1 infection of highly resistant and of multidrug-resistant (MDR) HIV-1 variants that harbor resistance-conferring mutations to two or three classes of ARVs. Studies have shown that these viruses display in vivo replication competence that is often comparable to that of drug-sensitive species and, moreover, that they are able to establish persistent infections in the absence of antiretroviral drug pressure [170–182]. The use of HIV-1 genotyping in this clinical setting may allow for earlier detection of HIV-1 MDR variants and, therefore, increase the likelihood for improved therapeutic outcomes during chronic infection. Furthermore, such testing may help to reduce overall the spread of HIV-1 drug resistance.

As mentioned previously, the use of interpretative algorithms in conjunction with HIV-1 genotyping has facilitated prediction of drug resistance from the plethora of mutational patterns that are frequently associated with failing antiretroviral regimens. Two types of computer-based algorithms have been developed for analysis of HIV-1 genotypic data; these are rule-based algorithms and a virtual phenotype [183]. Rule-based algorithms are derived from knowledge of in vitro drug susceptibility assays, the relationship between specific resistance-associated mutations and virological responses in HIV-1 infected patients, and expert opinion [184]. The virtual phenotype, on the other hand, utilizes databases that correlate various mutational patterns with actual in vitro phenotypic resistance and clinical response in order to infer the level of drug resistance (i.e., sensitive, intermediate, or resistant) to ARVs that is displayed by a viral isolate on the basis of its HIV-1 genotype [183]. Discordant results among widely used interpretative algorithms, in which a viral isolate is scored as sensitive by one program and resistant by another, are frequent. This situation constitutes an important limitation of current technology and, moreover, underscores the technical challenges associated with the coding and interpretation of complex patterns of drug resistance mutations.

Several studies have shown that discordance between various algorithms is greatest with NRTIs, with the exception of 3TC, as compared to NNRTIs and most PIs, where the level of disagreement is usually less [185–187]. For example, in one study that examined the Stanford University Database (HIV db), Bayer Diagnostics TRUGENE (BDT), and the Virco VirtualPhenotype (VP) HIV-1 genotyping programs, discordant results for interpretation of drug susceptibility to ddI, ddC, d4T, and ABC were reported in excess of 50 and 40 % of cases in comparisons between the HIV db and VP and BDT and VP algorithms, respectively [186]. In contrast to these findings, concordant scores for 3TC were obtained with all three genotyping programs in >90 % of cases studied [186]. It has also been suggested that the discordance that exists between algorithms reflects a need for increased clinical validation and better consensus in interpretation of drug resistance data during the development of these tools, especially for some drugs such as NRTIs [185, 186].

In addition, the use of phenotypic drug resistance assays in conjunction with genotyping may be of further predictive value in some situations [184, 188]. Unlike genotyping, phenotypic tests represent a more direct method for detection of HIV-1 drug resistance that is based on changes in the 50 % inhibitory concentration (i.e., IC_{50}) for a particular ARV in regard to a viral isolate in comparison with a HIV-1 reference strain [7, 184, 189, 190]. However, discordances between genotypic and phenotypic tests are not uncommon and can arise as a result of several circumstances [82, 184, 191]. In instances of either genotypic-phenotypic discordance or disagreements between different HIV-1 genotypic interpretative systems, access to expert advice on HIV-1 drug resistance may be invaluable in order to help reduce uncertainty with respect to decisions about a subsequent therapeutic regimen.

Lastly, genotypic drug resistance testing has also been successfully implemented for other chronic viral infections (i.e., HBV, CMV, and HSV) and may also hold promise for guiding therapy choice decisions to improve treatment outcomes for certain types of cancer. The development and future availability of antiviral therapy for additional viral diseases as well as the identification of novel molecular markers for cancer are likely to be key determinants in regard to the extended utilization of genotypic drug resistance assays.

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Part XIII

Public Health Issues of Drug Resistance

Antimicrobial Resistance: An International Public Health Problem

87

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1 Introduction

In recent years, the problem of antimicrobial resistance has been recognized and addressed by international, regional, and national public health agencies, authorities, and professional societies [1–4]. Antibiotics have saved millions of lives and have enabled many other medical advances since their discovery and introduction into clinical practice. The worsening problem of antimicrobial resistance now jeopardizes many of these advances. However, action plans to minimize this threat have been developed by many public health agencies around the globe [5–8]. This focus is likely to increase among public health agencies in the coming years. Several facets of the problem involve the relationship between human and animal use of antimicrobial agents [3, 9]. This chapter summarizes the components of the cost of resistance from a public health perspective and contrasts this perspective to other societal perspectives. It then reviews strategies at several different levels of responsibility, ranging from the patient care provider to international agencies. Finally, it considers appropriate public health responses according to the resources available for control. In an era of globalization, antimicrobial resistance represents an international concern that demands a concerted effort from multiple health and industry sectors. Public health must be at the forefront of these efforts. Antimicrobial resistance is widely recognized as a complex international problem. Antibiotics have saved millions of lives and have enabled many other medical advances since their discovery and introduction into clinical practice. The worsening problem of antimicrobial resistance now jeopardizes many of these advances [1, 2, 4, 7, 8, 10–13]. The number of pathogens resistant to multiple classes of antimicrobials has increased worldwide [14]. There have been reports of infections with *Pseudomonas aeruginosa* and *Acinetobacter* species which are resistant to all available antibiotics [15]. Other pathogens, such as *Neisseria gonorrhoeae*, that were previously relatively easy to treat, are now becoming much more difficult to treat due to antimicrobial resistance [16]. There is also evidence that resistant organisms that were previously confined to the acute care hospital setting are now sources of community-acquired infections [17]. Resistance is not only an issue for bacterial pathogens like staphylococci, enterococci, *Escherichia coli*, *Klebsiella*, *Neisseria*, and *Mycobacterium tuberculosis* but also the problem of resistance is increasingly being recognized in nonbacterial pathogens like *Candida*, HIV, malaria, and influenza [14].

The worldwide increase in the prevalence of resistance is a concern because it threatens both the optimal care of patients with infections and the viability of current healthcare systems [18]. For individual patients, antimicrobial drug resistance has a clear impact on patient morbidity and mortality [5, 19, 20]. Of concern for healthcare systems is the economic impact of resistance [20–23]. In the United States, resistance is especially costly for the healthcare system and for the third-party payers that support such systems [20, 22]. Costs are also burdensome for national-based healthcare programs [23]. The incremental cost of caring for patients infected with resistant organisms has several aspects [19, 20, 22]. As the prevalence of resistance increases, physicians often substitute older and less expensive drugs for newer and more expensive agents [24]. Such costs in the United States are absorbed only in part by third-party payers, which often reimburse on the basis of head count, diagnosis-related groups, or other formulas unrelated to specific services provided to an individual patient. Thus, most costs

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associated with resistant infections must be absorbed by the healthcare system itself [25]. As the prevalence of drug-resistant organisms increases, these additional costs will mount and become a threat to the financial stability of local, regional, and national healthcare systems, many of which are already struggling to survive.

2 Public Health as a Perspective on Resistance

Public health involves the population as a whole. It focuses on what we as a society do to assure conditions in which we can be healthy [26]. The goal of public health, the health of a population, can be distinguished from the goals of medical care, the health of a patient [27]. The public health goal, fueled by an aim of social good, is much broader in scope. It can encompass the health of neighborhoods, cities, countries, or even the entire world [21]. Using the public health perspective of health, a long time frame for evaluation is usually appropriate. Since antimicrobials can both prevent and treat infections in society, society considers them a valuable resource [28]. As resistance diminishes this resource, a societal goal should be to minimize resistance and therefore to reduce the forces that produce resistance [6, 29, 30].

All use of antimicrobials increases the likelihood of resistance developing [13]. From a societal viewpoint, the use of antimicrobials to appropriately treat infections would be an appropriate rate of depletion of this valuable resource [25, 31]. Overuse or misuse of antimicrobials would be an inappropriately increased depletion of this resource [32]. The costs of resistance from a public health perspective can be summarized as those resulting from treatment of patients infected with resistant organisms, those resulting from treatment of patients not infected with resistant organisms, and those resulting from antimicrobial use in agriculture, animal breeding, aquaculture, and industry (Table 87.1).

The impact of antimicrobial resistance includes an increase in morbidity, mortality, and added costs for patients with resistant organisms. The added costs include those derived from the use of scarce healthcare resources: the care of patients with infections of resistant organisms, preventing transmission, maximizing appropriate empiric therapy, and resistance surveillance. In addition, excess loss of productivity and excess intangible costs such as patient and physician anxiety, pain, suffering, and inconvenience are also increased with antimicrobial resistance. Antimicrobial drug markets may also be affected by increasing resistance. Older, cheaper, and more narrow-spectrum drugs often become less useful, while the marketability of newer, more expensive, and more broad-spectrum drugs is favored [33].

2.1 Morbidity and Mortality

Numerous studies have demonstrated that antimicrobial-resistant organisms are associated with a higher morbidity and mortality than susceptible organisms. This has been shown for many different types of resistant organisms [34–40]. Invasive infections with methicillin-resistant *Staphylococcus aureus* (MRSA) have frequently been shown to be associated with a higher mortality and an increase in hospital length of stay when compared to methicillin-susceptible *Staphylococcus aureus* (MSSA) [35, 36, 38]. Similar associations are found when looking at enterococci. Vancomycin-resistant enterococci (VRE) bloodstream infections are associated with a higher mortality than vancomycin-susceptible enterococci bloodstream infections [38]. Although prior studies had not shown a higher mortality with penicillin-resistant pneumococcal infections [38, 41], a more

Table 87.1 Factors contributing to the public health impact of antimicrobial resistance

Resulting from antimicrobial use in infected patients with resistant organisms	Added deaths[a]
	Added pain/suffering/inconvenience/anxiety
	Added costs for increased hospital stay: resulting work absence and loss of productivity
	Added costs for antimicrobial drug purchase (use of more expensive agents)
	Added costs for diagnostic and therapeutic procedures dealing with initial treatment and complications
	Added costs for infection control activities
	Loss of markets for old drugs (minus gain in markets for new drugs)
Resulting from antimicrobial use in patients not infected with resistant organisms	Added costs for substitution of a drug in empiric treatment because resistant organisms may be present (usually broader coverage, selecting for new emergence of resistance)
	Added costs for substitution of a drug in empiric treatment because resistant organisms may be present (usually more expensive)
	Added costs for infection surveillance
	Added anxiety about treatment failure
Resulting from nonhuman antimicrobial use (animals, aquaculture, agriculture, industry, etc.)	Emergence of resistance in human populations by transfer of resistance determinants from nonhuman settings

[a]Beyond that of a similar infection with a susceptible organism

recent meta-analysis has shown penicillin-resistant pneumococcal infections to be associated with a higher mortality compared to penicillin-susceptible infections [38, 42].

Similar outcomes have been noted for antibiotic-resistant gram-negative infections. *Escherichia coli* and *Klebsiella pneumonia*e strains resistant to extended-spectrum cephalosporins, multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains, and carbapenem-resistant *Acinetobacter* infections have all been associated with an increased length of stay and mortality when compared to their more susceptible counterparts [34, 35, 37, 43]. Specifically, this mortality is noted to be higher when study antibiotic-resistant gram-negative bacilli in the setting of septic shock [40]. One plausible reason for this finding is the difficulty in selecting an appropriate empiric antibiotic regimen in the era of highly drug-resistant gram-negative infections. It is well established that the duration of hypotension without appropriate antimicrobial therapy is a crucial factor in the probability of survival in septic shock [44].

In addition to gram-positive and gram-negative pathogens, drug-resistant tuberculosis has also been shown to have a higher mortality compared to drug-susceptible tuberculosis [39]. For all different types of drug-resistant infections, an increase in mortality affects more than just the patient when examined using the public health perspective. Although difficult to quantify, the consequences of premature death include a loss of productivity (number of productive years lost multiplied by the average yearly productivity) and the pain and suffering of family members and friends [25].

2.2 Added Healthcare Costs

In addition to an increase in morbidity and mortality, antimicrobial resistance is associated with added healthcare costs. These costs correspond to the direct and indirect costs of patient care (healthcare professionals' time, medications, devices, tests, administration, space, utilities, and patient travel costs) [45]. For infections that require hospital admission, these costs have been estimated by comparing total hospital expenditure for patients infected with resistant microbes to total hospital expenditure for those infected with susceptible organisms. These studies sometimes control for confounding factors such as comorbidities and severity of illness. In a 2012 study, hospitalizations with a resistant organism were on average US$15,626 more expensive than hospitalizations with a susceptible organism. This study included *Staphylococcus aureus*, *Enterococcus*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* [20]. Findings were similar in a 2009 study with the added cost ranging from US$18,588 to 29,069 [22].

In addition to hospitalization costs, healthcare costs can be estimated by looking at length of stay, which can be used as a surrogate measure. Length of stay has been found to be longer for resistant strains of *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterococcus* compared to susceptible strains. These increases in length of stay have ranged from 3.3 to 39.6 days in various studies of various organisms [20, 22, 34, 36, 37].

Tuberculosis is another example of an infection in which the drug-resistant strain has clearly been associated with higher healthcare costs compared to the drug-susceptible strain. A recent study showed multidrug-resistant (MDR) tuberculosis cases to cost US$134,000 on average compared to extensively drug-resistant tuberculosis cases at US$430,000. Both of these numbers are compared to an average cost of US$17,000 for non-MDR tuberculosis [46].

The costs of infection control activities and antimicrobial stewardship programs should also be considered because these programs largely exist due to antimicrobial resistance. These expenses include personnel costs, equipment costs, surveillance costs, and microbiology laboratory costs [21]. Antimicrobial resistance also leads to the phenomenon of resistance-induced antimicrobial substitution [21, 24]. In the setting of empiric therapy, when treating septic shock, for instance, the physician feels obligated to attempt to cover all possible pathogens. As antimicrobial resistance worsens, the number of drugs that are needed increases. The cost of the medications also often increases, and new drugs are often more broad spectrum. In addition to the direct costs of this phenomenon, the overall problem of antimicrobial resistance is also worsened [21, 24].

3 Contrasts Between the Public Health View of Resistance and Other Views

There are multiple stakeholders when it comes to antimicrobial resistance. These various groups have different viewpoints about resistance and its impact (Table 87.2) [21]. The view of antimicrobial resistance from a public health perspective will be contrasted with the view of physicians and other medical providers, patients, healthcare businesses (both providers and payers), and pharmaceutical companies.

3.1 Physicians

Physicians and other healthcare providers are focused on treating individual patients [21]. They seek to cure disease and alleviate suffering by treating medical conditions. The time frame of most patient-provider relationships is short (at least in regard to treating infections). From the perspective of physicians, the loss of effectiveness of a single antimicrobial agent would typically be of little concern assuming

Table 87.2 Differing perspectives on the importance of antimicrobial resistance[a]

	The public	Medical professional	Patient	Healthcare business	Industry
Focus	Population	Individual	Individual	Care group	Potential clients
Desired outcome	Maximize health	Absence of disease	Absence of disease	Reduce cost of care	Increase sales
Time frame	Long	Short	Short	Short	Short, long
Motivation	The social good	Professionalism	Personal well-being	Profit	Profit
Approach	Reduce the forces leading to resistance	Treatment	Treatment	Cost containment	Develop new drugs, keep old drugs viable

[a]Adapted in part from [18]

there were other antimicrobials that could be used. However, as microbes accumulate resistance mechanisms that render multiple classes of antimicrobials ineffective, medical providers will become alarmed. Providers may be left with alternative therapies that are more toxic or no therapies at all. An example of this situation is the spread of carbapenemase-producing gram-negative bacteria throughout the world [47–51]. As the incidence of these very resistant pathogens increases, medical providers will very much take notice.

3.2 Patients

Patients are interested in antimicrobial resistance to the point that it affects their personal well-being. They would be concerned to know that antimicrobial-resistant infections are associated with a higher mortality than susceptible infections [34–40]. They may also be concerned about the increases in cost associated with resistance (especially if patients pay retail prices for their healthcare).

3.3 Healthcare Businesses

For the administrators who control healthcare system financial resources (both systems that provide healthcare services and the agencies that pay for such services), the major impact of resistance is the increasing healthcare costs associated with resistance [19–23]. Managers of these groups are also concerned about a reduction in morbidity and mortality, but they look to accomplish this goal in a fiscally efficient manner. They aim to minimize the costs of increasingly scarce financial resources. Antimicrobials are usually a cost-effective method of caring for patients who these groups are responsible for [25]. Measures that must be taken to deal with antimicrobial resistance may lead to incremental increases in the costs of drugs, diagnostics, and therapeutic services. The institution must also fund costs for personnel time, supplies, and equipment for institutional programs to deal with resistance (infection control programs, antimicrobial stewardship programs, etc.).

One example of a healthcare system intervention to deal with antimicrobial resistance and increasing costs was the decision in October 2008 for the Centers for Medicare and Medicaid Services to discontinue payments to hospitals for certain hospital-acquired infections. This decision was made in an attempt to curb costs and reduce the rates of hospital-acquired infections. Unfortunately (at least so far), this policy doesn't appear to have reduced infection rates in the United States [52, 53].

3.4 Industry

The focus for pharmaceutical firms, diagnostic instrument manufacturers, and other industry groups providing products for treatment and prevention of infectious diseases (antimicrobial agents, products to stimulate host defenses, vaccines, etc.) is similar in some ways to that of healthcare business. In this case, however, the clients of interest are the potential users of their products, both directly (patients) and indirectly (healthcare systems, governments, etc.). Product sales are the desired outcome, and a short-term view of sales is part of their outlook. However, industry also must take a longer view of the subject and consider the impact of resistance as a potential opportunity for introduction and sales of new products. This sometimes leads to antagonistic and conflicting views of the problem. On the one hand, the firms wish to maintain the life of their current antimicrobial products, a goal that is threatened by new patterns of antimicrobial resistance. On the other hand, resistance may make render competitor's products obsolete. It may also open up new potential markets for either new drugs or new uses of old drugs [54]. An interesting phenomenon is the reemergence of old antimicrobials like colistin that were previously seldom used due to high rates of toxicity that have found a new market due to increasing antimicrobial resistance [55]. Thus, the consequences of resistance to industry are varied and depend on the individual situation. Diagnostic instrument manufacturers, for example, may benefit by antimicrobial resistance as their products may become more in demand.

3.5 Summary: A Dramatic Difference in Viewpoints

It is clear that different stakeholders concerned about antimicrobial resistance have different focuses, motivations, and approaches to the problem. This discrepancy between the public health perspective and the perspective of others' informs how these different groups act.

4 Influences on Resistance and Control Strategies

The forces that influence the rates of antimicrobial resistance are several (Fig. 87.1). Antibiotic resistance is a naturally occurring phenomenon, and antibiotic resistance genes are present in nature long before those antimicrobials are used in humans. This is because environmental organisms often produce substances very similar (if not identical) to antimicrobials, and bacteria have evolved to survive these naturally occurring substances [56]. The spread and proliferation of antimicrobial use is certainly heavily influenced by human behavior. Attempts to minimize antimicrobial resistance include appropriate antibiotic prescribing, antimicrobial stewardship programs, infection control programs, education, limiting use of antimicrobial use outside of human populations, and novel antimicrobial drug development [57, 58]. Different individuals and groups have different focuses to combat antimicrobial resistance (Table 87.3). While certain principles for controlling antimicrobial resistance apply to all organisms, other principles are organism specific [59]. For example, the prevention strategies for controlling the spread of gram-negative antimicrobial resistance are different than those for controlling resistance in HIV. In the paragraphs that follow, the strategies that various interested parties use to combat antimicrobial resistance will be examined.

4.1 Providers

Regardless of which resistant organisms are of interest, healthcare providers are crucial to combating antimicrobial resistance. Providers have a responsibility to attempt to educate their patients about the issue of antimicrobial resistance [7]. In addition to education, medical providers have a tremendous impact on antimicrobial resistance by their prescribing behavior. Antibiotic overuse, misuse, and underuse are all examples of inappropriate use of antimicrobials that contribute to resistance [60]. Antibiotics are overused when prescribed for noninfectious inflammatory processes like pancreatitis, when given for an unnecessarily long duration or when used to treat bacterial colonization rather than infection. Antibiotics are misused when unnecessarily broad-spectrum agents are chosen or when antibiotics aren't appropriately narrowed after culture results return. Antibiotics are underused when dosing is inappropriately used or when antibiotics are prematurely discontinued [60]. Despite providers' best intentions, the inappropriate use of antibiotics is rampant. Many hospitals have developed antimicrobial stewardship programs (usually lead by medical providers) to attempt to optimize the use of antimicrobials [61–63].

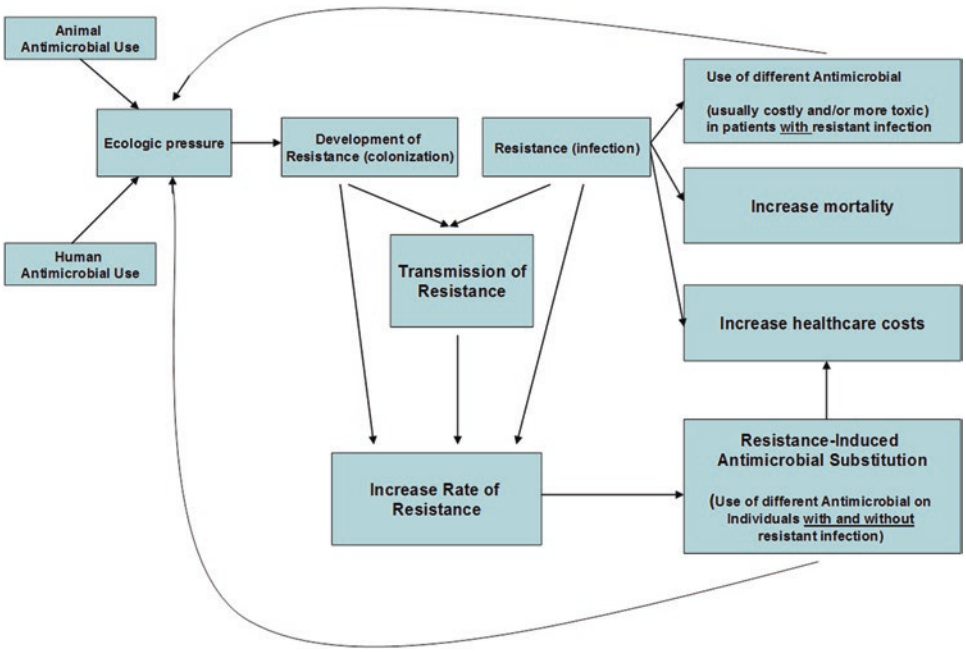

Fig. 87.1 Forces influencing antimicrobial resistance and its consequences

Table 87.3 Possible control strategies for controlling resistance, by levels of responsibility within the public health and healthcare systems

Strategy	Provider level	Local health department/ hospital level	Regional health department level	National level	International level
Patient education	Conduct	Provide materials	Provide materials	Provide materials	Provide materials
Prescriber education	Self-education	Provide materials	Provide materials	Provide materials	Provide materials
Resistance and antimicrobial use of surveillance	Report cases	Aggregate and disseminate resistance and antibiotic use summaries	Aggregate and disseminate resistance and antibiotic use summaries	Aggregate and disseminate resistance and antibiotic use summaries	Aggregate and disseminate resistance and antibiotic use summaries
Treatment	Implement	Community programs	Provide guidelines and resources; ensure drug supply/quality	Provide guidelines and resources; ensure drug supply/quality	Provide guidelines and resources; ensure drug supply/quality
Preventing spread	Implement isolation, quarantine, and vaccination	Contact tracing, vaccination campaigns	Provide guidelines and resources, vaccination campaigns	Provide guidelines and resources, vaccination campaigns	Provide guidelines and resources
Research	Study individual patients or a group of patients (case reports and case series)	Study effective provision of services	Study effective provision of services	Support local and regional studies; study drug, diagnostic test, and vaccine development	Support local and regional studies; study drug, diagnostic test, and vaccine development

4.2 Local Health Departments and Hospitals

Local health departments and hospitals should participate in education, generating useful tools and disseminating them to providers and patients. As mentioned previously, antimicrobial stewardship programs (usually run by hospitals) have an important role in attempting to optimize the use of antimicrobials [61–63]. Over the past decade, the number of antimicrobial stewardship programs in the United States has increased [64]. The goals of antimicrobial stewardship programs are several. They seek to reduce the rates of antimicrobial resistance (or at least slow the increase). They also seek to improve the care of patients (ensuring infections are appropriately treated), to minimize adverse drug toxicities, and to decrease the incidence of *Clostridium difficile* infection. They may also have the ability to reduce healthcare costs [65]. The possible strategies to achieve these goals are several. Antimicrobial stewardship programs have attempted to develop clinical guidelines, educate providers and patients, require prescription approval, and use computer-based decision support systems [65, 66].

In addition to antimicrobial stewardship programs, hospitals and local health departments play a significant role in surveillance. Using *Neisseria gonorrhoeae* as an example, local health departments play a critical role in the surveillance of drug-resistant strains. For this infection, diagnosis in the clinical setting is usually made by nucleic acid amplification tests (which cannot detect resistance). Thus, local health departments have a role in continuing to collect cultures to monitor for drug-resistant *N. gonorrhoeae* [67]. Tuberculosis is another infection where surveillance for drug resistance is important. Optimally, in all cases of tuberculosis, the isolate should undergo drug susceptibility testing. This is especially imperative when the patient is failing therapy or in locations with very high rates of resistance [68].

4.3 Regional, National, and International Health Organizations

All of these groups have similar responsibilities in the effort to control resistance. One important role is antimicrobial resistance surveillance. They should aggregate, summarize, and disseminate surveillance reports to help providers make patient care decisions and to help governments and health organizations make policy decisions [69–74]. These groups also have a role in generating "calls to action" and mobilizing the political will to bring about legislation that tackles the problem of antimicrobial resistance [75–78].

Another role that national and international organizations have is in the development of guidelines to reduce antimicrobial resistance. One example of such guideline development is the Centers for Disease Control and Prevention's Ventilator-Associated Pneumonia Surveillance Definition Working Group. This group was formed to improve ventilator-associated pneumonia surveillance with the ultimate aim of reducing such events [79–81].

5 Public Health Perspectives of Antimicrobial Resistance According to Resource Limitation Levels

Since antimicrobial resistance is both a cause and a consequence of resource utilization, forces that drive the emergence and spread of resistance are clearly different according to the

Table 87.4 Public health perspectives of antimicrobial resistance according to resource limitation levels

Resource limitation level	Extreme resource limitation	Significant to moderate resource limitation	Minimal to moderate resource limitation	Minimal resource limitation
Resource use	Minimal to none	Some (inconsistent and/or insufficient)	Inappropriate (excessive use and poor compliance)	"Perfect" use
Antimicrobial use and consequence	No antimicrobial use— minimal antimicrobial resistance (naturally occurring)	Inconsistent antimicrobial use (interrupted supply, suboptimal dosing, use of counterfeit drugs, and nonprescription antimicrobial use)—excessive emergence of resistance	Excessive use of antimicrobials (including those not used by humans) and excessive use of broad-spectrum agents—excessive emergence of resistance	Appropriate (indication and dosing) and consistent (uninterrupted supply) use of good quality antimicrobials—unavoidable emergence of resistance
Infection control activity and consequence	No infection control activities—minimal resistance transmission	Inconsistent and incomplete infection control activities—excessive resistance transmission	Inappropriate use of infection control activities (noncompliance, slow implementation, etc.)—excessive resistance transmission	Appropriate use of isolation practices and other infection control strategies—minimization of resistance transmission
Public health consequences	Excess mortality due to treatable infections, excessive public health costs	Excess morbidity, mortality, and excess healthcare costs caused by infections due to resistant organisms	Excess morbidity, mortality, and excess healthcare costs caused by infections due to resistant organisms	Unavoidable but justifiable emergence of resistance and public health costs
Possible responses	Scale up antimicrobial agents, surveillance, vaccination, and infection control strategies (limited by resource availability)	Optimize appropriate and consistent use of good quality antimicrobials, surveillance, vaccination, and infection control practices	Optimize appropriate antimicrobial use, surveillance, vaccination, infection control practices, drug development, vaccine development, and diagnostics development	Surveillance, drug development, vaccine development, and diagnostics development
Setting	Extremely poor or low-income countries	Mainly middle-income countries	Mainly high-income countries	Nonexistent (utopia)

different levels of resource limitation. Similarly, the public health consequences and the appropriate public health policy responses are different for each scenario. Table 87.4 shows a somewhat simplistic categorization of the emergence and spread of resistance, its public health consequences, and the possible responses according to resource limitation levels.

5.1 Settings with Extensive Resources

For comparison purposes, let's suppose a utopia where there is minimal resource limitation, antimicrobials are always used appropriately, and infection control practices are timely and adequately implemented. In this scenario, some resistance will emerge unavoidably because of the use of antimicrobials, but its spread will be limited as a consequence of good infection control strategies. The excess resistance and costs derived from antimicrobial use are justified since deaths from infection are being prevented by the rational use of antimicrobials. In this scenario, the benefit derived from antibiotic use outweighs the risks of emerging resistance. Resources can also be allocated to optimize active surveillance and to develop new diagnostic tests, new antimicrobials, and new vaccines. Of course, this utopia does not exist.

5.2 Settings with Minimal Resources

In settings in which resource limitation is extreme, antimicrobials are not available, and antimicrobial resistance only emerges as a natural phenomenon. For the 1 billion people in the world who live on less than $1.25 per day, paying for antibiotics may not be possible [3]. Excessive deaths result from treatable infections due to a lack of antimicrobials. In these settings, scaling up antimicrobial use, vaccination, surveillance efforts, and infection control activities should be done.

5.3 Settings with Significant Resource Limitations

This scenario may predominate in many of the low-resource countries and some rural areas of high-income countries. In these settings, antimicrobials are available, but are frequently misused due to overprescribing, use without a prescription or self-medication, and lack of diagnostic testing [82–84]. As a consequence of these practices, antimicrobial resistance is excessive. Additionally, infection control practices and antimicrobial stewardship programs have not been optimized, so the emerging resistance is easily transmitted [85]. In this scenario, the infections caused by resistant organisms lead to an

increase in morbidity, mortality, and healthcare costs. Appropriate public health responses for this scenario include optimization of the use and supply of good quality antimicrobials, active resistance surveillance, prioritization of vaccination, and improved infection control practices.

5.4 Settings with Minimal to Moderate Resource Limitations

This situation predominates in the high-income countries throughout the world and in high-complexity medical centers. In these settings, antimicrobials are readily available. However, antimicrobials are often used inappropriately. This includes excessive use and use of agents that are unnecessarily broad [60]. Antimicrobials are also used commonly in animals, agriculture, aquaculture, and industry [9, 76]. The ecologic pressure created by excessive antimicrobial use leads to an excessive amount of resistance. In these settings, significant resources are devoted to infection control practices [21], but these practices are often not followed or implemented appropriately. The net effect is excessive transmission of resistance. The excessive resistance leads to an increase in resistant organism-related mortality and an increase in healthcare costs. Appropriate public health responses include optimization of antimicrobial use [61–63], active resistance surveillance, improved vaccination, and improved infection control practices. Resources can also be directed toward new drug, diagnostics, and vaccine development.

6 Conclusion: Antimicrobial Resistance in the Era of Globalization—An International Concern for Public Health

Human migration, animal and vector movement, and food markets may facilitate the spread of antimicrobial resistance across almost any geographic or political boundary [76]. Methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* were first reported in the United Kingdom. After becoming widespread in North America and Europe, the same resistance patterns were noted in Asia. Today, novel gram-negative carbapenem resistance mechanisms such as OXA-48 and NDM-1 (New Delhi metallo-β-lactamase) were first discovered in Asia and have since spread to Europe and North America [86]. The global spread of antimicrobial resistance demands effort of multiple health and industry sectors (in both low-resource and high-resource countries) to strengthen multinational/international partnerships and regulations. Public health must be at the forefront of these efforts.

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Hospital Infection Control: Considerations for the Management and Control of Drug-Resistant Organisms

88

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1 Introduction

The prevalence of hospital-acquired, antibiotic-resistant organisms has increased significantly over the last 20 years. Data from the National Healthcare Safety Network (NHSN) report published in 2013 revealed an alarming proportion of drug-resistant pathogens [1]. The NHSN system report represents data from more than 4000 medical facilities throughout the United States. Reports of hospital-acquired infections and microbiology data from participating institutions are published annually. From the sample, in 2009–2010, 43.7–58.7 % of all *S. aureus* isolates were resistant to methicillin, depending on the site of infection. *Enterococcus faecalis* and *Enterococcus faecium* were 6.2–9.8 % and 62.3–82.6 % vancomycin resistant, respectively [1]. An increase in drug-resistant staphylococci and enterococci has also been reported in Europe and South America [2–4].

As hospital-acquired infections with drug-resistant pathogens become increasingly more common and endemic, healthcare systems have taken various infection control measures to limit both their frequency and spread (Table 88.1, summary). Three parameters define the prevalence of drug-resistant bacteremia: how much enters the institution from outside, how much is selected by antibiotic use and misuse, and how much spreads from person to person [5]. The early recognition and isolation of incoming patients harboring resistant pathogens, appropriate antibiotic control programs, and assiduous infection control are necessary to minimize cross infection. Within the infection control domain, there may be specific efforts to minimize patient, healthcare worker (HCW), and environmental reservoirs and efforts to create meticulous hand hygiene and glove and gown use. In addition, surveillance systems for infection with hospital-acquired pathogens are essential for establishing endemic rates and for defining outbreaks. Aggressive surveillance for asymptomatic reservoirs may be of value but is not without controversy. Other considerations for an infection control program include hospital design considerations and antibiotic control programs.

1.1 The Importance of Patient and Healthcare Worker Colonization with Drug-Resistant Pathogens: Reservoirs for Infection

Colonization serves as a significant reservoir of drug-resistant, hospital-acquired pathogens. Patient colonization by drug-resistant pathogens such as VRE and MRSA has been well described. Thirty to 50 % of healthy adults have nasal colonization with *S. aureus*, with 10–20 % persistently colonized [6, 7]. Both methicillin-sensitive *S. aureus* (MSSA) and MRSA isolates can be persistent colonizers. Colonization with MRSA has been well documented in various healthcare settings. It has been reported that 25 % of patients admitted to a hospital will become nasally colonized with *S. aureus* [8]. This figure varies widely based on different populations and risk factors. Rates as high as 40–60 % have been reported in select populations including patients with diabetes and HIV. Certain populations are predisposed to colonization with *S. aureus* at the time of admission. Dupeyron et al. prospectively analyzed *S. aureus* colonization in a cohort of 551 cirrhotic patients. Screening nasal and rectal swabs were performed within 48 h of admission to the

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Table 88.1 Summary of selected infection control measures for the management and control of drug-resistant organisms

Infection control measure	Rationale	Comment
Nasal decontamination with mupirocin	• 25 % of patient admitted to a hospital will become nasally colonized with *S. aureus* • Compared with MSSA colonization, both MRSA colonization and MRSA acquisition during hospitalization increased the relative risk of infection	• In one prospective study, the use of intranasal mupirocin in a surgical cohort was effective in reducing the frequency of *S. aureus* hospital-acquired infections only in patients previously colonized with *S. aureus* • Mupirocin decreased the rate of *S. aureus* infections in hemodialysis patients
Chlorhexidine bathing	• Colonization of bacteria on patients' skin leads to environmental contamination • Environmental contamination increases the risk of transmission of hospital-acquired infections	• Chlorhexidine bathing decreases rates of colonization and hospital-acquired infections • Chlorhexidine-impregnated cloths provide a convenient no-rinse option • Studies revealed no significant toxicity associated with chlorhexidine bathing • Use is not approved for infants <2 months old
Environmental decontamination	• The inanimate environment can be contaminated with MRSA, *C. difficile*, VRE, and drug-resistant gram-negative rods. This is a potential reservoir for cross-transmission of hospital-acquired pathogens via the hands of HCWs	• All healthcare facilities should develop policies for the terminal and periodic disinfection of patient care areas and environmental services • This policy should include input from infection control practitioners, industrial hygienists, and environmental services supervisors • Ultraviolet light and hydrogen peroxide vapor are useful options for whole-room terminal cleaning
Hand hygiene	• Hand hygiene is the single most effective method to limit the spread of drug-resistant pathogens and hospital-acquired infections • Multiple opportunities exist in the hospital environment for the contamination of healthcare worker hands including direct patient care and contact with environmental surfaces	• Increased accessibility to hand hygiene agents is associated with improved compliance • Medicated hand washing agents are bactericidal (alcohol, chlorhexidine gluconate, triclosan) and effectively reduced bacterial counts on the hands • Chlorhexidine has the advantage of producing a residual antibacterial effect, thereby limiting hand recontamination until the time of the next hand hygiene episode • Sustained improvements in hand hygiene compliance should be achieved through a multimodal approach, which includes efforts that stress increased use of accessible, easy to use, medicated hand hygiene products, coupled with a hospital-wide, administration-backed, high-priority hand hygiene campaign • Novel hand hygiene technologies are emerging as useful methods for monitoring hand hygiene compliance
Gloves	• Gloves should be worn to prevent healthcare worker exposure to blood-borne pathogens and to prevent contamination of hands with drug-resistant pathogens during patient care activities	• Even with proper glove use, hands may become contaminated during the removal of the glove or with micro-tears that allow for microorganism transmission • Glove use should not be used as a substitute for hand hygiene
Gowns	• Several studies have documented colonization of healthcare worker apparel and instruments during patient care activities without the use of gowns	• The use of gloves *and* gowns is the convention for limiting the cross-transmission of hospital-acquired pathogens; however, the incremental benefit of gown use, in endemic settings, may be minimal
Healthcare worker apparel	• Contamination of healthcare worker apparel occurs throughout the course of a normal work day • Biological plausibility suggests that contaminated apparel could lead to transmission of organisms between patients	• Expert guidance by SHEA recommends implementing hospital-wide policies that include "bare below the elbows," restriction of white coats during patient care activities, and frequent laundering of apparel
Contact precautions	• Contact precautions are for selected patients who are known or suspected to harbor certain infections	• Contact precautions are commonly employed for the endemic control of MRSA, VRE. *C. difficile*, and multidrug-resistant gram-negative rods • Contact precautions are typically employed along with other infection control measures during hospital outbreaks of drug-resistant infections • Controversies in contact precautions include low compliance rates, increased rates of adverse events and anxiety/depression, and decreased satisfaction of care among patients on contact isolation

(continued)

Table 88.1 (continued)

Infection control measure	Rationale	Comment
Screening for asymptomatic patient colonization with drug-resistant pathogens	• Some authorities advocate active surveillance cultures to identify the reservoirs of MRSA and VRE • The goal of active surveillance is to identify every colonized patient so that infection control interventions such as contact isolation and cohorting can be implemented to reduce the risk of cross-transmission	• This measure is controversial • The majority of the studies had multiple interventions and major methodological weaknesses. As such, the quality of evidence in many studies was considered weak • The use of strict isolation practices may have a detrimental impact on the process and quality of patient care
Antibiotic control program	• Prolonged antibiotic prophylaxis with cephalosporins was an independent risk factor on logistic regression analysis for infections with cephalosporin-resistant gram-negative rods • Enteric VRE colonization has been associated with cephalosporin use • MRSA colonization has been associated with fluoroquinolone use	• The degree in which antibiotic pressure directly contributes to the cross-transmission of hospital-acquired infections remains poorly defined • All healthcare facilities are encouraged to implement multidisciplinary antibiotic stewardship teams, which should include a physician, pharmacist, clinical microbiologist, and infection preventionist

hospital. The investigators reported carriage rates of 19 % for MSSA and 16 % for MRSA. When comparing nasal carriers vs. non-carriers, the investigators documented a greater frequency of prior MRSA bacteremia and urinary tract infections, respectively, 8.3 % vs. 0.8 % and 11.4 % vs. 0.6 %. Additionally, the colonizing MRSA strain matched the invasive strain by pulsed-field gel electrophoresis [9].

In a different prospective series, however, only 2.7 % of the isolates were identified as MRSA [10]. Using a case control study and multivariate analysis to determine risk factors for MRSA colonization, independent predictors for colonization with MRSA were prior admission to a nursing home (OR 16.5) and a prior hospitalization of greater than 5-day duration within the past year [10].

Surveillance in nursing home settings reveals an increasing prevalence of *S. aureus* colonization. A prospective study in the mid-1980s by Sheckler et al. failed to document MRSA colonization in a cohort of community-based nursing homes [11]. Another study of community-based nursing homes from the early 1990s revealed 24 % of patients with *S. aureus* colonization, while 8.7 % of all patients were colonized with MRSA [12]. Lee et al. reported *S. aureus* colonization and infection in a 149-bed skilled nursing facility over a 1-year period. In this series, nasal and stool or rectal screening cultures were done on admission and then on a quarterly basis for a year. At the conclusion of the study, 35 % of all patients were colonized with *S. aureus* at least once during the period of analysis. Of the positive cultures, 72 % were MSSA, 25 % were MRSA, and 3 % were mixed phenotype. Only a minority of patients colonized developed an infection with *S. aureus*. The authors reported no association between MRSA colonization and frequency of *S. aureus* infection [13].

MRSA colonization has been studied in the intensive care setting. Garrouste-Orgeas et al. prospectively studied MRSA colonization and infection in a medical-surgical ICU of a tertiary care medical center [14]. In this prospective, observational study, cultures were obtained within 48 h of hospitalization, then weekly thereafter. Five percent of all patients were colonized with MRSA at the time of admission, and 4.9 % were newly colonized with MRSA during the course of their ICU stay. After multivariable analysis, factors associated with MRSA infection were severity of illness (HR 1.64), male gender (HR 2.2), and MRSA colonization (HR 3.84). However, MRSA colonization was not associated with increased mortality [14]. Overall, 10 % of patients in the cohort were colonized with MRSA. A similar rate of MRSA colonization has been documented by other investigators [15].

Co-colonization or coinfection with multidrug-resistant pathogens has been reported in several different populations. A point prevalence survey of antimicrobial-resistant pathogens in skilled care facility residents revealed a high rate of MRSA colonization. Of the 177 patients surveyed, 24 % were colonized with MRSA. Additionally, ESBL-producing organisms were discovered in their patient population, including *K. pneumoniae* (18 %), *E. coli* (15 %), and VRE (3.5 %). As these patients were asymptomatic, the investigators discovered a large, unrecognized pool of antimicrobial-resistant pathogens in their nursing home population [16]. Warren et al. determined the occurrence of co-colonization and coinfection with VRE and MRSA among medical patients in a medical ICU of a tertiary care medical center. Screening cultures were obtained in adults requiring at least 48 h of intensive care therapy. The study evaluated 878 consecutive patients. Of these, 40 % were either colonized or infected with VRE, 4.4 % were either colonized or infected with MRSA, and 9.5 % had either co-colonization or coinfection with MRSA and VRE. Risk factors for co-colonization or coinfection were increasing age, prior hospitalization within the preceding 6 months, and admission from a long-term care facility [17]. In a study of patients at high-risk

wards at an urban academic center, almost 30 % of patients carrying VRE were co-colonized with MRSA [18].

1.2 The Impact of Colonization Status on Hospital-Acquired Infections

An association between MRSA colonization and the subsequent development of MRSA hospital-acquired infections exists. Pujol et al. prospectively analyzed the relationship of MRSA nasal colonization and bacteremia [19]. During a 1-year period in an ICU, nasal swabs were obtained on all patients within 48 h post admission and then weekly. Thirty percent of all patients were nasal *S. aureus* carriers: 17 % with MSSA and 13 % with MRSA. Bacteremia was observed in 38 % of the MRSA carriers and 9.5 % of the MSSA carriers. Using Cox proportional hazard modeling, the relative risk (RR) of *S. aureus* bacteremia was 3.9 when comparing MRSA to MSSA nasal carriers [19].

Other investigators have confirmed the significance of MRSA colonization and its predilection for subsequent infection. Davis et al. investigated MRSA colonization at hospital admission and its subsequent effect on MRSA infection rates [20]. Nares cultures were obtained on admission on patients admitted to various hospital units, including medical, surgical, and trauma ICUs. The patients were followed for the study period and then 1 year thereafter. Nasal colonization with MSSA far exceeded that with MRSA (21 % vs.3.4 %). However, 19 % of patients with MRSA colonization at admission and 25 % with subsequent colonization developed infection with MRSA. Reported infections included line sepsis, bacteremia, and skin and soft tissue infections. Compared with MSSA colonization, both MRSA colonization and MRSA acquisition during hospitalization increased the relative risk of infection (RR 13 and RR 12) [20].

Nasal carriage of both MRSA and MSSA has been associated with increased risk of vascular access-related infections in patients with type II diabetes on dialysis. In this series, nasal swabs were performed in 208 patients enrolled for long-term hemodialysis between 1996 and 1999 [21]. Persistent nasal carriage was defined as two or more positive cultures. Diabetic patients had higher MSSA and MRSA carriage rates (54 and 19 %) than nondiabetics (6 %). Overall, 73 % of all diabetic patients were colonized nasally with either MRSA or MSSA. Additionally, when compared to nondiabetic hemodialysis patients, the relative risk for vascular access-associated bloodstream infection was significantly greater [21].

Lastly, published data suggest that healthcare workers colonized with drug-resistant pathogens may be associated with cross-transmission and hospital-acquired infections. Wang et al. investigated a hospital-acquired outbreak of MRSA infection initiated in a surgeon carrier [22]. Over a 4-month period, five patients who had undergone open-heart surgery developed surgical wound infections and mediastinitis with MRSA. Investigation by the infection control team led to MRSA nasal screening of all ICU staff and of the surgical team. Pulsed-field gel electrophoresis technology was employed for isolate typing. Of the five hospital-acquired MRSA infections, all had the same attending surgeon and 2–3 assistant surgeons. Surveillance cultures of the staff were all negative save for one assistant surgeon, present in all of the five cases. The typing profile of the surgeon's isolate was identical to that of three of the cases. The remaining two isolates were lost and hence not typed; however, these were presumed to be identical to the others owing to the same antibiogram [22]. Other investigators have reported healthcare colonization and its effect on cross-transmission and subsequent MRSA infection and colonization. Boyce et al. reported the spread of MRSA within a hospital. A healthcare worker with chronic sinusitis was the purported source [23]. In addition, outbreaks of MRSA infections in a burn unit have implicated nursing staff as sources [24, 25].

1.3 Strategies for Staphylococcal Decolonization

Given the importance of *S. aureus* as a hospital-acquired pathogen, decolonization of carriers has been attempted in various populations. Early investigations employed both topical and systemic therapy for the eradication of *S. aureus* nasal colonization. In the 1980s, in experimental studies, it was shown that mupirocin was effective in reducing nasal carriage of volunteers with methicillin-sensitive *S. aureus* [26]. Subsequently it was shown that mupirocin was active against methicillin-resistant strains of *S. aureus* [26]. In the early 1990s, Darouich et al., as part of a multidisciplinary approach, attempted to control the spread of MRSA within a spinal cord unit [27]. Eleven patients in the spinal cord unit were colonized with MRSA. The sites of colonization varied but included nares, axilla, tracheostomy site, urethra, wounds, and urine. Ten of the colonized patients received a 2-week course of 100 mg of minocycline twice daily and 600 mg of rifampin once daily. The remaining patient was treated for only 1 week with the minocycline/rifampin combination. For those that were nasally colonized, nasal mupirocin ointment was applied twice daily for 5 days. The authors reported eradication of MRSA colonization in 10 of the 11 patients [27].

Subsequent data suggest that for nasal MRSA, mupirocin alone may be sufficient for decolonization. In one, 6-month, two-step, prospective study from France, the efficacy of nasal mupirocin for the prevention of *S. aureus* nasal carriage was assessed [28]. In the first 4 months, all patients in the surgical ICU were cultured without the nasal decontamina-

tion protocol. Nasal and surgical wound swabs and tracheal secretions were collected on admission and then once weekly. In the following 2 months, all patients admitted to the SICU were given twice daily intranasal mupirocin for 1 week. In the comparison, 31.3 % of untreated patients and 5.1 % of mupirocin-treated patients subsequently acquired nasal *S. aureus* while in the surgical ICU. In addition, nasal carriers were more commonly colonized in the bronchopulmonary tract and surgical wounds (62 %) than were nonnasal carriers (14 %). When compared to the nontreatment group, the bronchopulmonary tract infection rate was reduced in the group receiving mupirocin treatment. Thus, in a surgical ICU cohort, the use of prophylactic mupirocin treatment reduced the rate of both MRSA nasal colonization and subsequent MRSA colonization bronchopulmonary infection [28]. Additionally, the use of mupirocin has successfully decreased the rates of *S. aureus* infections in dialysis patients, even though most of these isolates were methicillin sensitive [29].

Intranasal mupirocin has been employed to prevent postoperative *S. aureus* infections. Perl et al. conducted a randomized, double-blind, placebo-controlled study to determine the efficacy of mupirocin in both the reduction of surgical site infections and in the prevention of other hospital-acquired infections [30]. A total of 3864 patients were included in an intention to treat analysis, and of these, 891 patients (32.1 %) were *S. aureus* colonized in the anterior nares. The cohort underwent either general, gynecologic, neurologic, or cardiothoracic surgery. At the conclusion of the study, 2.3 % of the mupirocin recipients and 2.4 % of the placebo recipients had *S. aureus* infections at the surgical site. However, in a subset analysis of *S. aureus* nasal carriers who received mupirocin, there was a statistically significant reduction in the rate of *S. aureus* hospital-acquired infections, 4.0 % versus 7.7 % for recipients of placebo. Thus, in this analysis, the use of intranasal mupirocin in a surgical cohort was effective in reducing the frequency of *S. aureus* hospital-acquired infections only in patients previously colonized with *S. aureus*. For patients known to be nasal carriers of *S. aureus*, consideration should be given to the preoperative application of mupirocin.

The above studies used a targeted approach to decolonization of patients colonized with MRSA, which requires active detection and isolation of the organism. This approach can be costly, both directly and indirectly [32]. Universal decolonization, which involves the broad use of infection prevention practices throughout populations that are at high risk of hospital-acquired infections, is favored by some as a preferred approach[31]. Huang et al. conducted a pragmatic, cluster-randomized trial to assess which approach is superior [32]. The study randomized 74,256 patients from 43 hospitals into three groups. The group that underwent universal decolonization had significantly lower rates of MRSA-positive clinical isolates when compared to either screening and isolation or targeted decolonization groups. Universal decolonization also resulted in decreased rate of bloodstream infections due to any pathogens. There was no significant difference in the number of MRSA bloodstream infections between the groups.

1.4 The Role of Chlorhexidine (CHG) Bathing for Prevention of Hospital-Acquired Infections

Chlorhexidine is an antiseptic that has activity against a broad spectrum of organisms, including gram-negative bacteria, gram-positive bacteria, and fungi [33]. Chlorhexidine bathing has been employed as a means to decrease bacterial burden on patient skin. Bathing or showering with a 4 % solution is effective in reducing bacterial density on the skin of patients [34, 35]. Recently, cloths impregnated with 2 % CHG have become widely available as a no-rinse option as well. One study demonstrated that 2 % CHG cloths may perform superiorly to topical application of 4 % CHG [35]. Chlorhexidine-impregnated cloths have also demonstrated effectiveness in reducing bacterial burden of multidrug-resistant organisms like *K. pneumonia* and MDR GNR on skin surfaces [36]. In 2012, Karki and Cheng published a systematic review that assessed the impact of CHG bathing (with CHG-impregnated cloths) on the incidence of healthcare-associated infections and colonization. The authors included 20 studies in the analysis: 15 quasi-experimental studies, 3 cohort studies, 1 crossover study, and 1 randomized controlled study. The final analysis demonstrated reduced rates of MRSA and VRE colonization and reduced rates of hospital-acquired infections with CHG bathing. There were no reports of significant toxicity for patients who underwent daily CHG bathing [37]. Subsequently, a multicenter, cluster-randomized, nonblinded crossover trial also concluded that CHG bathing reduces rates of colonization and CLABSI. The overall rate of acquisition of multidrug-resistant organisms was lower with the use of CHG-impregnated cloths than with the use of nonantimicrobial washcloths (5.10 cases per 1000 patient days versus 6.60 cases per 1000 patient days, respectively). This same study demonstrated significantly reduced rates of hospital-acquired bloodstream infections with the use of CHG-impregnated washcloths versus nonantimicrobial cloths (4.78 cases vs. 6.60 cases per 1000 patient days, respectively). Interestingly, central line-associated fungal bloodstream infections were also reduced with CHG bathing [38].

Two studies support the use of CHG bathing in children as well. One quasi-experimental study that included adult and pediatric patients found a significant reduction in *Clostridium difficile* infections with the use of CHG bathing. Compared to the baseline period when no CHG bathing was done, all cohorts that used CHG bathing had a lower relative risk of *C.*

difficile infection [39]. A multisite, cluster-randomized, crossover trial which was conducted in ten pediatric ICUs and involved 4947 patients demonstrated a reduction in the rate of bacteremia in patients receiving daily CHG bathing compared to those receiving standard bathing practices. Although reduction in the rate of bacteremia using intent to treat analysis was not statistically significant, the per protocol analysis did reveal a significant reduction in the rate of bacteremia. No serious adverse events were reported. However, more patients in the intervention group reported skin irritation [40]. Of note, these studies assessed tolerability and effectiveness of CHG in children greater than 2 months of age. Chlorhexidine is not currently approved by the US Food and Drug Agency for use in children less than 2 months of age due to the possibility of irritation or chemical burns [41].

Some concern for development of bacterial resistance to CHG and selection of resistant organisms with the use of CHG exists. There is a paucity of data on this topic, but reduced in vitro susceptibility to CHG have been reported [42, 43]. One study demonstrated higher MICs to CHG among a multidrug-resistant strain of *K. pneumonia*. Of the multidrug-resistant *K. pneumonia* isolates, 99 % had MICs >32 μg/mL, compared with 52 % of other *K. pneumonia* strains [42].

1.5 Environmental Contamination

It is well documented that patients colonized or infected with drug-resistant pathogens, such as MRSA, VRE, or multidrug-resistant gram-negative rods, contaminate the inanimate environment. Contaminated objects can include but are not limited to floor, bed linens, patient gowns, overbed tables, bedrails, urinary containers, enteral feeding tubes, light switches, bathroom faucets, IV pumps, telephones, and blood pressure cuffs [23, 44–46]. In addition to objects in patient rooms, contamination can extend beyond the immediate patient care area. Devine et al. surveyed two acute care hospitals (A and B) in the United Kingdom with a focus on contamination of ward-based computer modules [47]. In total, 24 % of sampled computer terminals were positive for MRSA. Five of the six positive computer terminal cultures were from hospital A. In contrast to hospital A, the infection control team of hospital B reviewed handwashing compliance regularly with doctors and nurses. Hospital B also reported a greater rate of paper towel consumption, a surrogate marker for hand hygiene compliance. Although the direction of transfer is impossible to define from such studies, the data suggest that inanimate reservoirs have the potential to contaminate the hands of healthcare workers. Furthermore, hand hygiene compliance may be essential in minimizing this risk of environmental contamination [47]. Contamination of gowns and gloves from hospital personnel (those performing nursing care activities on colonized patients and those with no direct patient contact) has also been documented [23].

The environment likely represents a potential source for healthcare worker hand contamination, an important step in the cross-transmission of hospital-acquired pathogens. A study by Duckro et al. gave credence to this idea [48]. Cultures were obtained from the intact skin of 22 patients colonized with VRE and from various environmental sites before and after routine care by 98 healthcare workers. Cultures were obtained from the hands of the HCWs before and after patient care, and pulsed-field gel electrophoresis typing of the isolates was performed. In this analysis, VRE was transferred from contaminated sites in the environment or on the patient's intact skin to clean, previously non-contaminated environmental and body sites via the HCW in 10.6 % of the opportunities. Of these 16 VRE transfer sites, 12 were patient body sites [48]. These data suggest that the hospital environment is a potentially important reservoir for cross-transmission of drug-resistant pathogens.

As patients colonized with resistant pathogens can contaminate the environment, proper environmental disinfection is an important step for minimizing the risk or cross-transmission. An extensive review of approved disinfectants and environmental cleaning practices is beyond the scope of this chapter. However, several general principles are of note. Terminal cleaning of patient rooms should aim to minimize the persistence of drug-resistant pathogens. Hospital environmental services personnel should clean the bed frame and handrails, mattress, and all other patient room furnitures with an Environmental Protection Agency (EPA)-approved disinfectant and use according to manufacturers' guidelines [49]. Suction containers should be removed and prepared for disposal or reprocessing, and all other reusable equipment should be decontaminated using an (EPA)-approved disinfectant. The bathroom in an isolation room should be thoroughly cleaned and disinfected, with particular attention paid to the sink, toilet, and doorhandle areas. Environmental surfaces with a high degree of patient body and hand contact such as bedrails, doorknobs, bathrooms, light switches, and wall areas should be cleaned with greater frequency and not exclusively at the time of patient discharge.

Traditional room decontamination may not be sufficient to eliminate environmental bioburden. Therefore, alternate methods for terminal disinfection of patient rooms are needed. Ultraviolet (UV) light irradiation has the ability to inactivate a wide range of biological agents. Rastogi et al. studied the effectiveness of UVC light for decontamination of three hospital surfaces (aluminum bed railings, stainless steel operating tables, and laboratory coats) [50]. *Acinetobacter baumannii* was inoculated onto small coupons of each of the three types of materials. Fifteen minutes of

UVC light exposure (at a fluence of 90 J/m [2]) resulted in ≥4-log reduction and complete killing of organisms on the two metal surfaces. However, UVC light was ineffective for laboratory coat disinfection [50]. In addition to inadequate penetration of fabrics, the use of UV light for whole-room disinfection has the disadvantage of providing only "line-of-site" killing.

Like UV irradiation, hydrogen peroxide vapor (HPV) inactivates a wide range of biological agents through production of oxygen free radicals. Its effectiveness was demonstrated in one prospective cohort intervention study [51]. The intervention (HPV) was implemented after routine cleaning and disinfection of rooms with a quaternary ammonium compound. All rooms were previously occupied by patients with known infection or colonization with multidrug-resistant organisms. Patients admitted to rooms that underwent HPV decontamination were 64 % less likely to acquire any multidrug-resistant organisms than those that were in rooms with no HPV decontamination. Specifically, patients in rooms with HPV decontamination were 80 % less likely to acquire VRE [51].

Several potential strategies exist for monitoring compliance and assessing environmental hygiene. Boyce et al. compared three methods of monitoring with a prospective observational study of 100 hospital rooms [52]. In this study, five high-touch surfaces were marked with different brands of fluorescent markers prior to terminal cleaning and were checked after cleaning with a black light to assess whether the marker had been partially or entirely removed. Aerobic colony counts (ACCs) and adenosine triphosphate (ATP) bioluminescence assays were performed on the same surface before and after terminal cleaning. The ATP method was much less likely than either fluorescent markers or ACC to classify a room as clean. This result is not surprising since ACC measures only contamination by aerobic bacteria, whereas ATP bioluminescence assays detect many ATP-containing organic substances such as secretions, blood, and food. The authors concluded that each method has utility for different situations. Fluorescent markers are simple to implement and are useful for providing feedback to housekeepers regarding adequacy of cleaning. Aerobic colony counts provide a quantitative measurement of surface contamination and provide information about specific organisms causing contamination, but are costly and time consuming. Advantages of ATP bioluminescence assays are ease of use, rapid results, and provision of quantitative measurements that can be used for trends and feedback [52].

All healthcare facilities should develop policies for the terminal and periodic disinfection of patient care areas and environmental services. This policy should include input from infection control practitioners, industrial hygienists, and environmental services supervisors.

2 Hand Hygiene

Hand hygiene, either by conventional handwashing or disinfection, is the single most effective method to limit the spread of drug-resistant pathogens and hospital-acquired infections [53]. Conceptually, the cross-transmission of hospital-acquired pathogens is summarized as follows [54]:

- Organisms present either on the patient's skin or from the inanimate environment must be transferred to the hands of the healthcare worker.
- Hospital-acquired pathogens must be capable of surviving on the hands of the healthcare worker.
- Hand hygiene must be either inadequate or omitted.
- The contaminated hands of the healthcare worker must then come into contact with another patient or into contact with an inanimate surface that will later come into contact with the patient.

The microorganisms of the hand can be divided into transient flora and resident flora [55]. The resident flora is typically of low virulence pathogens such as *Micrococcus*, coagulase-negative *Staphylococcus*, and *Corynebacterium*. These organisms are difficult to remove by handwashing yet are rarely pathogenic except when introduced to the patient by invasive procedures. Transient flora is acquired largely by contact with either the patient or an inanimate object, is loosely attached to the skin, and is easily removed by handwashing [55]. These organisms include MRSA, VRE, and MDR GNR. Additionally, these bacteria are important causes of hospital-acquired infections.

Numerous studies have shown that multiple opportunities exist in the hospital environment for the contamination of healthcare worker hands. Hospital-acquired pathogens can be recovered from a variety of patient care scenarios. Patient contact, including contact with wounds and intact skin, can result in healthcare worker hand contamination [56–67]. Areas of high hospital-acquired pathogen concentration on patient skin include the axillae, trunk, perineum, inguinal region, and hands [59, 61, 62, 64, 66–68]. As previously mentioned, the inanimate environment is a source of contamination.

Healthcare workers should practice hand hygiene before and after each patient contact. Methods of hand hygiene include washing with plain soap and water, or using an antibacterial agent such as alcohol, chlorhexidine gluconate, or triclosan as either detergent washes or waterless hand-rubs. Conventional soap and water may have various shortcomings and barriers to compliance. Although soap and water can remove loosely adherent transient skin, these agents have minimal antimicrobial activity [54]. For effective bacterial reduction, a 30 s hand rub is recommended; unfortunately, this time length of handwashing is rarely practiced. In

addition, several studies have demonstrated that handwashing with both plain soap and water can result in skin irritation, dryness, and a paradoxical increase in microbial counts on the skin [69–73]. Medicated handwashing agents are bactericidal (alcohol, chlorhexidine gluconate, triclosan) and effectively reduce bacterial counts on the hands. Moreover, chlorhexidine has the advantage of producing a residual antibacterial effect, thereby limiting hand recontamination until the time of the next hand hygiene episode [74].

At least one study supports the effectiveness of chlorhexidine as a hand antiseptic agent with regard to infection control endpoints. Doebbling et al. compared different hand hygiene agents with the end result of hand hygiene compliance observation and the reduction of hospital-acquired infections in an intensive care unit setting [75]. During an 8-month period, a prospective, multiple crossover trial was conducted in three intensive care units. The trial involved 1894 adult patients exposed to alternate months of either chlorhexidine or 60 % alcohol solution with the optional use of a non-medicated soap. A greater frequency of hospital-acquired infections was seen with the combination of alcohol and soap compared to the chlorhexidine hand hygiene agent (202 vs. 152). However, during periods of chlorhexidine use, there was a decrease in the rate of hospital-acquired infections and an increase in the observed frequency of hand hygiene compliance coupled with a volume of chlorhexidine consumption that exceeded that of the alcohol-based agent. The difference in hospital-acquired infections may have been partly due to increased compliance with hand hygiene practices. Regardless, owing to their bactericidal properties, medicated hand hygiene agents, including chlorhexidine, alcohol, and triclosan, should be highly considered especially in environments with elevated rates of drug-resistant pathogens.

Unfortunately, data on healthcare worker hand hygiene practice are discouraging. The reasons for poor compliance are multiple and have been studied by numerous investigators. Observational studies of hand hygiene compliance report compliance rates of 5–81 % [76–108]. Factors cited that may influence poor adherence with hand hygiene include insufficient time, understaffing, patient overcrowding, lack of knowledge of hand hygiene guidelines, skepticism about handwashing efficacy, inconvenient location of sinks and hand disinfectants, and lack of hand hygiene promotion by the institution [54].

In the intensive care units, where critically ill patients are particularly susceptible to hospital-acquired infections, hand hygiene is poor. A British study performed a detailed survey of hand hygiene practices in 16 ICUs [55]. Additionally, 381 (non-nurse) healthcare professionals were observed for hand hygiene compliance. Compliance with hand hygiene and proper glove use ranged from 9 to 25 %. Survey responses suggested that poor compliance with hand hygiene in the ICU was secondary to multiple issues including ineffective communication of infection control recommendations, insufficient promotion of hand antisepsis, and a deficiency of infection control education [55]. Poor compliance with hand hygiene was similarly observed by Kaplan et al. in a tertiary care American hospital [81]. Physician compliance with hand hygiene was 19 %, while compliance by the nursing staff was 63 %. Greater compliance with hand hygiene was observed among the nursing staff with a 1:1 bed to sink ratio than those with a greater bed to sink ratio (76 % vs. 51 %) [81].

Efforts to improve hand hygiene both in the ICUs and hospital-wide likely require simultaneous interventions on multiple levels. In a study by Bischoff et al. where alcohol-based hand sanitizers were introduced to an ICU, the greatest increment in hand hygiene compliance was observed when the hand sanitizer to healthcare worker ratio went from 1:4 to 1:1, thereby underscoring the importance of accessibility [82]. As such, the CDC now suggests promoting alcohol-based hand sanitizer access both by bedside dispensers and healthcare worker pocket-sized dispensers [54]. Pittet and colleagues improved overall compliance with hand hygiene by implementing a hospital-wide program with special emphasis on bedside, alcohol-based hand disinfection. The campaign ran from December 1994 to December 1997 and consisted primarily of hand hygiene promotion through large, conspicuous posters promoting hand hygiene throughout patient care areas. The project was supported and heavily promoted by senior hospital management. Additionally, alcohol-based handrub solutions were distributed in large amounts, mounted on beds/walls, and given to healthcare workers to encourage pocket carriage for convenience of use. During this time frame, seven institution-wide hand hygiene observational surveys were performed twice yearly. Additional measures included hospital-acquired infection rates, the rate of MRSA infections, and overall consumption of handrub disinfectant. In this 3-year study, 20,000 opportunities for hand hygiene were observed. Compliance with hand hygiene improved from a baseline of 44 % in 1994 to 66 % in 1997. Of note, hand hygiene improved markedly among nursing staff but remained poor for physicians. Additionally, over the study period, the overall prevalence of hospital-acquired infections decreased from 16.9 to 9.9 %, MRSA transmission rates decreased from 2.16 to 0.93 episodes per 10,000 patient days, and the consumption of alcohol-based hand rub increased from 3.5 to 15.4 L per 1000 patient days [109]. Unfortunately, as multiple interventions were employed simultaneously, the relative effect of each component was difficult to properly assess. Thus, although the most efficient and effective means for sustained improvements in hand hygiene compliance have yet to be defined, measures should at least include efforts that stress increased use of accessible, easy to use, medicated hand hygiene products, coupled with a hospital-wide,

administration-supported, high-priority hand hygiene educational and promotional campaign.

2.1 Hand Hygiene Bundles

Bundles are commonly used multimodal approaches in infection prevention practice that aim to improve patient care and outcomes. They combine several interventions concurrently in order to optimize outcomes more than any one intervention could achieve alone. Possible components of a hand hygiene bundle include administrative support, education and training, availability of hand hygiene resources (e.g., hand sanitizer, soap, etc.), and ongoing monitoring and feedback of hand hygiene compliance [110]. One commonly used bundle that is promoted by the WHO includes administrative support toward improved hand hygiene, access to alcohol-based hand rub (ABHR), performance feedback, education, and reminders [111]. Several studies have assessed the use of bundles in order to improve hand hygiene compliance. A recent meta-analysis reviewed the literature with the aim to assess utility of hand hygiene bundles [112]. Forty-six studies were included in the final analysis. There were 39 quasi-experimental, four cluster-randomized, and two randomized controlled trials. Two bundles were associated with improved hand hygiene compliance. One bundle included education, reminders, feedback, administrative support, and access to ABHR, while the other included education, reminders, and feedback. Interestingly, increasing the number of interventions in a hand hygiene bundle was not associated with improved compliance. This review was limited by study heterogeneity. Furthermore, most studies were quasi-experimental in design, which are subject to bias. Robust randomized controlled trials assessing hand hygiene bundles are lacking. Currently underway is a multicenter randomized controlled trial with the aim to identify an optimal hand hygiene bundle [113]. Combinations of three interventions (hand-hygiene point-of-use reminder signs to serve as an environmental cue to action, individual hand sanitizers, and healthcare worker hand cultures) will be assessed.

2.2 Emerging Technologies for Monitoring Hand Hygiene Compliance

Monitoring of hand hygiene provides important information about baseline and ongoing rates of compliance among healthcare workers. Several different methods of monitoring have been tried. Direct observation is the traditional method of monitoring and provides detailed information about adherence to the various components of hand hygiene (e.g., proper technique and compliance before and after patient contact). However, the reliability of direct observation is limited by observer bias as well as the Hawthorne effect [114, 115]. This practice is also time consuming and expensive to carry out. The use of novel hand hygiene technology has become a recent topic of interest and represents a possible alternative to direct observation. Measuring product consumption and electronic monitoring systems have been studied. Boyce recently published a thorough review of these emerging technologies [116]. Measurement of product consumption is accomplished via volume or weight of product used or amount of product purchased. Most studies have shown a direct relationship between amounts of product consumed and observed compliance rates [76, 117–119]. However, several other studies have shown no correlation between product consumption and observed hand hygiene rates [120, 121]. One prospective observational study compared direct observation, product usage, and electronic counting devices as methods of monitoring in a tertiary care hospital 40-bed ICU in Brazil. There were 2249 opportunities for hand hygiene observed with an overall compliance rate of 62.3 %. Direct observation did not correlate with the amount of product used. The authors concluded from this study that direct observation is an inaccurate method of monitoring [120]. Another quasi-experimental study by Morgan et al. similarly concluded that direct observation did not correlate with dispenser counts [121]. Despite this conflicting data, monitoring of product usage is likely to be a useful adjunct to monitoring by direct observation. Monitoring of product usage is less time consuming and less labor intensive, but also provides less detail about each episode. Once baseline product usage for an institution is established, trends can be followed.

Product use can also be monitored with electronic counting devices. These devices record a hand hygiene event every time sanitizer is dispensed. They supply additional important data, including frequency of use, and specific date, time, and location of use. One quasi-experimental study suggested that electronic counting may be a better method of monitoring than direct observation. Over a period of 30 weeks, 424,682 dispenser counts, 338 h of human observation, and 1783 room entries were recorded. Hand hygiene rates were monitored before and after feedback intervention, which included posters displaying unit-specific compliance rates and educational sessions for healthcare workers. Rates significantly increased according to electronic counters (average count/patient day increased 22.7 in the NCICU and 7.3 in the CCU), but were not significantly changed according to direct observation [121]. Larson et al. studied hand hygiene compliance (with the use of electronic counting devices) in response to changes in the hospital's organizational culture [122]. In this quasi-experimental study in two mid-Atlantic hospitals (one hospital received the intervention, while the other served as the control), 860,567 hand hygiene events were recorded over a period of 8 months. The intervention implemented in the study hospital included establishment of leadership support

and role modeling of proper hand hygiene, positive deviance, and feedback to units of current compliance rates. While the hand hygiene rate increased in both hospitals, the difference was greater in the intervention hospital. In addition to improvements in hand hygiene, rates of VRE infections were significantly reduced in the intervention group compared to the control group (85 % vs. 44 %, respectively) [122]. Other studies demonstrated that counting devices provide useful information about patterns of sanitizer use [123, 124]. For example, higher rates of sanitizer use outside of patient rooms than inside patient rooms were recorded [123]. Touch-free dispensers were preferred over manual dispensers [124].

Other technologies, such as dedicated hand hygiene systems and real-time location devices, target hand hygiene at the individual level. Marra et al. conducted a two-phase trial [125]. The first phase assessed baseline rates of hand hygiene using an electronic counting device. The second phase used real-time feedback with a wireless identification device (badge) that flashes red when the healthcare worker approaches the patient bed and has not performed hand hygiene and flashes green when hand hygiene has been performed. There was a significant increase in hand hygiene after implementation of the real-time feedback technology (74.5 episodes/patient day prior to intervention vs. 90.1 episodes/patient day during intervention) [125]. Another two-phase study used real-time feedback in the form of three consecutive beeps and the prerecorded voice prompt, "Please wash your hands," when healthcare workers failed to comply with hand hygiene upon entering or exiting a patient room. Hand hygiene improved from 36.3 % during phase 1 (prior to intervention) to 70.1 % during phase 2 (after intervention) [126]. Other studies reported similar improvements in hand hygiene rates after implementation of feedback technology [127, 128].

Another novel hand hygiene technology is real-time location systems. These systems use technologies such as Wi-Fi, active radio-frequency identification (RFID), infrared, and ultrasound to communicate information from special badges worn by healthcare workers. They have the advantage of being able to locate individual healthcare workers and the dispensers they access. This data is communicated back to a central server for real-time analysis [116]. Pineles et al. compared direct observation of hand hygiene to an RFID system. When compared to recorded data by the RFID system, direct observation was only 52.4 % accurate [129].

Video monitoring has also been used to assess healthcare worker hand hygiene compliance. Armellino et al. used video monitoring as a way to remotely assess hand hygiene in a medical ICU prior to and during a feedback period. Hand hygiene rates were 6.5 % during the 16-week pre-feedback period and 81.6 % during the 16-week post-feedback period. More importantly, the increase was maintained through 75 weeks at 87.9 % [130]. This study was extended to the surgical ICU and achieved similar results [131]. Video monitoring, similar to direct observation, requires significant man-hours, but may have the advantage of improved accuracy.

Possible barriers to implementing these technologies include upfront and maintenance costs, and healthcare worker buy-in. While these new technologies may improve hand hygiene monitoring accuracy and healthcare worker compliance rates, they continue to have shortcomings. They do not provide the level of detailed monitoring achieved by direct observation, such as hand hygiene technique, and hand hygiene practices prior to aseptic procedure or when hands are soiled.

2.3 The Use of Gloves and Gowns to Limit Cross-Transmission of Hospital-Acquired Pathogens

Gloves should be worn to prevent healthcare worker exposure to blood-borne pathogens and to prevent contamination of hands with drug-resistant pathogens during patient care activities. Nevertheless, even with proper glove use, hands may become contaminated during the removal of the glove or with micro-tears that allow for microorganism transmission [132]. Glove use should not be used as a substitute for hand hygiene. The promotion of glove use may increase compliance with hand hygiene protocols. A recent study by Kim and colleagues observed the rate of hand disinfection with glove use and patient isolation [133]. In this prospective, observational study, hand hygiene and glove use compliance rates were measured in two ICUs of a tertiary care hospital. Over 40 h of observation and 589 opportunities for hand disinfection were noted. Overall hand hygiene compliance was 22 %. The investigators found a statistically significant, positive association between glove use and subsequent hand disinfection (RR 3.9). Isolation precautions did not significantly increase hand hygiene compliance. For infection control purposes, glove use should be promoted as a means of limiting hand contamination with drug-resistant pathogens such as MRSA and VRE. Additionally, glove use and hand hygiene should be promoted concurrently.

2.4 Gowns

Gowns have been used as part of contact precaution protocols to limit the spread of hospital-acquired pathogens. Several studies have documented colonization of healthcare worker apparel and instruments during patient care activities without the use of gowns [134–136]. One study by Boyce et al. demonstrated the efficacy of disposable gowns in the prevention of HCW clothing contamination [136]. In another study, Srinivasen et al. prospectively measured the effect of gown and glove use in a 16-bed medical ICU of a tertiary care medical center. Over a 3-month period, all admissions

to a medical ICU were screened for VRE by perirectal swab. Patients who were culture positive for VRE were isolated by hospital policy, requiring the use of gown and glove for patient care. For the following 3 months, precautions were changed to glove use alone. The VRE acquisition rate was 1.8 cases per 100 patient days at risk in the gown/glove group and 3.78 per 100 patient days during glove use alone [137].

Not all studies, however, support the routine use of gowns for infection control measures. In addition, with regard to the endpoint of colonization and cross-transmission, there may be little incremental benefit to gown use over proper glove use and hand hygiene alone. Pelke et al. studied the effect of gowning in a neonatal intensive care unit over an 8-month time frame employing an alternating 2-month gowning and non-gowning cycles. The outcomes of interest were colonization patterns, necrotizing enterocolitis, respiratory syncytial virus, other hospital-acquired infections, mortality, and handwashing. The investigators failed to document any significant difference between the gowning and non-gowning cohorts with respect to the rates of bacterial colonization, infection type, or mortality. In addition, no significant difference in hand hygiene practice was observed [138].

Other investigators have compared gown use in addition to gloves and the effect on hospital-acquired transmission of VRE. Slaughter et al. compared the universal gloving versus universal gown and glove use on the acquisition of VRE in a medical intensive care unit. This prospective study involved 181 consecutive admissions. Half of the 16-bed ICU was designated for universal gown and glove use during patient care activities, and the other half was universal gloving for patient care activities. Rectal surveillance cultures were taken daily from patients along with monthly environmental cultures of bed rails, bedside tables, and other common objects in patient rooms. The investigators found no superiority in the universal use of gowns and gloves versus use of gloves only in preventing the rectal colonization of VRE in a medical ICU cohort [102]. Trick and colleagues compared the impact of routine glove use versus contact isolation on the transmission of multidrug-resistant bacteria in a skilled nursing home environment [139]. Over an 18-month period, all residents admitted to the skilled care unit of an acute and long-term care facility were randomly allocated to two different contact isolation precautions (gown and glove use) vs. routine glove use during patient care. No differences were observed in the transmission of antimicrobial-resistant bacteria, including MRSA, VRE, and extended spectrum beta-lactamase producing *K. pneumonia* and *E. coli* between the two study groups. Of note, greater compliance with proper glove use and hand hygiene was seen in the routine glove use section [139]. Harris et al. conducted a cluster-randomized trial among 20 medical and surgical ICUs. All healthcare workers in the ten ICUs assigned to the intervention groups were required to wear gloves and gowns for all patient contact and when entering any patient room. The ten ICUs in the control groups continued to follow their usual standard of care, which involved contact precautions (gloves and gowns) for patients known to be infected or colonized with multidrug-resistant organisms. Surveillance cultures for MRSA and VRE were performed at the time of ICU admission and at the time of discharge. A total of 92,241 swabs were collected for surveillance cultures from 26,180 patients. Intervention and control ICUs experienced a decrease in patient acquisition of antibiotic-resistant organisms between the baseline and study periods. The difference in change was not statistically significant between the groups, however. In this same study, universal glove and gown use resulted in increased room-exit hand hygiene compliance (62.9% pre-intervention vs. 78.3% post-intervention). Of potential concern, healthcare worker room entry was also decreased (5.24 entries per hour pre-intervention vs. 4.28 entries per hour post-intervention). However, no change in the rate of adverse events was experienced [140]. Thus, although the use of gloves *and* gowns is the convention for limiting the cross-transmission of hospital-acquired pathogens, the incremental benefit of gown use, in endemic settings, may be minimal.

2.5 The Role of Healthcare Worker Apparel in Hospital-Acquired Transmission of Pathogens

Contamination of healthcare worker apparel has been well documented [141–147]. The most commonly isolated organisms include *Staphylococcus aureus*, *Enterococci* (including VRE), and gram-negative organisms. Although evidence that transmission of organisms occur via contaminated clothing is lacking, there remains concern that healthcare worker apparel can act as a fomite for transmission of harmful organisms. The Society for Healthcare Epidemiology of America (SHEA) recently issued expert guidance regarding healthcare worker attire in acute care hospitals [148]. Despite the lack of firm evidence, these recommendations confer low cost and low likelihood of harm. Based on thorough review of the literature and expert opinion, they offered guidance for voluntary adoption of the following policies:

- Bare below the elbows (BBE): This is defined as wearing short sleeves, no wristwatch, no jewelry, and no ties while performing clinical duties. While direct evidence of transmission of organisms from clothing to patients is lacking, this practice is supported by biological plausibility and low risk of harm, according to the authors.
- White coats: If white coats are used, facilities should provide access to two or more coats with easy access to on-site laundering. Hooks should also be provided by the facility as a place where white coats can be hung during patient contact.

- Frequency of laundering: Clothes that come into contact with patients should be laundered daily, while white coats should be laundered at least weekly and when soiled. Preferably, items laundered at home should go through a hot-water wash cycle (with bleach if feasible), followed by a dry cycle. Less frequent laundering of clothing may be indicated for healthcare workers who engage in direct patient contact less often.
- Footwear: For healthcare worker safety, shoes with closed toes, low heels, and nonskid soles should be worn. This practice also confers less risk of exposure to blood, other potentially infectious materials, and injuries due to sharp objects.
- Identification: Identification badges should be worn by healthcare personnel and be clearly visible at all times for identification and security purposes.
- Other recommendations: Equipment used on multiple patients, such as stethoscopes, should be cleaned between patients. No other recommendations were made on additional personal items such as cellular phones, pagers, or other clothing items. In general, any item that comes into contact with multiple patients should be cleaned in between patients.

3 Contact Precautions

Contact precautions prevent spread of organisms from an infected patient through direct (touching the patient) or indirect (touching surfaces or objects that that been in contact with the patient) contact. This type of precaution requires the patient either be placed in a private room or be cohorted with a roommate with the same organism. Healthcare workers should don gloves upon entering the room. After patient care or environmental contact, the gloves should be removed and hand hygiene should be performed prior to leaving the room. In addition, the use protective gowns have been advocated to decrease the risk of healthcare worker garment contamination. Patient care items used for a patient in contact precautions, such as a stethoscopes and blood pressure cuffs, should not be shared with other patients unless they are properly cleaned and disinfected before reuse. Patients should be restricted to the isolation room [150].

Contact isolation is recommended for diarrheal illnesses of infectious origin and for infections with *C. difficile*. Traditionally, contact precautions have also been recommended for patients with drug-resistant pathogens such as MRSA, VRE, and multidrug-resistant gram-negative rods. However, controversies with the use of contact precautions exist.

3.1 Controversies in Contact Precautions

Effectiveness of contact precautions has been exhibited in outbreak situations [151–153]. Extrapolation of these results has led to the use of contact precautions as a control measure for transmission of multidrug-resistant organisms, such as MRSA, VRE, and multidrug-resistant gram-negative rods, in healthcare settings. While some studies have documented reduced rates of transmission of drug-resistant organisms when contact precautions are used, others fail to show this association [140, 161–163]. In addition to conflicting outcomes, well-designed, robust studies are lacking.

Several studies have noted suboptimal compliance with contact precautions. A large prospective cohort study analyzed compliance with various components of contact isolation practices, including hand hygiene prior to donning gloves, gowning, using of gloves, doffing of gown and gloves, and hand hygiene after removal of gown and gloves. Out of the 1013 healthcare workers observed, only 28.9 % adhered to all components of contact precautions [161]. Another prospective observational study involving a 900-bed tertiary care teaching hospital observed 73 % overall compliance with routine gown use. Specifically, healthcare workers were 76 % compliant, while visitors were 65 % compliant [162]. On the other hand, it has been argued that gown use may actually improve hand hygiene compliance. Golan et al. studied this hypothesis with an interventional study in two ICUs in the same tertiary care hospital. The intervention ICU eliminated the use of gowns for contact precautions, while the other ICU continued with the usual use of both gowns and gloves for contact precautions. Of concern, a very low rate of overall hand hygiene compliance was observed (10.1 % before patient care, 35.6 % after patient care, and only 5 % both before and after patient care). Hand hygiene compliance was no different between the intervention and control groups [163]. Another observational study demonstrated improved rates of hand hygiene on exiting the room of patients on contact precautions (63.2 %) vs. patients not on contact precautions (47.4 %), $p < 0.001$ [164]. Notably, a recent prospective cohort study found that as the burden of isolation increased from ≤20 to >60 %, hand hygiene compliance upon room entry decreased from 43.6 to 4.9 % [161].

Of some concern, adverse events associated with the use of contact precautions have been documented. In a prospective cohort study, Saint et al. reported in a prospective cohort study that patients on contact precautions are examined by the attending physician less often than their non-isolated controls (35 % vs. 73 %, respectively) [165]. In 2009, Morgan et al. conducted a review of the literature on adverse outcomes related to contact precautions. Four main outcomes were recognized. Isolated patients experienced less contact with healthcare workers, delays in care and increases in adverse events, increased anxiety and depression, and more dissatisfaction with care [164]. Since that time, several other reports have reinforced these results. A prospective observational study reported that patients on contact precautions had 36.4 % fewer hourly healthcare worker visits and 17.7 % less patient contact time with healthcare workers. These patients also had fewer outside visitors. Another study compared 150

patients on contact precautions to 300 controls. The patients on isolation were more likely to experience preventable adverse events, such as falls and pressure ulcers, experience less satisfaction with care, and have less physician progress notes [166]. Another study surveyed 1876 adult patients. Those on contact precautions had higher depression and anxiety scores [167]. Additionally, patients on contact precautions experience more medication errors, such as erroneous insulin and anticoagulant administration [168].

4 Measures to Control Hospital-Acquired Outbreak of Drug-Resistant Pathogens

Data published by the CDC report that more than 70 % of bacterial pathogens implicated in hospital-acquired infections are resistant to at least one commonly used anti-infective [169]. In addition, current evidence suggests that the proportion of MRSA and VRE attributable to cross-transmission is significant. Transmission of clonal MRSA strains within a healthcare setting has been confirmed by pulsed-field gel electrophoresis and has occurred in various healthcare settings including general hospital wards, neonatal intensive care units, and surgical intensive care units [155, 170–178]. Similarly, the clonal transmission of VRE within healthcare settings has been documented via molecular typing [179–189].

There is no one size fits all approach to the control of hospital-acquired drug-resistant pathogens such as MRSA or VRE. The literature is replete with reports of intervention and programs to limit the spread of drug-resistant pathogens. These examples, occurring in diverse patient populations such as hospital wards, intensive care units, and neonatal units, typically involve different combinations of multiple interventions such as surveillance cultures, pulsed-field gel electrophoresis typing of isolates, patient isolation, cohorting, gloving, gowning, antibiotic restriction, and healthcare worker decolonization [10, 190–195]. The best approach for controlling the hospital-acquired spread of pathogens such as MRSA or VRE should take into account the frequency of transmission of hospital-acquired infection, the reservoirs, the patient risk factors, and the resources of the healthcare system for implementation of varied infection control measures.

5 Screening for Asymptomatic Patient Colonization with Drug-Resistant Pathogens

As the incidence of both patient infection and colonization with drug-resistant pathogens such as MRSA or VRE has increased, the management of this phenomenon has evolved. Aggressive strategies include screening to detect asymptomatic carriers and the strict use of isolation measures to control spread. Nevertheless, there has been much debate about the rationale and efficacy of this practice to control the endemic spread of potential hospital-acquired pathogens.

In the latest guidelines by the Society for Healthcare Epidemiology of America (SHEA) for the prevention and spread of antibiotic-resistant pathogens, the use of active surveillance cultures to identify the reservoirs of MRSA and VRE is strongly recommended [196]. The ultimate goal of active surveillance is to identify every colonized patient so that infection control interventions such as contact isolation and cohorting can be implemented to reduce the risk of cross-transmission. As per the SHEA guidelines, these active surveillance cultures are indicated at the time of hospital admission for patients at high risk for carriage of MRSA and/or VRE [196–201]. For patients with ongoing or prolonged hospitalization, or high risk for VRE or MRSA carriage due to hospital location, underlying comorbidities, and concurrent antibiotic therapy, periodic re-culturing is recommended, typically on a weekly basis [176, 187, 202–209]. Furthermore, for facilities with high endemic rates of VRE or MRSA, as determined by surveillance of high-risk patients, an institution-wide survey should be conducted so that these patients are identified and placed in either contact isolation or cohorted [196].

However, a recently published review of isolation policies by the British National Health Service highlighted the strong evidence for the effectiveness of different isolation and screening policies for MRSA [210]. Data were extracted from articles reporting infection control mechanisms, policies, and interventions for MRSA-related outcomes, including colonization or infection. From 4382 abstracts, 254 full article appraisals were made with 46 papers included in the final review. Of the 46 studies, 18 included the use of isolation wards, 9 used nurse cohorting, and 19 involved other isolation policies including multiple combinations of different interventions such as patient cohorting in single or multiple occupancy rooms, strict use of gown, glove and mask, changes in antibiotic formulary, screening on admission and weekly thereafter, prompt patient discharge, mupirocin for decolonization, hand hygiene education with and without feedback to healthcare workers, and antibiotic restriction. Although the review concluded that concerted efforts, including isolation, can reduce the rates of MRSA in both endemic and epidemic settings, several other findings were noteworthy. The majority of the studies had multiple interventions and major methodological weaknesses such as lack of measures to prevent bias, the absence of consideration for confounding, and inappropriate statistical analysis. As such, the quality of evidence in many studies was considered weak, many alternative and plausible explanations for the reduction in MRSA could not be excluded, and the role and impact of isolation measures were not assessed by well-designed studies.

At least one recently published, well-designed, prospective study evaluated the efficacy of single room and cohort isolation for MRSA in the intensive care unit setting [211]. In this 1-year analysis conducted in the intensive care units of two teaching hospitals, MRSA screening was performed both on admission and then weekly for all patients. During the first 3 months and the last 3 months, all MRSA-positive patients were moved either to a single occupancy isolation room or cohorted with other MRSA-positive patients. During the middle 6-month period, MRSA-positive patients were not placed in isolation or cohorted unless they were co-colonized with another multidrug-resistant pathogen. Patient characteristics, hand hygiene compliance, and MRSA acquisition rates were similar in the periods when patients were moved and not moved. Using Cox proportional hazard modeling to control for confounders such as gender, age, APACHE II score, antibiotic use, number of intravascular catheters, and colonization pressure, no significant reduction in MRSA acquisition was observed between the two groups [211].

The use of strict isolation practices may have a detrimental impact on the process and quality of patient care. Evans et al. prospectively observed surgical patients in both the ICU and on a general surgical floor. In both the ICU and surgical floor, surgical patients on contact isolation had fewer healthcare worker visits and less contact time overall despite a higher severity of illness as measured by APACHE II score [212]. Stelfox et al. studied the quality of medical care received by patients isolated for MRSA-related infection control precautions using a case control study design. Although isolated and control patients had similar baseline characteristics, isolated patients were twice as likely as non-isolated patients to experience adverse events during their hospitalization. These adverse events included supportive care measures and process of care measures such as days with incomplete or absent vital signs and days without documented nursing and physician progress notes. Additionally, patients on MRSA contact isolation expressed greater dissatisfaction with the quality of their treatment [166]. Similarly, Saint and colleagues observed, in a prospective cohort study of two in-patient medical services, that patients on contact isolation were half as likely to be examined by an attending physician as non-isolated patients [165].

Contact isolation may have a detrimental psychological impact on patients. One cross-sectional matched case control study compared contact isolated versus non-isolated elderly patients [213]. The level of depressive and anxiety symptoms exhibited by the contact isolation group exceed that of the noncontact isolation group. Catalano et al. prospectively studied the impact of contact isolation on anxiety and depression in non-critically ill hospitalized patients [214]. Patients on contact isolation for either MRSA or VRE were compared to other hospitalized patient with infectious diseases not requiring isolation. All patients were evaluated with the Hamilton Anxiety and Depression Rating Scale at baseline and then later during the hospital course. Although no significant differences in baseline anxiety and depression scores were noted, for patients in contact isolation, statistically significant higher scores on both scales were reported later during the course of hospitalization.

Thus, the optimal strategy for control of endemic, resistant pathogens such as MRSA or VRE is yet to be defined. Aggressive measures involving surveillance cultures for colonized patient reservoirs may not effectively reduce the rate of pathogen cross-transmission. Additionally, surveillance cultures with consequent the implantation of isolation measures may have the impact of increased patient depression and anxiety, and may be detrimental to the both the process and quality of care.

6 Antibiotic Control Programs and Surveillance for Hospital-Acquired Infections

The implications of widespread antibiotic use, including the impact on public health, are beyond the scope of this chapter. The reader is referred to other chapters within this textbook for further information on the topic. Although the degree to which antibiotic pressure directly contributes to the cross-transmission of hospital-acquired infections remains poorly defined, several studies and observations are worth mentioning. Harbath and colleagues prospectively studied surgical site infections in cardiovascular surgical patients. In this cohort, prolonged antibiotic prophylaxis with cephalosporins was an independent risk factor on logistic regression analysis for infections with cephalosporin-resistant gram-negative rods [215]. Additionally, in a prospective, non-randomized, cohort study in a neonatal ICU, a change to a new empiric antibiotic regimen resulted in a decrease in colonization or infection by gram-negative organisms resistant to the standard or prior empiric regimen [216]. Donskey and colleagues showed that enteric VRE colonization was significantly associated with colonization pressure, presence of feeding tube, and cephalosporin use [217]. Similarly, MRSA colonization has been associated with antibiotic use. A significant risk factor for prolonged MRSA colonization, as defined by multivariate regression analysis, was fluoroquinolone use [218]. Additionally, using an ecologic study design, investigators from Belgium reported a direct association between fluoroquinolone use and MRSA infections [219]. Consideration should be given to antibiotic restriction and control programs in the event of elevated rates of hospital-acquired drug-resistant pathogens.

According to the Society for Healthcare Epidemiology of America (SHEA), antimicrobial stewardship refers to coordinated interventions designed to improve and measure the appropriate use of antimicrobials by promoting the selection

of the optimal antimicrobial drug regimen, dose, duration of therapy, and route of administration [220]. All healthcare institutions in the United States are urged to adopt antimicrobial stewardship programs. The goal of these programs is to improve clinical outcomes by optimizing antimicrobial use in order to minimize toxicity, reduce adverse events, and reduce selective pressure that leads to antibiotic resistance. In 2012, SHEA, the Infectious Diseases Society of America (IDSA), and the Pediatric Infectious Diseases Society (PIDS) issued a joint policy statement regarding antimicrobial stewardship [221]. Several recommendations are given.

First, antimicrobial stewardship programs should be required through regulatory mandates (through a combination of state and federal mandates, and via the Centers for Medicare and Medicaid Services (CMS)). However, objectives should be flexible enough so that resource-limited facilities are able to maintain participation in such programs. Requirements of a program should include:

- A multidisciplinary antimicrobial stewardship team, which is physician directed and has at least one team member trained in antimicrobial stewardship. At the least, the team should include a physician, a pharmacist, a clinical microbiologist, and an infection preventionist.
- A medication formulary limited to non-duplicative antibiotics of clinical need.
- Institutional guidelines for the use of antibiotics for management of common clinical syndromes.
- Methods for detecting and eliminating the use of antibiotics in a manner that is redundant, inappropriate, or inadequate (e.g., the use of antibiotics for the treatment of nonbacterial illness and the use of antibiotic regimens that are either too broad, not broad enough, or not appropriately targeted for the pathogen).
- Processes for monitoring antibiotic use for internal benchmarks.
- Periodic distribution of facility-specific antibiograms to clinicians.

In addition, CMS should require institutions to report to the National Healthcare Safety Network's (NHSN) Antimicrobial Use and Resistance option of the Medication-Associated Module, conduct prospective surveillance and concurrent interventions to optimize antimicrobial use, establish national benchmarking of antimicrobial use at the institutional level based on acuity of care and patient mix, and report other indicators of effective antimicrobial use such as incidence rates of drug-resistant organisms and *C. difficile* infections.

Second, validated antimicrobial stewardship interventions do not exist for ambulatory healthcare settings. Therefore, pilot projects should be designed to develop and implement antimicrobial stewardship interventions in these settings. National organizations, such as the Agency for Healthcare Research and Quality (AHRQ), CMS, National Institutes for Health (NIH), and the Centers for Disease Control and Prevention (CDC) should provide funding for such projects. Suggested areas of research include integration of clinical decision support into electronic health records and e-prescribing systems. Once validated, interventions should become CMS requirements.

Third, mechanisms should be put into place to educate physician trainees (e.g., medical students, residents, and fellows) about antibiotic resistance and antimicrobial stewardship. Furthermore, practicing clinicians should receive education as well. Educational materials can be distributed through specialty societies, the FDA, and individual institutions, for example.

Fourth, there should be a national system for collecting antimicrobial use data, which can then be used to benchmark institutions. The data could potentially be used as part of an incentive-based payment system.

Fifth, research on antimicrobial stewardship is needed in order to understand antimicrobial resistance and how interventions affect it. This is best accomplished via translational research. Research should focus on:

- Development of a standard definition of appropriate and inappropriate antimicrobial use, measures of use, and the factors that contribute to misuse. In addition, standardized data collection tools should be developed for purposes of measurement and interpretation of antimicrobial use.
- Determination of the most effective and cost-efficient means of implementing antibiotic stewardship programs in various settings, using robust study design.
- Development and validation of process and outcome measures that allow comparison of antimicrobial use within and across healthcare settings. Measures may include surrogate markers of effective and appropriate antibiotic use, such as rates of infections due to drug-resistant organisms and *C. difficile* infections, adverse effects of antibiotics, and hospital/ICU length of stay.
- Understanding how generic versus trade name antimicrobial agents affects use.
- Evaluation of the impact of rapid diagnostic tests and biomarkers, such as procalcitonin, on the use of antibiotics, and whether or not unnecessary antibiotic use (e.g., for viral infections) is decreased.

7 Conclusion

The prevalence of hospital-acquired, antibiotic-resistant pathogens has increased significantly over the last 20 years. Hospital infection control programs are seen as increasingly important for the control of antibiotic-resistant organisms. Strategies to control the spread of hospital-acquired infections by drug-resistant pathogens are multiple. The patient, the healthcare

worker, and the environment are reservoirs for drug-resistant pathogens. For high-risk patients colonized with MRSA, such as surgical candidates and those in intensive care units, decolonization with nasal mupirocin should be considered. Patients colonized with resistant pathogens such as MRSA, VRE, and drug-resistant gram-negative rods can contaminate the environment. As such, all healthcare facilities should develop policies for the terminal and periodic disinfection of patient care areas and environmental services. Cross-transmission of hospital-acquired pathogens by the hands of healthcare workers has been well documented. Meticulous hand hygiene should be practiced with medicated handwashing agents (alcohol, chlorhexidine gluconate, triclosan) that are bactericidal and effectively reduced bacterial counts on the hands. Measures to promote hand hygiene compliance should include efforts that stress increased use of accessible, easy to use, medicated hand hygiene products, coupled with a hospital-wide, administration-backed, high-priority hand hygiene campaign. Glove use is beneficial in limiting the contamination of healthcare worker hands but is not a substitute for hand hygiene. Concerns about the contamination of personnel clothing with hospital-acquired pathogens has led to the use of gowns for patients in contact isolation. The incremental benefit of gowns and glove use may be minimal. Transmission-based precautions are useful for the control of hospital-acquired infections and include contact, airborne, and droplet precautions. Aggressive surveillance for asymptomatic reservoirs may be of value but is not without controversy including questions about efficacy and effect on quality of care. Other considerations for an infection control program include antibiotic control programs and surveillance systems for infections with hospital-acquired pathogens. This type of surveillance is essential for establishing endemic rates, defining outbreaks, and developing institution-specific antibiograms. In the end, the purpose of a hospital infection surveillance program is to define endemic rates, recognize outbreaks, and obtain data of value in recognizing the extent and causation of the infections. This data is later applied for the planning and implementation of risk reduction policies and interventions.

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Controlling the Spread of Resistant Pathogens in the Intensive Care Unit

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1 Introduction

As is detailed carefully throughout this text, antimicrobial resistance has surfaced as a major challenge to modern medicine in the twentieth and twenty-first centuries. The challenges presented by burgeoning antimicrobial resistance are magnified in the intensive care unit (ICU), where aggressive, invasive care of severely ill patients sets up a perfect storm for resistant pathogens. ICU patients frequently develop nosocomial infections, which are often severe, difficult to treat, and, in some populations, recurrent [1]. Extensive exposure to antimicrobial agents, colonization with nosocomial bacteria, and prolonged immunosuppression put ICU patients at high risk for infection from resistant pathogens. Some of the most aggressive resistant pathogens have become endemic in hospital environments, and many of these pathogens have established residence in intensive care units [2–4]. Examples of such pathogens are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus*, and some carbapenem-resistant Gram-negative bacilli (e.g., *Klebsiella pneumoniae*, *Enterobacter* spp., *Acinetobacter baumannii*). The rise of extensive drug resistance and pan-resistance among nosocomial Gram-negative bacteria has made controlling the spread of these organisms in the ICU environment even more important. In addition, some consider *C. difficile* infection a multidrug-resistant pathogen because it is selected out by antibacterial therapy.

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This chapter discusses the special issues relating to the ICU that make antimicrobial resistance a major problem confronting critical care clinicians. The chapter addresses (1) reservoirs of infection in the ICU, (2) common nosocomial infections encountered by intensivists, (3) resistant pathogens that particularly affect ICU patients, and (4) approaches to preventing infections caused by resistant pathogens in the ICU setting.

2 Reservoirs

The nosocomial reservoirs for resistant organisms vary by the pathogen and the clinical setting. Organisms can be transmitted from person to person on the hands of healthcare personnel or spread via contaminated surfaces or equipment. All persons are colonized with bacteria on the mucous membranes and skin and within the fecal flora. The flora of hospitalized patients quickly change during hospitalization, often incorporating locally endemic multidrug-resistant bacteria [5]. The source of these new flora may be the patient, a provider, or the inanimate environment of the ICU. Little evidence suggests that hospital visitors are a significant source of multidrug-resistant bacterial transmission, though family members who provide extensive care to colonized patients certainly may become colonized themselves [6–8]. The endogenous flora of hospitalized patients may become more resistant with antibiotic exposure or may acquire new resistant pathogens that are spread within the ICU. Patients colonized with resistant bacteria are potential reservoirs for transmission to other patients via the hands of healthcare personnel or contamination of the environment. Identification and isolation of colonized patients, hand hygiene, environmental disinfection, and other infection control precautions are key to preventing or interrupting this cycle of transmission.

Staphylococci are often carried on the skin and mucous membranes of staff and patients [9]. *Staphylococcus aureus* may colonize several sites on the body, including the face, hands, throat, axillae, and groin, but are most frequently

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found in the epithelium of the anterior nares [10]. Patients who are colonized and/or infected with resistant staphylococci can serve as reservoirs for the spread of these organisms within healthcare institutions [11]. Studies suggest that 30–60 % of healthy adults carry *S. aureus* and that 10–20 % of these individuals are chronically colonized [12, 13]. Many patients are identified as nasal carriers of *S. aureus*, including methicillin-resistant strains, at the time of hospital admission [14, 15]. Healthcare personnel have higher rates of MRSA carriage than are found in the general population, with as many as 44 % of healthcare personnel carrying *S. aureus* and up to 15 % carrying MRSA in some studies [16–19]. The primary route of MRSA transmission within the hospital appears to be from patient to patient, with healthcare personnel the likely vector, carrying the organisms on their hands. In addition, some studies have suggested that resistant staphylococci can establish an inanimate environmental reservoir and can persist on contaminated objects in the environment. When these objects are used for subsequent patients, they may serve as vehicles of transmission for the resistant pathogens as a result of the patient having direct contact with the contaminated objects or a healthcare worker handling the object and then touching the patient [20–22]. Some respected investigators believe that environmental or fomite spread may be substantially underestimated as a potential nosocomial route of transmission of resistant organisms.

Some pathogens (e.g., *Clostridium difficile*, *Enterococcus faecium*, resistant Gram-negative organisms, etc.) can be carried in the fecal flora of patients. The bacteria may not invade and cause infection unless the intestinal epithelium is damaged, the intestinal microbiome is subjected to antibiotics, or both. Antibiotic pressure can give multidrug-resistant bacteria a selective advantage (e.g., the administration of antimicrobial agents to which the majority of the fecal flora—and especially the fecal anaerobes—are susceptible, but to which the pathogen is not). Similarly, organisms can be carried as part of the cutaneous flora, causing few problems until the normal flora are perturbed by external forces, such as antimicrobial agents.

In the ICU environment, healthcare personnel who exercise inadequate hand hygiene may carry multidrug-resistant bacteria on their hands; carriage may be transient but last long enough to spread the bacteria with facility to the ICU environment or directly to patients. Personnel can have longer-term carriage of bacterial pathogens under long or artificial fingernails, and the recurrent role of long and artificial nails in outbreaks has led the Centers for Disease Control and Prevention (CDC) to recommend against them [23].

2.1 The Inanimate Environment

Certain organisms have a proclivity for establishing reservoirs in the inanimate environment in healthcare settings. Some such organisms find moist places in the environment and establish residence in biofilms. Examples include *Stenotrophomonas maltophilia*, species of *Pseudomonas*, *Aeromonas*, *Sphingomonas*, and others. Other organisms are resilient and able to tolerate a range of conditions of temperature and humidity, including spores of *Clostridium difficile*, *Acinetobacter* species, and enterococci. The latter hardy bacteria may survive for months in the hospital environment and, in the absence of effective disinfection, may spread to patients long after they were shed, creating a prolonged transmission cycle. Multidrug-resistant bacteria, once established in an environmental reservoir, may cause recurring clustered infections in the ICU. In multiple ICU outbreaks with multidrug-resistant bacteria, the outbreak organism has been identified in sink drain biofilms. Although circumstantial evidence may implicate the sink drain colonization in the outbreak, transmission from sink drains to patients has not been definitively proven [24–27]. In cases of sporadic infection and even some outbreaks, identifying the precise environmental reservoir may be extremely difficult [28].

3 Major Infectious Disease Syndromes Commonly Encountered in the ICU

Whereas virtually any infectious syndrome may occur in patients hospitalized in the ICU, several categories are worthy of special mention because of the frequency with which they occur, as well as the frequency with which these syndromes are associated with resistant pathogens: catheter-associated bloodstream infections, ICU-acquired nosocomial pneumonia, *Clostridium difficile* infection, and sepsis in immunosuppressed patients. The intensivist must be particularly attuned to the pathogenesis of these infectious syndromes in the ICU patient population, as well as the factors that increase the likelihood that these infections will involve resistant organisms.

Most, if not all, of these syndromes are by-products of medical progress. We are able to prolong life though the use of aggressive chemo- and immunotherapies, the use of sophisticated life-support devices, and other invasive diagnostic and therapeutic approaches. Use of each drug or device is associated with increased risks for complications, including infection. Seriously ill ICU patients often sustain repeated bouts of infection and are therefore exposed to multiple courses of antibacterial, antiviral, and antifungal agents. With inadequate host defenses and multiple invasive devices in place, these patients are essentially incubators for microbial resistance.

3.1 Central Venous Line-Associated Bloodstream Infection (CLABSI)

The occurrence of CLABSI in ICU patients has become commonplace. Microorganisms reach the circulation via the catheter insertion site, along hubs [29, 30], junctions, and

connectors, and via intrinsic or extrinsic contamination of the infusion fluid. Insertion site colonization and infection are facilitated by conditions that favor the growth and proliferation of skin flora, thereby accelerating the migration of organisms from the skin surface along the catheter insertion tract. Contamination may occur at the time of insertion or, more likely, weeks to months later. This type of contamination will most commonly result in colonization along the external surface of catheter and is facilitated by fibrin sheath/platelet deposition on the external catheter surface and organism-produced biofilm at the catheter surface in the circulatory channel. Similarly for contamination introduced into the system via the catheter hub and the catheter's junctions and connectors, the resident skin flora are the most common pathogens producing device-associated infection. Again, the source of these organisms may be the patient, a healthcare provider, or the ICU environment. These organisms are typically introduced into the system at the time the device is being manipulated. This pathway is more likely to produce colonization of the catheter lumen. Because infection is introduced as the device is being manipulated, this route of infection becomes increasingly important as a source of infection as the duration of catheterization increases. The likelihood of contamination and colonization may relate to the design of the device and also will be facilitated by fibrin sheath production, platelet deposition, and/or biofilm development on the catheter surface.

Contamination introduced via the infusion fluid itself occurs less commonly. Such contamination may be intrinsic (i.e., due to contamination during manufacture or processing) or extrinsic (i.e., contamination introduced at the time the fluid is hung or at the time additives are injected into the container).

3.2 Ventilator-Associated Pneumonia

The reservoir for ventilator-associated pneumonia is again most commonly the patient's own oropharyngeal flora. Patients' oral flora change quickly, often in critically ill patients within 24 h of hospitalization, from the normal, primarily anaerobic flora to an oral flora that is dominated by aerobic Gram-negative bacilli and *S. aureus* [7]. When the patient is intubated and placed on a ventilator, risk for pulmonary infection increases dramatically. The endotracheal tube itself contributes to this risk. Direct inoculation through the respiratory apparatus may occur, either as a result of cross-contamination or from breaks in sterile technique.

The inner lumen of an endotracheal tube also rapidly develops a biofilm containing microorganisms [31, 32], such as aerobic Gram-negative rods and *S. aureus*, at very high concentrations. This biofilm can be inoculated directly into the lower respiratory tract either by ventilatory flow or by inserting suction catheters through the tube and producing infectious emboli [33, 34]. Additionally, in the critically ill, supine, ventilated patient, oral secretions pool in the oropharynx and subglottic space above the tracheal tube cuff, forming a reservoir of secretions contaminated with the altered flora [33, 34]. Without measures to drain subglottic fluid, leakage of pooled secretions around the cuff occurs almost uniformly in these patients.

If the patient has a nasotracheal tube or has had a nasogastric tube inserted, the risk of nosocomial sinusitis is increased. In a patient with a substantially altered mental status, such sinus infections often are unsuspected and undiagnosed. Predominant pathogens for these sinus infections are aerobic Gram-negative bacilli. More importantly, the development of nosocomial sinusitis increases the risk of ventilator-associated pneumonia by a factor of four [35, 36].

Positioning of the patient is also associated with risk for ventilator-associated pneumonia. Aspiration of gastric contents occurs four times more frequently when the patient is in the supine position, rather than when the head of the patient's bed is elevated at a 45° angle [37]. Isolation of the same organisms from the stomach, pharynx, and endobronchial samples occurred in 32 % of semirecumbent patients in one study compared with 68 % of patients in the supine position [38]. Unfortunately, gastric reflux occurs irrespective of body position in mechanically ventilated patients who have nasogastric tubes.

Ventilated patients are frequently placed on proton pump inhibitors to decrease gastric acidity to reduce the risk for gastric hemorrhage. Decreased gastric acidity (which is clearly appropriate for ventilated patients) increases the microbial colonization of the stomach [39]. Enteral feedings (often administered to such patients) also increase the risk for gastric colonization with Gram-negative bacilli. The use of either continuous or intermittent enteral feeding increases gastric pH and is associated with an 80 % risk for Gram-negative colonization of the stomach [40]. Conversely, the maintenance of adequate nutritional status is clearly associated with a reduced risk for ventilator-associated pneumonia, and enteral nutrition is clearly the route of choice for these patients. In ICU patients who have received multiple courses of empiric and/or therapeutic antimicrobials, the likelihood that the organism colonizing the stomach is a multidrug-resistant pathogen is increased substantially. VAP caused by multidrug-resistant bacteria prolongs ICU stay compared with VAP caused by antibiotic-susceptible organisms [41].

3.3 Catheter-Associated Urinary Tract Infections

Urinary tract infections are among the most common of all healthcare-associated infections. The overwhelming majority (approximately 75 %) of these infections are related to the use of indwelling urethral catheters [42]. The use of such

indwelling catheters is extremely common in the ICU setting. Catheter-associated urinary tract infection (CAUTI) rates among adult patients in ICUs reported to the CDC's National Healthcare Safety Network have been reported to be between 1.2 and 4.7 infections per 1000 urinary catheter-days [43]. Factors associated with a risk for CAUTIs among adult ICU patients include the duration of catheterization, increased age, female sex, and failure to maintain a closed drainage system [44].

3.4 Sepsis in Immunosuppressed ICU Patients

Immunosuppressed patients lack some of the normal physical barriers to infection. Impaired integrity of the skin and mucous membranes that accompanies some immunosuppressed states allows these surfaces to become portals of entry for pathogens that colonize the skin or intestinal tract. The skin and mucous membrane damage that accompanies radiation therapy, chemotherapy, burns, graft-versus-host disease, surgery, trauma, and many other conditions facilitates colonization with nosocomial pathogens. Again, ICU patients have been exposed to multiple courses of antimicrobials, and the likelihood that the organisms colonizing the skin are multidrug-resistant pathogens (e.g., MRSA, VRE, Gram-negatives) is increased substantially. Following radiation therapy or chemotherapy, patients' oral, pharyngeal, and intestinal mucous membranes experience accelerated apoptosis without cell renewal, ultimately resulting in an ulcerative phase (mucositis) that may permit entry of nosocomially acquired microflora into the circulation. This ulcerative phase is followed by a healing phase that restores the integrity of the mucous membrane barrier. Additionally, the administration of antimicrobials may facilitate colonization of the gut with resistant pathogens. Additional possible portals of entry for resistant nosocomial pathogens include the respiratory tract, the genitourinary tract (particularly if the tract has been instrumented), and a variety of others.

For all of these reasons, these patients are at extreme risk for infection. Pathogens causing infections in these patients may originate from the patient's endogenous flora, from the hands of their healthcare providers, from fomites and equipment, from the inanimate healthcare environment, and even from the air. As rough approximations, about 80 % of bacterial pathogens causing infection in neutropenic patients originate from patients' endogenous flora, and approximately half of patients' endogenous microbial flora are acquired nosocomially. For the reasons noted above, the normal flora of the oropharynx, the skin, and the lower gastrointestinal tract are perturbed, and, particularly because of the frequent exposures to broad-spectrum antimicrobials, resistant organisms play an increasingly important role in colonization and infection in this setting. Resistant pathogens frequently encountered in the ICU causing these infections include MRSA, VRE, and multidrug-resistant Gram-negative bacteria, including carbapenemase-producing strains and organisms that have other mechanisms of resistance.

4 Resistant Pathogens of Particular Interest to ICU Staff

Certain resistant pathogens are worthy of special mention as particularly problematic for patients hospitalized in the ICU. Whereas a wide range of bacterial, viral, and fungal pathogens can affect patients hospitalized in the ICU, four bacterial pathogens have emerged as particularly challenging for critical care staff in the past two decades: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium*, *Clostridium difficile*, and highly resistant Gram-negatives, including carbapenemase-producing *Acinetobacter baumannii* and carbapenemase-producing Enterobacteriaceae. ICU infections associated with each of these pathogens will be discussed in more detail.

4.1 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

Resistant *Staphylococcus aureus* organisms are often spread to patients from the hands of healthcare personnel; these organisms may be acquired from an infected or colonized patient and then transferred to another patient when hand hygiene procedures are inadequate to remove the organisms. In addition, unlike other resistant nosocomial organisms, MRSA is also spreading extensively in the community setting—such that many patients now may be colonized due to acquisition outside the healthcare setting. The ICU environment, with its attendant urgencies and immediacy of care, is an ideal environment for the spread of MRSA. Thus, starting in the 1980s, MRSA became predominant pathogens in the ICU [11]. As noted above, resistant staphylococci can also establish transient residence on objects in the environment and be spread from these objects to patients, often via healthcare personnel's hands [20–22]. Since staphylococci are primarily considered skin and nares colonizers, environmental or fomite spread of resistant staphylococci may be substantially underestimated as a route of nosocomial transmission.

Resistant staphylococci, as is the case for relatively susceptible staphylococcal organisms, possess essentially the same number of toxins and virulence factors and, hence, are aggressive human pathogens, capable of producing significant infections in even immunologically normal patients. In the ICU setting, MRSA is primarily encountered as a pathogen causing skin and soft tissue infections, wound

infections, CLABSIs, and, somewhat less frequently, respiratory infection. The propensity for MRSA to cause CLABSIs is well established. Several studies have demonstrated that resistant staphylococcal infections are associated with prolongation of hospitalization and increased costs of hospitalization [45–50].

The critical care practitioner has several options for treatment of MRSA infections. Antimicrobial selection should be governed by disease severity, susceptibility patterns, clinical response to therapy, and cost. Current parenteral therapeutic options include vancomycin, linezolid, daptomycin, ceftaroline, teicoplanin, and telavancin. An occasionally overlooked but nonetheless important therapeutic intervention for resistant staphylococcal infections is the assurance of adequate drainage of purulent fluid collections.

4.2 Vancomycin-Resistant *Enterococcus* (VRE)

Vancomycin-resistant *Enterococcus faecium* (VRE) was first detected in Europe, as early as 1987, but its appearance was preceded by substantial resistance to other antimicrobials (e.g., resistances to β-lactam antibiotics, such as ampicillin, as well as extremely high-level resistance to aminoglycosides) among enterococcal isolates.

In North America, VRE is a significant nosocomial pathogen. Colonization with VRE is common in the ICU, especially among chronically ill, critically ill, and immunocompromised patients who have prolonged hospitalization and have received multiple courses of broad-spectrum antimicrobials. Because the organism can be carried on healthcare personnel's hands and survives well in the inanimate environment, cross-transmission in the complex ICU environment has become a substantial problem over the past 15 years.

In US hospitals, and particularly in US ICUs, the inanimate environment is likely a significant source of VRE transmission. Hayden and colleagues demonstrated that VRE was highly prevalent in the inanimate environment in their ICU and also subsequently demonstrated that reducing environmental contamination had a statistically significant effect on the spread of VRE in their ICU [51]. As discussed elsewhere in this text, the increasing use of vancomycin and antimicrobial drugs that target anaerobes has likely applied substantial antimicrobial pressure on enterococcal isolates in US ICU patients [52]. To date, to our knowledge, no community reservoir for VRE has been identified in the United States.

Unlike MRSA, VRE is not a very aggressive pathogen. Nonetheless, due to the dramatically immunosuppressed state of many twenty-first century critically ill patients, the frequency with which ICU patients receive multiple courses of broad-spectrum antimicrobials, and the extent to which such patients are exposed to invasive techniques, these pathogens commonly cause infection, particularly in tertiary referral centers. In 1993, the prevalence of VRE had increased 20-fold in the ICUs of US hospitals participating in the National Nosocomial Infections Study (NNIS) [53]; however, in recent years the proportion of enterococcal infections resistant to vancomycin has plateaued at approximately 30 % [54].

Although several antimicrobial agents have activity against VRE, resistance to some of those agents has made treatment of VRE infections difficult. Agents currently marketed with efficacy against VRE include linezolid, an oxazolidinone, and daptomycin, a lipopeptide. Telavancin, a lipoglycopeptide that is approved for treatment of skin and soft tissue infections, has low potency against VanA strains of VRE [55]. The combination streptogramin, quinupristin-dalfopristin, has fallen out of favor because of its poor side effect profile. Tigecycline, a glycylcycline, is not generally used for enterococcal infections because of lack of evidence of clinical efficacy against VRE and an overall increased mortality among its recipients [56]; its use may be appropriate as a component of salvage therapy for those without better treatment alternatives.

4.3 *Clostridium difficile* Enterocolitis

Clostridium difficile enterocolitis is an extremely common sequela of broad-spectrum antimicrobial therapy. Approximately 3 % of healthy adults [57] and 14–40 % of hospitalized patients are colonized with *C. difficile* (usually in the metabolically inactive spore form) [58–61]. Because of *C. difficile's* remarkable ability to persist in the environment and resist standard cleaning and disinfecting agents in its spore form, transmission from the hospital environment may play a significant role in the organism's nosocomial spread. Development of *C. difficile* infection typically occurs after exposure to broad-spectrum antimicrobial agents. Other risk factors include colonization density of *C. difficile* infection in the patient care unit and underlying gastrointestinal disease (such as inflammatory bowel disease [62, 63] and intestinal graft-versus-host disease [64]), as well as gastric acid suppression [65].

The past decade has seen a dramatic resurgence of *C. difficile*-associated disease in North America and Europe, such that it has become the most prevalent healthcare-associated pathogen in the United States [66]. Although the rate is lower in Europe [67], variation in testing may underestimate its prevalence in that part of the world [68]. *C. difficile* infection is particularly severe in older adults, in whom the vast majority of attributable mortality occurs [69]. Toxins, A and B, are primarily involved in the pathogenesis of *C. difficile*-associated disease; however, a hypervirulent strain that emerged in the past decade contains a binary toxin, increased toxin production, and high-level resistance to fluoroquinolones

[70]. Polymorphisms in the *tcdC* gene, which downregulates toxin production, may explain toxin production that is 16–23 times higher than that of other strains [71]. Experts have speculated that widespread fluoroquinolone use may have selected this strain and led to its emergence. The increased virulence of this now-dominant hypervirulent strain (BI/NAP1/027) has been associated with higher rates of fulminant and fatal *C. difficile* infection [70, 72].

Environmental persistence combined with the substantial antimicrobial agent use among ICU patients and other host factors that place ICU patients at risk makes *C. difficile* one of the most challenging ICU pathogens to control.

4.4 Carbapenemase-Producing Gram-Negative Bacteria

Gram-negative bacteria harboring carbapenemase enzymes have emerged over the past decade and disseminated around the globe, changing dramatically the epidemiology of nosocomial infections in many countries. The predominant bacterial etiology of ICU-acquired bloodstream infections is, in many centers, shifting from Gram-positive to Gram-negative organisms, with a high proportion of multidrug-resistant Gram-negative strains [73, 74]. Although a wide range of Gram-negative species can harbor plasmid-borne genes for carbapenemase enzymes (discussed in Chapter 56), the most common in North America and Europe are *Klebsiella pneumonia* and *Enterobacter* species. Globally, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are also important pathogens whose multidrug-resistance is often, but not always, attributable to carbapemenase genes.

Clinically significant carbapenemase genes include bla_{KPC}, $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}23}$, and the metallobetalactamase genes $bla_{NDM\text{-}1}$ bla_{VIM}, and bla_{IMP}. The genes that encode these enzymes are often found in organisms that already harbor other resistance genes, such as extended-spectrum beta-lactamases, and the carbapenemase thus confers extensive or even pan-drug resistance. These bacteria have caused numerous ICU-based outbreaks in every inhabited continent and profoundly affected the epidemiology of ICU-acquired infection in patients of all ages in countries with the highest prevalence of carbapenemase-producing organisms [73, 75–78].

Infections caused by Gram-negative bacteria that are carbapenem resistant due to the presence of carbapenemase or other mechanisms of resistance are frequently very difficult to treat. In contrast to multidrug-resistant Gram-positive infections, for which typically several antibiotics retain activity, treatment options for resistant Gram-negative infections may be drastically narrowed, frequently leaving one, two, or no antibiotic options (typically, these organisms may be initially susceptible to colistin and an aminoglycoside). Colistin, a polymyxin antibiotic previously of primarily historical interest and limited use, has become the last line of therapy for many Gram-negative species. The drug has substantial side effects, including the potential for significant neurotoxicity and renal toxicity, particularly in critically ill patients and especially when administered with aminoglycosides or other nephrotoxic drugs that may be necessary to treat these bacteria. Colistin-resistant strains have developed during therapy [77] and have caused nosocomial outbreaks [79]. Because resistant Gram-negative organisms disproportionately affect susceptible hosts, the mortality rate from infection with carbapenemase-producing bacteria in highly immunocompromised hosts is reported to be 40–80 % [77, 80, 81].

As is the case for other healthcare-associated bacteria, highly resistant Gram-negative bacilli are likely transmitted on the hands of healthcare personnel, with a probable lesser role of environmental contamination in nosocomial spread. Transmission is rampant in long-term acute care hospitals in some geographic areas. Patients transferred to hospitals from those facilities may arrive with colonization or infection; if surveillance or clinical cultures do not identify their carriage, those patients can serve as the sources of nosocomial spread. The organisms join the fecal flora of susceptible patients who may subsequently develop infection or become reservoirs for transmission to other patients, via the personnel or environment of the hospital. Without development of novel antimicrobial treatments or implementation of better infection control, these organisms are likely to pose an enormous challenge for intensivists for years to come.

4.5 Multidrug-Resistant *Acinetobacter baumannii*

Acinetobacter baumannii, an aerobic, nonfermenting, Gram-negative coccobacillus, has, in the past decade, become a formidable problem for intensivists. Although other *Acinetobacter* species can cause infections in the community or hospital, *Acinetobacter baumannii* is predominantly a healthcare-associated pathogen and, worldwide, typically harbors significant antimicrobial resistance. *A. baumannii* is a particular problem in surgical and medical ICUs. *A. baumannii* has been estimated to be responsible for two to 10 % of all Gram-negative infections in intensive care units in both the United States and Europe [82]. *A. baumannii* was frequently identified as a significant wound pathogen in US troops returning from the Middle East with battlefield injuries [83], leading to nosocomial transmission in US military healthcare facilities [84, 85]. The pathogen is ubiquitous in some parts of the world, where it has become a dominant cause of infection in ICUs [86].

A. baumannii has the remarkable ability to develop durable antimicrobial resistance with alarming speed; resistance genes can be acquired from transposons, integrons, or plasmids carrying large clusters of resistance genes. It increasingly hosts plasmids that carry the carbapenemase genes described above, among its mechanisms of resistance. Additionally, in several other respects, *A. baumannii* is also a formidable pathogen. For example, atypical of most Gram-negative bacilli, *A. baumannii* is able to withstand long periods of desiccation and can therefore persist in the inanimate environment of the hospital ICU. In fact, *A. baumannii* has been found contaminating a wide range of patient equipment, including ventilators, mattresses, pillows, beds, gloves, pumps, and other electrical equipment in the ICU [87]. The nosocomial reservoir for *A. baumannii* is unclear and may be quite diverse. Candidate reservoirs for this problematic pathogen include healthcare workers' hands and skin, hospital food, the inanimate hospital environment and hospital equipment, and even arthropods (though arthropods are an unlikely reservoir for transmission) [88, 89].

Acinetobacter baumannii can be the responsible pathogen for several infectious syndromes in patients hospitalized in the ICU, including bacteremia, pneumonia (including ventilator-associated pneumonias), meningitis, urinary tract infection, as well as wound infections [90]. Surveillance data from the CDC show that resistance to carbapenems among *Acinetobacter* isolates increased from 0 % in 1986 to 42 % in 2003 to more than 60 % in 2010 [91, 92].

Because of the remarkable ability of these organisms to acquire multidrug resistance rapidly, the therapy of infections caused by *A. baumannii* is quite challenging. As described above, colistin has become one of the most commonly used agents for treatment of multidrug-resistant *A. baumannii* infection. Therapy must be individualized and must be guided by the antimicrobial susceptibility and the patient's clinical progress.

5 Measures to Prevent and Control Infection in the ICU

5.1 General Infection Control Measures

Several agencies and organizations have issued guidelines for controlling the spread of resistant pathogens in the healthcare setting [92, 93]. Some of these guidelines are general in nature, whereas others address specific pathogens. The Society for Healthcare Epidemiology of America (SHEA) has issued guidelines that focus on the prevention of MRSA and *C. difficile* infections as well as device-related and other healthcare-associated infections [93]. Guidelines published by the Healthcare Infection Control Practices Advisory Committee (HICPAC) of the CDC describe the tools and methodology for controlling nosocomial spread of carbapenem-resistant bacteria [94]. Recommendations for infection control interventions from these and other organizations have proven worthwhile in limiting the transmission of these and other resistant organisms in the healthcare setting [95]. Principles designed to control the nosocomial spread of pathogens are described in detail in Chapter 88; however, some of these principles deserve special emphasis for addressing these important issues in the ICU.

The HICPAC guideline advocates a sensible "two-tiered" approach to the management of resistant pathogens [96], suggesting that the control of resistant pathogens is a dynamic process that requires a systematic approach tailored to the problem and the unique healthcare setting. When the practitioner is faced with the emergence of a resistant pathogen problem that cannot be controlled with standard or traditional infection control measures, additional control measures should be selected from a second tier of interventions that include interventions from the following categories: administrative measures/adherence monitoring, staff education, antimicrobial stewardship, surveillance, infection control precautions, environmental measures, and decolonization [96]. Decisions to increase control activities should be based on the individual circumstance [96].

With respect to administrative controls, among the most important is establishing and assuring strong administrative support for clear policies and procedures, grounded in science, that definitively delineate organizational expectations for techniques to be followed routinely in the management of specific infection syndromes. Several studies have argued that administrative engagement and support were critical to controlling the spread of resistant pathogens in the ICU [97–99]. Several infection control interventions require substantial administrative investment, among them: (1) Using information systems to provide important "real-time" data (e.g., alerts, warnings, feedback about adherence data) to healthcare providers at the point of care (2) Assuring the provision of appropriate hospital infrastructure and supplies (e.g., adequate quantities of hand hygiene products, sufficient number and placement of hand washing sinks and hand rub dispensers in the ICU and throughout the facility) (3) Assuring education and ongoing training of ICU staff (4) Providing appropriate staffing levels to meet intensive care needs [100, 101] (5) Assuring the development and implementation of infection control policies and procedures in the ICU (e.g., use of masks, gowns, and gloves and use of contact isolation precautions for multidrug-resistant pathogens) and providing oversight to assure adherence to these infection control policies, procedures, and practices [96]

A second infection prevention principle that is worthy of emphasis for the ICU setting is antimicrobial stewardship. The potential for antimicrobial misuse and abuse is greater in the ICU than perhaps any other locus in the healthcare institution.

Although rarely implemented as a single strategic intervention, several studies have demonstrated at least a temporal association between antimicrobial restriction and control of resistance [102–105]. Although emergence and dissemination of the hypervirulent and fluoroquinolone-resistant NAP1/B1/027 strain in the past decade have been linked to widespread fluoroquinolone use [106], cephalosporin and clindamycin administration likewise places patients at elevated risk for subsequent development of *C. difficile* infection [107]. Targeted reduction in use of broad-spectrum antimicrobial agents has, in some settings, significantly reduced rates of *Clostridium difficile* infection [108, 109]. Although a comprehensive discussion of antimicrobial stewardship is beyond the scope of this chapter, interventions to try to improve antimicrobial stewardship have used several different approaches. A SHEA/IDSA collaboration has outlined guidelines for antimicrobial stewardship, addressing, among other topics, education, formulary restriction, prior approval systems, streamlining empiric regimens, regimen cycling or rotation, the use of computer-assisted programs to provide relevant point of use information to the provider, and comprehensive programs that combine some or all of these strategies (discussed in detail in Reference) [110].

A third infection control strategy or intervention that is worthy of additional discussion is the use of surveillance cultures for resistant pathogens, which are recommended to be used in a targeted fashion dependent on the local and institutional epidemiology of multidrug-resistant bacteria and the origin of admitted patients [111]. The importance and efficacy of using blanket microbiologic surveillance as an intervention to minimize transmission of resistant pathogens remain controversial. Whereas the strategy is intuitively appealing and has been shown to be effective in some models [112], as well as in some clinical settings (including ICU settings) [113], widespread use of this strategy is both costly and labor intensive. By screening all patients and identifying those colonized or infected with resistant pathogens, the intensivist can manage the affected patients aggressively with isolation precautions. A major problem with many of the studies that have used active surveillance cultures is that the strategy is not studied as an independent intervention. Almost all of the published studies purporting to show a benefit of prospective surveillance cultures have implemented this strategy as one of several interventions in an outbreak setting. In all these studies, one cannot determine which of the interventions produced the benefit. The lack of well-controlled studies on the issue of active microbiologic surveillance has been the source of ongoing criticism of this intervention. Surveillance cultures may be useful as a component of "secondary prevention" following a transmission event or outbreak in the ICU [114].

5.2 Syndrome-Specific Infection Control Measures

Whereas the principles outlined above relate to general infection control practices and procedures that have specific relevance to the ICU setting, specific interventions have also been developed to address the four major nosocomial infection syndromes frequently encountered in the ICU (discussed above).

5.2.1 Preventing Device-Associated Bacteremia in the ICU

Several strategies have been specifically directed at limiting the access of organisms to the intravascular device at the catheter insertion site. The use of sterile technique during insertion, attention to the detail of sterile technique when entering or manipulating the system, and rigorous attention to details of appropriate hand hygiene all contribute to reductions in device-associated bacteremia rates in the ICU. Other techniques that have been shown repeatedly to be effective in reducing device-associated bacteremia rates include the use of maximal sterile barrier precautions during the process of catheter insertion, cutaneous antisepsis with chlorhexidine/alcohol, avoidance of femoral vein insertion in adults, and daily review of need for having the line in place, with prompt removal when it is no longer essential [115]. Sites implementing this type of evidence-based approach experienced substantial success in reducing rates of device-associated bacteremia [116–118].

In addition to the following strict precautions during catheter insertion, meticulous care and maintenance of catheters are critical to preventing late CLABSIs. Daily baths with 2% chlorhexidine gluconate reduce the rate of CLABSIs in ICU patients [119–122]. Scrubbing the hubs, needleless connectors, or injection ports of lines and their tubing for at least 5 s with chlorhexidine/alcohol prior to accessing the line, using chlorhexidine-impregnated catheter dressings, and antiseptic caps or hubs are all recommended preventive measures [115].

Other techniques have been suggested in select patient populations. For example, the use of antiseptic or antibiotic-impregnated catheters has been effective in some, but not all, studies and is thus recommended for use when CLABSI rates are high or in individual patients who have high risk of infecting an intravascular site or who have few remaining intravenous access sites [115]. Antibiotic lock therapy, used for catheter salvage following a CLABSI, can be used for CLABSI prevention in patients who have long-term hemodialysis catheters and those who have high risk of infecting an intravascular site or who have few remaining intravenous access sites [115].

The use of needleless connectors for catheters is a topic of unsettled controversy. Needleless connectors were introduced in the 1990s to reduce the use of needles to access catheters and thus avoid needlestick injuries to healthcare personnel that could result in parenteral exposure to bloodborne pathogens. The earlier design, split-septum devices are accessed by a blunt cannula and have free flow and simple internal structure due to lack of a valve. Newer connector designs contain mechanical valves with positive, negative, or neutral displacement and connect via Luer lock with a syringe or tubing. The complexity of the valve structure or the hub may promote formation of biofilm and bacterial colonization. A number of reports document increased CLABSI rates after introduction of the mechanical valve devices [123] [124–128]. Early in their use, improper use due to inadequate training and education of healthcare personnel was blamed for the higher rate of infections [129]; more recently, clinical trials of needleless connectors CLABSIs have shown variable impact on CLABSI rates [123, 128–131], possibly related to differences in study design, patient populations, device used, and training of healthcare personnel in care of needleless connectors. Strategies for disinfection of needleless access ports and use of antimicrobial-impregnated components have had variable success in reducing microbial contamination and infection rates [132, 133]. Chlorhexidine gluconate disinfection appears to be the most effective disinfectant with the added benefit of a residual antimicrobial effect [134]. Whereas utilization of these connectors has become widespread in ICUs due to their ease of use, we do not yet have clinical data pointing to optimal design for CLABSI prevention. Pending more definitive data, practitioners must redouble efforts at meticulous adherence to catheter care and disinfection guidance with needleless connectors and catheters of all types.

5.2.2 Preventing Ventilator-Associated Pneumonia

Strategies have been developed to address the various pathogenetic mechanisms associated with risk for ventilator-associated pneumonia. To address the issues that relate to the rapid changes in hospitalized patients' microbial flora, guidelines have emphasized hand hygiene at all appropriate opportunities. Well-designed clinical trials from Europe have demonstrated a reduction in ICU-associated respiratory tract infections and increased 28-day survival with the use of selective oral decontamination and selective digestive decontamination, which employ use of prophylactic topical, oral, and intravenous antimicrobial agents to reduce the microbial burden in the oropharynx and gastrointestinal tract [135]. Although the interventions have become the standard of care in the Netherlands, North American intensivists have yet to adopt the strategies, in part due to concerns about fostering antimicrobial resistance. (These studies have largely been conducted in ICUs that have low background levels of antimicrobial resistance.) Although two longitudinal studies in ICUs employing selective oral decontamination or selective digestive decontamination have shown no increased antimicrobial resistance, [136, 137] a larger multicenter, randomized Dutch study demonstrated low rates of resistance but slowly progressive development of aminoglycoside resistance with use of selective digestive decontamination [138].

To address the risks associated with the endotracheal tube itself, noninvasive ventilation strategies have been developed, as well as approaches to decreasing the subglottic pooling of secretions. When possible, one should avoid nasotracheal or nasogastric intubation because of the risk for precipitating bacterial sinusitis that increases the risk for pneumonia. Staff should minimize sedation, should avoid unnecessary manipulation of ventilator circuitry/tubing, and should perform spontaneous breathing trials daily while lifting sedation to assess the need for continued intubation. To minimize the risk for aspiration, the head of the patient's bed should be elevated to 45°, and endotracheal tubes with subglottic drainage ports should be used if intubation is anticipated to last more than 2–3 days [139]. Guidelines now recommend against some interventions that were previously favored by some intensivists. Some examples are oral care with chlorhexidine gluconate and use of antimicrobial-coated endotracheal tubes, which have not been associated with improved outcome [139].

5.2.3 Preventing Catheter-Associated Urinary Tract Infections in Patients in the ICU

Preventing CAUTI involves a team approach in the ICU, beginning with the creation and implantation of clear institutional guidelines for catheter use, insertion, and maintenance. Specific indications for catheter use and catheter removal should be developed. Staff should be educated about the indications, aseptic insertion, need for catheter monitoring, and importance of prompt removal of urinary catheters. Only trained, dedicated personnel should insert urinary catheters. ICU staff should meticulously track and document catheter status in each patient's medical record. Institutions should develop protocols that assist in the identification of catheters that are no longer needed and that assure the prompt removal of these catheters. Similarly, in concert with the institution's healthcare epidemiology program, ICU staff should develop and implement a policy requiring mandatory periodic review of the necessity for continued catheterization, for example, the use of institutional triggers or reminders that mandate the assessment of the continued need for catheterization and the use of automatic stop orders or the daily review of each catheter focused on the institutional indications for use and/or removal [140].

Several interventions that have been proposed for CAUTI prevention have not, in our view, been shown to be reliably beneficial (or in some instances have actually been detrimental). Among these interventions are the following: the routine use of antimicrobial/antiseptic-impregnated catheters; screening for asymptomatic bacteriuria among ICU patients who have indwelling catheters; treating asymptomatic bacteriuria in catheterized ICU patients; routine use of catheter irrigation, with or without antimicrobials in the irrigating solution; the use of systemic antimicrobials as CAUTI prophylaxis; and routinely changing catheters at some defined interval.

5.2.4 Preventing Infection in Immunocompromised Patients in the ICU

Preventing healthcare-associated infections among severely immunocompromised patients hospitalized in the ICU is a formidable challenge. For the myriad reasons outlined in the pathogenesis section above, the effects of underlying diseases and therapies leave immunosuppressed patients highly vulnerable to infections from within and without. Basically, the intensivist and the ICU staff must pay attention to the details of all aspects of infection control, emphasizing hand hygiene, administrative controls, aggressive early diagnosis, and appropriate empiric therapy; maintaining a high index of suspicion for yeast and filamentous fungal infection and appropriate antibacterial and antifungal chemoprophylaxis; and maintaining constant vigilance about the potential for the development of infection caused by one or more of the aggressive resistant pathogens that are described above, keeping in mind that the source of these resistant pathogens may be the patient, a provider, or the healthcare environment. Such immunosuppressed patients are at substantially increased risk for many of the pathogens that are described above—MRSA, VRE, *Acinetobacter baumannii*, and other MDR organisms.

Other strategies that may be of use in preventing infections in immunocompromised patients in certain settings include the use of a totally protected environment and selective decontamination of oral and gastrointestinal flora (in some instances protecting the patient's anaerobic flora).

In the final analysis, ICU staff must maintain vigilance for resistant pathogens. Given the difficulty of treating infections due to increasingly resistant pathogens in the ICU, prevention is of paramount importance. Hand hygiene with the use of soap and water or alcohol-based hand rubs is crucial in this setting to prevent spread of resistant organisms. Targeted surveillance for organisms of epidemiological significance may help contain the reservoirs of these pathogens. Adhering to other infection control precautions, maintaining meticulous care of invasive devices, minimizing devices and device days, and judicious use of antimicrobial are all key to reducing the incidence of infections with multidrug-resistant organisms in the ICU.

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Implications of Antibiotic Resistance in Potential Agents of Bioterrorism

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Linda M. Weigel and Stephen A. Morse

1 Introduction

One of the latest challenges to global public health is the deliberate dissemination of biological agents via a number of different routes, including air, water, food, and infected vectors to affect the health of humans and livestock. Congress has addressed this challenge with respect to human health by providing funding to the Centers for Disease Control and Prevention (CDC) to enhance the ability of the nation's epidemiology and laboratory systems to respond to the deliberate release of a biological agent [1]. A Strategic National Stockpile (SNS, formerly called the National Pharmaceutical Stockpile) was also established to provide large quantities of essential medical materiel to states and local communities during such an emergency. The SNS contains antibiotics as well as chemical antidotes, antitoxins, life-support medications, intravenous administration kits, airway maintenance supplies, and medical/surgical items [2]. The broad spectrum antibiotics in the SNS play an important role in providing postexposure prophylaxis and treatment for individuals exposed to or infected with a bacterial agent as a result of a deliberate release. The antibiotics in the SNS were selected, in part, for their effectiveness based on current antimicrobial susceptibility data for each bacterial species. Conventional susceptibility testing methods may require one to several days, depending on the growth characteristics of the species. However, recent revelations suggest that a priority of the former Soviet Union offensive biological weapons program was the development of recombinant organisms that were resistant to common therapies [3–5]. For example, bacterial agents targeted for preparedness efforts, such as *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Burkholderia* spp., *Brucella* pp., and *Coxiella burnetii*, require biosafety level 3 (BSL-3) containment and practices, which are usually not found in clinical laboratories, but are necessary for safely performing antimicrobial susceptibility tests. Many of these bacteria are intrinsically resistant to one or more antimicrobial agents, and isolates with acquired resistance have been reported. Although genetic analyses may provide some clues to susceptibility profiles, in numerous cases the resistance phenotype does not correlate with the genotype. All of these bacteria have been genetically engineered for antimicrobial resistance by introduction of resistance genes or by selection of resistant mutants by in vitro passage. With the increased potential for deliberate dispersal of antimicrobial resistant pathogens, rapid determination of the antimicrobial susceptibility of a bioterrorism agent has become essential for selection and distribution of effective prophylactic or therapeutic treatments to ensure an appropriate public health response in the event of an outbreak or deliberate release of one of these biothreat agents.

The objectives of this chapter are to examine issues concerning antimicrobial susceptibility testing and antimicrobial resistance in selected bacterial agents that have been identified for public health preparedness efforts.

1.1 Definitions

The use of a biological agent is often characterized by the manner in which it is employed. For the purposes of this article, **biological warfare** has been defined as a specialized type of warfare conducted by a government against a target; **bioterrorism** has been defined as the threat or use of biological agents (or toxins) by individuals or groups motivated by political, religious, ecological, or other ideological objec-

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tives [6]. Criminals may also be driven by psychopathologies to use biological agents. When criminals use biological agents for murder, extortion, or revenge it is called a **biocrime** [6]. Terrorists are distinguished from criminals based on motivation and objectives.

2 Threat Agents

Many biological agents can cause illness in humans, but not all are capable of affecting public health and medical infrastructures on a large scale [7]. In order to bring focus to public health preparedness activities, the CDC convened a meeting in June 1999 of national experts to review the criteria for selecting bacterial, viral, and toxin agents that posed the greatest threat to civilians and to help develop a list of these agents for public health preparedness efforts. The considerations for inclusion on the "Critical Agents List" included the ability of the agent to be widely disseminated either by aerosol or by other effective means; the ability of the agent to be transmitted from person to person; the ability of the pathogen to provoke fear; and special public health preparedness needs such as vaccines, therapeutics, enhanced surveillance, and diagnostics [7]. The Critical Agents List [1] includes viruses, toxins, and bacteria; however, due to this chapters focus on antimicrobial resistance and that the control of viral biothreat agents is generally not focused on the use of antiviral agents for prophylaxis, this chapter will cover only the critical bacterial agents (Table 90.1). No priority was assigned within the categories and that the list did not rank the probability of deliberate use of an agent. This effort occurred before the Department of Homeland Security (DHS) was established. Nevertheless, all of the bacterial agents discussed in this chapter have been subjected to DHS Risk Assessments and bacterial threat assessments.

All of the bacterial agents discussed in this chapter are also classified as select agents. Select agents are microorganisms (bacteria or viruses) or toxins, which since 1997 have been declared by the US Department of Health and Human Services (HHS) or by the US Department of Agriculture (USDA) to have the potential to pose a severe threat to public health and safety. For administration purposes, select agents are divided into three categories: (1) HHS select agents and toxins (those affecting humans); (2) USDA select agents (those affecting plants or animals); and (3) overlap (HHS and USDA) select agents and toxins (those affecting humans and animals). HHS select agents discussed in this chapter are *Coxiella burnetii*, *Francisella tularensis*, and *Yersinia pestis*. The overlap select agents discussed in this chapter are *Bacillus anthracis*, *Brucella* spp., *Burkholderia mallei*, and *Burkholderia pseudomallei*. The CDC administers the Select Agent Program (SAP) for HHS while the Animal and Plant Health Inspection Service (APHIS) administers the USDA Select Agent Program. Both CDC and APHIS regulate the overlap agents. The SAPs regulate laboratories which may possess, use, or transfer select agents within the USA. In the wake of the 2001 anthrax attack and several high-level program reviews, the select agent regulations were reviewed and subsequently revised in 2012 to identify a group of select agents designated as Tier 1 agents. Tier 1 select agents have a documented risk of causing a high consequence event based on the following criteria: (1) ability to produce a mass casualty event or devastating effects to the economy; (2) communicability; (3) low infectious dose; and (4) history of or confirmed interest of a terrorist group in weaponization. Tier 1 agents discussed in this chapter include *B. anthracis*, *F. tularensis*, *Y. pestis*, *B. mallei*, and *B. pseudomallei* [8]. Laboratories working with Tier 1 select agents have increased biosecurity requirements including periodic personnel suitability assessments.

Some diseases caused by Tier 1 select agents are also exceedingly uncommon. For example, the first reported case of *B. mallei* infection (i.e., glanders) in the U.S. since 1949 occurred in 2001 in a microbiologist with insulin-dependent diabetes [9]. Despite the patient's history of working with *B. mallei*, both the clinical and laboratory diagnoses were

Table 90.1 Critical bacterial agents for public health preparedness[a]

Agent	Disease
Category A[b]	
Bacillus anthracis	Anthrax
Yersinia pestis	Plague
Francisella tularensis	Tularemia
Category B[c]	
Coxiella burnetii	Q fever
Brucella species	Brucellosis
Burkholderia mallei	Glanders
Burkholderia pseudomallei	Melioidosis
Subset of category B spread by food and water	
Salmonella spp.	Salmonellosis
Shigella dysenteriae	Bacillary dysentery
Escherichia coli O157:H7	Hemolytic uremic syndrome
Vibrio cholerae	Cholera
Category C[d]	
Multidrug-resistant *Mycobacterium tuberculosis*	Tuberculosis

[a]Modified from [1]

[b]Other Category A agents: Variola major, Filoviruses (e.g., Ebola and Marburg), Arenaviruses (e.g., Lassa and Junin), *Clostridium botulinum* neurotoxins

[c]Other Category B agents: Alphaviruses (e.g., Venezuelan, Eastern and Western encephalomyelitis viruses), Staphylococcal enterotoxin B, Ricin from *Ricinus communis*, *Clostridium perfringens* epsilon toxin, *Cryptosporidium parvum*

[d]Other Category C agents: Yellow fever virus, Tickborne encephalitis complex (flavi) viruses, Tickborne hemorrhagic fever viruses, Nipah and Hendra Complex viruses, Hantaviruses

delayed highlighting the difficulties of identifying these rare infections.

Select agents not included in the Tier 1 category also have some potential for large-scale dissemination, but generally cause less severe illness and death than the Tier 1 agents. Such agents have been weaponized in the past, or are being considered as weapons by some state-sponsored programs [3, 10]. They also could be used to contaminate food or water sources. In addition, many of these agents are relatively easy to obtain and therefore are more likely to be used in the setting of a biocrime or bioterrorism [11].

The Risk Assessments and bacterial threat assessments conducted by DHS support the issuance of the bacterial threat determinations that inform the federal government's medical countermeasure decisions and the need to develop or acquire effective medical countermeasures under the Project Bioshield Act of 2004.

3 Bioterrorism Preparedness and Response

3.1 Laboratory Response Network

Because there is only a small window of opportunity during which prophylaxis or other control measures can be implemented to reduce the morbidity and mortality associated with a bioterrorism event, the public health response must be rapid to be effective [12]. The Laboratory Response Network (LRN) was created in order to facilitate the rapid identification of threat agents [13]. The LRN was established in 1999 by the CDC, in concert with the Association of Public Health Laboratories (APHL) and with collaboration from the Federal Bureau of Investigation (FBI) and the United States Army Medical Institute of Infectious Diseases (USAMRIID) to address the extremely limited national infrastructure of diagnostic testing laboratories competent to deal with biological terrorism that existed at that time.

This national system is designed to link state and local public health laboratories with other advanced-capacity clinical, military, veterinary, agricultural, and water- and food-testing laboratories, including those at the federal level, building upon the existing interactions of nationwide public health laboratories and their complementary disease surveillance activities [14].

The LRN consists of laboratories that operate in either a sentinel or reference capacity, with the latter characterized by progressively stringent safety, containment, and technical proficiency capabilities [13]. Sentinel laboratories are, for the most part, hospital and clinical laboratories because it is likely that in the aftermath of a covert bioterrorism attack, patients will seek care at widely dispersed hospitals, some of which would house such laboratories [14]. Sentinel laboratories participate in the LRN by ruling out the presence of a critical agent or referring suspected critical agents (Table 90.1) encountered in their routine work to a nearby LRN reference laboratory. Protocols and algorithms, which are available on the Internet (www.asm.org or www.bt.cdc.gov), have been developed to make this process as rapid as possible. Reference laboratories can perform tests to detect and confirm the presence of a threat agent. They are primarily local and state public health laboratories, employing both biosafety level 2 (BSL-2) facilities where BSL-3 practices are observed (i.e., for culture and identification of *Mycobacterium tuberculosis*), and public health laboratories with full BSL-3 facilities. The LRN reference laboratories use protocols and reagents that have been standardized and validated for the identification, and characterization of threat agents. Characterization of bacterial agents isolated from clinical samples includes determining antimicrobial susceptibility and resistance, a procedure that is not performed at all LRN laboratories due to the extensive training and experience required for visual interpretation of results. However, a rapid susceptibility test for *B. anthracis* has been developed that is based on real-time PCR to detect growth or inhibition of growth by antibiotics [15].

There are also national LRN laboratories that have the ability to generate and analyze whole genome sequences. These federal laboratories identify agents in samples submitted by the reference laboratories and can also identify recombinant (e.g., chimeras) or genetically engineered microorganisms that may only be characterized by whole genome sequence analysis.

3.2 Epidemiological Investigations

Bioterrorism events can be characterized by two types of scenarios: overt (announced) and covert (unannounced). The deliberate nature of an intentional release will often be obvious, as in the case of multiple mailed letters containing highly refined anthrax spores [16]. The letter received and opened in a Senator's office in the Hart Senate Office Building is an example of an overt attack. Some forms of bioterrorism may be more covert, such as the deliberate contamination of salad bars in the Dalles, Oregon, with *Salmonella typhimurium*, which sickened more than 751 persons [17].

The LRN has a dual function in that it has the ability to detect and respond not only to agents released intentionally but also to those that occur naturally, a capacity that warrants emphasis because it will generally not be known at the time of detection whether the outbreak is intentional or natural. A few examples involving the critical bacterial agents will suffice. In the first, the outbreak on Martha's Vineyard of primary pneumonic tularemia in 11 patients in the summer of

2000 may have indicated a deliberate aerosol release of *F tularensis* type A. However, the epidemiologic investigation suggested that infection was associated with lawn mowing and brush cutting, activities that could aerosolize the organism from the environment [18]. Second, the occurrence of plague in a couple visiting New York City in November 2002 was highly unusual and suggested the possibility of bioterrorism because these infections occurred outside the area where plague is endemic in the United States [19]. On initial consultation with medical personnel, the couple reported that they had traveled from Santa Fe County, New Mexico, where routine surveillance conducted by the New Mexico Department of Health had identified *Y pestis* in a dead wood rat and fleas collected several months earlier on their New Mexico property. One day after the patients were evaluated, the New Mexico Department of Health and CDC investigated the couple's New Mexico property and a nearby hiking trail where rodents and fleas were collected. The results of pulsed-field gel electrophoresis and multiple-locus variable-number tandem-repeat assays (MLVA) on isolates from one of the patients and from seven flea pools suggested that the *Y. pestis* infection was most likely acquired on the couple's property. Third, a case of inhalation anthrax in a male drum maker who resided in New York City in February 2006 raised the specter of bioterrorism. However, the epidemiologic investigation determined that the source of exposure was spores on dried goat hides brought back from Cote d'Ivoire [20].

An epidemiological investigation may identify indicators, one or more of which were noted in the examples above, that raise the level of suspicion that an outbreak may have been caused intentionally. These epidemiologic clues include the following as enumerated by Treadwell et al. [21]:

- A single case of disease caused by an uncommon agent (e.g., inhalation or cutaneous anthrax, glanders) without adequate epidemiologic explanation.
- The presence of an unusual, atypical, or antiquated strain of an agent or antibiotic resistance pattern.
- Higher morbidity and mortality in association with a common disease or syndrome, or failure of such patients to respond to standard therapy.
- Unusual disease presentation, such as inhalation anthrax or pneumonic plague.
- Disease with an unusual geographic or seasonal distribution (e.g., plague in a nonendemic area).
- An unexpected increase in the incidence of stable endemic disease, such as tularemia or plague.
- Atypical disease transmission through aerosols, food, or water, in a mode suggesting sabotage (i.e., no other possible explanation).
- Several unusual or unexplained diseases coexisting in the same patient without any other explanation.
- Unusual illness that affects a large, disparate population (e.g., respiratory disease in a large heterogeneous population may suggest exposure to an inhaled biologic agent).
- Illness that is unusual (or atypical) for a given population or age group (e.g., outbreak of measles-like rash in adults).
- Unusual pattern of death or illness among animals that is unexplained or attributed to an agent of bioterrorism that precedes or accompanies illness or death in humans.
- Unusual pattern of death or illness in humans that precedes or accompanies illness or death in animals, which may be unexplained or attributed to an agent of bioterrorism.
- Agents of an unusual illness isolated from temporally or spatially distinct sources that have a similar genotype.
- Simultaneous clusters of similar unusual illness in noncontiguous areas, domestic or foreign.
- Large numbers of unexplained diseases or deaths.
- Large numbers of ill individuals who seek treatment at about the same time (point source with compressed epidemic curve).

4 Critical Bacterial Agents

For the majority of bacterial agents, vaccination has not been a major strategy in pre-event preparedness for the general population; the one exception is anthrax where a combination of vaccine and antimicrobials is recommended for post-exposure prophylaxis. Thus, it is imperative that the antimicrobial susceptibility patterns of suspected bacterial agents of bioterrorism be determined so that effective prophylactic or therapeutic treatment can be administered [22]. However, all of the Tier 1 bacterial agents and many of the other bacterial select agents (Table 90.1) require BSL-3 containment and practices, which are usually not found in the sentinel and other clinical laboratories, but which are necessary for performing antimicrobial susceptibility studies. Some of these bacteria have intrinsic resistance to one or more antimicrobials. In addition, many of these bacterial agents can be genetically engineered by introduction of genes required for antimicrobial resistance [22] or by selection for resistant mutants by in vitro passage on low levels of antimicrobial agents [23, 74]. Thus, the antimicrobial susceptibility pattern of a microorganism encountered as a result of an intentional release is not necessarily predictable.

The deliberate introduction of antimicrobial resistance markers or selection of strains resistant to therapeutically useful antimicrobial agents is generally considered to be a prohibited experiment. However, the introduction of antimicrobial resistance markers may be justifiable under certain circumstances [24]. For example, DNA manipulation for genetic studies on virulence factors often requires the use of

plasmids with antimicrobial resistance genes as markers for selection. In such cases, the antimicrobial resistance genes used should not confer resistance to antibiotics used for the treatment of infections caused by that organism [25]. Nevertheless, laboratory mutants with resistance to antimicrobial agents used for treatment (e.g., ciprofloxacin) have been generated for studies on the molecular basis and for detection of fluoroquinolone resistance [23, 26, 27]. Most laboratories use attenuated or avirulent strains for these purposes.

4.1 Detection of Resistance

Procedures employed for susceptibility testing may generate aerosols that pose a high risk of laboratory-acquired infections. Therefore, antimicrobial susceptibility testing of the critical bacterial agents should be performed only in designated LRN laboratories or at the CDC. Trained personnel, BSL-2 or BSL-3 laboratory facilities (depending on the organism), and personal protective equipment are required to work with these organisms. In addition to the hazards associated with the mechanics of performing susceptibility testing, the working conditions in a BSL-3 environment contribute to difficulties in obtaining accurate and consistent results. Mohammed et al. [28] recognized this issue, commenting that visual evaluation of growth in broth microdilution assays or the ability to see single colonies or light films of growth on agar plates is complicated by the necessity of reading susceptibility results through the glass barrier of a biological safety cabinet. Visibility may be further compromised when laboratory personnel are using power-assisted respirators with face shields.

Susceptibility testing methods include the conventional methods of disk diffusion, agar dilution, broth microdilution, and Etest. There is also the rapid susceptibility method for *B. anthracis* mentioned above, which is currently being modified for other Tier 1 bacterial select agents. Many factors influence antimicrobial susceptibility test results. Among these are: (1) inoculum density, which has been described as the single most important variable in susceptibility testing [29]; (2) the pH, electrolyte concentration, and composition of the medium; (3) time and temperature of incubation; and (4) growth characteristics of the strain to be tested. Growth characteristics are critical because a typical susceptibility testing medium, such as Mueller-Hinton broth or agar, must be either enhanced with specific supplements (i.e., *Francisella*) or changed to a specific medium (i.e., and *Brucella*) to support growth of some bacterial agents. Not all procedures are appropriate for every species. For example, results of disk diffusion tests are not reliable for slow-growing organisms [29] such as *F. tularensis* and *Brucella* spp., and none of these in vitro culture methods support the growth of *C. burnetii*.

The intrinsic resistance mechanisms in each species, the potential for additional naturally acquired resistance mechanisms among individual strains, and the possibility of engineered resistance are compelling reasons for susceptibility testing to guide therapy and postexposure prophylaxis in the event of a natural outbreak or intentional release of these organisms. Guidelines for broth dilution susceptibility testing of *B. anthracis*, *Brucella* spp. *B. mallei*, *B. pseudomallei*, *F. tularensis*, and *Y. pestis* are available in the Clinical and Laboratory Standards Institute (CLSI) document M45-A2 [30]. The guidelines provide testing conditions, quality control recommendations, and set breakpoints for MIC (μg/ml) interpretations of susceptibility and resistance.

Interpretation of in vitro susceptibility data for facultative (e.g., *Brucella* spp., *F. tularensis*, *B. mallei*, *B. pseudomallei*, *Y. pestis*) or obligate intracellular pathogens (e.g., *C. burnetii*) requires consideration of multiple factors that may affect the in vivo activity of the agent. These factors include the ability of the antimicrobial agent to enter an infected host phagocyte and the microenvironment within the eukaryotic intracellular space where the organism resides. The uptake and accumulation (pharmacokinetics) of the various classes of agents by phagocytes are dependent upon the chemical structure of the agent. The intracellular concentration of an antimicrobial agent is expressed as C_c/C_e, the ratio of the cellular (C_c) and extracellular (C_e) concentrations. Therefore, $C_c/C_e > 1$ indicates a higher concentration (accumulation) within the eukaryotic cells of the host. The slightly acidic cytosol works against the intracellular accumulation of weakly acidic antimicrobial agents, such as β-lactams [31] thus $C_c/C_e < 1$. However, even zwitterionic β-lactams (e.g., ampicillin) and many cephalosporins do not accumulate intracellularly, suggesting that additional factors are involved in the exclusion of β-lactams from intracellular cytosol and compartments [32]. Macrolides, however, accumulate in many types of cells [33–37]. This class of antimicrobial agents has a weakly basic character which enables much higher concentrations of drug to accumulate within the acidic (pH = 5) phagolysosomes than in the cytosol [38, 39]. Among the macrolides, C_c/C_e values at equilibrium range from 4 to 10 for erythromycin to 40–300 for azithromycin [32]. Fluoroquinolones accumulate very quickly in cells [40–42], while aminoglycosides accumulate in the cell so slowly that early studies concluded this class of antimicrobial agents did not enter eukaryotic cells [43]. However, over a period of several days, aminoglycoside concentrations within macrophages have been shown to increase to 2–4 times the concentration outside the cell [44–46]. There are limited data on the intracellular accumulation of tetracyclines [45, 47] or sulfonamides [48]. It is important to note that the various methods used for determining the intracellular concentrations and, as a result, the C_c/C_e values may result in conflicting data between studies.

In addition to intracellular accumulation, the intracellular activity (pharmacodynamics) of an antimicrobial agent must be considered. Both the infecting microbe and the antimicrobial agent may exert unknown influences on the infected host cell [49]. The pH within the cytosol or phagolysosome will affect the antimicrobial activity of some agents more than others. The general consensus is that fluoroquinolones, macrolides, and tetracyclines should have activity against intracellular bacteria, and that β-lactams and aminoglycosides show little or no activity against intracellular bacteria. However, there are examples of β-lactam and aminoglycoside therapies that are known to be effective against intracellular infections. These include the use of β-lactams for the treatment of listeriosis and the use of aminoglycosides for the treatment of brucellosis, plague, tularemia, and tuberculosis [50–53].

4.1.1 Genomic Analysis for Determination of Possible Intrinsic Resistance

A number of genome sequences have been completed for the bacterial agents listed in Table 42.1 and are publicly available [54]. The annotated genomes can be searched for genes associated with resistance to antimicrobial agents (Table90.2). The information obtained in this manner is important but has limitations. Genome annotations are produced by computer algorithms to identify putative protein-coding regions based on the search of databases for sequence homology. Many of the genes thus identified have not been verified by laboratory methods. Also, in vitro susceptibility studies are necessary to ascertain whether potential resistance genes are expressed and the product is functional. In addition, there may be considerable variability in the resistance genes among different strains that is not reflected in the available genome sequence(s) of one or a few strains. Furthermore, genome data may not reflect recent acquisition of antimicrobial resistance. Nevertheless, genomic data complements in vitro susceptibility data and may provide important information on the characterization of a specific strain, including the genetic basis for resistance or susceptibility.

4.2 *Bacillus anthracis*

4.2.1 General Characteristics

B. anthracis, the etiologic agent of anthrax, is a facultative anaerobe, spore-forming, nonmotile, nonhemolytic, gram-positive rod [55] that grows rapidly (doubling time of ~30 min.) on most microbiologic media. Vegetative cells sporulate in the presence of oxygen. The spore is infectious and is highly stable in the environment where it is frequently found

Table 90.2. Antimicrobial resistance genes identified in the annotated genomes of Category A and B bacterial agents[a]

Genome	Resistance genes
Bacillus anthracis[b]	Aminoglycosides
	aacC7, aminoglycoside *N*-acetyltransferase
	str, aminoglycoside 6-adenylyltransferase
	Aminoglycoside phosphotransferase
A0039	β-Lactams
	bla1, β-lactamase (penicillinase)
	bla2, β-lactamase (cephalosporinase)
	Metallo-β-lactamase family protein
	*mecR*1, methicillin resistance
Ames	Chloramphenicol
	cat, chloramphenicol acetyltransferase
	bmr, chloramphenicol resistance protein
Ames Ancestor	Glycopeptides
	vanW, vancomycin B-type resistance protein
	vanZ, teicoplanin resistance
CNEVA 9066 (France)	Macrolides
	Macrolide 2-phosphotransferase
	Macrolide efflux protein
	Macrolide glycosyltransferase
Kruger B	Tetracyclines
	tet(V), putative tetracycline efflux
Sterne	Others
	bacA-1, *bacA*-2, bacitracin resistance
	bmr1, bicyclomycin resistance
Vollum	*fosB*-1, fosmidomycin resistance
	vgaB, pristinamycin resistance
Western N. America USA6153	*emrA*, multidrug resistance
	qac, quaternary ammonium compound resistance
	Multidrug resistance protein, Smr family
Brucella abortus	β-Lactams
	Putative β-lactamase
	Metallo-β-lactamase family proteins
9-941	Macrolides
	Macrolide efflux protein
	Others
	fsr, fosmidomycin resistance
	qacH, quaternary ammonium compound resistance
Brucella melitensis	Aminoglycosides
	Aminoglycoside phosphotransferase
	β-Lactams
	β-Lactamase
	Metallo-β-lactamase family proteins

(continued)

Table 90.2. (continued)

Genome	Resistance genes
Biovar Abortus	Macrolides
	Macrolide efflux protein
	Tetracyclines
	tet(B), tetracycline efflux
	Others
	Multidrug resistance efflux protein
	norM, probable multidrug resistance
	Fosmidomycin resistance protein
	Florfenicol resistance protein
	Bleomycin resistance protein
	qacE, qacH, quaternary ammonium compound
	marC, multiple antibiotic resistance
	Bicyclomycin resistance
	fusB, fusC; fusaric acid resistance
Brucella melitensis	Aminoglycoside
	Aminoglycoside phosphotransferase
	cat, chloramphenicol acetyltransferase
16M	Others
	emrB/qacA, macrolide efflux protein Florfenicol resistance
	fosB, fosfomycin resistance
	Multidrug resistance protein
	norM, putative multidrug resistance
Brucella suis	Metallo-β-lactamase
	norM, putative multidrug resistance protein
1330	*fsr*, fosmidomycin resistance
	Fosfomycin resistance family protein
Burkholderia mallei	Aminoglycosides
	aac(*6′*)-*Iz*, aminoglycoside 6-acetyltransferase
ATCC 23344	β-Lactams
	Metallo-β-lactamase
	penA (class A β-lactamase)
	Others
	Fosmidomycin resistance protein
	Fusaric acid resistance protein
	norM, putative multidrug resistance protein
Burkholderia pseudomallei	Aminoglycosides
	Aminoglycoside phosphotransferase

(continued)

Table 90.2. (continued)

Genome	Resistance genes
1710b	β-Lactams
	β-Lactamase
	Metallo-β-lactamase
	oxa β-lactamase
	Macrolides
	macA, *macB* (macrolide-specific ABC-type efflux)
	Tetracyclines
	Tetracycline resistance protein, class A (efflux)
	Others
	Bleomycin resistance protein
	emrA, emrB; multidrug resistance
	fsr, fosmidomycin resistance
	Fusaric acid resistance
	mdtA, mdtB, mdtC; multidrug resistance
	qacE, quaternary ammonium compound resistance
Burkholderia pseudomallei	Aminoglycosides
	Aminoglycoside acetyltransferase
K96243	β-Lactamase
	blaA (class A β-lactamase)
	oxa β-lactamase
	Metallo-β-lactamase
	Putative class B β-lactamase
	Tetracyclines
	Putative tetracycline efflux protein
	Others
	Bleomycin resistance protein
	emrB, multidrug resistance
	fsr, fosmidomycin resistance
	Fusaric acid resistance protein, putative
	mexB, putative multidrug resistance
	norM, multidrug resistance
	qacE, quaternary ammonium compound resistance
Francisella tularensis subsp. *tularensis*	β-Lactams
	blaA (class A β-lactamase)
	β-Lactamase
	Metallo-β-lactamase (putative)
Schu 4	Tetracyclines
	tet (multidrug transporter)
	bcr/cflA, drug resistance transporter
	Fusaric acid resistance protein, putative
Coxiella burnetii	Aminoglycosides
	aacA4, aminoglycoside 6-acetyltransferase

(continued)

Table 90.2. (continued)

Genome	Resistance genes
RSA 493	β-Lactams
	β-Lactamase
	Metallo-β-lactamase family protein
	Others
	Multidrug resistance protein
Yersinia pestis	β-Lactams
	ampG, ampE, ampD β-lactamase induction proteins
CO92	Macrolides
	Macrolide efflux protein, putative
	Others
	bacA, bacitracin resistance, putative
	bicR/bicA, probable drug resistance translocator
	emrA, emrB, emrD-2; multidrug resistance
	marC, multidrug resistance
	qacE, quaternary ammonium compound resistance
	tcaB, multidrug resistance
	vceA/vceB, multidrug resistance
Yersinia pestis	*bacA,* bacitracin resistance protein
KIM	*bcr,* bicyclomycin resistance
	emrA, emrD-2, emrE multidrug resistance
	farB, drug resistance translocase
	Fosmidomycin resistance protein
Yersinia pestis	β-Lactams
	ampD1, ampE, ampG, ampG1 (β-lactamase induction proteins)
Biovar Medievalis 91001	β-Lactamase
	Metallo-β-lactamase family proteins
	Predicted Zn-dependent β-lactamase
	Others
	bcr, bicyclomycin resistance
	bssH bicyclomycin resistance (sulfonamide resistance)
	emrA/emrB, multidrug resistance
	Fusaric acid resistance
	*marC*2, multiple antibiotic resistance
	qacE, quaternary ammonium compound resistance
	ydeF, putative multidrug resistance

[a]*TIGR-CMR* The Institute for Genomic Research-Comprehensive Microbial Resource at www.cmr.tigr.org
[b]Identified genes, gene copy numbers, and chromosomal locations vary among the annotations of *B. anthracis* whole genome sequences

in soil surrounding the carcasses of infected livestock and wildlife. Following inoculation of a susceptible host, the spores germinate, and the resulting vegetative cells multiply in long, bamboo-like chains that are characteristic of the organism. Optimal growth is achieved at 37 °C, and growth does not occur at temperatures ≥43 °C. *B. anthracis* cells are encapsulated in infected tissues or when grown under appropriate in vitro culture conditions.

Two plasmids, pXO1 and pXO2, are associated with virulence in *B. anthracis.* pXO1 carries the genes (*pagA*, *lef*, and *cya*) that are required for expression of the anthrax toxin components, protective antigen, lethal factor and edema factor [56]. pXO2 encodes three genes (*capA*, *capB*, and *capD*) that are required for production of the antiphagocytic poly-D-glutamic acid capsule [57]. Elevated temperature and CO_2 concentration, and specific nutrients are considered to be physiological signals for *B. anthracis* that lead to the germination of spores. Both toxin production and capsule formation are enhanced by growth in 5 % CO_2 or in a medium supplemented with bicarbonate. The CO_2/bicarbonate response is specific (not due to buffering capacity or decreased oxygen concentration) and results in a 20- to 25-fold increase in capsular gene transcription as well as a 5- to 8-fold increase in toxin production. Expression of toxin genes is further enhanced by growth of *B. anthracis* at 37 °C.

B. anthracis is a pathogen of herbivores, and human infection is usually accidental, resulting from contact with spores in contaminated meat or on hides. In humans, the disease may present as cutaneous, inhalational, gastrointestinal, or injectional anthrax, based on the route of infection. Cutaneous anthrax occurs following introduction of spores through a break in the skin. The lesion progresses from a papule to a characteristic eschar, a firm, dry, black lesion that is accompanied by extensive edema. Antibiotics will not alter the progression of the lesion but will prevent systemic infection. Gastrointestinal anthrax may affect either the oropharyngeal area (resulting in sore throat, dysphagia, fever, and regional lymphadenopathy) or the intestine (characterized by nausea, vomiting, fever, and bloody diarrhea). In the absence of effective therapy, infection can rapidly lead to toxemia followed by shock and death. The mortality rate of gastrointestinal anthrax is >50 % with death occurring 2–5 days after onset of symptoms. Inhalational anthrax is a usually rapidly fatal disease with death occurring 2–7 days postexposure, depending on the number of spores inhaled. Initial symptoms may be mild, but if left untreated a rapid succession of sudden shock, collapse, and death all occur within a matter of hours. At the time of death the blood may contain as many as 10^9 bacilli per milliliter in untreated patients [9]. Injectional anthrax has emerged among persons who inject drugs that are contaminated with spores of *B. anthracis*. This form of the disease is more

severe than conventional cutaneous anthrax and may progress to septic shock, meningitis, and death [58].

4.2.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

The antimicrobials used for postexposure prophylaxis and for treatment of the various forms of anthrax are listed in Table 90.3. Historically, penicillin has been the drug of choice for treatment of anthrax. Several susceptibility studies have been published [28, 59–62] most of which were conducted since the intentional release of *B. anthracis* in 2001 (Table 90.4.). Comparison of the MICs determined in these studies has been difficult because there was no standardized testing method, nor were there any interpretive criteria available for *B. anthracis*. Most studies relied on breakpoints published for *Staphylococcus aureus* to interpret the data. Mohammed et al. [28] addressed this issue in a comparison of broth microdilution and Etest agar gradient diffusion methods and found that most of the results for the two methods were comparable, with the exception of penicillin. The Etest MIC result for a penicillin-resistant isolate of *B. anthracis* was consistently in the susceptible range, having 4–9 doubling dilutions difference when compared with the MIC from the broth microdilution method. The MIC results for other agents used for treatment or prophylaxis of anthrax, such as ciprofloxacin and doxycycline, indicate good in vitro activity against *B. anthracis*.

Although *B. anthracis* is generally susceptible to penicillin, penicillin-resistant strains as well as treatment failures with penicillin have been reported [63–65]. Based on laboratory results with geographically diverse groups of strains, the prevalence of naturally occurring penicillin-resistant *B. anthracis* is estimated to range from 3 to 11.5 % [61, 62]. Two β-lactamase genes, located approximately 900 kb apart, have been identified in the chromosome of *B. anthracis* [66]. The *bla1* gene, which encodes a group 2a penicillinase, is usually not expressed. The *bla2* gene, which encodes a cephalosporinase similar to a group 3 *Bacillus cereus* metalloenzyme, is poorly expressed. The genes for both enzymes have been cloned and shown to confer resistance to β-lactams when expressed in *E. coli* [67]. The basis for susceptibility of *B. anthracis* to penicillin, in spite of the presence of two β-lactamase genes, was reported by Ross et al. [68] to be associated with an extracytoplasmic function (ECF) sigma factor, σ^P, and the anti-sigma factor, RsiP. When functional RsiP is produced, σ^P is sequestered and not available to interact with RNA polymerase, effectively preventing the efficient transcription of the β-lactamase genes. In a naturally occurring penicillin-resistant strain, a nucleotide deletion in *rsiP* was identified that resulted in a truncated, nonfunctional protein. As a result, σ^P is not sequestered by RsiP, and the

Table 90.3. Antibiotics used in the treatment of infections caused by selected critical bacterial agents

Disease	Antibiotic	Reference
Anthrax	Ciprofloxacin[a]	
	Levofloxacin[a]	[26, 27]
	Doxycycline[a]	
	Tetracycline[a]	
	Penicillin[a]	
	Amoxicillin (a) superscript	
	Ampicillin	
	Imipenem	
	Meropenem	
	Vancomycin	
	Rifampin	
	Chloramphenicol	
	Clindamycin	
	Clarithromycin	
Tularemia	Streptomycin[a]	[28]
	Gentamicin[a]	
	Doxycycline[a]	
	Tetracycline[a]	
	Chloramphenicol[a]	
	Ciprofloxacin[a]	
	Levofloxacin[a]	
Plague	Streptomycin[a]	[29]
	Gentamicin[a]	
	Doxycycline[a]	
	Tetracycline[a]	
	Ciprofloxacin[a]	
	Levofloxacin[a]	
	Chloramphenicol[a]	
	Trimethoprim-sulfamethoxazole[a]	
Brucellosis	Doxycycline[a]	
	Tetracycline[a]	[30]
	Gentamicin[a]	
	Streptomycin[a]	
	Rifampin	
	Trimethoprim-sulfamethoxazole[a]	
Glanders	Ceftazidime[a]	[31]
	Imipenem[a]	
	Doxycycline[a]	
	Tetracycline[a]	
Melioidosis	Ceftazidime[a]	[31, 32]
	Imipenem[a]	
	Trimethoprim-sulfamethoxazole[a]	
	Chloramphenicol	
	Doxycycline[a]	
	Tetracycline[a]	
	Amoxicillin/clavulanic acid[a]	
Q fever	Doxycycline	[33]
	Ciprofloxacin	
	Rifampin	
	Erythromycin	

[a]Interpretive guidelines for susceptibility or resistance available from CLSI [176]

two β-lactamases are expressed constitutively. These data also provide an explanation for why expression of *bla1* and *bla2* is not inducible when a β-lactam antibiotic is present.

B. anthracis exhibits intrinsic resistance to both second- and third-generation cephalosporins that is not associated with β-lactamase activity. Chen et al. [66] demonstrated that a laboratory-generated mutant of *B. anthracis* Sterne, lacking both *bla1* and *bla2*, remained resistant to cefepime, ceftazidime, and cefpodoxime (MICs >32, >128, >16 μg/ml, respectively). In vitro susceptibility results (Table 90.4.) indicated that <10 % of isolates tested were susceptible to cephalosporins [59].

B. anthracis is highly resistant to aztreonam (Table 90.4.) and exhibits decreased susceptibility to macrolides such as erythromycin. Using susceptibility breakpoints for *S. aureus*, two studies [28, 62] found 97 and 85 % of the MICs to be intermediate for erythromycin when testing isolates of *B. anthracis*. A strain of *B. anthracis* from Korea was reported to possess the *ermJ* macrolide resistance determinant [69] which, if expressed, would confer resistance to macrolides, lincosamides, and streptogramin B. However, the MICs for these antimicrobial agents were not included in the report.

B. anthracis is naturally resistant to trimethoprim and sulfamethoxazole [70]. The organism appears to be susceptible to rifampin in vitro; however, in an in vivo murine model, treatment with rifampin did not significantly increase the survival rate of infected mice [71]. There have been no reports of naturally occurring *B. anthracis* with resistance to aminoglycosides, doxycycline, or fluoroquinolones. However, *B. anthracis* has been shown to acquire resistance determinants in its natural environment, the rhizosphere of grass plants [72], and coexisting soil-dwelling bacteria are known to harbor an extensive reservoir of resistance determinants [73]. Thus, the potential for natural acquisition of additional antimicrobial resistance genes should not be overlooked.

Whole genome sequences have been determined for numerous strains of *B. anthracis*, 39 of which are designated as complete (gapless chromosome) in the National Center for Biotechnology Information (NCBI) database [74]. These include strains A0039, Ames, Ames Ancestor, HYU01, Sterne, Vollum, Western North America USA6153, CNEVA 9066 (France), and Kruger B. A search of the annotated sequences for several of these strains revealed numerous potential resistance genes in each strain (Table 90.2.). In addition to the known β-lactamase genes, *bla1* and *bla2*, putative resistance determinants for aminoglycosides, chloramphenicol, macrolides, and a tetracycline were noted. As is the case with penicillin, the presence of a resistance determinant does not necessarily confer the resistance phenotype. For example, genes for chloramphenicol acetyltransferase (*cat*) and a chloramphenicol resistance protein (*bmr*) have been identified in the genomes of all of the strains that have been sequenced (Table 42.2.), yet results of in vitro susceptibility studies show that this organism remains susceptible to chloramphenicol (Table 90.4.). The gene and/or the encoded protein may be incomplete or nonfunctional, or mutations in the regulatory elements controlling transcription or translation may prevent or limit expression.

Engineered Resistance

Resistance to several antimicrobial agents has been genetically introduced or resistant mutants have been selected by in vitro passage. For example, fluoroquinolone-resistant mutants of *B. anthracis* have been selected in vitro by serial passages on medium containing increasing concentrations of fluoroquinolones [23, 26, 27, 75, 76]. Point mutations in the resistant organisms were found in the quinolone-resistance determining regions (QRDR) of *gyrA, parC*, and *gyrB*. MICs were increased by 16- to 2048-fold for ofloxacin, ciprofloxacin, levofloxacin, and moxifloxacin [77]. In gram-positive bacteria, such as *S. aureus* and *Streptococcus pneumoniae*, the first mutation detected is generally found in *parC* [78]. However, first-step mutants of *B. anthracis* harbored point mutations in *gyrA* and second-step mutants acquired either a mutation in *parC* or an additional mutation in *gyrA*. Otherwise the resulting amino acid substitutions within the QRDR were in the same position and with similar changes to those found in other gram-positive bacteria. For GyrA, the most frequent change observed was Ser85-Leu and for ParC, Ser81-Phe (or Tyr). Amino acid changes detected at the Glu89 position of GyrA were highly variable [27, 75, 77].

Tetracycline resistance has been transferred to *B. anthracis* by the introduction of plasmids or transposons. Resistance to tetracycline, doxycycline, and minocycline was reported following introduction of pBC16, a plasmid that was originally obtained from *B. cereus* strain GP7 [79–81]. Pomerantsev and Staritsyn [81] introduced a recombinant plasmid, pCET, which encodes the *tet*(L) gene, into the Russian anthrax vaccine strain STI-1. The *tet*(L) gene confers resistance to tetracycline but not to minocycline or glycylcyclines [82]. Resistance to tetracyclines has also been transferred to *B. anthracis* following transposon mutagenesis using Tn*916* [83] and Tn*917* [84]. Strains with point mutations resulting in streptomycin and rifampin resistance have been isolated following UV mutagenesis [84].

A multidrug-resistant strain of *B. anthracis* was engineered by Stepanov et al. [85] by the introduction of a plasmid, pTEC, into the vaccine strain STI-1. The new strain, designated STI-AR, was resistant to penicillin, rifampin, tetracycline, chloramphenicol, macrolides, and lincosamides. Stable inheritance of the plasmid and resistance phenotype was confirmed for this strain.

Other resistance genes reported to have been introduced into *B. anthracis* include *ermC* on pE194, which encodes resistance to macrolides [86]; *aad9* on a recombinant plasmid designated pDC, which encodes resistance

Table 90.4. Selected antimicrobial susceptibility studies by Etest, broth microdilution, and agar dilution for *Bacillus anthracis*

	Doganay and Aydin (1991)[a]		Mohammed et al. (2002)[b]		Coker et al. (2002)[c]		Cavallo et al. (2002)[d]		Turnbull et al. (2004)[e]	
	Agar dilution, $n = 22$		Broth microdilution, $n = 65$		Etest, $n = 25$		Agar dilution, $n = 96$		Etest, $n = 76$	
Antimicrobial agent	MIC[f] range	% S–I–R[g]	MIC range	% S–I–R	MIC range	% S–I–R	MIC range	% S–I–R	MIC range	% S–I–R
Amikacin	0.03–0.06	100–0–0								
Amoxicillin	0.015–003	ND					0.125–16	88.5–0–11.5		
Amox/clav	0.015–0.015	100–0–0							0.016–0.5	100–0–0
Azithromycin									1–12	26–64–10
Aztreonam	>128	0–0–100					1–>128	0–0–100		
Cefaclor					0.125–0.75	100–0–0				
Cefotaxime	8–32	4.5–13.5–82							3–>32	1–1–98
Cefoxitin							1–64	74–15.3–10.7		
Ceftazidime	128–256	4.5–0–95.5								
Ceftriaxone	16–32	9–50–41	4–32	22–78–0			4–64	0–100–0		
Cefuroxime	16–64	4.5–9–86.5			6–48	4–76–20				
Cephalexin					0.38–2	100–0–0				
Cephalothin							0.125–32	83.2–12.2–4.6		
Chloramphenicol	1–2	100–0–0	2–8	100–0–0			1–4	100–0–0		
Ciprofloxacin	0.03–0.06	100–0–0	0.03–0.12	100–0–0	0.032–0.38	100–0–0	0.03–0.5	100–0–0	0.032–0.094	100–0–0
Clindamycin	0.5–1	95.5–4.5–0	≤0.5–1	94–6–0			0.125–1	100–0–0		
Doxycycline					0.094–0.38	100–0–0	0.125–0.25	100–0–0		
Erythromycin			0.5–1	3–97–0			0.5–4	95.4–4.6	0.5–1	15–85–0
Gatifloxacin							0.125–0.125	100–0–0		
Gentamicin	0.03–0.25	100–0–0					0.125–0.5	100–0–0	0.064–0.5	100–0–0
Imipenem							0.125–2	0–0–100		
Levofloxacin							0.03–1	100–0–0		
Nalidixic acid							0.125–32	94.8–4.2–1		
Ofloxacin	0.03–0.06	100–0–0					0.06–2	99–1–0		

(continued)

Table 90.4. (continued)

	Doganay and Aydin (1991)[a]		Mohammed et al. (2002)[b]		Coker et al. (2002)[c]		Cavallo et al. (2002)[d]		Turnbull et al. (2004)[e]	
	Agar dilution, $n = 22$		Broth microdilution, $n = 65$		Etest, $n = 25$		Agar dilution, $n = 96$		Etest, $n = 76$	
Antimicrobial agent	MIC[f] range	% S–I–R[g]	MIC range	% S–I–R	MIC range	% S–I–R	MIC range	% S–I–R	MIC range	% S–I–R
Penicillin			≤0.06–128	97–0–3	≤0.016–0.5	88–0–12	0.125–16	88.5–0–11.5	≤0.016–>32	97–0–3
Pefloxacin							0.03–1	100–0–0		
Piperacillin	0.125–0.5	100–0–0					0.25–32	99–1–0		
Rifampin			≤0.25–0.5	100–0–0			0.125–0.5	100–0–0		
Streptomycin	1–4	ND					0.5–2	100–0–0		
Teicoplanin							0.125–0.5	100–0–0		
Tetracycline			0.03–0.06	100–0–0					0.016–0.094	100–0–0
Tobramycin	0.25–1	100–0–0			0.25–1.5	100–0–0				
Vancomycin	0.25–1	95.5–4.5–0	0.5–2	100–0–0			0.25–2		0.75–5	99–1–0

[a]Doganay: Mueller-Hinton agar, 37 °C/overnight [60]; *n*, number of strains
[b]Mohammed: cation-adjusted Mueller-Hinton broth, 35 °C/16–24 h [32]
[c]Coker: tryptic soy agar with 5 % sheep blood, 37 °C/overnight [60]
[d]Cavallo: Mueller-Hinton agar, 37 °C/18 h [61]
[e]Turnbull: Mueller-Hinton agar, 36 °C/18–20 h [62]
[f]*MIC* minimal inhibitory concentration in μg/ml
[g]*S* susceptible, *I* intermediate, *R* resistant; based on breakpoints for *S. aureus* (Interpretive criteria for ciprofloxacin, doxycycline, penicillin, and tetracycline available from CLSI since 2003) New breakpoints for penicillin and for amoxicillin were published by CLSI in 2016.

to spectinomycin [87]; and chloramphenicol acetyltransferase (pC194/*cat*), which confers resistance to chloramphenicol [80].

4.3 *Yersinia pestis*

4.3.1 General Characteristics

Y. pestis is a member of the family Enterobacteriaceae and the etiologic agent of plague. This small Gram-negative coccobacillus is facultatively anaerobic and nonmotile. The organism grows relatively slowly, forming small colonies (0.1–1.0 mm in diameter) after 24–48 h. *Y. pestis* grows on most laboratory media [88] over a wide range of temperatures, with optimal growth at 28 °C. To achieve the visible growth required for broth microdilution susceptibility testing, incubation for up to 48 h may be required.

Plague is a zoonotic disease. The classic model of the transmission *Y. pestis* from fleas to mammals was described by Bacot in 1915. In this model, the vector was the oriental rat flea (*Xenopsylla cheopis*). Ingestion of *Y. pestis* by the fleas during a blood meal from an infected animal results in infection of the flea. In *X. cheopis*, *Y. pestis* multiplies in the alimentary canal eventually forming clumps of bacteria. These clumps block attempts to feed by ingesting another blood meal. In this model, transmission results when a blocked infected flea attempts to feed on another animal or human. Contaminated mouth parts or regurgitation of infected material results in infection of the new host. Twelve to sixteen days are required for the flea infection to progress to the point where the blockage results in an infectious vector, a time frame that is not consistent with the rapid spread of plague during epizootics or pandemics. However, recent reports suggest that *Y. pestis* can also be transmitted by unblocked fleas. Eisen et al.[87a] investigated the transmission of plague from *Oropsylla montana*, a flea that infests squirrels and is the primary vector of *Y. pestis* to humans in North America. These fleas rarely became blocked, are immediately infectious, and efficiently transmit *Y. pestis* for 4 days following an infected blood meal. The dynamics of this flea model are consistent with the rapid rates of transmission necessary to support enzootic and epizootic spread of *Y. pestis*.

Animal reservoirs of *Y. pestis* include many rodents, especially rats, as well as squirrels, and prairie dogs. The most common type of human infection, bubonic plague, is characterized by bubos (acute lymphadenitis) that result from spread of the organism from the flea bite through the bloodstream to the lymph nodes where it grows to large numbers. However, human plague may also present as pneumonia, septicemia, or as meningitis [89].

In humans and animals *Y. pestis* is a facultative intracellular organism. While largely destroyed by polymorphonuclear (PMN) white blood cells, *Y. pestis* cells that are engulfed by monocytes will grow intracellularly and become resistant to phagocytosis by both types of phagocytes [51].

4.3.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

Mortality rates from plague are high in the absence of effective antimicrobial therapy. Fortunately, most isolates of *Y. pestis* are susceptible to antimicrobial agents that are active against gram-negative bacteria. The antimicrobial agents used for the treatment of the various forms of plague are listed in Table 90.3.. *Y. pestis* appears to be susceptible to β-lactams in vitro (Table 90.5.); however, penicillin and cephalosporins are considered to be ineffective for therapy [51]. This may be due to the facultative intracellular nature of *Y. pestis* or to the induced expression of resistance genes (Table 90.2) in the human host. Streptomycin is the drug of choice for treatment of plague, but the availability of this drug is limited[54a] [162]. Gentamicin is an acceptable alternative (MIC_{90} = 0.5–1 μg/ml, Table 90.5.). Both doxycycline and ciprofloxacin have been shown to be effective therapeutic agents.

In 1997 a multidrug-resistant strain of *Y. pestis* was isolated from a patient in Madagascar [90]. This isolate was resistant to many of the drugs recommended for therapy and prophylaxis. The resistance phenotypes and associated genes included ampicillin, TEM-1 β-lactamase; chloramphenicol, *catI;* kanamycin, *aph(3′)-I;* streptomycin and spectinomycin, *aad(3″);* sulfonamides, *sulI*; tetracycline and minocycline, *tet* (D). A 150-kb broad-host-range conjugative plasmid, pIP1202, most likely originating from Enterobacteriaceae, was found to be responsible for the multidrug resistance. The plasmid was highly transferable in vitro, raising concerns that the incidence of multidrug-resistant *Y. pestis* may increase in future outbreaks of plague.

High-level resistance to streptomycin, an agent used for the treatment of plague in many countries outside of the United States, has recently been reported for another clinical isolate of *Y. pestis* from Madagascar [91]. The resistance determinant was located on a 40-kb conjugative plasmid, pIP1203, that could be transferred to other strains of *Y. pestis* at high frequencies. Molecular analysis identified the resistance genes as *aph(3″)-Ib* and *aph(6)-Id. Y. pestis* strains harboring the multidrug resistance plasmid, pIP1202, and the streptomycin resistance plasmid, pIP1203, were of different ribotypes. Furthermore, pIP1202 and pIP1203 belonged to different plasmid incompatibility groups. These results indicate that these two strains arose independently and that there are at least two different resistance plasmids present in strains of *Y. pestis* found in Madagascar.

Wong et al. [92] reported that while 20 % of 92 *Y. pestis* isolates from diverse sources were resistant to rifampin and imipenem, all of the strains were susceptible to antimicrobial agents recommended for treatment and prophylaxis. *Y. pestis* is usually highly susceptible to trimethoprim, although published reports from Russia indicate that resistance to trimethoprim is a natural marker for a variant of *Y. pestis* recovered from voles [93].

Currently, the NCBI database [74] lists >200 *Y. pestis* genomes in various stages of assembly and annotation. Of these, the genome sequences of 27 strains that are indicated as complete (closed chromosome) include: CO92, KIM 10+, biovar Microtus strain 91001 (previously designated as biovar Medievalis), Pestoides F, Nepal 516, Antiqua, Angola, D106004, D18308, Z176003, Harbin 35, and A1122. Putative resistance genes in the annotated genome sequences from several strains (Table 90.2.) included β-lactamase genes, a macrolide-specific efflux system, and a sulfonamide resistance gene.

Engineered Resistance

Genetic studies of *Y. pestis* often require the introduction of plasmids with antimicrobial resistance genes as markers for selection. Most laboratories use the KIM strain of *Y. pestis*, which is avirulent due to the loss of the Lcr (low-Ca^{2+} response) plasmid. Laboratory mutants of *Y. pestis* with resistance to ciprofloxacin have been generated for studies on the detection of fluoroquinolone resistance [94]. Serial passage on medium containing increasing concentrations of ciprofloxacin led to ≥40-fold increase in the MIC among spontaneous mutants of *Y. pestis* KIM 5 [95]. As with most gram-negative species, following the first round of selection, sequence analysis revealed *gyrA* point mutations in codons for two amino acids: Ser83-Ile (or -Arg) and Gly81-Asp (or -Cys). Only first-round mutants were selected in this study, so the importance of topoisomerase IV (*parC*) mutations, which are usually detected in second-round mutants of gram-negative bacteria, is not known.

Russian scientists purportedly developed antibiotic-resistant strains of *Y. pestis* as biological weapons [96]. Ryzhoko et al. reported on the use of β-lactamase-producing strains containing plasmids RP-1 (TEM-2), R57b (OXA-3), and R40a (resistance to carbenicillin) [97]. Further studies by this group employed the use of an

Table 90.5. Selected antimicrobial susceptibility studies for *Yersinia pestis*

	Bonacorsi et al. (1994)[a]		Smith et al. (1995)[b]		Frean et al. (1996)[c]		Wong et al. (2000)[d]		Frean et al. (2003)[e]	
	Agar dilution, $n = 18$		Agar dilution, $n = 78$		Agar dilution, $n = 100$		Etest, $n = 92$		Agar dilution, $n = 28$	
Antimicrobial agent	MIC range[f]	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
Amoxicillin	0.12–0.5	0.5			≤0.03–0.25	0.12				
Ampicillin			0.125–0.5	0.5			0.094–0.38	0.38		
Azithromycin			4–32	32						
Cefotaxime	≤0.03	0.03			≤0.03	≤0.03				
Cefixime							0.006–0.032	0.023		
Ceftazidime							0.016–0.19	0.125		
Ceftriaxone	≤0.03	0.03	0.008–0.031	0.031			0.006–0.032	0.023		
Chloramphenicol			0.5–4	4	0.06–2.0	1	0.25–4.0	2		
Ciprofloxacin			0.008–0.031	0.062					0.016–0.031	0.031
Clarithromycin									4–>32	>32
Doxycycline	0.25–1	1	0.25–1	1	≤0.03–4.0	1	0.125–2.0	1.5	0.25–0.5	0.5
Erythromycin					≤0.03–>16	16			16–32	32
Gentamicin	0.25–1	0.5	0.25–1	1			0.19–1.0	0.75		
Imipenem							0.094–>32	>32		
Levofloxacin					≤0.03–0.06	≤0.03				
Ofloxacin	0.06–0.12	0.12	0.031–0.25	0.25	≤0.03–0.12	≤0.03				
Penicillin			0.25–2	2						
Rifampin			2–8	8	≤0.03–8.0	8	2–32	16		
Streptomycin	2–8	4	4–8	4	≤0.03–2.0	0.5	1.5–4	3		
Tetracycline			0.5–4	4	≤0.03–2.0	2				
TMP-SMX[g]			0.5/2–1/32	1/16	≤0.03/0.59–0.06/1.18	0.06/1.18	0.012–0.047	0.032		

[a]Mueller-Hinton agar, 28 °C/48 h [177]
[b]Mueller-Hinton agar [178]
[c]No information on agar or incubation [179]
[d]Mueller-Hinton agar with sheep blood, 35 °C/overnight [92]
[e]28 °C/48 h [180]
[f]*MIC* minimal inhibitory concentration (μg/ml)
[g]*TMP-SMX* trimethoprim-sulfamethoxazole

avirulent strain, *Y. pestis* 363 Monr, with resistance to the aminoglycosides streptomycin, kanamycin, gentamicin, and amikacin [98]. Mutant strains with resistance to rifampicin (designated as strain Rifr) and nalidixic acid (strain Nair) also have been described. Mutants of the strain Nair were cross-resistant to fluoroquinolones such as ciprofloxacin, ofloxacin, pefloxacin, and lomefloxacin [98, 99]. A study reported in 2004 mentions the use of strains designated as *Y. pestis* EV Rifr R(SmTc) and *Y. pestis* 231 R(SmTc), both of which are apparently resistant to streptomycin and tetracyclines [100]. Another report indicates that aminoglycoside (gentamicin-kanamycin) resistance genes were transferred to *Y. pestis* by transduction using a P1-type bacteriophage [101].

4.4 *Francisella tularensis*

4.4.1 General Characteristics

F. tularensis, the etiologic agent of tularemia, is divided into three subspecies, tularensis, holarctica, and mediasiatica. These subspecies differ in both their virulence and their geographical distribution [102]. The taxonomy of the genus *Francisella* has undergone changes over the past few years. The pathogenic species *F. tularensis* subsp. *tularensis* (highly virulent, type A tularemia) is currently divided into three genetically distinct clades A1a, A1b, and A2 based on pulsed-field gel electrophoresis (PFGE) and whole genome single nucleotide polymorphism (SNP) typing [103]. *F. tularensis* subsp. *holarctica* (responsible for the less severe type B tularemia) was divided into four clades based on SNPs and insertion/deletion mutations (indels). The clades were designated as BI, BII, BIII, and BIV by Volger et al.; an additional clade, BV, was subsequently identified by Karlsson et al. [104, 105]. For humans, the most virulent type is *F. tularensis* subsp. *tularensis*. Culture of this microorganism in vitro presents a high risk of a laboratory-acquired infection. The organism is a small, pleomorphic, aerobic, gram-negative coccobacillus that stains poorly. Growth is very slow and requires an agar medium supplemented with cysteine. The optimal growth temperature is 37 °C. Single colonies may require 2–3 days to appear, and therefore may be overgrown by other bacteria before detection.

F. tularensis is widely found in animal reservoirs. Tularemia, also known as rabbit fever or deerfly fever, results from transmission of *F. tularensis* to humans by biting arthropods, contact with infected animals, or exposure to contaminated aerosols, food, or water [106]. The low infectious dose, which is estimated to be ten organisms by aerosol [107], prior weaponization, and the potential for widespread dispersion make *F. tularensis* an agent of concern for bioterrorism.

4.4.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

The antimicrobial agents that are commonly used to treat the various forms of tularemia are listed in Table 42.3. Because *F. tularensis* is a facultative intracellular bacterium that resides and replicates inside host cells, usually macrophages, susceptibility data (Table 42.6) do not necessarily correlate with effective therapy. Antimicrobial agents that are bactericidal in vitro may be bacteriostatic in vivo. Relapse following antimicrobial therapy is not uncommon and may be attributable to the protective intracellular location of the microorganism.

F. tularensis is inherently resistant to most β-lactam antibiotics (including penicillin, cephalosporins, carbapenems) and to azithromycin [106, 108] and is usually resistant to vancomycin and sulfonamides [109] (Table 42.6). The use of cephalosporins, for which MIC data indicate excellent activity against *F. tularensis* in vitro, has resulted in treatment failures [110]. The NCBI database indicates that whole genome sequences have been completed (gapless chromosome) for 14 strains of *F. tularensis*, subsp. *tularensis*, for 8 strains of *F. tularensis*, subsp. *holarctica*, and one strain of *F. tularensis*, subsp. *mediasiatica*. Annotation of the genome sequence of *F. tularensis* subsp. *tularensis* Schu 4 indicates the presence of genes that encode a class A β-lactamase and a metallo-β-lactamase enzyme (Table 42.2.). Mutagenesis studies suggest that one of the *bla* genes (*blaA*) is either not expressed or has little activity; expression of the other *bla* gene (*blaB*) does not account for the full measure of resistance to β-lactams [111]. Other factors such as cell membrane permeability or targets (penicillin-binding proteins) with low affinity for β-lactam antimicrobial agents may contribute to this resistance phenotype. A *tet* gene described as a multidrug transporter was also noted in the genome annotation.

Engineered Resistance

Numerous plasmid vectors have been developed for various studies of *F. tularensis*. Many of these have been derived from a cryptic plasmid, pFNL10, originally isolated from the *F. novicida*-like strain F6168. Ligation of pFNL10 to pBR328 produced a derivative, pFNL100. This recombinant plasmid, which replicates in both *F. tularensis* and *E. coli*, conferred resistance to ampicillin, tetracycline, and chloramphenicol and was shown to be stably inherited by *F. tularensis* [112]. Additional constructs also have produced stable plasmids with various combinations of resistance genes. A hybrid plasmid, pSKEFT5, derived from pFNL10 and encoding the resistance gene for chloramphenicol was developed for mutagenesis of *F. tularensis*. Shuttle vectors have also been constructed for use in either *E. coli* or *F. tularensis*. These vectors confer resistance to either tetracycline and chloramphenicol [113] or to kanamycin and either tetracycline or ampicillin [114]. Another plasmid, pOM1, also derived from pFNL10, is a 4.4-kb plasmid that encodes *tet*(C) for tetracycline resistance [115].

In addition to plasmids, Lauriano et al. [116] described an allelic exchange method using linear PCR products that include the *ermC* gene, which, after introduction and recombination, result in *F. tularensis* strains that exhibit resistance to erythromycin.

4.5 *Burkholderia pseudomallei*

4.5.1 General Characteristics

B. pseudomallei is a small, motile, irregular staining gram-negative bacillus, and is also a facultative intracellular pathogen. *B. pseudomallei* grows well on simple media, including

Table 90.6. Selected antimicrobial susceptibility studies for *Francisella tularensis*

	Johansson et al. (2002)[a]		Ikaheimo et al. (2000)[b]		Baker et al. (1985)[c]	
	Etest, $n = 24$		Etest, $n = 38$		Broth microdilution, $n = 15$	
Antimicrobial agent	MIC range [4]	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
Ampicillin					>8	>8
Azithromycin	0.064–2	ND	>256	>256		
Aztreonam					4.0–>32	>32
Cefotaxime					≤0.12–4.0	4
Cefoxitin					≤0.25–16	8
Cefpirome			>256	>256		
Ceftazidime			>256	>256	≤0.5–1.0	≤0.5
Ceftriaxone			>32	>32	0.5–16	8
Cephalothin					≤0.25–8.0	>8
Chloramphenicol	0.25–1	ND	0.125–0.5	0.38	≤0.25–4.0	1
Ciprofloxacin	0.016–0.064	ND	0.008–0.023	0.016		
Clindamycin					1.0–>2.0	>2
Doxycycline	0.125–2	ND				
Erythromycin	0.125–2	ND			0.5–2.0	2
Gentamicin	0.032–0.25	ND	0.38–1.5	1	0.25–2.0	2
Imipenem			>32	>32		
Levofloxacin	0.016–0.064	ND	0.008–0.023	0.016		
Linezolid	1–16	ND				
Meropenem			>32	>32		
Methicillin					≤0.12–>4	>4
Oxacillin					≤0.06–>2	>2
Penicillin					4.0–>8	>8
Piperacillin					≤0.5–>64	>64
Piperacillin-tazobactam			>256	>256		
Rifampin	0.125–2	ND	0.094–0.38	0.25	≤0.03–1.0	1
Streptomycin	0.032–2	ND	0.25–4.0	4	≤0.5–4.0	4
Tetracycline			0.094–0.5	0.38	≤0.25–2.0	2
Tobramycin			0.5–2.0	1.5	≤0.12–4.0	2
Vancomycin					>16	>16

[a]The study included 20 human isolates and 4 animal isolates; 8 isolates of *F. tularensis tularensis*, each from a different state in the United States, and 16 isolates of *F. tularensis holarctica*; on Mueller-Hinton II agar supplemented with 1 % isoVitaleX and on cysteine heart agar supplemented with 9 % chocolatized sheep blood; 37 °C/48 h/ambient air. MICs for subspecies *tularensis* and *holarctica* were similar for each agent tested [181]
[b]All isolates were identified as *F. tularensis*; cysteine heart agar supplemented with 2 % hemoglobin; 35 °C in 5 % CO_2, overnight or two nights [182]
[c]Strains were selected from the Centers for Disease Control collection; most isolates were from the southeastern and southwestern areas of the Untied States. Cation-adjusted Mueller-Hinton broth supplemented with 0.1 % glucose, 2 % IsoVitaleX; 35 °C/CO_2 for 24 h [108]

nutrient, blood, and MacConkey agars, but does not grow on deoxycholate citrate or Salmonella-Shigella agars. After overnight incubation on nutrient agar at 37 °C, the colonies are 1–2 mm in diameter. Culture and manipulation of *B. pseudomallei* presents a risk to laboratory personnel; all procedures involving live cultures should be performed in a BSL-3 laboratory. A natural saprophyte, it is found in soil and water in Southeast Asia and northern Australia where the disease (melioidosis) is endemic. Human infection usually occurs by entry of the organism through skin abrasions, although aerosol inhalation or ingestion is also possible. Although person-to-person transmission has been documented, it is very rare [117]. Melioidosis is difficult to treat, requiring prolonged courses of antibiotics. The clinical response to treatment is slow, and relapse is common.

4.5.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

The therapeutic agents used to treat the various forms of melioidosis are listed in Table 42.3. *B. pseudomallei* is usually susceptible to carbapenems, β-lactam-β-lactamase inhibitor combinations, ceftazidime, co-trimoxazole, and tetracyclines (Table 90.7) [118–122]. Treatment is provided in two phases: the intensive phase requires intravenous therapy

for 10–14 days (ceftazidime if the isolate is susceptible), and the eradication phase, which consists of one or more oral drugs (co-trimoxazole and doxycycline) for at least 3 months. *B. pseudomallei* is intrinsically resistant to many aminoglycosides, β-lactams, fluoroquinolones, and macrolides [123]. Multidrug resistance efflux systems have been implicated in antimicrobial resistance; AmrAB-OprA, specific for aminoglycosides and macrolides, and BpeER-OprC, implicated in trimethoprim resistance [122–124]. Whole genome sequence analysis of *B. pseudomallei* has revealed the presence of at least three β-lactamase genes, encoding class A, C, and D enzymes (Table 90.2). Mutation or overexpression of the PenA (class A) β-lactamase results in resistance to amoxicillin-clavulanic acid and to ceftazidime, both of which are mainstays in therapy. Chloramphenicol-resistant strains have been recognized since 1988 [119]. CeoA, a multidrug resistance efflux pump which confers resistance to chloramphenicol, has been identified among the list of resistance genes on the *B. pseudomallei* genome sequence in ARDB-Antibiotic Resistance Genes Database [125]. Also identified is the aminoglycoside phosphotransferase, Aph3-Ia, which confers resistance to gentamicin B, kanamycin, and neomycin. In a study of 199 clinical isolates from Thailand, fewer than 20 % of the strains were susceptible to trimethoprim-sulfamethoxazole and kanamycin [126]. The emergence of resistance to doxycycline, ceftazidime, amoxicillin/clavulanic acid, and co-trimoxazole has been documented during prolonged therapies [118].

The NCBI database [74] indicates that 49 genome sequences of *B. pseudomallei* strains are designated as complete (closed chromosomes). The unusually large genome of this organism is comprised of two chromosomes with a combined size of approximately 7.3 Mb. In the annotation of genes, β-lactamases, metallo-β-lactamase family proteins, macrolide efflux proteins, and a putative tetracycline efflux protein have been identified (Table 42.2).

Engineered Resistance

Resistance to several antimicrobial agents has been engineered by Russian scientists by introduction of natural and recombinant plasmids. Abaev et al. [127] reported efficient and stable transfer of naturally occurring plasmids into *B. pseudomallei*; RSF1010 (streptomycin and sulfonamide resistance), pSa (*aacA4*—gentamicin and kanamycin resistance, *aad2*—streptomycin and spectinomycin resistance, *sul1*—sulfonamide resistance), RP4 (*aphA*—aminoglycoside resistance, *tetA* and *tetB*—tetracycline resistance), and R15 (resistant determinants not described). In the same study, derivatives of RSF1010 were not successfully maintained. Plasmid pOV13, containing the genes for streptomycin, kanamycin, and tetracycline resistance, was transferred into *Burkholderia* spp. by Zakharenko et al. [128].

4.6 *Burkholderia mallei*

4.6.1 General Characteristics

B. mallei, the etiologic agent of glanders, is a small, nonmotile, aerobic gram-negative bacillus. It grows less well than *B. pseudomallei* on nutrient agar, forming colonies 0.5–1 mm in diameter in 18 h at 37 °C. In vitro growth of *B. mallei* presents a risk to laboratory personnel; all procedures involving live cultures should be performed in a BSL-3 laboratory. The organism is genetically very similar to *B. pseudomallei*, but has evolved as an obligate pathogen of equines. Consistent with this host adaptation, *B. mallei* has a significantly smaller genome (5.8 mb), which is considered to be a result of gene deletions as it evolved from a *B. pseudomallei* ancestor. Overall about 1000 fewer genes remain on the chromosomes since *B. mallei* evolved from a lifestyle that required survival in both the environment and a broad range of mammalian hosts to equine-limited infections. At one time glanders was widespread throughout the world. Today the disease has been essentially eliminated from equine populations in the United States and Canada but is still found in the Middle East, Asia, Africa, and South America [129]. Although glanders is now rarely seen in humans, the infection can be fatal and, like melioidosis, treatment is prolonged and clinical cures are difficult to achieve. Person-to-person transmission of glanders has been documented but is rare. The disease was first described by Aristotle as a "disease that originates in the region of the head, and thick and reddish discharge comes from the nostrils" [130]. In equines it is characterized by chronic nasal discharge, with enlargement and induration of lymphatics and lymph nodes. The disease progresses to nodules, pustules, and ulcers on the flanks and extremities. Discharges from the nostrils and ulcers are sources of transmission to other animals as well as humans [131].

B. mallei was used as a biological weapon to infect horses during the American Civil War, World War I, and World War II [132]. In older literature glanders is referred to as farcy. Both *B. mallei* and *B. pseudomallei* have qualities that would make them potential biological weapons. They have high infectivity, can be disseminated into the environment where they will survive for long periods, and have the capacity to cause severe disease with a high mortality.

4.6.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

Few *B. mallei* antimicrobial susceptibility studies have been published, which probably reflects both the scarcity of clinical isolates and the hazardous nature of this microorganism. *B. mallei* is intrinsically resistant to many antimicrobial agents including β-lactams, macrolides, and aminoglycosides (Table 42.8). Although most strains are highly resistant

Table 90.7 Selected antimicrobial susceptibility studies for *Burkholderia mallei*

	Thibault et al. (2004)[a]		Heine et al. (2001)[b]				Kenny et al. (1999)[c]	
	Agar dilution, *n* = 15		Broth microdilution Etest, *n* = 11				Broth microdilution, *n* = 17	
Antimicrobial agent	MIC range[d]	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
Amikacin	1–128	64	0.5–4	2	0.25–1	0.5		
Gentamicin	0.125–128	128	0.25–1	0.5	0.047–0.125	0.094	0.063–0.5	0.5
Streptomycin			2–8	4				
Tobramycin			0.25–16	0.5				
Clindamycin	>128	>128						
Azithromycin			0.25–1	1	0.094–0.75	0.5	0.25–16	4
Erythromycin	0.25–2	1						
Clarithromycin			4–16	4				
Ofloxacin	0.125–32	2	0.5–8	8	0.023–3	1	0.5–8	8
Ciprofloxacin	0.5–16	4	≤0.03–4	1	0.008–0.5	0.25	0.25–8	8
Levofloxacin	0.125–4	1						
Amoxicillin	16–128	64	>64	>64				
Amox/clav	0.125–8	4	1–4	4	0.125–0.5	0.25	1–8	8
Ampicillin			32–64	64	2–16	6	1–>64	>64
Piperacillin	0.125–8	8	1–8	8	0.125–1	0.38	4–16	16
Imipenem	0.125–0.5	0.5	0.12–1	0.25	0.064–0.19	0.125	0.125–0.25	0.25
Ceftazidime	1–4	2	1–6	4	0.125–1	0.5	2–16	8
Cefotaxime	0.5–32	16	4–6	16				
Cefotetan			16–>64	32	2–32	16		
Cefoxitin	4–>128	>128						
Cefuroxime			32–64	64	1.5–16	6	8–>64	>64
Cefazolin			32–>64	>64				
Ceftriaxone			16–64	16	1–32	12		
Aztreonam	4–128	64	32–>64	32	2–32	12		
Sulfamethoxazole			0.25–>64	16			1–>64	>64
Co-trimoxazole	1–4	4	0.25–64	32	0.003–0.25	0.125	0.063–>64	>64
Trimethoprim			1–32	16			0.125–64	32
Doxycycline	0.125–0.5	0.25	≤0.5	0.12	≤0.016–0.094	0.032	0.125–4	2
Rifampin	0.25–16	4	2–16	8			1–16	16
Chloramphenicol	0.125–8	4	4–64	32	0.25–24	8	1–>64	>64
Quinupristin-dalfopristin			1–32	32				

[a]Selected strains from China, Turkey, Hungary, Iran, and India collected over a period of 1920–1966 from man and animals; Mueller-Hinton agar, 37 °C/48 h [123]

[b]Seven NCTC strains and four ATCC strains; broth microdilution in cation-adjusted Mueller-Hinton broth; 37 °C/overnight; Etest on Mueller-Hinton agar incubated 37 °C/18–24 h [183]

[c]Ten ATCC strains and seven strains from Central Veterinary Laboratories, Weybridge, UK. Broth microdilution in cation-adjusted Mueller-Hinton broth, 37 °C/36 h [133]

[d]*MIC* minimal inhibitory concentration in μg/ml

to ampicillin, the combination of ampicillin with a β-lactamase inhibitor such as clavulanic acid results in very low MICs in vitro. The percentage of gentamicin-resistant strains varies from 0 to 19% [133]. Some antimicrobial agents that are active against *B. mallei* in vitro are clinically ineffective, most likely due to the intracellular location of the organism. Ceftazidime has been used successfully for treatment of glanders; however, a resistant isolate has been reported [134]. *B. mallei* is usually susceptible to imipenem, doxycycline, and minocycline. Many strains are susceptible to erythromycin but resistant to clindamycin. The NCBI database [74] currently lists 12 strains for which the whole genome sequence has been completed (closed chromosomes). Antimicrobial resistance genes identified and listed in the ARDB include those associated with resistance to chloramphenicol, aminoglycosides, and macrolides (Table 42.2).

Engineered Resistance

Studies of the pathogenesis and genetics of *B. mallei* have often included the introduction of plasmids with antimicrobial resistance markers. These include resistance to gentami-

cin, kanamycin, streptomycin, tetracycline, chloramphenicol, trimethoprim, and bleomycin. The development of a multidrug-resistant strain by Russian scientists has been reported; however, the resistance genes that were introduced were not specified [3]. Abaev et al. [127] described successful introduction of natural plasmids RSF1010, pSA, R15, and RP4 in *B. mallei* (as described above for *B. pseudomallei*). Unlike *B. pseudomallei*, several derivatives of these plasmids were stably maintained in *B. mallei*.

4.7 *Brucella* spp.

4.7.1 General Characteristics

Brucella spp., the etiologic agents of brucellosis, are small gram-negative coccobacilli. These nonmotile, aerobic organisms are facultative intracellular bacteria that grow slowly and require complex media containing serum or blood. Many strains require CO_2 for growth. Nomenclature has traditionally been based on host preferences and pathogenicity. The six classical species (and their hosts) include *B. melitensis* (goats, cows, sheep), *B. abortus* (cattle), *B. suis* (swine), *B. canis* (dogs), *B. ovis* (sheep), and *B. neotomae* (rodents). Debates have occurred as to whether these organisms should be reclassified as biovars of a single species on the basis of high levels of genetic relatedness by DNA-DNA hybridization. However, the current approach is to consider phylogenetic-evolutionary data based on genotyping as well as host preferences, virulence, and pathogenicity as the criteria for classification using species instead of biovars [135]. Molecular methods used for genotyping include multilocus sequence analysis, multiple-locus variable-number tandem-repeat (VNTR) analysis (MLVA), and analysis of single nucleotide polymorphisms (SNPs) [136]. Based on these methods, additional species have been suggested: *B. ceti* (whales and other cetaceans), *B. pinnipedialis* (seals), *B. microti* (from common vole, *Microtus arvalis*), and *B. inopinata* BO1 and BO2 [137].

These bacteria are highly infectious and are distributed worldwide. Brucellosis is a debilitating disease characterized by undulant fever, myalgia, arthralgia, night sweats, and malaise. Numerous names for the disease include Malta fever, Mediterranean fever, and undulant fever. Infection with *B. melitensis*, the most virulent species for humans, may be

Table 90.8 Selected antimicrobial susceptibility studies for *Burkholderia pseudomallei*

	Yamamoto et al. (1990)[a]		Smith et al. (1996)[b]		Sookpranee et al. (1991)[c]		Ashdown (1988)[d]	
	Agar dilution, $n = 97$		Agar dilution, $n = 100$		Agar dilution, $n = 199$		Broth microdilution, $n = 100$	
Antimicrobial agent	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
Ampicillin					0.25–>512	32		
Amp/sul					0.25–128	8		
Amoxicillin							>64	>64
Amox/clav			0.5–8	4			2–>64	4
Penicillin	0.39–3.13	1.56						
Piperacillin					0.25–16	2	1–4	2
Cefepime	3.13–50	12.5						
Cefotaxime	0.78–12.5	3.13					2–8	8
Ceftazidime	0.39–3.13	1.56	0.25–32	2	0.125–16	2	1–8	4
Ceftriaxone							2–8	8
Imipenem	0.2–1.56	0.78	0.12–1	0.5	0.06–4	0.5	0.25–2	1
Meropenem	0.39–3.13	0.78	0.25–1	1				
Aztreonam,	6.25–50	25			8–>256	32	2–16	8
Nalidixic acid	3.13–>200	50						
Ofloxacin	0.78–12.5	6.25						
Ciprofloxacin	0.78–6.25	3.13			0.125–16	8	0.5–16	8
Tetracycline	0.78–12.5	12.5						
Minocycline	0.78–3.13	3.13						
Chloramphenicol	6.25–>200	25						
Rifampin	3.13–25	25						
SXT	0.78–25	12.5						

[a]Human isolates: 27 from Ubon-Rajathanee, Thailand (1989); 70 from Nonthaburi, Thailand (1981–1989); Medium: Mueller-Hinton agar, incubated 37 °C/20 h, [119]

[b]Human isolates from Ubon Ratchathani, Thailand, collected during 1991–1992 [184]

[c]Human isolates from Khon Kaen, Thailand; Mueller-Hinton agar [126]

[d]Human isolates from northern Australia, Mueller-Hinton broth [121]

acquired by inhalation, consumption of contaminated food such as unpasteurized dairy products, or contact with infected animals. If acquired during pregnancy, the infection leads to early or midterm abortion. Rare instances of person-to-person transmission have been recorded, either by sexual contact [138] or by transfer of tissue, including blood and bone marrow [139]. Laboratory-acquired infection with *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* is a significant problem and results from accidental ingestion, inhalation, injection, and mucosal and skin contamination. The infectious dose is estimated to be between 10 and 100 organisms [140]. Procedures involving *Brucella* cultures should be performed in a BSL-3 laboratory. Extended, combination antimicrobial therapy is required, and relapse frequently occurs following treatment.

4.7.2 Antimicrobial Susceptibility and Resistance

Intrinsic Resistance

The antimicrobial agents used to treat brucellosis are listed in Table 42.3. The recommended regimen requires combination therapy with doxycycline and an aminoglycoside such as streptomycin or gentamicin, or doxycycline and rifampin for 6–8 weeks. In vitro, *Brucellae* are usually susceptible to tetracyclines, aminoglycosides, fluoroquinolones, and rifampin (Table 42.9) [141–143]. Erythromycin ($MIC_{90} > 8$) and vancomycin ($MIC_{90} > 16$) generally have poor activity [141, 144]. As with other intracellular bacteria, the in vivo efficacy of antimicrobials may not correspond with in vitro test results. Additional factors include penetration and accumulation of the agent within the host cell and the effect of low pH in the phagolysosome where the organism resides. A multidrug efflux pump, NorMI, has been identified in *B. melitensis* [145] (and Table 42.2). The substrate profile for this type of pump includes fluoroquinolones [146]. Although the clinical impact, if any, has yet to be established, efflux mechanisms may reduce susceptibility to an antimicrobial agent, allowing time for selection of mutations that increase the level of resistance.

The NCBI database [74] lists numerous whole genome sequences of *Brucella* spp that have been completed (closed chromosomes). Among the completed genomes are 7 strains of *B. melitensis*, 12 strains of *B. abortus*, 5 strains of *B. canis*, and 15 strains of *B. suis*. The genome consists of two chromosomes with a total size of approximately 3.2–3.3 Mb.

Resistance genes have been identified in the annotated genomes (Table 42.2). The putative resistance genes differ among the species. Several efflux systems were detected in *B. melitensis*, but none in *B. suis*. Macrolide and tetracycline resistance genes were also noted in the *B. melitensis* genome, but not *B. suis*. However, the *B. suis* sequence included β-lactamase genes and a chloramphenicol resistance determinant not identified in the *B. melitensis* genome.

Engineered Resistance

Reports from the former Soviet Union document the introduction of antimicrobial resistance genes in *Brucella*. The plasmid pOV13, which confers resistance to streptomycin, tetracycline, and kanamycin, was described as being stably inherited by *Brucella*, as well as *Pseudomonas* spp. (i.e., *Burkholderia*) [128]. *B. abortus* strain 19-BA was selected for resistance to rifampicin and then transformed with the plasmid pOV1. The resulting strain was resistant to rifampicin, tetracycline, doxycycline, ampicillin, and streptomycin [147].

4.8 *Coxiella burnetii*

4.8.1 General Characteristics

Coxiella burnetii, the etiologic agent of Q fever (Q originating from query to investigate an outbreak of fever of unknown origin), is a small gram-variable coccobacillus from the family *Coxiellaceae*, which contains the genera *Coxiella* and *Rickettsiella* [148]. This organism is an obligate intracellular parasite that grows in cytoplasmic vacuoles of animal cells, primarily macrophages. Historically, *C. burnetii* could be cultivated only in eukaryotic host cell cultures or embryonated eggs, and the most rapid culture method, the shell vial technique, required 7–10 days [149]. However, in 2009 a cell-free medium designated as Acidified Citrate Cysteine Medium (ACCM) was developed by systematically evaluating the metabolic requirements using expression microarrays [150]. The authors report a 2.5–3 log increase in genome equivalents as an indicator of growth in the complex nutrient medium after 6 days of culture in a microaerophilic (2.5 % oxygen) environment.

Small colony variants (SCVs) of *C. burnetii*, which resemble chlamydial elementary bodies, are common and apparently represent a stage of the developmental cycle [151]. SCVs are highly resistant to heat, drying, and chemicals such as 10 % bleach, 5 % Lysol, and 5 % formalin [152]. SCVs have been shown to survive pasteurization and can survive for months in milk or dried feces. *C. burnetii* cells, including SCVs, are highly infectious as an aerosol. Natural routes of transmission to humans include inhalation of contaminated dust or hay, direct contact with infected animals, contaminated milk or other dairy products, and body lice. Unlike human infections, ticks play a role in transmission to animals. The infectious dose for humans is about ten organisms [153]. Q fever may present either as an acute infection, usually febrile pneumonia or hepatitis, or as a persistent, chronic disease that often includes endocarditis. The environmental stability of the organism and the low infectious dose are the reasons this agent has been considered as a potential bioweapon [154, 155].

Table 90.9 Selected antimicrobial susceptibility studies for *Brucella melitensis*

	Baykam et al. (2004)[a]		Akova et al. (1999)[b]		Trujillano-Martín et al. (1999)[c]	
	Etest, $n = 37$		Broth microdilution, $n = 43$		Agar dilution, $n = 160$	
Antimicrobial agent	MIC range[d]	MIC_{90}	MIC range	MIC_{90}	MIC range	MIC_{90}
Co-trimoxazole	0.047–3.0	1.5				
Ceftriaxone	0.125–1	0.5				
Doxycycline	0.016–0.094	0.064	≤0.125–8	≤0.125	0.12–0.25	0.25
Rifampin	0.19–1.5	1.0	1–32	2	0.5–1	1
Erythromycin			0.5–256	128		
Azithromycin			≤0.126–4	1		
Streptomycin			0.25–8	2	4–16	8
Ciprofloxacin	0.064–0.50	0.19	≤0.125–8	2	0.25–1	1
Ofloxacin			≤0.125–4	1	1–2	2

[a]Human blood isolates collected between 2000 and 2003, Ankara, Turkey. Medium: Mueller-Hinton agar supplemented with 5 % sheep blood; incubated 35 °C/48 h [185]
[b]Human isolates from blood or bone marrow, collected between 1991 and 1994, Ankara, Turkey. Medium: Mueller-Hinton broth supplemented with 1 % PoliVitex, adjusted to pH 7.0, incubated 35 °C/48 h [186]
[c]Human blood isolates collected during 1997 from Salamanca, Spain. Medium: Mueller-Hinton agar supplemented with 1 % hemoglobin and 1 % PoliVitex [187]
[d]*MIC* minimal inhibitory concentration in µg/ml

4.8.2 Antimicrobial Susceptibilities and Resistance

Methodology

Historically, susceptibility studies of *C. burnetii* were performed with infected embryonated eggs or with cell cultures [156, 157]. The organism replicates to high numbers, but the doubling time is estimated to be 12–20 h [158]. These culture methods are labor- and time-intensive and are not easily adaptable for multiple antimicrobial agents. Two alternative methods are now in use. The shell vial assay [159] is a modified cell culture technique that facilitates testing of multiple antibiotics. The second method, quantitative real-time PCR, detects the number of copies of a *C. burnetii*-specific gene in the culture as an estimate of the growth [160, 161]. Both of these methods require 6–7 days of bacterial growth in cell cultures. Currently there are no reports of susceptibility testing studies that employ the cell-free medium described above.

The centrifugation-shell vial technique employs a shell vial (manufactured by Sterilin, Feltham, England) containing a human embryonic lung (HEL) fibroblast cell monolayer. The inoculum of *C. burnetii* is added and subjected to low-speed centrifugation (700 × *g*) to bring the bacteria in contact with the HEL monolayer. After 6 days of growth to allow 30–50 % of the cells to become infected, the cell culture medium is replaced with medium containing a specific concentration of an antimicrobial agent. The medium/antimicrobial solution is replaced daily during 6 days of incubation at 37 °C in 5 % CO_2. Cell numbers are determined by indirect immunofluorescence using anti-*C. burnetii* rabbit serum and fluorescently labeled goat-anti-rabbit antibodies. The number of *C. burnetii* cells in the test is compared with positive and negative controls (HEL cell cultures with and without *C. burnetii* infection, respectively) to determine if the strain is susceptible (absence of infected cells), intermediate (fewer than 10 % cells infected), or resistant (normal growth in presence of antibiotic).

Brennan and Samuel [160] reported on the determination of antimicrobial susceptibility of *C. burnetii* by real-time PCR. In this method, the antimicrobial dose response curve is based on the number of copies of the *com1* gene from *C. burnetii* as determined semiquantitatively by real-time PCR. A similar method was used by Boulos et al. [161] using the gene for superoxide dismutase (*sod*). Both studies used murine macrophage cell lines that were infected with *C. burnetii*. A standard curve of the number of gene copies was established by real-time PCR for *C. burnetii*-infected cells grown without antibiotics. The effect of antimicrobials on the growth of *C. burnetii* was determined by the difference in the number of gene copies in the presence of the antibiotic when compared with the growth curve of the control culture.

Intrinsic Resistance

Doxycycline is the treatment of choice for the acute form of Q fever, although fluoroquinolones appear to be useful as an alternative [162]. Treatment of persistent infections is problematic, requires extended therapy, and relapse may occur after antimicrobial agents are withdrawn. Combination therapy consisting of doxycycline with either ofloxacin or rifampin for a period of 3 years has been recommended as treatment. The use of hydroxychloroquine, an alkalinizing compound of the phagolysosome vacuole, has also been recommended to achieve bactericidal activity [163, 164]. Results from representative susceptibility studies using the methods described above are shown in Table 42.10.

The whole genome sequences of four strains of *C. burnetii* are listed as complete in the NCBI database [74, 165]. Gene sequences for putative β-lactamase and metallo-β-lactamase family proteins have been identified in the annotated genome (Table 42.2). Both genes are located on the chromosome. There is also an aminoglycoside acetyltransferase identified as *aacA4*. Different strains of *C. burnetii* are genetically heterogeneous with variations in both chromosomal and plasmid DNA. There is usually a single plasmid in *C. burnetii*. However, among strains there are considerable differences in the size (34 to >50 kb) and the gene arrangement on the plasmid. There are reports of significant differences in the susceptibility profiles of distinct isolates [166, 167] and also in isolates from acute vs. chronic disease [168]. These data suggest that susceptibility testing of *C. burnetii* isolates may be beneficial for selecting appropriate antimicrobial therapy.

Engineered Resistance

Although the requirement for growth in tissue cultures and the slow generation time have limited genetic studies of *C. burnetii*, fluoroquinolone-resistant mutants have been selected in vitro. Two reports indicate that MICs of 8–16 μg/ml of ciprofloxacin [169] and 32–64 μg/ml pefloxacin [170] were attained by in vitro selection. Tetracycline-resistant strains have also been developed in laboratory studies [171].

Table 90.10 Selected antimicrobial susceptibility studies of *C. burnetii*

Antimicrobial agent	Raoult et al. [149] 1991	Boulos et al. [143] 2004		Gikas et al. [170] 1998
	S–I–R (no. isolates), n = 13	Range of MICs in μg/ml, n = 2		Range of MICs in μg/ml, n = 8
	Shell vial assay[a]	Real-time PCR	IFA[b]	Shell vial assay
Amikacin	R (13)			
Amoxicillin	R (13)			
Ciprofloxacin	S (5); I (8)	2–4[c]	4–8	4–8
Clarithromycin				2–4
Chloramphenicol	S (10); I (3)			
Co-trimoxazole	S (13)	8–16	8	
Doxycycline	S (13)	2–4	1–2	1–2
Erythromycin	I (7); R (6)	2–4	4–8	
Gentamicin		>10	>10	
Ofloxacin	S (12); I (1)	2	1–2	1–2
Rifampin	S (13)	4	2	
Tetracycline	S (13)			

[a] *S* susceptible (no growth), *I* intermediate (reduced growth), *R* resistant (normal growth)
[b] *IFA* immunofluorescence antibody assay
[c] Range of MICs in μg/ml

5 Conclusions and Future Considerations

Antimicrobial resistance remains an important factor to be considered in efforts to prepare and respond to the intentional release of an infectious agent. While the antimicrobial susceptibility of the bacterial agent is a concern for infections resulting from either a naturally occurring outbreak or bioterrorism, there is a growing concern that genetic engineering could be used to make a normally susceptible microorganism resistant to one or more of the antimicrobial agents commonly used for therapy. Conventional antimicrobial susceptibility testing requires time to isolate a pure culture of the organism and 1–3 days (depending on the organism) for growth in susceptibility tests. Rapid detection of potential resistance is essential for effective therapeutic and prophylactic treatment. To address this concern, molecular assays involving standard PCR or microarrays have been developed to detect the presence of resistance determinants [172, 173]; however, the presence of a known resistance gene does not necessarily correlate with phenotypic resistance. Mutations associated with fluoroquinolone resistance can also be detected by rapid DNA sequence analysis or hybridization assays such as microarrays. Because the resistance determinant(s) may be unknown or not previously found in a particular organism, oligonucleotide microarrays have been developed that can identify all known resistance determinants [174, 175]. However, phenotypic resistance can only be determined by analysis of the isolate in the presence of an antibiotic. A rapid method for determining phenotypic susceptibility or resistance has been developed using real-time PCR to detect growth or no growth. This method decreases the time to results, after an isolate has been obtained, to 6 h for *B. anthracis* [15], 8 h for *Yersinia pestis*, and 8 h for *B. pseudomallei* (unpublished data). These rapid methods were developed for the Laboratory Response Network as a preparedness measure for outbreaks or a bioterrorism event. As new technologies become available, the rapid tests may become widely used. Although the availability of annotated genomes has provided information on resistance genes that are present in the sequenced strains, phenotypic susceptibility testing remains essential to determine the clinical significance, if any, of any resistance genes identified and to detect new resistance determinants or mechanisms of resistance as they emerge and spread through microbial populations.

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Internet Resources on Antimicrobial Resistance

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1 Introduction: Significance of Internet Resources on Antimicrobial Resistance

A few years ago, we compiled a list of World Wide Web (WWW) addresses of sites of major international networks that present data regarding resistance to commonly used antimicrobial therapeutic agents. The relevant article was published and is an open-access educational resource available at http://cid.oxfordjournals.org/content/43/5/630.long [1].

This chapter is mainly based on the published article [1]; however the lists of Internet addresses have been recently revised in order to provide reliable and up-to-date information.

Our lists of World Wide Web resources of data from surveillance studies on antimicrobial resistance may be useful to practitioners, especially infectious diseases specialists, as well as to scientists with a research interest in the field of antimicrobial resistance. Such educational and informative World Wide Web resources are potentially helpful because of the growing problem of antimicrobial resistance that has become a significant public health concern worldwide [2]. This refers practically to all types of pathogens, including viruses, bacteria, mycobacteria, fungi, and parasites. Previous studies have shown the impact of antimicrobial resistance on various outcomes including mortality, morbidity, and cost and length of hospitalization [3–5].

The Infectious Diseases Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) have recently published their concerns regarding the considerable proportion of clinical isolates that are resistant to most antimicrobial agents [6, 7]. To combat this phenomenon, the mandatory implementation of antimicrobial stewardship programs has been proposed by IDSA [8]. Among the various clinically important bacteria, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Enterococcus* spp., *Acinetobacter* spp., *Pseudomonas* spp., and *Klebsiella* spp. represent major pathogens that cause high incidence of infections that are resistant to treatment with antibiotics of many antimicrobial classes [9–13]. Of particular concern recently is the increasing incidence of community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) in most countries [14, 15] as well as the epidemic of multidrug-resistant *Acinetobacter baumannii* infections in several countries, especially in patients in the intensive care unit (ICU) setting [16].

When practicing medicine during this era of easy international travel and because transfer of patients between hospitals in different countries is not rare, the clinician and especially the infectious diseases specialist should have easily available epidemiological data regarding the antimicrobial resistance. In addition, investigators studying various aspects of the problem of antimicrobial resistance also benefit enormously from the availability of such data. Thus, both clinicians and investigators benefit by knowing the proportion of clinical isolates that are resistant to various antimicrobial agents in their community, hospital, area, country, continent, as well as around the globe, because the cross continental travel of both humans and goods causes the spread of antibiotic-resistant bacteria from one country to another.

Advances of modern technology including the development of the Internet and the World Wide Web have given the opportunity to clinicians and researches to have immediate access to continuously updated information in various scientific fields. Thus, the collection and update of ongoing surveillance antimicrobial resistance data from various sources has been made possible [17]. As a useful guidance tool to practitioners and researchers, we sought to compile a list of

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major networks' web pages/sites that provide valuable World Wide Web links that offer additional information relevant to the problem of antimicrobial resistance.

2 Methodology of WWW Resource Selection

We gathered information regarding the relevant World Wide Web resources by making use of Internet search engines (Google, Bing, and Yahoo). We used as keywords the abbreviated names of major antimicrobial surveillance systems/projects that were known to us (i.e., EARS-Net, NARMS, STRAMA, DANMAP, etc.). Also, we performed searches of the PubMed database, Current Contents, and the World Wide Web for information regarding additional relevant sources by using the following keywords: resistance, antimicrobial resistance, surveillance, network, program, and project. In addition, we reviewed the information provided in the initially identified sources to find additional World Wide Web links that contained data relevant to antimicrobial resistance.

We chose to include in our lists dependable English-language web pages, which we categorized into three groups: those that presented antimicrobial resistance data from major international networks, those that presented antimicrobial resistance data from major national networks, and those that provided links to other international surveillance organizations/associations that study antimicrobial resistance. Regarding the first group of web pages, the web pages that were finally presented in our assessment were selected from a very extensive catalogue, by the criterion of providing international surveillance data (more than two countries involved). In the second group, we included representative major national networks websites. For both groups strong selection criteria were comprehensive and evidence-based information, as well as ease of access to that information. In the third group, we included websites providing links to the most commonly visited web pages by infection experts.

Although we managed, through our gathering strategy, to review most of the major international and national networks' websites/pages, it is inevitable that some were overlooked, while for some others, the authors decided that did not fulfill the criteria to be enlisted.

3 Internet Resources on Antimicrobial Resistance from Major International Networks

In Table 91.1, we listed 15 web pages/sites of 11 major international networks that present data of antimicrobial resistance, either as interactive database or as reports of international antimicrobial resistance surveillance systems. We accessed each of the web addresses and verified that they contain data from surveillance studies on antimicrobial resistance.

4 Internet Resources on Antimicrobial Resistance from Major National Networks

In Table 91.2, a catalogue of 15 representative major national networks web pages, which present data of drug-resistant microorganisms either in the form of interactive databases or as annual surveillance reports, is shown. We can verify that the web addresses presented in the table are easily accessible and contain comprehensive and valuable antimicrobial resistance information.

5 Internet Links on Antimicrobial Resistance from Major Networks

In Table 91.3, we present seven major networks' web pages/sites providing numerous of valuable web links to international organisms/associations that conduct research on antimicrobial resistance and/or suggest guidelines for infection control as well as for prudent use of antibiotics. We accessed each of the links included in this table and verified that they contain information relevant to the field of antimicrobial resistance.

6 Limitations in the Selection of Relevant Internet Resources

The goal of our effort was to provide to clinicians and investigators immediate access to a collection of World Wide Web resources that include updated information regarding the antimicrobial resistance patterns of clinical isolates from patients of various parts of the world. We acknowledge that the lists we present are far from exhaustive. Rather, they should be regarded as a subset of relevant World Wide Web resources that include readily available information on antimicrobial resistance.

We need to highlight the significance of the numerous national antimicrobial resistance surveillance projects that are monitoring the resistance pattern of clinical isolates from patients, within the borders of each country. The investigators related to some of these projects report their national level data in scientific publications. In addition, a minority of data related to these efforts is included in regional websites. Although the presentation of each and every one of the various websites of the national antimicrobial surveillance networks of each country would be valuable, it was considered to be out of the scope of this project.

Table 91.1 Summary of major international networks web pages/sites presenting data of antimicrobial resistance

Title/subject	Web address	Contents/objective	Source
Drug resistance	http://www.who.int/drugresistance/en/	Antimicrobial Resistance: Global Report on Surveillance (latest issue 2014). Information on *E. coli*, *K. pneumoniae*, *S. aureus*, *S. pneumoniae*, *Non-typhoidal Salmonella* spp., *Shigella* spp., *N. gonorrhoeae*, *TBC*, *Malaria*, *HIV*, *Influenza*, *Invasive Candidiasis.*	World Health Organization (WHO)
European Antimicrobial Resistance Surveillance Network (EARS-Net)	http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/Pages/index.aspx	Interactive database and Surveillance Reports (latest issue 2012). Information on *E. coli*, *E. faecalis*, *E. faecium*, *K. pneomoniae*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae*.	European Centre for Disease Prevention and Control (ECDC)
Antibiotic/antimicrobial resistance	http://www.cdc.gov/drugresistance/index.html	Drug resistance, surveillance systems and educational projects.	Centers for Disease Control and prevention (CDC)
Healthcare-associated Infections (HAI)	http://www.cdc.gov/hai	Drug-resistant organisms, prevention and control, campaigns, lab practices.	CDC
National Antimicrobial Resistance Monitoring System (NARMS) for enteric bacteria	http://www.cdc.gov/narms/	Antimicrobial Resistance among enteric bacterial isolates from humans. Interactive database and reports (latest issue 2013).	CDC for NARMS
NARMS	http://www.fda.gov/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/ucm059089.htm	Antimicrobial resistance among enteric bacterial isolates from retail meat. Publications and reports (latest issue 2011).	Food and Drug Administration (FDA) for NARMS
Reservoirs Of Antibiotic Resistance (ROAR) network	http://www.roarproject.org/	Registration required for database access. ROAR publications available.	Alliance for the Prudent use of Antibiotics (APUA)
European Surveillance of Antimicrobial Consumption Network (ESAC-Net)	http://www.ecdc.europa.eu/en/activities/surveillance/esac-net/pages/index.aspx	Among others, interesting interactive database on antibiotic consumption in the European countries.	ECDC
Gonorrhoea Antimicrobial Resistance Programme (GASP)	http://www.wpro.who.int/hiv/topics/gasp/en/	Information on antimicrobial resistance of *N. gonorrhoeae* in countries of the western Pacific region.	WHO
European Surveillance of Antimicrobial Resistance (ESAR)	http://www.esbic.de/esbic/ind_esar.htm	The web site provides results of overall resistance. (Has not been updated since 1999)	European Society of Clinical Microbiology and Infectious Diseases (ESCMID)
Asian Network for the Surveillance Of Resistant Pathogens (ANSORP)	http://www.ansorp.org/	Publications regarding antibiotic resistance provided online.	Asia Pacific Foundation for Infectious Diseases (APFID)
Resistance surveillance website	http://www.bsacsurv.org/	Interactive database containing information on antimicrobial resistance for respiratory tract infections as well bacteremias.	British Society for Antimicrobial Chemotherapy—Data from UK and Ireland (BSAC)
ProMED-mail	http://www.promedmail.org/?p=2400:1000	The global electronic reporting system for outbreaks of emerging infectious diseases and toxins, open to all sources.	International Society for Infectious Diseases (ISID)
Global Antibiotic Resistance Partnership (GARP)	http://www.cddep.org/map	Interactive database containing information on antimicrobial resistance of multiple pathogens.	The Center for Disease Dynamics, Economics and Policy (CDDEP)—Bill and Melinda Gates Foundation
Antimicrobial Resistance Management (ARM) Program	http://www.armprogram.com/TrendCrystalReport.aspx?Region=National&OrganismID=1	Interactive database containing information on antimicrobial resistance of *E. faecalis*, *E. faecium*, *S. aureus*, *S. pneumoniae*, *E. coli*, *H. influenzae*, *P. mirabilis*, *P. aeruginosa.*	University of Florida —Data from USA

All Internet addresses last assessed in September 2014

Table 91.2 Summary of representative major national networks web pages/sites presenting data of antimicrobial resistance

Country	Title/subject	Web page address	Source of information	Contents
Australia	*Communicable Diseases Intelligence* (CDI) journal	http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-pubs-cdi-cdiintro.htm	Australian Government. Department of Health.	Electronic journal containing reports on surveillance of communicable diseases in Australia.
Canada	Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)	http://www.phac-aspc.gc.ca/cipars-picra/pubs-eng.php	Government of Canada. Public Health Agency of Canada.	Provides CIPARS annual reports (latest issue 2011).
Canada	Canadian Antimicrobial Resistance Alliance (CARA)	http://www.can-r.com/	Association of Medical Microbiology and Infectious Disease (AMMI Canada), Canadian Bacterial Surveillance Network (CBSN), University of Manitoba and others.	Interactive database, information on antimicrobial resistance of multiple pathogens.
Denmark	Danish Integrated Antimicrobial resistance Monitoring and Research Program (DANMAP)	http://www.danmap.org/	Statens Serum Institute.	DANMAP annual reports (latest issue 2012).
Great Britain	Resistance Surveillance website	http://www.bsacsurv.org/	British Society for Antimicrobial Chemotherapy (BSAC).	Focuses on respiratory infections and bacteremia.
Greece	The Greek system for surveillance of antimicrobial resistance (WHONET Greece)	http://www.mednet.gr/whonet/	National School of Public Health (NSPH), Hellenic Center for Disease Control and Prevention – Ministry of Health (HCDCP).	Interactive Database.
Japan	Infectious Diseases Surveillance Center (IDSC)	http://idsc.nih.go.jp/index.html	Japanese National Institute of Infectious Diseases (NIID).	Infectious agents surveillance monthly reports.
Netherlands	NethMap	http://www.swab.nl/english	Dutch Working Party on Antibiotic Policy (SWAB), National Institute for Public Health and the Environment (RIVM).	Reports on consumption of antimicrobial agents and antimicrobial resistance. (latest issue 2014).
New Zealand	Public Health Surveillance—Antimicrobial Resistance	https://surv.esr.cri.nz/antimicrobial/antimicrobial_resistance.php	Institute of Environmental Science and Research (ESR), New Zealand Ministry of Health.	Reports on antimicrobial resistance of multiple pathogens.
Norway	NORM Surveillance Program for Antimicrobial Resistance	http://www.vetinst.no/eng/Publications/NORM-NORM-VET-Report	Norwegian Institute of Public Health, University Hospital of North Norway.	Reports on consumption of antimicrobial agents and antimicrobial resistance. (latest issue 2012)/
Philippines	Antimicrobial Resistance Surveillance Program	http://www.ritm.gov.ph/	Research Institute for Tropical Medicine (RITM), Philippines Department of Health.	Reports on antimicrobial resistance of multiple pathogens (latest issue 2013).
Russia	Antimicrobial Resistance in Russia	http://www.antibiotic.ru/index.php?doc=73	Institute of Antimicrobial Chemotherapy (IAC) of the Smolensk State Medical Academy (SSMA), Ministry of Health of Russian Federation, Interregional Association for Clinical Microbiology and Antimicrobial Chemotherapy (IACMAC).	Provides information on resistance of nosocomial and community-acquired pathogens (has not been updated since 2004).
Sweden	Swedish Strategic Program for the Rational use of Antimicrobial Agents and Surveillance of Resistance (STRAMA)	http://en.strama.se/dyn//,92,4.html	Swedish Reference Group for Antibiotics (SRGA), the Medical Products Agency, the National Board of Health and Welfare, the Swedish Institute for Infectious Disease Control (SMI) and others.	Provides surveillance data as well as link to interactive database.
Thailand	National Antimicrobial Resistance Surveillance Center, Thailand (NARST)	http://narst.dmsc.moph.go.th/	National Institute of Health of Thailand.	Reports on antimicrobial resistance of multiple pathogens (latest issue 2014).
USA	CDC Surveillance systems	http://www.cdc.gov/drugresistance/surveillance.html	Centers for Disease Control and Prevention (CDC), Department of health and human services.	Links and publications of US national surveillance systems.

Countries are presented in alphabetical order. All Internet addresses last assessed in September 2014

Table 91.3 Summary of major networks web pages/sites providing valuable web links on antimicrobial resistance

Title/subject	Web address	Contents/objective	Source
Antibiotic/antimicrobial resistance-related links	http://www.cdc.gov/drugresistance/resources.html	Extensive list of links US-national and also international on antimicrobial resistance.	CDC (Centers for Disease Control and Prevention)
Organisms and specific disease issues/links	http://www.who.int/medicines/areas/rational_use/DC_Organism_or_Disease_Specific_Issues/en	Web links containing information classified by disease and pathogen type.	WHO (World Health Organization)
National/international networks on antimicrobial resistance	http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net/external_sites/Pages/external_sites.aspx	Provides links to national European and international networks web pages on antibiotic resistance.	ECDC (European Centre for Disease Prevention and Control)
Antimicrobial resistance	https://www.gov.uk/government/collections/antimicrobial-resistance-amr-information-and-resources#tools-and-resources	Related topics and links.	Public Health England/ Department of Health/UK Government
Nordic Society of Clinical Microbiology and Infectious Diseases (NSCMID)	http://nscmid.org/links	Links to Scandinavian and other international organizations that study antimicrobial resistance.	NSCMID
Related communicable diseases surveillance links	http://www.health.gov.au/internet/main/publishing.nsf/Content/cda-cdilinks.htm	Links to communicable disease surveillance sites.	Australian government, department of health and ageing
ReAct—Reference Library	http://www.reactgroup.org/resource-center/6-reference_library	Links to scientific articles and reviews on resistance data.	ReAct—Action on Antibiotic Resistance

All Internet addresses last assessed in September 2014

We believe that efforts for the continuous update of information of databases reporting the findings of surveillance studies of antimicrobial resistance should be encouraged and supported financially. The toll of infections due to multidrug-resistant pathogens is too high to ignore the significance of various types of studies on antimicrobial resistance.

7 Conclusion

Advances of modern technology including the development of the Internet and the World Wide Web have given the opportunity to clinicians and researches to have immediate access to continuously updated information in various scientific fields. We tried to compile a list of World Wide Web resources of data from surveillance studies on antimicrobial resistance that may be useful to practitioners, especially infectious diseases specialists, as well as to scientists with a research interest in the field of antimicrobial resistance.

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