Ivano de Filippis *Editor*

Molecular Typing in Bacterial Infections, Volume I

Second Edition

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Editor

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Second Edition

Editor
Ivano de Filippis
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Part I

Oral and Respiratory Pathogens

Corynebacterium: Molecular Typing and Pathogenesis of *Corynebacterium diphtheriae* and Zoonotic Diphtheria Toxin-Producing *Corynebacterium* Species

1

Verônica Viana Vieira, Juliana Nunes Ramos,
Louisy Sanches dos Santos,
and Ana Luíza Mattos-Guaraldi

1.1 Introduction

Molecular typing techniques have been successfully applied for determining virulence potential, origin, and routes of diphtheria and atypical invasive infections, confirming endemicity, outbreaks, and trace cross-transmission caused by *Corynebacterium diphtheriae* and zoonotic toxin-producing *Corynebacterium* species.

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1.2 Clinical Significance and Epidemiology of Diphtheria Toxin-Producing *Corynebacterium* spp.

Corynebacterium diphtheriae is a major etiologic agent of classic respiratory diphtheria, including local pharyngeal symptoms and systemic manifestations, mainly caused by the action of diphtheria toxin (DT). Symptoms typically begin 2 to 5 days after infection. *C. diphtheriae* usually localizes in the upper respiratory tract, ulcerates the mucosa, and induces the formation of an inflammatory pseudomembrane. Systemic toxicity increases as the pseudomembrane spreads from the tonsillopharyngeal area. A form of malignant diphtheria is associated with extensive "membranous pharyngitis" plus massive swelling of the tonsils, uvula, cervical lymph nodes, submandibular region, and anterior neck (the so-called bull neck of toxic diphtheria). Acute disease of the respiratory tract usually involves one or more of the following: tonsillar zones, larynx, soft palate, uvula, and nasal cavities or, less commonly, in the stomach or lungs. The exceedingly potent DT is absorbed into the circulation, and lesions may also occur in vital organs, including the heart (myocarditis), nervous system, and kidneys, potentially resulting in death [1].

Diphtheria toxin is an extracellular protein that inhibits protein synthesis and ultimately exerts death of susceptible eukaryotic cells. DT was the first member to be identified of a group of bacterial protein toxins that act by ADP ribosylation of a target protein. DT contains a toxic A subunit (active toxin with enzymatic activity) and the receptor binding B subunit. The B subunit (fragment) facilitates translocation of the A subunit from the phagosome to the cytosol, followed by separation, allowing full activity of the A subunit on its target protein elongation factor-2 (EF-2). EF-2 transfers polypeptidyl-transfer RNA from acceptor to donor sites on the ribosome of the host cell. The A subunit catalyzes the transfer of adenine, ribose, and phosphate from NAD to EF-2 (ADP ribosylation), inactivating EF-2 and turning off inhibiting protein synthesis. DT causes local destruction at the site of membrane formation and may be also absorbed into the bloodstream and distributed, resulting in systemic complications including demyelinating neuritis and myocarditis. The *tox* gene that encodes DT is present in β and ω corynephages, and DT is only produced by *C. diphtheriae* isolates that harbor *tox*$^{+}$ corynephages. Although the *tox* gene is part of the phage genome, the regulation of DT expression is under bacterial control, as the corresponding iron-sensing regulator DtxR is encoded by a gene on the *C. diphtheriae* chromosome [2–4].

Cutaneous diphtheria is the most common nonrespiratory clinical manifestation of infection due to *C. diphtheriae* strains. The disease is characterized by the presence of shallow skin ulcers, usually chronic, which can occur anywhere on the body, mostly on the legs, feet, and hands. This type of diphtheria may cause pain, redness, and swelling similar to other bacterial cutaneous infections. Cutaneous diphtheria is likely to be diagnosed less quickly than respiratory infection due to the nonspecific clinical appearance and often coinfections with pathogens, mostly *Staphylococcus aureus* and *Streptococcus pyogenes.* Cutaneous diphtheria frequently occurs in warm tropical climates and is normally associated with colonization of preexisting

skin lesions, including surgical wounds, burns, impetigo, psoriasis, leishmaniotic ulcers, and insect bites [5, 6].

In the United Kingdom, cases of travel-related cutaneous diphtheria were reported, including patients with high diphtheria vaccination coverage and from tropical countries. The authors emphasized that with increasing travel to and from diphtheria-endemic countries, more cases may occur. These lesions are an important reservoir of infection and can cause respiratory and cutaneous infections in contacts as well as outbreaks. In several outbreaks, secondary transmission has been higher in contacts of patients with cutaneous infection than in those with respiratory tract infection. Cutaneous diphtheria may also cause greater environmental contamination, through dust and fomites. The potential for secondary transmission leads to a large number of contacts requiring follow-up, especially children at school [6]. Therefore, awareness of clinicians and microbiologists of the importance of obtaining swab specimens from any chronic nonhealing skin lesions in patients who have traveled to or a disease-endemic area is necessary, especially in tropical countries. Wound swab samples from these patients should be examined for diphtheria toxin-producing *Corynebacterium* species. Early diagnoses and reporting are crucial to trigger effective public health control measures (skin ulcers, which can occur anywhere on the body and are usually chronic) [5–8].

In many countries, diphtheria is considered an infrequent disease once there are treatment and diphtheria toxoid-containing vaccines to prevent it. Since 1990, diphtheria reemerged in the Russian Federation and spread to all Newly Independent States (NIS) and Baltic states. Awareness of characteristics of the largest diphtheria epidemic in the last decades that seized several European countries should be used to help predict the spread of future epidemics. The epidemic demonstrated conclusively the potential susceptibility of adults to diphtheria in the vaccine era. Important characteristics included, among several other factors, the emergence of distinct epidemic clonal group, a progressive spread of disease from urban centers to rural areas. However, epidemic diphtheria outbreaks remain poorly understood and continues to challenge industrialized and developing countries. Higher risk of acquiring *C. diphtheriae* infections and potentially life-threatening complications may be possible related to inadequately immunized or unimmunized conditions of persons and travelling from/to countries with endemic diphtheria [5, 9–12].

Although immunization is one of the most successful and cost-effective health interventions known, there are still many regions of the world with low vaccine coverage. Diphtheria caused by *C. diphtheriae* is still endemic worldwide, mostly among developing countries, including Nigeria, Venezuela, India, and Brazil. Diphtheria resurgence or epidemic outbreaks remain an important cause of morbidity and mortality that may occur in places where vaccination programs are not maintained or there is a proportion of adults susceptible to disease due to decline in antibody levels provided by vaccine, especially in low socioeconomic and health conditions areas (Fig. 1.1) [14–21]. Previous investigations reported an epidemic outbreak in Dhule, a predominantly tribal and rural district in Northern Maharashtra, India, with diphtheria cases mostly observed among adolescents (10–15 years), despite poor immunization coverage (below 50%) [17]. A diphtheria outbreak was

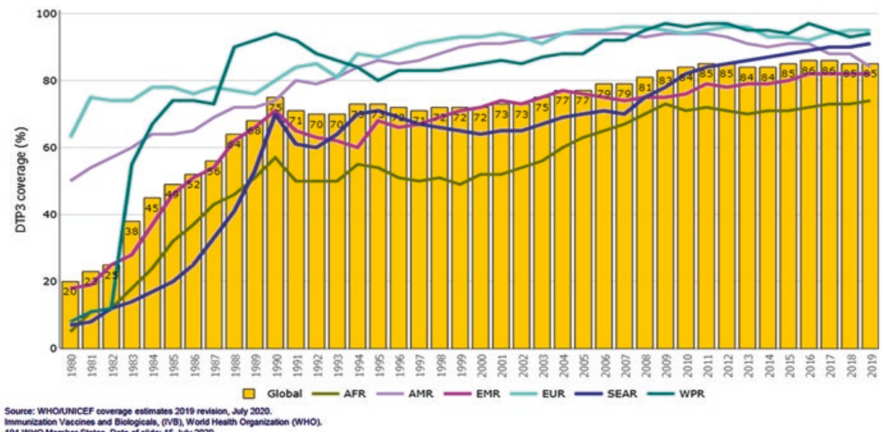

Fig. 1.1 Diphtheria global immunization 1980–2019. Global coverage from three doses of a diphtheria toxoid-containing vaccine (DTP) at 85% in 2019 [13]

also verified in villages of three municipalities of Maranhão, a northern state from Brazil. Most cases occurred in partially or completely immunized patients, including pharyngitis without pseudomembrane formation [22].

Clinical features of diphtheria in partially vaccinated patients may still be similar to those that were observed in the pre-vaccine era. However, mass vaccination has also altered clinical features of some diphtheria cases, independent of immunization status and age of individuals. Therefore, health professionals should be aware of the possibility of atypical cases of DT-producing *C. diphtheriae* infections, including pharyngitis without pseudomembrane formation. Cases of coincidental respiratory diphtheria with infectious mononucleosis were also reported [5, 17, 22, 23].

C. diphtheriae has been increasingly reported not only as the etiological agent of diphtheria but also as the causative agent of atypical invasive infections. *C. diphtheriae* was originally characterized as an extracellular pathogen with local growth pharynx mucosa. During the last decades, cases of atypical and/or invasive infections caused by both non-DT-producing and DT-producing *C. diphtheriae* strains have been reported, such as pneumonia, arthritis, endocarditis, bacteremia, and catheter-related infections, including cancer patients, leading patients to death in varied opportunities independent of age and sex. Septicemia, renal failure, and/or arthritis are frequently reported in patients with *C. diphtheriae* endocarditis [24–30].

C. diphtheriae is usually transmitted by respiratory droplets, direct contact, and fomites when individuals are at home or during occupational activities, especially in laboratory and hospital environments. In 1941, during the pre-vaccine era, the occurrence and persistence of diphtheria bacilli in floor dust of hospital wards and the resultant contamination of the air were verified. DT-producing *C. diphtheriae* strains were found capable to survive in dust and clothing for an extended period of time [31]. Once considered a strictly human pathogen, *C. diphtheriae* strains have been found to be able to infect animals, including cows, horses, and cats [32–35].

Changes of varied aspects in the epidemiology of diphtheria pathogens have been occurring worldwide. Starting in the middle of the 1980s, DT-producing and non-DT-producing *Corynebacterium ulcerans* have been increasingly identified as the etiologic agent of diphtheria of zoonotic nature and extrapharyngeal infections in varied industrialized and developing countries, including immunized individuals in the American continent [8, 12, 36–40]. Similar to *C. diphtheriae* strains, *C. ulcerans* strains were found to produce clinical syndromes of the lower respiratory tract, such as pneumonia and pulmonary granulomatous nodules, independent of DT production [36, 41–44]. At first, zoonotic diphtheria cases were mainly restricted to rural populations and associated with contact with dairy cattle and consumption of unpasteurized dairy products. Lately, *C. ulcerans* has been increasingly isolated as emerging zoonotic agent from companion animals such as cats and dogs. Therefore, there is a potentially large reservoir of infection with little knowledge about the risks of zoonotic transmission, since *C. ulcerans* strains were already found among animals from farms, domestic and natural settings [8, 37–39, 45].

Since the epidemic in European countries during the 1990s, the number of diphtheria cases due to *C. ulcerans* was found to exceed the number of reported cases related to *C. diphtheriae* [45]. Although *C. ulcerans* have been increasingly recognized in several industrialized countries as an emerging zoonotic pathogen, its capacity to cause disease in humans, including among the inhabitants of urban centers, is still often neglected [38]. Detection of *C. ulcerans* strains in Canada, during the period of 2006–2019, showed that 77% of the isolates were from humans and mostly obtained from cutaneous sites and 23% were from animals – mink (lung), dog (ear), and cat and horse (abscess and skin) – comprising 45% DT-producing strains [39]. In Brazil, a case of concurrent zoonotic diphtheria by *C. ulcerans* and infectious mononucleosis (IM) was first reported in the literature [46]. Moreover, a case of fatal pulmonary disease caused by a unusual penicillin and clindamycin resistant non-DT-producing *C. ulcerans* disseminated from primary nonhealing lesions on lower legs was documented. Both legs of the elderly patient with *C. ulcerans*-invasive infection presented skin ulcers covered by yellowish membranes [36]. Previous studies also reported the isolation of *C. ulcerans* strains from nares and/or skin wounds of asymptomatic dogs (companion dogs or kept in animal shelter), from Duque de Caxias and Niterói cities, located at the metropolitan area of Rio de Janeiro [37, 47].

Similar to *C. ulcerans*, *Corynebacterium pseudotuberculosis* is also a zoonotic etiologic agent and also considered a public health concern. *C. pseudotuberculosis* is a diphtheria toxin-producing pathogen of medical, veterinary, and biotechnological interest that mainly affects small ruminants, causing caseous lymphadenitis (CLA), throughout the world and generates significant economic losses. Sheep and goats are the most common animals infected within the broad spectrum of hosts in which *C. pseudotuberculosis* causes clinical disease. This zoonotic pathogen may also infect bovines, pigs, and equines. Therefore, contamination of meat and milk by *C. pseudotuberculosis* may possibly occur, putting children and adult consumers at risk. However, human infections due to *C. pseudotuberculosis* remain apparently

rare and have been mostly reported among those with close contact to animals, including farm workers and travelers to rural areas [48–50].

In many countries, routine procedures for identification of DT-producing *Corynebacterium* spp. are uncommonly undertaken by diagnostic laboratories due to a low prevalence of diphtheria cases alongside the difficulties of diagnosis through conventional biochemical tests and the ever-increasing need for cost-effectiveness. This fact contributes to justify the low number of reported cases of human infections by zoonotic *C. pseudotuberculosis* strains over the years [49].

In a previous investigation of zoonotic potential of *C. pseudotuberculosis*, a summary data from all 33 cases of human infections reported over a period of 42 years (from 1966 to 2008) showed that a main group of non-DT-producing *C. pseudotuberculosis* strain-infected patients presented a characteristic of lymphadenopathy. Profiles of most of these patients included adult males, 21–40 years old, and previously exposed to raw milk or meat, farm animals (mostly sheep), and/or rural areas. Only two cases involved clinical presentations other than the characteristic lymphadenopathy: from the United States, a 28-year-old (male) veterinary student who worked with equines, diagnosed with eosinophilic pneumonia, and from China, a 63-year-old (male) with ocular infection post-retinal reattachment intervention. Until the present moment, only one case of human infection due to DT-producing *C. pseudotuberculosis* was reported. The zoonotic pathogen was isolated in the United Kingdom from the aortic root vegetation of an intravenous drug user with endocarditis [50–53].

1.3 Treatment and Prevention of DT-Producing *Corynebacterium* spp. Infections

Antibiotics are needed to kill DT-producing *Corynebacterium* spp., eliminate diphtheria toxin production, limit carriage that may persist even after clinical recovery, and prevent further transmission from asymptomatic carriers and colonization of close contacts. Penicillin and erythromycin have long been the drugs of choice for the eradication of DT-producing strains of *Corynebacterium* spp. The World Health Organization (WHO) has recently added azithromycin as part of the standard antibiotics for these pathogens. However, the increasing problems of resistance to penicillin, oxacillin, erythromycin, and other drugs including rifampicin, tetracycline, and clindamycin are examples of challenges confronting both industrialized and developing countries. Resistance to ß-lactams should also be considered in invasive infections, since failure to eliminate *C. diphtheriae* in cases of endocarditis treated with penicillin has been reported. Data emphasize the need for a continuous survey of antibiotic susceptibility for these pathogens, especially in tropical and developing countries where diphtheria is endemic and invasive infections may occur [20, 22, 28, 46, 54–57].

In cases of classic and zoonotic diphtheria, patients with severe infections should be immediately admitted to a hospital *intensive care unit* and given diphtheria *antitoxin (DAT)*, consisting of *antibodies* isolated from the *serum* of horses that have

been challenged with diphtheria toxin. Since antitoxin does not neutralize toxin that is already bound to tissues, delaying its administration increases risk of death. Therefore, the decision to administer diphtheria antitoxin is based on clinical diagnosis, and should not await laboratory diagnosis [20]. Administration of diphtheria vaccine is recommended during convalescence because diphtheria infection does not always confer immunity [53].

In the early 1880s, *C. diphtheriae* was first visualized in stained specimens from pseudomembranes and shown to be the cause of diphtheria isolated by bacteriologists Edwin Klebs and Friedrich Loeffler. In 1890, Emil von Behring isolated the first diphtheria antitoxin from blood samples of an infected horse. A few years later, William H. Park and Anna W. Williams isolated a *C. diphtheriae* strain that produced an unusually large amount of diphtheria toxin, later named the Park-Williams no. 8 (PW8) strain. Since the 1920s, a diphtheria toxoid vaccine has been produced from diphtheria toxin treated with formalin to inactivate the toxicity and to maintain the immunogenicity of the protein. *C. diphtheriae* PW8 is lysogenized by two copies of corynephage ω^{tox+}, suggesting that the enhanced DT synthesis is due to a gene dosage effect of the *tox* gene [3].

Protection against diphtheria is mainly due to the development of neutralizing toxin antibodies. Diphtheria antitoxin production, primarily of IgG type, can be induced by absorption of native toxin during clinical infection or in the carrier state or by immunization with diphtheria toxoid. It is believed that a circulating diphtheria antitoxin level of 0.01 IU/ml, as determined by the neutralization test in animals or in cell culture, provides clinical immunity against disease. The outcome of revaccination of adults depends on several factors, including the immunization schedule, potency, and time since the last dose of toxoid [58, 59]. In developing countries where diphtheria is endemic, the process of maintaining immunity usually operates through natural mechanisms, including frequent skin infections caused by *C. diphtheriae*. Nowadays, adults might become susceptible to diphtheria due to reduced opportunities of subclinical infections. Since diphtheria infection may also occur among previously vaccinated persons, the immunity gap observed among adults should be closed by regular diphtheria boosters [9, 60–62].

1.4 Identification of DT-Producing *Corynebacterium* spp. in Diagnostic Laboratories

Accurate and fast diphtheria laboratory diagnosis is not only a matter of acute patient management but also an important issue in public health due to international notification and management requirements, and there is an urgent need for a reliable, robust, and fast laboratory method for diagnosing DT-producing *Corynebacterium* spp., especially in the light of the continuing loss of laboratory expertise even in national reference laboratories for diphtheria [5, 7, 8, 22, 63].

Phenotypic characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* may be performed by conventional biochemical assays and semiautomatized systems, including API Coryne System (bioMérieux). However, it takes at

least 16 hours after isolation of suspicious colonies from screening plates of catalase-positive irregular Gram-positive bacilli (IGPB) [7, 22, 29, 36, 64].

In many countries, routine procedures for identification of *C. diphtheriae* and *C. ulcerans* are not commonly undertaken by diagnostic laboratories due to a low prevalence of diphtheria cases in recent years alongside the ever-increasing need for cost-effectiveness. Consequently, many diagnostic laboratories have suspended screening for diphtheria etiologic agents, further increasing the potential for missed and delayed diagnoses. Therefore, screening tests remain currently essential for the presumptive identification of these pathogens in clinical microbiology laboratories [5, 8, 22, 28, 65].

The use of DNase screening test provided a substantial improvement in the existing standard identification algorithm of DT-producing *Corynebacterium* spp. of routine diagnostic laboratories. DNase assays have been useful for differentiating DNAse-positive *C. diphtheriae* and *C. ulcerans* from DNAse-negative *C. pseudotuberculosis* and other suspected pathogenic corynebacteria, particularly in the surveillance of cases of diphtheria, asymptomatic carriers, and invasive infections in endemic or epidemic areas with unfavorable economic conditions [64]. The reverse CAMP test is particularly a screening assay effectively used as part of the identification of *C. ulcerans* and *C. pseudotuberculosis* zoonotic pathogens. A reverse CAMP test is based on the inhibition of hemolytic activity of beta-hemolysin from *S. aureus* through the production of phospholipase D by *C. ulcerans* and *C. pseudotuberculosis* [36].

The application of molecular techniques for the identification of bacterial pathogens has been expanded for use in clinical microbiology laboratories. Molecular procedures have been also proposed for the identification of *Corynebacterium* species. Improvements should become widely available for the rapid and precise detection of DT-producing *Corynebacterium* spp., including direct analysis of swabs and other clinical samples, as already done with *C. diphtheriae* and *C. ulcerans* in some laboratories [66–68].

1.5 MALDI-TOF Assays

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is one of the most recently established technologies used for species identification based on the protein composition of microbial cells. MALDI-TOF MS has been increasingly applied worldwide in routine analysis of clinical microbiology laboratories due to easy procedure, rapid results (15 minutes), and accurate identification of several bacterial species, including misidentified pathogens in specific clinical specimens. MALDI-TOF MS became a powerful tool that has initiated a revolution in the clinical microbiology laboratory for identification of nosocomial pathogens, including *Corynebacterium* spp. Misidentification of some human-pathogenic clinical isolates commonly occurs when using conventional and commercially available methods in microbiology laboratories. The ability to rapidly identify bacterial species, including rarely described as pathogens in specific

clinical specimens, may help to study the clinical burden resulting from the emergence of these species as human pathogens and MALDI-TOF MS may be considered an alternative to DNA-based methods in clinical laboratories. Due to the difficulties of diagnosis in the laboratory routine through conventional biochemical tests, MALDI-TOF MS also represents an important alternative method for the identification of *C. diphtheriae*, *C. ulcerans*, and *C pseudotuberculosis* strains [39, 46, 69–71].

1.6 DNA-Based Methods

The timely and precise diagnosis of corynebacterial infections, especially those involving DT-producing strains, is indispensable for the patient management and for establishment of surveillance and control strategy of the disease. Consequently, different molecular methods, such as end-point and real-time PCR (polymerase chain reaction), have been used since the 1990s for the characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* [7, 49, 66, 68, 72–78]. Since the investigation of the toxigenic potential of clinical isolates is one of the most critical aspects of diphtheria diagnosis, conventional end-point PCR assays targeting the *tox* gene were the first to be developed. Subsequently, the *tox* gene detection was combined with species identification PCR targets, as *dtx*R (*d*iphtheria *t*oxin *r*epressor gene) from *C. diphtheriae*, in multiplex assays [7, 49, 66, 68, 72, 73]. Since detection of the *tox* gene only provides presumption of toxigenicity, additional phenotypic investigations such as Elek test and Vero cell cytotoxicity assays have been currently used to demonstrate DT production by *Corynebacterium* strains [7, 66, 74].

Multiplex PCR represents a fast, simple, and reliable methodology for identification and differentiation between DT-producing and non-DT-producing strains of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis*. In Brazil, an mPCR protocol was developed, and it has been used for clinical diagnosis and epidemiological and virulence research, during the last decade. Direct analyses of swabs and other clinical samples have also been done. Brazilian mPCR allows the detection of *tox* gene from potentially DT-producing *Corynebacterium* spp., in addition to 16S rRNA from both *C. pseudotuberculosis* and *C. ulcerans*, *pld* from *C. pseudotuberculosis*, *dtx*R from *C. diphtheriae*, and *rpo*B from *Corynebacterium* spp. [22, 27, 37, 46, 49].

Real-time PCR instruments are increasingly common in public health laboratories, mostly in industrialized countries. Real-time PCR (qPCR for quantitative PCR) presents some advantages than classical PCR, including faster data collection, low contamination risks, and high sensitivity, especially for pathogen detection in host carriers and clinical samples which often contain components that inhibit PCR [69, 79].

During the last decades, different qPCR assays have been developed for detection of the *tox* gene and/or identification of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* directly from clinical samples. However, similarly to end-point PCR,

currently available qPCR assays allow only the detection of *tox* gene from *Corynebacterium* spp. strains. However, the confirmation of DT expression still requires phenotypic investigations [66, 69, 75, 80, 81].

1.7 rpoB Gene Sequencing Technique

The genus *Corynebacterium* is a heterogeneous group of species comprising human and animal pathogens and environmental bacteria. It is defined on the basis of several phenotypic characters and the results of DNA-DNA relatedness and, more recently, 16S rRNA gene sequencing. The *rpoB* gene, encoding the beta-subunit of RNA polymerase, has emerged as a core gene candidate for phylogenetic analyses and identification of corynebacteria, especially when studying closely related isolates. However, the 16S rRNA gene is not polymorphic enough to ensure reliable phylogenetic studies and needs to be completely sequenced for accurate identification. Previous studies verified that higher proportions (91%) of corynebacterial isolates were positively identified by partial *rpoB* gene determination than by that based on 16S rRNA gene sequences [82–84].

1.8 Diphtheria Toxin-Producing Group Becoming Diverse: A Novel *C. diphtheriae* Complex

C. diphtheriae was historically classified into four biovars – *gravis*, *mitis*, *intermedius*, and *belfanti* – based on biochemical phenotypic testing [4, 85]. Recent investigations documented *C. diphtheriae* to be genetically heterogeneous and that genomics does not support the use of biovars to reliably classify diphtheria bacilli isolates, since *C. diphtheriae* strains within a certain biovar were found to be genetically more distant than between biovars [3, 86, 87].

A switch in populations causing endemic infections from DT-producing to non-DT-producing isolates in the 1990s and the 2000s and other countries with vaccination coverage has been documented as a direct consequence of the large-scale use of diphtheria toxoid. In Brazil, several *C. diphtheriae* isolates were found capable of degrading sucrose, a phenotypic characteristic rarely described in other parts of the world. Results of *rpo*B sequence analysis confirmed all sucrose-fermenting isolates as *C. diphtheriae* species. Sucrose-fermenting and DT-producing *C. diphtheriae* strains were predominantly isolated from human respiratory tract of diphtheria patients. However, cases of endocarditis due to sucrose-fermenting *C. diphtheriae* phenotypes were also observed in South American countries [28, 88, 89].

During the 1980s and 1990s, studies dealing with *C. diphtheriae* biovar belfanti were occasionally reported in literature. *C. diphtheriae* biovar *belfanti* were mostly isolated from human respiratory samples. In Brazil, the case of pulmonary infection in a cancer patient was reported [90]. The *tox* gene, which codes for diphtheria toxin, was infrequently reported in isolates of biovar *belfanti* [7, 90–93].

Taxonomic status of *C. diphtheriae* biovars has been increasingly investigated [91, 93, 94]. Phylogenic analysis described two lineages of non-DT-producing *C. diphtheriae* biovar belfanti obtained from 18 countries, covering a time period from 1957 to 2006 [91]. In France, the number of non-DT-producing *C. diphtheriae* biovar *belfanti* increased between 1977 and 2011, and it is the most frequent biovar recovered in recent years. Non-DT-producing belfanti isolates were mostly isolated from human respiratory samples, including from a woman presenting with rhinitis. However, there were also found *belfanti* phenotypes isolated from blood, skin, and bone lesions. Phylogenic analyses of French non-DT-producing *C. diphtheriae* biovar *belfanti* human isolates were distributed among three distinct lineages. Almost all belfanti isolates belonged to a single clonal complex. A third new lineage was composed of a single clinical isolate (rhinitis) and phylogenetically distant from other two partially studied belfanti lineages from France [91, 94].

Recent investigations by genomic sequencing, biochemical, and chemotaxonomic analyses indicated that *C. diphtheriae* biovar belfanti represents a branch that is clearly demarcated from *C. diphtheriae* biovar *mitis* and *gravis*. Data, including the inability to reduce nitrate, allowed to differentiate biovar *belfanti* from other *C. diphtheriae* strains. Consequently, the name *Corynebacterium belfantii* sp. nov. for the group of nitrate-negative strains, previously considered as *C. diphtheriae* biovar *belfanti*, was recently reported [95].

In a further study, it was proposed that *C. diphtheriae* taxon should be subdivided into two subspecies, *C. diphtheriae* subsp. *diphtheriae* and nitrate-negative *C. diphtheriae* subsp. *lausannense* [93]. However, given that *C. belfantii* was validly published a few months before the taxonomic proposal *C. diphtheriae* subsp. *lausannense* was validated, the latter subspecies was suggested to be a heterotypic synonym of *C. belfantii* [92].

Most recently, a group of clinical isolates previously identified as *C. diphtheriae* biovar *belfanti* strains isolated from human cutaneous or peritoneum infections and from one dog were characterized by genomic sequencing, biochemical analysis, and MALDI-TOF assays as *Corynebacterium rouxii* sp. nov. for the novel group. Phenotyping data revealed an atypical negative or heterogeneous intermediate maltose fermentation reaction for both human and animal isolates. Atypical, maltose-negative, and DT-negative *C. diphtheriae* biovar *belfanti* isolated from domestic cats, including with severe otitis, was also previously described in the United States [33, 92].

C. pseudotuberculosis are classified into biovars *equi* and *ovis* based on the ability to convert nitrate to nitrite, due to genetic characteristic that includes the presence of the nitrate reduction operon: *equi*, nitrate-positive strains, and *ovis*, nitrate-negative strains. Disease caused by *C. pseudotuberculosis* biovars has different clinical manifestations in the susceptible hosts, and biovar identification is important for understanding the epidemiology of infection and consequently for disease control. *C. pseudotuberculosis* biovar *equi* strains are etiologic agents of ulcerative lymphangitis in horses, cows, camels, buffaloes, and occasionally humans. *C. pseudotuberculosis* biovar *ovis* strains are the causative agents of caseous lymphadenitis (CLA) in small ruminants, mostly ovine and caprine herds. CLA

causes important economic losses in ovine and caprine herds by reducing wool, meat, and milk production. Cases of human infections due to non-DT-producing *C. pseudotuberculosis* biovar *ovis* were more frequently described worldwide than cases by biovar *equi*. Lymphadenitis in human hosts due to *C. pseudotuberculosis* was also reported in literature, mostly occurring in those who were visitors or were occupationally exposed to animals in rural areas, especially sheep farms [78, 96, 97].

Only one case of DT-producing *C. pseudotuberculosis* was described in literature. The zoonotic pathogen was isolated from the aortic root vegetation of an intravenous drug user with endocarditis; this patient had no history of animal contact, and no possible source of infection was identified. This isolation occurred in the United Kingdom and biovar was not reported [53].

1.9 Pathogenomics of Potentially DT-Producing *Corynebacterium* spp.

Diphtheria toxin is one of the best investigated bacterial toxins and a leading virulence factor of toxigenic *C. diphtheriae* and *C. ulcerans* strains. Different investigations demonstrated that *tox* genes of *C. diphtheriae* strains showed similar nucleotide sequence identity. Phylogenetic analyses of *C. ulcerans* revealed diverse diphtheria toxin suggesting that *C. ulcerans* tends to acquire mutations more frequently than *C. diphtheriae*. Two possible explanations for this phenomenon are that *C. ulcerans* strains are maintained by various animals and have a phage-independent pathway to acquire the DT-encoding gene, increasing its diversity compared with *C. diphtheriae* [2, 3, 98, 99].

The diphtheria toxin repressor DtxR is known as an iron-dependent regulator that controls the transcription of the diphtheria toxin gene *tox* and a complex gene regulatory network involved in iron homeostasis. Variations of *dtx*R genes and in the regulatory network of DtxR might lead to differences in iron supply of the bacterial cell, thereby influencing the expression of the *tox* gene and the virulence of DT-producing *Corynebacterium* spp. [3, 100, 101]. Therefore, naturally occurring diversity of *tox* genes and variations on the expression of diphtheria toxin due to *dtx*R regulatory activities may exert influence on efficacy of diphtheria toxoid vaccine and diphtheria antitoxin for preventing and treating infections caused by DT-producing *Corynebacterium* spp. pathogens [3, 5, 99, 100].

The occurrence of diphtheria among immunized persons, as well as the increasing frequency of cases of atypical and invasive diseases, caused by non-DT-invasive clones also points the relevance of multiple virulence factors of the potentially DT-producing *Corynebacterium* spp. [26, 30, 37, 102–105]. In previously reported cases of invasive infections, non-DT-producing *C. diphtheriae* and *C. ulcerans* strains were found capable of expressing additional proteins with cytotoxic effects similar to Shiga-like toxins, characterized as ribosome-binding proteins (Rbps). Experimental evidence for the cytotoxic function of Rbps toxins were provided by the interaction of *C. diphtheriae* and *C. ulcerans* wild-type, mutant, and complementation as well as overexpression strains with invertebrate model systems,

Caenorhabditis elegans and *Galleria mellonella*, and on various animal and human macrophage and epithelial cell lines, including Vero cells [28, 36, 105, 106].

The dermonecrotic phospholipase D (PLD) exotoxin may be also produced by both zoonotic pathogens that have been investigated as a prominent virulence factor, especially for *C. pseudotuberculosis*. The *pld* gene encoding the phospholipase D is included among the subset of genes homologous for *C. pseudotuberculosis* and *C. ulcerans* species. Studies with *C. pseudotuberculosis* strains with inactivated PLD have convincingly demonstrated the necessity of PLD for establishment of diseases in animals, including caseous lymphadenitis [8, 46, 98, 107, 108]. However, a case of diphtheria due to *C. ulcerans* strain that is unable to express both PLD and DT activities was recently reported. These data emphasize that virulence mechanisms and pathogenic potential of *C. ulcerans* species may arise independent of PLD and DT production. *C. ulcerans* virulence potential and zoonotic pathogenicity traits need further investigation [36–38, 46, 98, 104, 105, 109].

Basic mechanisms and specific virulence determinants, other than DT, involved in the pathogenic potential of *C. diphtheriae* have been investigated for almost half a century [110]. Since 2003, data from whole-genome sequencing (WGS) of *C. diphtheriae*, including a pangenomic study with Brazilian clinical isolates from cases of classical diphtheria, endocarditis, and pneumonia, involving sucrose-fermenting *C. diphtheriae* strains, exposed horizontal gene transfer of virulence factors, such as adhesins, fimbrial proteins, and iron uptake systems [3, 111].

In an attempt to further investigate mechanisms that promote *C. diphtheriae* survival within different environmental conditions, infection and dissemination through host tissues, several features have been of concern [112–114]. A putative determinant (CDCE8392_813 gene), coding for tellurite ($TeO3^{2-}$) –resistance (TeR) was detected, and the influence on virulence attributes of *C. diphtheriae* strains was verified. Tellurium (Te) is a metalloid that exists as a trace component in natural environments. Although $TeO3^{2-}$ is toxic to most microorganisms, TeR bacteria, including *C. diphtheriae*, exist in nature. The presence of TeR determinants in pathogenic bacteria might provide selective advantages in the natural environment. The *C. diphtheriae* TeR-disrupted mutant strain expressed increased susceptibility to $TeO3^{2-}$ and reactive oxygen species (hydrogen peroxide) but not to other antimicrobial agents. Moreover, TeR determinants contributed to the survival of *C. diphtheriae* strains by using in vivo and in vitro models of infection [115].

The ability of biofilm formation on varied biotic and abiotic surfaces was also investigated. Low-dose antibiotics was reported to favor biofilm formation by *C. diphtheriae*, similar to observations for other human pathogens. *C. diphtheriae* strains expressed higher cell-surface hydrophobicity and biofilm formation on different abiotic surfaces in the presence of penicillin and erythromycin. Moreover, *C. diphtheriae* was also recognized as a potential cause of catheter-related infections, independent of DT production [29, 89, 102, 115–118].

C. diphtheriae was also found to express the ability to invade and survive within different types of human cells and the capacity to cause invasive bloodstream infections. Systemic complications of *C. diphtheriae* bacteremia are not unusual and include endocarditis, joint infections, and peripheral embolic disease. A

strain-dependent ability to induce osteomyelitis by DT-negative *C. diphtheriae* strains was probed by an in vivo assay using Swiss Webster mice, as first reported for *C. ulcerans* [27, 104].

Mechanisms of interaction with different cell types have been also investigated, such as erythrocytes, macrophages, and endothelial and epithelial cells [26, 27, 30, 102, 115, 119]. The pathogenic role of aggregative-adhering properties in *C. diphtheriae*-invasive disease was investigated. *C. diphtheriae* biovar *mitis* and *gravis*, isolated from cases of endocarditis, expressed aggregative adherence (AA) patterns to human epithelial cells. The predominance of localized (LA) and diffuse adherence (DA) patterns have been reported for *C. diphtheriae* strains mostly isolated from throat and skin lesions [26, 64]. *C. elegans* nematodes have been also applied as an infection model system for *C. diphtheriae* and *C. ulcerans* with invasive phenotypes [102, 115, 120–122].

During years of research, the adhesive properties of *C. diphtheriae* strains have been already defined as multifactorial, relying on specific and general mechanisms. Functions and mechanisms of action of fimbriae; non-fimbrial adhesins – 67-72p (DIP0733) and DIP2093 – trans-sialidase; hydrophobins; and sugar residues are already recognized at different levels, especially how they jointly participate in the adherence to the host cells and in the colonization of these cells during bacterial infection [113, 117, 123–125].

Genomic analysis of *C. diphtheriae* revealed the identification of three distinct pili clusters (spaABC, spaDEF, spaGHI) together with five sortase-encoding genes (srtA–E), which are essential for pilus assembly. Adherence rates are not strictly correlated with pili formation, and the pili repertoire of *C. diphtheriae* strains is highly variable. spaA-type is the pilus mostly detected among *C. diphtheriae* strains. As shown by genome comparisons, it is necessary to investigate various isolates on a molecular level to understand and to predict the colonization process of different *C. diphtheriae* strains [3, 123, 124, 126].

The DIP0733 was initially described as a non-fimbrial 67-72p protein responsible for the adherence of *C. diphtheriae* strains to human erythrocytes. Further studies demonstrated DIP0733 protein as a microbial surface component recognizing adhesive matrix molecule (MSCRAMM). The influence of DIP0733 in *C diphtheriae* interaction with human epithelial cells and macrophages in addition to the ability to induce host cell death, giving a signal for apoptosis in the early stages of infection, was also reported. These findings support the idea that DIP0733 is a multifunctional virulence factor of *C. diphtheriae* that enhances the ability to spread throughout the whole human body via the bloodstream [125, 127, 128].

1.10 Molecular Typing Methods for DT-Producing *Corynebacterium* spp.

Molecular typing methods are expected to be reproducible with high discriminatory power, stable and cost-effective, and easy to perform and interpret. Several typing methods have been developed to investigate epidemiological relationship of strains

in disease outbreaks. Investigation of outbreaks is possible due to phylogenetic analysis of origin of strains and patterns of local and global dissemination over the years, among other features [23, 129, 130].

In the past, epidemiological surveillance of diphtheria was limited, depended largely on phenotypic characterization of strains, first by differentiation into biotypes and subsequently by serotyping and phage and bacteriocin typing. At the request of the World Health Organization Regional Office for Europe, the European Laboratory Working Group on Diphtheria (ELWGD) was formed in July 1993 because of the re-emergence of diphtheria to epidemic levels in the Russian Federation and Newly Independent States. The main objectives were to form a network of laboratories for microbiological surveillance, to standardize laboratory diagnostic methods, and to understand the molecular epidemiology and characteristics of *C. diphtheriae* strains at that time. In 2001, the network was expanded to become a Diphtheria Surveillance Network (DIPNET) (http://www.dipnet.org) concerned with epidemiological and microbiological aspects of diphtheria and other infections caused by other DT-producing *Corynebacterium* species, including Brazilian scientists. The main purpose of DIPNET is to establish a Pan-European network of expertise for the prevention of diphtheria and other related infections across the EU Member States and beyond. Among the specific objectives of DIPNET are to (i) determine the disease prevalence and characteristics of toxigenic and non-toxigenic *C. diphtheriae* and *C. ulcerans* in a variety of populations with emphasis upon higher-risk countries, (ii) expand the DIPNET external quality assurance schemes for laboratory diagnosis to include epidemiological typing and serological immunity, and (iii) develop novel tools for integrated molecular epidemiological characterization so as to gain a clearer understanding of the spread of epidemic clones throughout the WHO European Region [3, 23, 108, 131–136].

Over the years, several molecular typing methods have been applied for *C. diphtheriae*, including ribotyping, pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), multilocus enzyme electrophoresis (MEE), multilocus sequence typing (MLST), and spoligotyping. Some of these genotyping approaches have been also used for epidemiologic investigations of both *C. ulcerans* and *C. pseudotuberculosis* zoonotic pathogens [92, 137–141].

1.10.1 Ribotyping Assays

Ribotyping methods, based on restriction patterns of ribosomal RNA genes, had been previously considered the gold standard procedure for *C. diphtheriae* epidemiological surveillance, due to its high discriminatory power, reproducibility, and optimal typeability [142–144]. At the beginning, two ribotypes were identified by the restriction enzyme BstEII and were named G and M, since they seemed to be related to biovars *gravis* and *mitis*, respectively. However, G ribotypes were found in *C. diphtheriae* biovar *mitis* strains, and M ribotypes were also found in *C. diphtheriae* biovar *gravis* strains. The ten most frequent ribotypes of *C. diphtheriae* strains from Russia during the period of 1984 to 1996 were shown in Fig. 1.2.

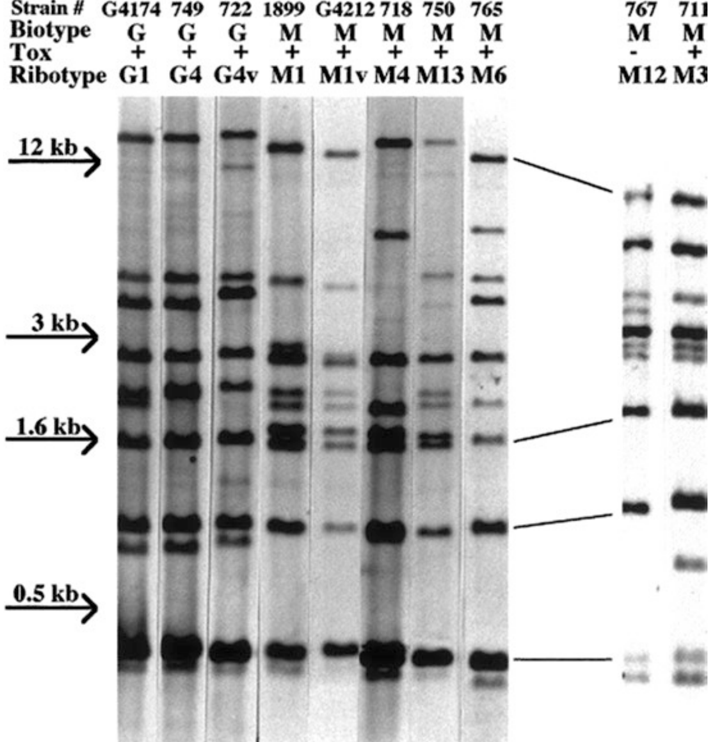

Fig. 1.2 Ribotyping assay based on restriction patterns of ribosomal RNA genes. Predominant riboprofiles identified in *Corynebacterium diphtheriae* strains (n = 156) from Russia during the period of 1984 to 1996. Tox, diphtheria toxin-producing. G, *gravis*. M, *mitis*. (Reprinted from Popovic et al. [142])

During an accurate interlaboratory comparative analysis, a revised nomenclature for the designation of ribotypes was proposed. Prefixes "G" and "M" were both replaced by prefix "D" (diphtheria). In 2004, an international ribotyping database was established at the Pasteur Institute and several collaborating laboratories supported by ELWGD/WHO. The ribotype nomenclature was revised and named using the geographical origin to reflect the location where one of the strains was isolated or studied. Eighty-six ribotypes were identified by the restriction patterns using BstEII digestion of the DNA [142, 145].

Ribotyping methods have been also used in studies of *C. ulcerans* zoonotic pathogen. In Japan, DT-producing *C. ulcerans* strains isolated from pharyngeal swabs of two patients attended at a hospital unit were indistinguishable by PFGE analysis and distinguished by ribotyping methods [146]. In 2016, a case of asphyxia death due to pseudomembrane caused by a DT-producing *C. ulcerans* strain, also recovered from the patient's domestic cat, was reported in a Japanese woman. Ribotyping analysis during this case in 2016 detected identical ribotype observed for *C. ulcerans* 0102 strain isolated from the first case in Japan during the year 2001

[57]. In a previous study, 9 different ribotypes, designated U1 to U9, were identified when evaluating 81 *C. ulcerans* strains, 50 of which were clinical isolates from the United Kingdom (90% toxigenic), 7 isolates from domestic cat, and the remaining from different places and sources of origin. The U1 ribotype was the predominant pattern found among human clinical isolates from the United Kingdom (20 isolates) and four isolates from other countries (Germany, France, Ukraine, and Italy). The seven domestic cat isolates also generated ribotypes found among human clinical isolates [147].

Limitations of ribotyping as a genotyping method for *C. ulcerans* were also reported. Genome sequence of *C. ulcerans* FH2016-1 isolated from the first fatal case described above was sequenced and compared with genomes of *C. ulcerans* strains of the first and second cases from Japan, 0102 and 0211, respectively. Although the analyses demonstrated a low variability between genomes, the isolate FH2016-1 was genetically distinct from 0102 and 0211, indicating that conventional ribotyping did not accurately reflect the strain with consequent inaccurate classification [148].

1.10.2 Pulsed-Field Gel Electrophoresis (PFGE) Technique

PFGE technique consists of the separation of DNA fragments with high molecular weight of bacterial genoma, obtained by using restriction enzymes [149]. PFGE technique consists of the separation of DNA fragments with high molecular weight of bacterial genoma, obtained by using restriction enzymes were used, and the *SfiI* restriction endonuclease was chosen, producing 18 to 25 DNA fragments ranging in size from 24 to 290 kb. In comparison with ribotyping, PFGE was not able to distinguish three ribotypes [137]. Thereafter, minor changes in the PFGE protocol were done, and both PFGE and ribotyping showed identical discriminatory ability. In that opportunity, all isolates grouped in one PFGE type were grouped in a ribotype, and vice versa. In addition to the PFGE protocol changes, some explanations seemed plausible for the results found [150]. A protocol using difference in three or more bands was used to distinguish *C. diphtheriae* PFGE types. In a later study, a difference in only two bands to distinguish the PFGE types of a limited number of *C. diphtheriae* strains was used [137, 150].

Nowadays, PFGE typing method has been scarcely used to investigate epidemiological relationship of *C. diphtheriae* strains in disease outbreaks. During the diphtheria outbreak in Northeastern Brazil in 2010, most of the confirmed cases occurred in partially or completely immunized children, including three fatal cases. Molecular analysis demonstrated the spread of predominant PFGE type related strains (Fig. 1.3) [22].

PFGE typing assays have been also used in investigations of origin, transmission, and dissemination of zoonotic *C. ulcerans* strains, especially in Japan. DT-producing *C. ulcerans* strains were isolated from pharyngeal swabs of two patients from the same hospital unit during 2001 and 2002 and were characterized by PFGE and ribotyping. The isolates could not be distinguished by PFGE;

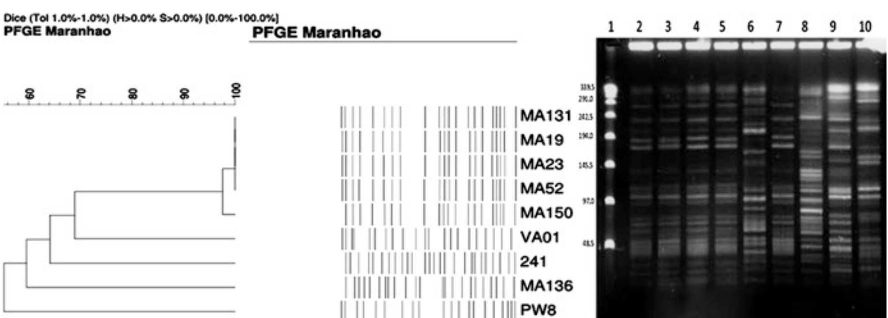

Fig. 1.3 Pulsed-field gel electrophoresis (PFGE) types of *Corynebacterium diphtheriae* biovar *intermedius* strains isolated from children with diphtheria living in the state of Maranhão, Brazil. Lane 1, λ DNA ladder PFGE marker; lanes 2–5, PFGE type Ia (MA19, MA23, MA52, MA131 strains, respectively); lane 6, PFGE type II (MA136 strain); lane 7, PFGE type Ib (MA150 strain). Other Brazilian DT-producing *C. diphtheriae* strains: lane 8, profile III (sucrose-positive TR241 biovar *mitis* strain); lane 9, profile IV (sucrose-negative VA01 biovar *gravis* strain). Toxoid vaccine producer strain: lane 10, profile V (PW8 strain). (Reprinted from Santos et al. [22])

however, ribotyping showed discriminatory results [146]. In contrast, a study of a fatal case due to a DT-producing C. *ulcerans* strain, PFGE analyses, and ribotyping of C. *ulcerans* strains from the patient and his cat belonged to the same molecular type [57].

1.10.3 Multilocus Enzyme Electrophoresis (MEE)

This electrophoresis technique detects amino acid substitutions capable of altering charge and structural conformation of cellular housekeeping enzymes. Each electromorph or mobility variants of the same enzyme are visualized in a starch gel matrix as bands with different migration rates. Twenty-seven enzymes are tested, and each electromorph is considered a different allele of the specific enzyme. An electromorph profile defines the electrophoretic type (ET) of each bacterial strain. The genetic distance of ET is calculated as a dendrogram generated by the average linkage method of clustering the ETs [151]. Several studies had used MEE methods to estimate *C. diphtheriae* genetic diversity and epidemiological features of endemic and epidemic diseases. Diphtheria epidemic in the 1990s, which initiated in the Russian Federation and dispersed to several European countries, was characterized by the simultaneous presence of different ET that were also detected in *C. diphtheriae* strains isolated during the pre-epidemic period. The majority of *C. diphtheriae* strains with D1 and D4 ribotype patterns (previously named G1 and G4) belonged to the clonal group called ET8 complex [139].

1.10.4 Random Amplification of Polymorphic DNA (RAPD) Assays

The RAPD technique aims to amplify random segments of DNA with single primers of arbitrary nucleotide sequence that may be used to construct genetic maps in a variety of species [152]. The RAPD assay for *C. diphtheriae* was recently standardized by using *C. diphtheriae* strains isolated worldwide. Initially, *C. diphtheriae* strains from Russia (1985–1994) were evaluated, and primers 3 and 4 classified the isolates previously ribotyped into 19 and 24 genotypic profiles, respectively. However, epidemic ribotypes D1 and D4 could not be differentiated by primers 3 and 4. Subsequently, 120 *C. diphtheriae* strains isolated from Russia (1994–1995), Kazakhstan (1996), and the Republic of Georgia (1995–1996) were included and presented RAPD profiles typical of the epidemic ribotypes D1 and D4 [153]. Difficulties in the standardization of RAPD assays for *C. diphtheriae* such as the use of crude DNA and different thermocyclers resulted in poor amplifications, and nonreproducible patterns were also verified [138]. RAPD assays have advantages for being simple, rapid, and inexpensive. However, RAPD assays for *C. diphtheriae* demonstrated low reproducibility in some opportunities [138, 150, 153].

In a previous study, DT-producing and non-DT-producing *C. diphtheriae* strains isolated from cases of infective endocarditis showed different RAPD profiles, demonstrating the invasive properties and circulation of these different clones in Brazil [28]. Analysis performed with purified DNA of several *C. diphtheriae* strains from 26 countries resulted in the differentiation of Eastern European epidemic ribotypes D1 and D4 corresponding to the RAPD profiles Rp1 and Rp4, respectively [143] (Fig. 1.4).

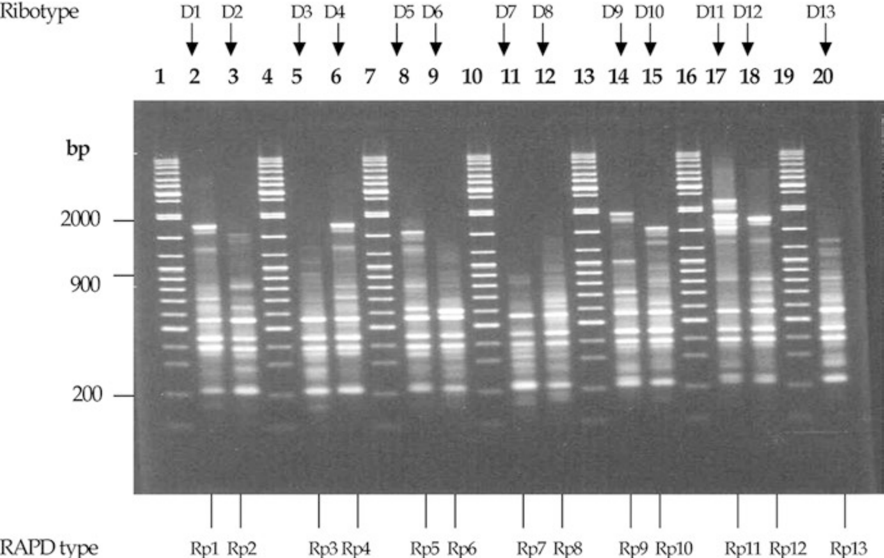

Fig. 1.4 Thirteen RAPD profiles of *C. diphtheriae* isolates illustrating the differentiation of epidemic ribotypes D1 e D4 as Rp1 and Rp4, respectively. (Reprinted from De Zoysa et al. [143])

1.10.5 Amplified Fragment Length Polymorphism (AFLP)

AFLP technique is based on PCR amplification of restriction fragments from total genomic DNA digestion, by using generic primers that do not require prior information of the target DNA sequence. DNA restriction and ligation of oligonucleotide adapters are made to form the binding sites. Subsequently, the selective amplification of the restriction fragments occurs, and finally the fragments are visualized in gel [154]. Evaluation of the AFLP technique was verified during a study conducted with *C. diphtheriae* strains ($n = 57$) presenting nine different ribotypes. A total of ten AFLP profiles were assigned to *C. diphtheriae* tested strains; however, it was not able to discriminate the predominant ribotype during the Eastern European epidemic. AFLP is a PCR fingerprint method easy to perform, rapid, inexpensive, and suitable for most laboratories. Moreover, the AFLP standards are representative of the complete genome [155]. However, AFLP method was less discriminatory than ribotyping in some studies [143, 156].

1.10.6 Multilocus Sequence Typing (MLST) Method

MLST assays have been widely used in different countries for molecular typing of circulating *C. diphtheriae* strains and investigations of epidemic outbreaks. The method aims to group strains related to cloned complex after sequencing and analyzing fragments of seven constitutive genes that encode essential functions for microbial metabolism. *C. diphtheriae* MLST scheme uses fragments of the following housekeeping genes: ATP synthase alpha chain (*atp*A), DNA polymerase III alpha subunit (*dna*E), chaperone protein (*dna*K), elongation factor G (*fus*A), 2-isopropylmalate synthase (*leu*A), 2-oxoglutarate dehydrogenase E1 and E2 components (*odh*A), and DNA-directed RNA polymerase beta chain (*rpo*B) [91, 144].

The PubMLST website hosts a collection of open-access, curated databases that integrate sequence data with phenotype information for many microbial species, including *C. diphtheriae* and *C. ulcerans*. Currently, more than 700 categorized types are deposited (October 2020) in the PubMLST database (http://pubmlst.org/cdiphtheriae/). In 2010, the MLST scheme was proposed for *C. diphtheriae* by using the sequences of the 7 constitutive genes described above, with a total of 150 tested strains (toxigenic isolates $n = 96$) from 18 different countries, during the period of 1957–2006. The results were consistent with previous ribotyping data, which was considered the "gold standard" typing method of *C. diphtheriae* for many years [91].

Although MLST is recognized as a valuable fast and simple PCR-based method used for the tracking the spread of important clones and evolutionary investigation of bacteria, this methodology has some limitations, such as the identification of hypervirulent clones, since the MLST data are based on changes in the core genome, while changes in the accessory genome are responsible for *C. diphtheriae* virulent variants. Each ST can be represented by toxigenic noninvasive, nontoxigenic invasive, and nontoxigenic noninvasive strains [157, 158]. Furthermore, some *C.*

diphtheriae strains with identical ST may differ in up to 290 genes; noncorrelated results between MLST and biotype tests may also occur [158, 159].

The ST-8 clone was responsible for the beginning of the Eastern European epidemic and spread of more than 157,000 registered cases of diphtheria, which resulted in approximately 5,000 deaths. Non-DT-producing profiles of ST-8 strains, previously isolated as toxigenic in Russia, were recently described in Poland. This change was attributed to the environmental pressure exerted by the increase in the number of vaccinated individuals. ST-8 continued to circulate after the epidemic period, as reported realized in Germany, which points to the persistence of ST-8 until today [158, 160, 161]. From 2016 to 2017 in Germany, there was an increase in the circulation of nontoxigenic *C. diphtheriae* characterized mostly by the ST-8. This ST is the most abundant found in the MLST database for *C. diphtheriae* and probably in Europe [160].

Since invasive infections caused by *C. diphtheriae* in vaccinated and non-vaccinated individuals have been reported in Brazil, a genetic relationship of *C. diphtheriae* strains isolated from classic diphtheria and invasive infections in Rio de Janeiro metropolitan area was investigated by using MLST. Four strains presented an atypical sucrose-fermenting ability and corresponded to new STs. Interestingly, a sucrose-fermenting *C. diphtheriae* biovar *mitis* strain, isolated in 1999 patient with endocarditis, formed a clonal complex with a DT-positive *C. diphtheriae* biovar *mitis* strain isolated in Argentina (1995) causing classic diphtheria, suggesting a *C. diphtheriae* biovar *mitis* clonal complex circulation in South America. Moreover, a sucrose-negative strain isolated from a case of endocarditis in 2003 generated an MLST profile that had been previously deposited in the database, ST128, a single locus variant (SLV) of ST-80, the clonal complex that comprises strains currently isolated in different countries including France (ST128) and Canada. These data indicated that *C. diphtheriae* clonal complexes comprising clinical strains related to diphtheria disease may also include invasive phenotypes [9, 28, 91, 94, 111, 162].

An MLST protocol *C. ulcerans* was based on protocol described for *C. diphtheriae*. The website PUBMLST comprises data for both species [91, 163, 164]. MLST methods have been also used in phylogenetic analyses, epidemiology, and zoonotic transmission investigations of *C. pseudotuberculosis* [55, 97, 163, 165].

1.11 In Silico-Based Approaches

Bacterial in silico typing based on repetitive DNA sequences has been also established with the objective of genomic characterization and epidemiological surveillance of diphtheria. Two approaches named CRISPR (clustered regularly interspaced short palindromic repeats) loci and VNTR (variable number tandem repeats) were investigated. In Poland, a study used the complete genome sequence of the NCTC 13129 *C. diphtheriae* strain to identify 75 VNTR loci, of which 14 were selected. Primers were designed, and PCR conditions were optimized to amplify the selected VNTR markers. Fourteen markers were tested and eight were considered

potentially useful. This approach showed discriminatory genotyping ability for the *C. diphtheriae* tested strains ($n = 28$), but the preliminary results were not compared with other genotyping methods [166].

CRISPR-based spoligotyping, defined as a genotyping technique to identify *C. diphtheriae* strains at the phylogeographic level, was also described. According to the authors, this typing methodology presents a high level of discrimination and may be employed to study local epidemiology. One of the limitations is the need for expensive equipment or the use of external services, but the comparison of results between laboratories is easy, and the data generated can be compared in a database. In a study conducted with *C. diphtheriae* of Russia and Belarus epidemic clone, the 156 strains tested were subdivided into 45 spoligotypes. A high level of discrimination in the study of the local epidemiology of diphtheria was observed. However, the three selected CRISPR loci were not present in all *C. diphtheriae* tested strains, and most of them had unique spaces in the leader sequence, indicating that they evolved independently after diverging from a common ancestor [141, 167, 168].

Whole-genome sequencing (WGS) has become an essential tool for molecular epidemiology of infectious disease studies. In recent years, WGS has become the gold standard of high-resolution typing methods, allowing the understanding of the molecular epidemiology and global transmission of pathogens. Genome sequencing remains expensive to be employed in routine genotyping. Nevertheless advances in *C. diphtheriae* genomics concern an increasing number of complete genomes in GenBank may benefit sequence-based genotyping methods as identification of SNPS, tandem repeats (VNTRs), and CRISPR-based spoligotyping. The development of more inexpensive and discriminatory methodologies for use in epidemiological studies will be crucial in our understanding of the molecular epidemiology and carriage of *C. diphtheriae* and *C. ulcerans* [23, 87, 144].

1.12 Conclusions

Molecular typing methods have become essential during the analysis of studies involving epidemiology outbreaks, endemic conditions, recurrent infections, transmission, and virulence potential of *C. diphtheriae* and zoonotic diphtheria toxin-producing *Corynebacterium* species. Application of any typing method depends on the objectives of the study, the level of resolution desired (species vs. strain), and the laboratory conditions and technical expertise available. In studies of complex epidemiological situations and strain-dependent virulence mechanisms, it is recommended not to rely on a single method but to use combinations of methods for strain identification and to interpret results within the context of the epidemiological background and evolution of once acquired pathogenicity features during vaccine era [22, 23, 38, 55, 74, 97, 143, 155].

Epidemiological investigations demonstrated the prevalence of *C. diphtheriae* biovars *gravis* and *mitis* in Eastern Europe and most of Brazilian outbreaks, respectively. Diphtheria cases and deaths caused by *C. diphtheriae* biovar *intermedius* were also documented in previously immunized individuals in India and during the

most recent diphtheria outbreak in Brazil [5, 17, 22, 169, 170]. Nowadays, molecular epidemiological investigations demonstrated the prevalence of different *C. diphtheriae* genotypes in specific geographic regions, including epidemic outbreak: Thailand (ST-243), South Africa (ST 379), and Malaysia (ST453) [171–173].

The occurrence of diphtheria among immunized persons and the increasing frequency of atypical infections caused by non-DT-producing clones indicated that other microbial factors should be used as one of the antigens in the potential vaccine development in the near future. In conclusion, molecular typing methods became a remarkable achievement in wide-ranging research to potentially DT-producing *Corynebacterium* species (*C. diphtheriae* complex), group of extremely dangerous human pathogenic species.

1.13 Summary

Corynebacterium diphtheriae is the leading causing agent of respiratory and cutaneous diphtheria, an acute disease with local and systemic manifestations, which remains as an important cause of morbidity and mortality in different continents. Diphtheria vaccination programs implemented in industrialized and developing countries led to an increasing number of atypical cases of diphtheria in addition to localized and systemic infections, including fully immunized adults. Changes in the clinical epidemiology and virulence features of diphtheria pathogens have been investigated. Cases of infections due to diphtheria toxin (DT)-producing and non-DT-producing *C. diphtheriae* and *Corynebacterium ulcerans*, a zoonotic pathogen, have been increasingly reported. The timely and precise diagnosis of DT-producing *Corynebacterium* strains is indispensable for the patient management and for establishment of surveillance and control strategy of disease. Different molecular methods, such as real-time PCR (polymerase chain reaction) and multiplex PCR, have been used for the characterization of *C. diphtheriae*, *C. ulcerans*, and *C. pseudotuberculosis* and the detection of the gene for DT (*tox*). Recent investigations by genomic sequencing and chemotaxonomic analyses reported DT-producing *C. diphtheriae* subsp. *lausannense* and *Corynebacterium belfantii* sp. nov. in addition to *Corynebacterium rouxii* sp. nov. Genotyping methods have been used as essential epidemiological tools for *C. diphtheriae* and *C. ulcerans* infection prevention and control, including pulsed-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and multilocus sequence typing (MLST) assays. Molecular typing methods are required in studies involving characterization, virulence potential, and susceptibility to antimicrobial agents of *C. diphtheriae* and *C. ulcerans* clinical isolates; origin, routes, and transmission of diphtheria and atypical invasive infections; and endemicity, outbreaks, recurrent infections, and trace cross-transmission caused by non-DT-producing and DT-producing *Corynebacterium* spp.

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Mycoplasma and *Ureaplasma*

2

Ken B. Waites, Li Xiao, Rose M. Viscardi, Sixto M. Leal Jr, and John I. Glass

2.1 Introduction

The bacteria commonly referred to as mycoplasmas are included within the phylum *Tenericutes*, class *Mollicutes* which is comprised of 4 orders, 5 families, 8 genera, and about 200 known species distributed among humans, animals, insects, and plants (Table 2.1). There are 17 mollicute species isolated from humans, excluding species of animal origin that have been detected in humans, usually in immunosuppressed hosts, but which are generally considered transient colonizers.

There are at least six species known to be of pathologic significance for humans, either as primary pathogens or opportunists: *Mycoplasma pneumoniae*, *M. hominis*, *M. genitalium*, *M. fermentans*, *Ureaplasma urealyticum*, and *Ureaplasma parvum. M. amphoriforme* is the most recently described *Mycoplasma* species isolated from humans. While the true extent of its role as a human pathogen has not yet been firmly established, evidence is accumulating for person-to-person transmission and that it can cause illness in immunosuppressed as well as immunocompetent persons [1, 2].

Some important characteristics of individual mollicute species that occur in humans are shown in Tables 2.2 and 2.3. This chapter will focus primarily on

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Table 2.1 Characteristics of class *Mollicutes*[a]

Order	Family	Genus	No. of species	Sterols required	Hosts	Genome size (kbp)	Mol% G + C of DNA	Defining features
Mycoplasmatales	*Mycoplasmataceae*	*Mycoplasma*	>100	Yes	Humans and animals	580–1350	23–40	
		Ureaplasma	7	Yes	Humans and animals	760–1170	25–32	Metabolizes urea
Entomoplasmatales	*Entomoplasmataceae*	*Entomoplasma*	6	Yes	Insects and plants	870–900	27–34	
		Mesoplasma	11	No	Insects and plants	825–930	26–32	
	Spiroplasmataceae	*Spiroplasma*	38	Yes	Insects and plants	780–2220	24–31	Helical morphology
Acholeplasmatales	*Acholeplasmataaceae*	*Acholeplasma*	18	No	Animals, insects, and plants	530–1350	26–36	
Anaeroplasmatales	*Anaeroplasmataceae*	*Anaeroplasma*	4	Yes	Animals	1500–1600	29–33	Obligate anaerobes
		Asteroleplasma	1	No	Animals	1500	40	Obligate anaerobes

[a]*Mollicutes* enumerated in this table do not include the noncultivable hemotrophic mycoplasmas and *Candidatus* phytoplasmas

Table 2.2 Characteristics of *Mycoplasma* species that colonize and infect humans

Species	Primary site of colonization		Metabolic substrate		Role in human diseases[a]
	Respiratory tract	Urogenital Tract	Glucose	Arginine	
M. amphoriforme[b]	+	?	+	–	Yes
M. buccale	+	–	–	+	No
M. faucium	+	–	–	+	No
M. fermentans	+	+	+	+	Yes
M. genitalium	?+	+	+	–	Yes
M. hominis	+	+	–	+	Yes
M. lipophilum	+	–	–	+	No
M. penetrans[c]	–	+	+	+	?
M. pirum[d]	?	?	+	+	No
M. orale	+	–	–	+	No
M. pneumoniae	+	–	+	–	Yes
M. primatum	–	+	–	+	No
M. salivarium	+	–	–	+	No
M. spermatophilum	–	+	–	+	No

[a]Some of the species listed as commensals, such as *M. salivarium* and *M. orale*, have occasionally been shown to produce invasive disease (e.g., arthritis) in persons with primary antibody deficiency or other immunosuppressed states, but they are still considered nonpathogenic overall

[b]*M. amphoriforme* is the newest human *Mycoplasma* species to be identified. It has been recovered from the respiratory tract of persons with primary antibody deficiency and chronic bronchitis or bronchiectasis as well as immunocompetent persons, but not in healthy persons thus far [1, 2, 6]. Whether it occurs in sites other than the respiratory tract has not been evaluated

[c]*M. penetrans* has been detected in the urine, rectum, and throats of homosexual men with human immunodeficiency virus infection. However, this mycoplasma has not been conclusively shown to produce significant disease in any patient population, despite the fact that it possesses potentially pathogenic features such as a prominent terminal attachment organelle and has proven ability to invade host cells [19]

[d]*M. pirum* was first characterized in 1985, but its natural host was unknown. This mycoplasma has been isolated from peripheral blood lymphocytes and urines in persons with human immunodeficiency virus infection and has been found by PCR in the rectums of homosexual men. It has also been detected in healthy persons and no conclusive evidence that it is a cause of human disease has been forthcoming [19]

methods for detection, identification, and typing of pathogenic mollicutes of humans and will not include information on the many mycoplasmal pathogens of animals.

2.2 Class *Mollicutes*

Mollicutes are smaller than conventional bacteria in cellular dimensions as well as genome size, making them the smallest free-living organisms known. Some species such as *M. pneumoniae* and *M. genitalium* also possess distinct terminal attachment organelles. Mollicutes cannot be detected by light microscopy, and they rarely

Table 2.3 Genomic characteristics of *Mycoplasma* and *Ureaplasma* species[a]

Species (no. of genomes sequenced)[b]	Genome size (kbp)	Protein-coding genes	Mol% G + C of DNA
M. amphoriforme (1)	1031	715	32
M. fermentans (5)	921–1120	690–938	27
M. genitalium (6)	560–580	346–515	31–32
M. hominis (23)	633–768	502–606	27
M. pneumoniae (89)	778–858	661–743	4
U. parvum (14)	630–773	334–601	25–26
U. urealyticum (18)	644–947	435–698	25–28

[a]Data obtained from https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/mycoplasma
[b]Number may include more than one sequence for the same strain, sometimes performed by different methods

produce visible turbidity in liquid growth media. They do not possess a cell wall, but like mammalian cells, they are bounded by phospholipid bilayer membranes. The permanent lack of a cell wall barrier makes mollicutes unique among prokaryotes. Lack of a cell wall also renders mollicutes insensitive to the activity of beta-lactam antimicrobials, prevents them from staining by Gram stain, and is largely responsible for their pleomorphic form. Most mollicutes are nonmotile, but some species, including *M. pneumoniae* and *M. genitalium*, show gliding motility. Mollicutes contain a single circular chromosome and a low G + C content. The extremely small genomes and limited biosynthetic capabilities explain their parasitic or saprophytic existence, their sensitivity to environmental conditions, and fastidious growth requirements. Type strains and clinical isolates of important mollicute pathogens of humans have undergone complete genomic sequencing in order to provide a better understanding of their comparative genomics and offer clues about pathogenic mechanisms at the molecular level.

Mollicutes require enriched growth medium supplemented with nucleic acid precursors, fatty acids, and amino acids. Except for acholeplasmas, asteroleplasmas, and mesoplasmas, mollicutes require sterols in growth media, supplied by the addition of horse or bovine serum. Although mollicutes can flourish in the osmotically stable environment of a eukaryotic host, they are extremely susceptible to desiccation. In addition to the phospholipid bilayer membrane, mollicute cells typically have an underlying protein cytoskeleton network that provides structural support. Some mollicutes, including *M. pneumoniae* and *Ureaplasma* spp., also elaborate extracellular capsular material [3, 4]. Growth rates in culture medium vary among individual species, with generation times of approximately 1 hour for *Ureaplasma* spp., 6 hours for *M. pneumoniae*, and 16 hours for *M. genitalium* [5]. Typical mollicute colonies vary from 15 to 300 μm in diameter. Colonies of some species, such as *M. hominis*, often exhibit a “fried egg” appearance owing to the contrast in deeper growth in the center of the colony with more shallow growth at the periphery, while others, such as *M. pneumoniae* and *M. genitalium*, produce spherical colonies. Whereas colonies of some mycoplasmal species may be observed with the naked

eye, those produced by ureaplasmas are typically 15–60 μm in diameter and require low-power microscopic magnification for visualization.

Mollicutes can be classified according to whether they ferment glucose, utilize arginine, or hydrolyze urea. Except for hydrolysis of urea, which is unique for ureaplasmas, these biochemical features are not sufficient for species distinction. Anaeroplasmas and asteroleplasmas, which occur in ruminants, are strictly anaerobic and oxygen-sensitive, while most other mollicutes are facultative anaerobes. The type of media, pH, metabolic substrates, and optimum temperature for in vitro cultivation and colony development can vary considerably for the many different species that may be encountered in various hosts. No tricarboxylic acid cycle enzymes, quinones, or cytochromes have been found in this class. The 16S rRNA gene sequences place the mollicutes into several distinct phylogenetic groups (Fig. 2.1) and have also shown that certain noncultivable hemotropic bacteria previously classified among the rickettsiae belong to class *Mollicutes* [2].

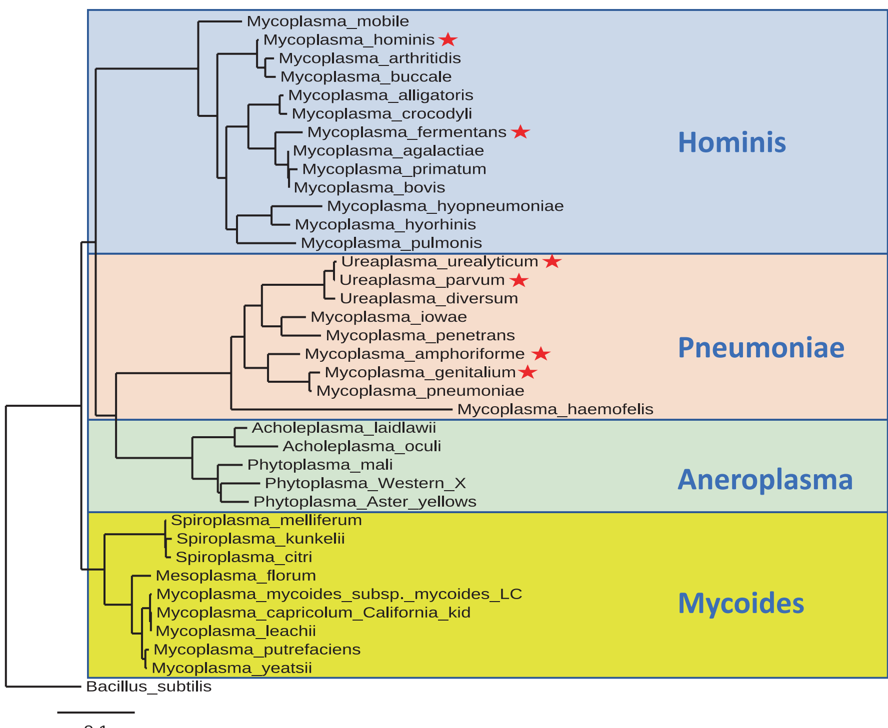

Fig. 2.1 Phylogenetic classification of mollicutes based on 16S rRNA gene. The six species of *Mycoplasma* and *Ureaplasma* that are proven human pathogens are noted with red stars. The four main taxonomic groups of the class *Mollicutes* are highlighted in different background colors

2.2.1 Genus *Mycoplasma*

The genus *Mycoplasma* is the largest and most important in the class, comprised of well over 100 known species, which may occur as commensals or cause significant diseases in animals or humans.

2.2.1.1 Mycoplasma amphoriforme

M. amphoriforme was first isolated in 1999 from a patient with X-linked agammaglobulinemia suffering from chronic bronchitis [6]. Subsequently, it has been detected in persons with primary antibody deficiencies with chronic bronchitis, as well as in immunocompetent adults with lower respiratory tract infections, but not in normal adult control subjects. *M. amphoriforme* has been detected on multiple occasions from patients with chronic respiratory symptoms, primarily a productive cough with mucopurulent sputum [7]. The full geographic distribution and pathogenic role of *M. amphoriforme* in humans have not been firmly established. Scanning electron microscopy has revealed that the pleomorphic flask-shaped cells of *M. amphoriforme* exhibit one or more polar extensions terminating in a knob-like structure whose shape is distinct from that of the *M. pneumoniae* attachment organelle [8]. *M. amphoriforme* exhibits gliding motility, but whether it possesses cytadherence properties associated with this terminal structure analogous to *M. pneumoniae* has not been investigated. *M. amphoriforme* grows poorly and very slowly on Hayflick's/SP4 agar after 2–3 weeks of incubation in air plus 5% CO_2. It produces spherical colonies similar in appearance to those of *M. pneumoniae.* Like *M. pneumoniae*, it is glycolytic [2].

2.2.1.2 Mycoplasma fermentans

M. fermentans can occur in many body fluids and tissues, but its primary site of colonization, mode of transmission, and pathogenic potential are incompletely understood. It has been detected in adults with an acute influenza-like illness [9] and in bronchoalveolar lavages, peripheral blood lymphocytes, and bone marrow from patients with acquired immunodeficiency syndrome (AIDS) and respiratory disease [10, 11]. Respiratory infection with *M. fermentans* is not necessarily linked with immunodeficiency, but it may also behave as an opportunistic pathogen. Several studies have implicated *M. fermentans* in a variety of human inflammatory arthritides including rheumatoid arthritis. Considerable interest arose in the 1990s regarding the possibility that *M. fermentans* could be a cofactor in the pathogenesis in AIDS and/or as an etiologic agent of fibromyalgia, Gulf War, and chronic fatigue syndromes. However, after several large-scale studies failed to demonstrate a conclusive role for this organism in these conditions, the belief that M. fermentans is pathogenic in such conditions has been abandoned [12]. *M. fermentans* is able to invade host cells and produces a potent immunomodulator, macrophage-activating lipopeptide 2, which stimulates macrophages to release cytokines that may increase neutrophilic infiltration locally where the organism resides [12]. *M. fermentans* does not have the specialized attachment organelle that occurs in some other species. Additional virulence factors may include plasminogen activation and other

membrane surface proteins that mediate cell fusion, cytadherence, and antigenic variation [13]. *M. fermentans* grows well on SP4 or Hayflick's agar supplemented with either arginine or glucose since it has the enzymatic systems to utilize both as substrates. It produces fried egg colonies after several days of incubation.

2.2.1.3 Mycoplasma genitalium

M. genitalium was initially isolated from men with urethritis in 1980 and is now known to be a significant cause of this condition as well as female cervicitis and pelvic inflammatory disease [14, 15]. *M. genitalium* possesses a terminal structure, the MgPa adhesin, which facilitates its attachment to epithelial cells. *M. genitalium* also attaches to spermatozoa and erythrocytes and invades epithelial cells with evidence of nuclear localization [15]. A family of repetitive DNA elements with homology to the MgPa adhesin gene provides a reservoir of sequence that could contribute to variation in the protein of the MgPa adhesin gene. Sequence divergence among strains of *M. genitalium* has been shown, and this antigenic variation may help avoid the host immune response and optimize adhesion [16]. Extensive variation of the MG192 sequence changes the antigenicity of the protein to allow immune evasion but also alter the mobility and adhesion ability of the organism to adapt to diverse host microenvironments, thus facilitating persistent infection [17]. *M. genitalium* is glycolytic, but cultivation is difficult and time-consuming, requiring up to several weeks for typical spherical colonies to emerge on agar, although subcultures may grow more rapidly on agar. The best culture media are SP4 broth and agar incubated at 37 °C in air plus 5% CO_2. Additional techniques designed to improve recovery of *M. genitalium* from clinical specimens have involved serial passages in Vero cells to allow adaptation for the organisms to grow in broth and eventually on agar [18]. Though various modalities have been developed to enhance the ability to detect *M. genitalium* in culture, the high failure rate and extremely slow growth make the culture approach impractical for diagnostic purposes.

2.2.1.4 Mycoplasma hominis

Approximately 21–53% of asymptomatic sexually active women may be colonized with this mycoplasma in the cervix or vagina, but the occurrence is somewhat lower in the male urethra [19]. It is often present concurrently with *Ureaplasma* spp. and is venereally and vertically transmissible. *M. hominis* is associated with a variety of conditions including pyelonephritis, pelvic inflammatory diseases, chorioamnionitis, postpartum endometritis, bacterial vaginosis, arthritis, osteoarthritis, wound infections, and several conditions in neonates including congenital pneumonia, meningitis, bacteremia, and abscesses. Systemic infections are usually, but not always, associated with immunocompromised hosts [19]. Henrich [20] demonstrated the presence of the variable adherence-associated (Vaa) antigen, which displays high-frequency phase and size variation that is believed to be a major adhesin of M. hominis and may also assist in evasion of host immune responses. Additional surface proteins such as OppA, an oligopeptide permease substrate-binding protein, are also believed to be involved in cytadherence and may also induce ATP release from cells, resulting in apoptosis [21]. The pathogenic potential of *M. hominis* is

complicated by the high degree of genomic and antigenic heterogeneity observed within the species [22]. *M. hominis* is a coccoid organism without a prominent polar attachment organelle. It is non-glycolytic and synthesizes ATP through the arginine dihydrolase pathway [21]. *M. hominis* grows well in SP4 broth or agar medium supplemented with arginine at 37 °C in 5% CO_2 in air, but it will also grow on A8 agar and in 10B broth. Colonies develop in 2–3 days and exhibit the typical fried egg appearance.

2.2.1.5 Mycoplasma pneumoniae

M. pneumoniae is a common cause of upper and lower respiratory tract infections in children and adults worldwide. Details of the epidemiology, pathogenesis, and clinical manifestations of *M. pneumoniae* infections have recently been reviewed by Waites et al. [23]. It is easily spread through respiratory droplets and can cause a wide array of clinical manifestations including pharyngitis, tracheobronchitis, and pneumonia. Extrapulmonary manifestations involving any of the major organ systems sometimes occur following primary respiratory infection either by direct spread or autoimmune effects. Attachment of *M. pneumoniae* to host cells in the respiratory tract of humans is a prerequisite for colonization and infection. Cytadherence is mediated by the P1 adhesin and other accessory proteins [24] and is followed by induction of chronic inflammation, and cytotoxicity mediated by hydrogen peroxide, which also acts as a hemolysin. *M. pneumoniae* stimulates B and T lymphocytes and induces formation of autoantibodies which react with a variety of host tissues and I antigen on erythrocytes, which is responsible for production of cold agglutinins. An ADP-ribosylating toxin with limited sequence homology to the pertussis toxin S1 subunit known as the community-acquired respiratory distress syndrome toxin (CARDS TX) causes vacuolation and ciliostasis in cultured host cells and is now considered a significant virulence factor in *M. pneumoniae* [24, 25]. Although mycoplasmas are generally considered to be extracellular pathogens, intracellular localization is now appreciated for *M. pneumoniae*, *M. fermentans*, *M. penetrans*, and *M. genitalium* [26]. Intracellular localization may be responsible for protecting the organisms from antibodies and antibiotics, as well as contributing to disease chronicity and difficulty in cultivation in some cases. *M. pneumoniae* appears in electron micrographs as pleomorphic rods 0.1–0.2 μm in width with a prominent polar attachment organelle [3]. *M. pneumoniae* can be cultivated at 37 °C in 5% CO_2 in air in SP4 medium containing glucose at pH 7.4–7.6. Spherical colonies develop after several days.

2.2.2 Genus *Ureaplasma*

The genus *Ureaplasma* comprises those members of the family *Mycoplasmataceae* that hydrolyze urea and use it as a metabolic substrate for generation of ATP. Shepard provided the first description of ureaplasmas when he cultivated them from the urethras of men with nongonococcal urethritis (NGU) [29]. Human ureaplasmas were originally considered to belong to a single species, *U. urealyticum*, until 2002 when

its two biovars were reclassified as the separate species *U. parvum* and *U. urealyticum* [28]. There are 14 known serovars. *U. parvum* contains the serotypes 1, 3, 6, and 14, while the remaining serovars 2, 4, 5, and 7–13 are assigned to *U. urealyticum* [2].

As many as 40%–80% of healthy adult sexually active women may harbor ureaplasmas in their cervix or vagina. The organisms are venereally as well as vertically transmissible. Their occurrence is somewhat less in the lower urogenital tract of healthy men (approximately 20%–29%) [29]. *U. parvum* is more common than *U. urealyticum* as a colonizer of the male and female urogenital tracts and in the neonatal respiratory tract [12]. Ureaplasmas reside primarily on the mucosal surfaces of the urogenital tracts of adults or the respiratory tracts in infants. Despite their frequent occurrence in the lower urogenital tracts of healthy persons, *Ureaplasma* spp. may cause a variety of clinical conditions including urethritis, chorioamnionitis, postpartum endometritis, preterm birth and pneumonia, bacteremia, abscesses, meningitis, and chronic lung disease in preterm infants [2, 19]. Ureaplasmas are opportunistic pathogens, particularly in hosts with impaired antibody production and have been associated with arthritis, osteomyelitis, pneumonia, and other systemic conditions in this setting. Along with *M. hominis*, *Ureaplasma* spp. have recently been implicated as a cause of fatal hyperammonemia in lung transplant recipients [30].

Ureaplasmas are capable of attaching to urethral epithelial cells, spermatozoa, and erythrocytes [12]. The adhesins have not been characterized completely, but current evidence suggests the receptors are sialyl residues and/or sulfated compounds [12]. A major family of surface proteins, the multiple banded antigens (MBA), is immunogenic during ureaplasma infections. MBAs have been used as a basis for the development of reagents for diagnostic purposes, but MBA expression is known to be phase variable, so false negatives using assays based on MBA protein or mba gene detection are possible [31]. Ureaplasmas produce nucleases, IgA protease, and release ammonia through urea hydrolysis, all of which are considered possible virulence factors [12]. An intact humoral immune response appears to be important in limiting invasion and dissemination of ureaplasma beyond mucosal surfaces. This is demonstrated by their tendency to cause chronic respiratory infections and arthritis in persons with hypogammaglobulinemia and to cause invasive disease in preterm neonates [12]. Variation in surface antigens may be related to persistence at invasive sites. Ureaplasmas typically appear as coccoid cells of about 0.2–0.3 μm diameter under electron microscopy but may be as small as 0.1 μm. *Ureaplasma* spp. grow rapidly in 10B broth and A8 agar and will produce colonies 15–60 μm colonies within 1–2 days after incubation at 37 °C in air plus 5% CO_2. Colonies appear brown and granular in the presence of $CaCl_2$ in A8 agar and may produce the fried egg effect.

2.3 Detection of Mollicutes by Traditional Methods

2.3.1 Culture

Culture is a quick and reliable method for detection of *Ureaplasma* spp. and *M. hominis* in clinical specimens. These organisms will produce color change in broth, and colonies will develop on agar within 1–3 days if suitable growth media and incubation conditions are provided. Culture has an additional advantage in that it provides an isolate that can be studied further and on which antimicrobial testing can be performed. However, for *M. fermentans*, *M. pneumoniae*, and especially *M. genitalium*, culture is seldom used since it is insensitive for detection and may require several days to weeks for evidence of growth. *M. amphoriforme* has been detected by culture conditions comparable to what are used for *M. pneumoniae*, but very limited experience suggests it grows slowly and very poorly in vitro, which probably accounts for the paucity of clinical isolates that have been reported thus far. Detailed methods for obtaining specimens and culturing mycoplasmas and ureaplasmas of humans in vitro have been described in other reference texts [2, 32].

2.3.1.1 Identification of Mollicutes Grown in Culture

For *Ureaplasma* spp., the appearance of granular colonies on A8 agar in the presence of $CaCl_2$ indicator is sufficient for genus identification. Growth of a mycoplasma from a clinical specimen cannot be conclusively identified to species level based on phenotypic appearance or biochemical activities. Formerly, there were several phenotypic methods used to identify mycoplasmas detected by culture in clinical specimens. However, they have now been replaced by molecular methods such as PCR which is discussed in a separate section.

2.3.2 Serological Detection

2.3.2.1 M. pneumoniae

Serological testing was the first method for detection of *M. pneumoniae* infections. Disadvantages of serology are the need for both acute and convalescent paired sera collected 2–3 weeks apart that are tested simultaneously for IgM and IgG to confirm seroconversion, difficulty in distinguishing current or recent infection from past infection, and the need to wait 1–2 weeks until detectable antibody develops. This is especially important in adults who may not mount an IgM response. Moreover, IgM can sometimes persist for several weeks to months; antibody production may also be delayed in some infections or even absent if the patient is immunosuppressed. Serological testing of *M. pneumoniae* has been described in depth elsewhere [23].

2.3.2.2 Urogenital *Mycoplasma* and *Ureaplasma* spp.

Despite investigations over several years aimed at developing serological assays for genital mycoplasma and ureaplasma infections, such assays have never been standardized and are not generally available or recommended for diagnostic purposes.

2.4 Molecular-Based Mollicute Detection, Identification, and Genotyping

Over the past three decades, hundreds of publications have described various nucleic acid amplification tests (NAATs) and their applications to detect mycoplasmas and ureaplasmas in clinical specimens. These assays enable detection of small numbers of extremely fastidious species such as *M. genitalium* that might never be detected otherwise and identification of organisms to the species level as in the case of ureaplasmas. Many different target genes have been used as described in subsequent sections. NAATs have also been adapted to detect antimicrobial resistance determinants and to analyze genetic relatedness of clinical isolates. Real-time PCR is capable of amplifying and simultaneously detecting and quantifying the target DNA molecule as it accumulates during the reaction in *real time* after each amplification cycle. Examples of detection systems include agarose gel electrophoresis, SYBR green, TaqMan probes, hybridization probes, molecular beacons, and microchip electrophoresis [33]. Other NAATs applied to mollicutes include nucleic acid sequence-based amplification (NASBA) [34], loop-mediated isothermal amplification (LAMP) [35], transcription-mediated amplification (TMA) [36], and recombinase polymerase amplification (RPA) [37]. Since organism viability does not have to be maintained for NAAT-based detection, specimen collection, handling, and transport are much simpler than for culture. Moreover, NAAT is inherently more sensitive than culture, when optimum conditions and gene targets are employed, making it an attractive alternative in many clinical and research settings. Real-time PCR can potentially provide results the same day a specimen is received and provide quantitative data to determine the bacterial load in a clinical specimen. This can be important for interpretation of results for organisms that are known to colonize asymptomatic persons. Some of the newer automated real-time PCR systems incorporate both DNA extraction and real-time PCR reactions such that no additional manipulation of a clinical specimen is required once it is placed into the instrument. This can be important from the standpoint that it substantially reduces risk for cross-contamination and lessens hands-on technologist time, further reducing turnaround time from when a specimen is received to when results can be reported. Both monoplex and multiplex PCR assays have been developed.

PCR and selective or whole-genome sequencing (WGS) are now popular methods for characterization of mollicutes and determining genetic relatedness, but other molecular-based methods provided some useful information before PCR-based methods were widely available. These include immunoblotting with monoclonal antibodies, two-dimensional gel electrophoresis or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), pulsed field gel electrophoresis

(PFGE), DNA-DNA hybridization, and restriction endonuclease cleavage. The general conclusion of most studies has been that many of these organisms exhibit a great deal of heterogeneity as a result of antigenic variation and gene transfer within and among species.

Currently, there are four commercial NAATs for detection of *M. pneumoniae* and one for *M. genitalium* that are FDA-cleared, and others are currently under review. Various methods for strain typing such as P1 typing, multilocus tandem variable repeats (MLVA), and multilocus sequence typing (MLST) have been applied to improve understanding of the epidemiology of mollicute infections.

2.4.1 *Mycoplasma* spp.

2.4.1.1 M. amphoriforme

Pitcher described the first PCR for detection of *M. amphoriforme* based on 16S r RNA [38]. Subsequently, Ling developed a quantitative real-time PCR targeting uracil DNA glycosylase (*udg*) and one targeting the variable region of 23S rDNA [1]. A TaqMan real-time PCR targeting the *M. amphoriforme* gene MAMA39_00510 was developed at the UAB Diagnostic Mycoplasma Laboratory and shown to be sensitive and specific. Primers and probes for the assay are Mam198-F: 5′-ATTT CCTTTTTTGCATTATGTCC-3′, Mam198-R: 5′-CTCGTTTTCTTTCACTTATG TT-3′, and Mam198-TP: 5′-FAM-CACTTTTTTTGTTTTCGCGGCTTTT-TAM RA-3′.

WGS of the type strain A39 and 19 M. amphoriforme isolates found that most isolates from the United Kingdom and two from France and Tunisia were closely related, while others were more diverse [7]. WGS data also showed that patients can be chronically infected with *M. amphoriforme* and that transmission of strains occurred [7]. Antibiotic resistance mutations accumulated in isolates taken from patients who received multiple courses of antibiotics.

2.4.1.2 M. fermentans

The most commonly used PCR target has been the 16S rRNA gene [39, 40] although other targets such as insertion sequence IS1550 and *malp* (macrophage-activating lipopeptide gene) [41] have been used. The UAB Diagnostic Mycoplasma Laboratory has adapted and validated a real-time PCR procedure to detect *M. fermentans* originally developed by Blanchard [40] for conventional PCR using the 16S rRNA gene target. Primers from the 16S rRNA gene are RNAF1: 5′-CAGTCGATAATTTCAAATACTC-3′ and RNAF2: 5′ GGCACCGTCAAAA CAAAAT-3′. Schaeverbeke genotyped seven strains isolated from synovial fluids of seven arthritis patients and compared them to three reference strains and a clinical isolate using arbitrarily primed PCR, conventional restriction enzyme analysis, PFGE, and Southern blotting. Four synovial fluid isolates were genetically related to the reference strain PG-18, while the remaining ones and a urethral isolate were related to reference strain K7 and *incognitus* strain. Campo [43] examined 21 *M. fermentans* strains using PCR, Southern blotting and DNA hybridization,

SDS-PAGE, and Western blotting with monoclonal antibodies and determined that there were two genotypes represented which differed with respect to the sites of insertion of the IS element, but not in the sequence of the element itself. The heterogeneity of *M. fermentans* supports the need for larger studies. The published genome sequences for *M. fermentans* may also facilitate design of better PCR assays [44].

2.4.1.3 M. genitalium

Lack of reliable cultivation techniques for *M. genitalium* has necessitated intense study of molecular-based techniques for detection and characterization, especially as evidence accumulates for its importance as an etiologic agent of urethritis, cervicitis, and pelvic inflammatory disease [14]. PCR assays for detection of *M. genitalium* developed in the early 1990s to amplify various fragments of the MgPa adhesin protein derived from the original G37 type strain [45, 46]. Additional PCR assays have targeted various regions of the MgPa operon as shown in Table 2.4. Other reports soon followed with a variety of formats for PCR assays using this gene target and 16S rRNA [47–49]. Techniques including TMA have also been utilized for epidemiological purposes [50–52]. As with other mollicutes, quantitative, rapid, real-time PCR assays have been developed that allow measurement of bacterial load in clinical specimens using targets such as MgPa, 16S rRNA, 115-kDa gene, and *gap* encoding glyceraldehyde-3-phosphate dehydrogenase [49, 52–58]. Ma [58] examined the three genes of the *M. genitalium* MgPa operon and nine repetitive sequences (MGPar) to investigate their sequence variability and its potential implication for both pathogenesis and development of diagnostic tools. Operon sequences as well as all MgPars differed from each other more than from the published G37 operon sequence at both the nucleotide and deduced amino acid levels. Due to concerns for use of the MgPa target because of variations among *M. genitalium* strains, the UAB Diagnostic Mycoplasma Laboratory adapted the real-time PCR assay described by Svenstrup [56] which targets the conserved housekeeping gene *gap* (NCBI accession no. U39710) in a primer and probe system. This target is different from other species and is present in the genome as a single copy [56]. The forward primers (10 μm) are mg-gap-605f: 5′-GTGCTCGTGCTGCAGCTGT-3′, and reverse primers (10 μm) are mg-gap-794r: 5′-GTCCATCTGTTGAACAAGT AAATCAAGC-3′. Probes (4 μm) consist of a fluorescein-labeled probe are mg-gap-669FL: 5′-TGTTGTTCCAGAAGCAAATGGCAAACTT-FL-3′ and (4 μm) Red640 probe mg-gap-700LC: 5′-LCRed640-GGGATGTCACTCCGTGTTCC AGTGT-phosphate-3′.

PCR-based systems for detection of *M. genitalium* alone or multiplexed with other sexually transmitted pathogens and/or other urogenital mollicute species are sold as kits in several countries using various formats, gene targets, and instrument platforms [59–63].

Hologic, Inc. (Marlborough, MA) developed TMA technology for commercial use targeting 16S rRNA for the detection of *M. genitalium* in clinical specimens [36, 64]. This assay was cleared for clinical diagnostic testing in the United States in January 2019, making it the first molecular diagnostic test for *M. genitalium* available for commercial use in this country. Since that time, an additional molecular

Table 2.4 Examples of diagnostic PCR assays targeting the MgPa operon of *Mycoplasma genitalium*

Forward primer (5′-3′)	Reverse primer (5′-3′)	Reference
MgPa-1, AGTTGATGAAACCTTAACCCCTTGG	MGpA-3, CCGTTGAGGGGTTTTCCATTTTTGC	[46]
Mg1 (outer) TGTCTATGACCAGTATGTAC Mg3 (inner) GTAATTAGTTACTAGTAGA	Mg2, CTGCTTTGGTCAAGACATCA	[45]
MgPa-476, ATGGCGAGCCTATCTTTGATCCTTTTAA	MgPa-903, TTACCTCCCCACTACTGTCCTTATGC	[195]
G3A, GCTTTAAACCTGGTAACCAGATTGACT	G3B, GAGCGTTAGAGATCCCTGTTCTGTTA	[196]
MGS-1, GAGCCTTTCTAACCGCTGC	MGS-4, GTTGTTATCATACCTTCTGAT MGS-2, GTGGGGTTGAAGGATGATTG	[197]
Mg1a, GGTTAACTTACCTTAGTGGCTTTGATC Mg3 (inner), GTAATTAGTTACTCAGTAGA	MGS-2, GTGGGGTTGAAGGATGATTG	[198]
ModMgPa1, TGAAACCTTAACCCCTTGG	ModMgPa3, AGGGGTTTTCCATTTTTGC	[199]
MgPaW1, AATGGAGCGATCATTACTAAC	MgPaWR1, CCTTGTTATCATACCTTCTGA	[200]
MgPa-355F, GAGAAATACCTTGATGGTCAGCAA	MgPa-432R, GTTAATATCATATAAGCTCTACCGTTGTTATC	[57]

test, the Roche Cobas TV/MG PCR (Roche Diagnostics, Indianapolis, IN) which detects both *M. genitalium* and *Trichomonas vaginalis* has also received FDA clearance. The Hologic Aptima assay displayed greater sensitivity than other lab-developed or CE-marked DNA-based tests in two comparison studies conducted in Europe [65, 66].

In *M. genitalium,* macrolide resistance has increased dramatically over the past several years [67]. Macrolide treatment failures for *M. genitalium* infections associated with mutations in domain V of the 23S rRNA gene that affect binding of the antibiotic at the target site are becoming more common over the past decade with pooled cure rates in 12 studies prior to 2009 of 85% compared to 67% in 9 studies since 2009 [68]. Resistance to the second-line drug, moxifloxacin, is also beginning to occur as a result of mutations in DNA gyrase and/or topoisomerase IV. According to a recent study from Alabama [67], 60% of African American couples had M. genitalium strains with mutations conferring macrolide resistance, 11% of whom also had strains with mutations conferring fluoroquinolone resistance highlighting the potential for potential treatment failure. The 2016 European guidelines on *M. genitalium* infections recommended complementing the molecular detection of *M. genitalium* with an assay capable of detecting macrolide resistance-associated mutations [69]. NAATs capable of detecting *M. genitalium* in clinical specimens simultaneous with detection of 23S rRNA mutations conferring macrolide resistance have been developed and used to study epidemiology of macrolide-resistant *M. genitalium* [70–75]. The *ResistancePlus MG* (SpeeDx, Australia) has been developed for commercial distribution in Australia and Europe and is under development for eventual sale in the United States. This PCR utilizes PlexZyme/PlexPrime technology for the detection of *M. genitalium* (MgPa adhesin gene) and the five predominant 23S rRNA macrolide resistance-associated mutations. *ResistancePlus MG* has been compared to lab-developed assays [76–79], and its performance was generally satisfactory. There are no commercial products for detection of fluoroquinolone resistance in *M. genitalium* as of early 2022 [67].

The UAB Diagnostic Mycoplasma Laboratory currently utilizes a real-time PCR assay that detects *M. genitalium* in urine and vaginal specimens and determines whether macrolide resistance-associated mutations are present, based on a modification of the PCR described by Touati [72]. Details of the assay are shown in Table 2.5. This PCR was shown to be superior to the PCR targeting *gap* gene [73] and enables simultaneous detection of *M. genitalium* and macrolide resistance-associated mutations in clinical specimens in a single procedure [73].

In 1999, Kokotovic typed *M. genitalium* isolates based on whole-genome fingerprinting involving selective amplification of restriction fragments obtained from purified DNA of cultured strains [80]. Typing methods that are not dependent on having a cultivated clinical isolate have since been developed because culture in vitro is so difficult. Molecular typing methods are based on PCR amplification of a specific genomic locus followed by DNA sequencing or restriction fragment length polymorphisms (RFLP) analysis. Specific methods have included short tandem repeat (STR) analysis of putative lipoprotein gene MG309 [81],

Table 2.5 Primers and probes used in real-time PCR for primary detection and identification of macrolide resistance in *M. genitalium*[a]

Sequence name	Reference name	Sequence	Reference
MGMR-F	F1-Mg	GAAGGAGGTTAGCAATTTATTGC	[72]
MGMR-R	R1-Mg	TTCTCTACATGGTGGTGTTTTG	[72]
MGMR-P1	Anchor probe	CGGGTGAAGACACCCGTTAGGC-fluorescein	[72]
MGMR-P2-2	Sensor probe	LC-Red 705-AACGGGACGGAAAGACCCCG-phosphate	[73]

[a]The MGMR PCR procedure developed at the UAB Diagnostic Mycoplasma Laboratory uses the fluorescence resonance energy transfer coupled with melting curve analysis method initially described by Touati [72] and performed on a LightCycler® 480 II (Roche Diagnostics, Indianapolis, IN). To improve the signal, the sensor probe was modified. Details of the PCR procedure that enables simultaneous detection of *M. genitalium* and macrolide resistance mutations are available in the publication by Xiao [73]

single-nucleotide polymorphisms (SNPs) in the rRNA operon [81], RFLP of the *mgpC* gene [82], and SNPs in the *mgpB* gene [54, 83].

In one study [83], a PCR assay based on the *mgp* gene of the MgPa operon using the MgPa-1/MgPa-3 primer set was applied to urogenital specimens from multiple countries. This method identified 29 different sequences from 52 unrelated patients and also demonstrated that 79 *M. genitalium*-positive specimens from 19 couples had concordant sequence types, indicative of sexual transmission. They were also able to demonstrate acquisition of new strains concordantly in sexually active couples. Occurrence of a few large clusters indicated either spread of certain strains or particularly common sequence types that were detected in specimens from different countries. These findings indicate the heterogeneity of *M. genitalium* from clinical specimens.

MLVA is based on variable number of tandem repeats (VNTRs) located in different genetic loci. Ma [84] used MLVA to identify 18 loci in the G37 *M. genitalium* reference strain containing short tandem repeat sequences (STRs) and determined that combination of *mgpB* SNPs and MG 309 STRs complements one another, thereby providing greater typing efficiency, and may define genetic relationships more accurately than other methods. We used MLVA to type clinical samples to study the concordance of the *M. genitalium* infection and occurrence of macrolide and fluoroquinolone resistance in 116 heterosexual couples [67]. MLVA typing of direct clinical samples not only reveals the source the infection but also tracks the spread of drug-resistant strains.

2.4.1.4 M. hominis

Conventional PCR assays for *M. hominis* have mainly utilized 16S rRNA and rDNA as gene targets [85–87]. Since heterogeneity has been reported in the 16S rRNA gene [88], other targets including the housekeeping genes such as *yidC*, *ftsY*, *tuf*, and *gap* have been used [89–91]. However, variations have been observed in clinical isolates in some of these genes that lead to false-negative results. The UAB

Diagnostic Mycoplasma Laboratory has utilized a TaqMan real-time PCR assay to detect *M. hominis* in clinical specimens based on RNA polymerase beta subunit (*rpoB*) gene. Primers and probe for this assay are forward primer MH rpoB-F: 5′-C0AACCAATATTAGAGCAACCAGA-3′, reverse primer MH rpoB-R: 5′-TCGCCRGCCTTTGAATGT-3′, and a locked nucleic acid (LNA) probe MH rpoB-P: 5′-[FAM]tca[+c]at[+t]ga[+a]ga[+a]caatc[BHQ1]-3′. Analysis of the genome for the *M. hominis* PG21 type strain indicated that this organism has undergone horizontal gene transfer with *Ureaplasma* spp., but not with *M. genitalium* [21]. Ladefoged and Christiansen [92] constructed physical and genetic maps of the genomes of five *M. hominis* strains obtained from different individuals. Genome sizes obtained by PFGE ranged from 704 to 825 kb. Restriction patterns varied greatly, and none of the strains had identical patterns. Other methods including serology, DNA-DNA hybridization, two-dimensional gel electrophoresis, SDS-PAGE, RFLP, random amplified polymorphic DNA (RAPD), and immunoblotting with monoclonal antibodies have also confirmed heterogeneity [93–97]. Blanchard [93] used conventional PCR to evaluate genetic variation within the 16S rRNA gene of 51 *M. hominis* isolates confirming high intraspecies conservation within this gene. However, 16S rRNA gene sequencing also documented differences at five positions when one isolate was compared to the PG21 reference strain. Jensen [94] studied 60 *M. hominis* isolates from pregnant women and their offspring using SDS-PAGE and PFGE and determined that sequential isolates from the same women were identical or nearly identical, suggesting adaptation to the host environment, whereas those from different women exhibited considerable variation with respect to both genomic and antigenic profiles. On the basis of restriction band patterns of protein P75, *M. hominis* can be divided into several groups. Férandon [89] used MLVA to evaluate 210 urogenital and extragenital isolates of *M. hominis* collected in France over two decades and confirmed a high degree of genotypic heterogeneity with 40 different MLVA types identified. Multiple isolations from the same person indicated persistence or relapse rather than new infection with a different strain. They were also able to confirm vertical transmission from mother to neonate in two instances and showed that the high rate (66%) of tetracycline resistance in this collection was not clonal.

2.4.1.5 M. pneumoniae

Gene targets for PCR assays have included 16S rRNA, 16S rDNA, P1 adhesin gene, *tuf* gene, *parE* gene, *dnak* gene, *pdhA* gene, ATPase operon, CARDS toxin gene, and noncoding repetitive element *repMp1* [23].

The UAB Diagnostic Mycoplasma Laboratory adapted the real-time PCR assay of Dumke targeting the *repMp1* noncoding DNA sequence for routine diagnostic use. Its theoretical advantage is that sensitivity may be improved by amplification of a multicopy gene [98]. The primers are MpLCrepF: 5′ TCTTTACGCGTTACG TATTC-3′ and MpCrepR: AGTGTGGAATTCTCTGGCA-3′. The probe consists of MpLCrepS: 5′FAM-CTGGTATAACCGGTTTGTTAAG-TAMRA-3′. This assay provides acceptable sensitivity and specificity when tested against several reference strains as well as a large group of specimens from patients with radiologically

proven pneumonias, representing both major P1 subtypes, who were positive for *M. pneumoniae* by serology, culture, and/or conventional PCR. PCR has also been shown to be advantageous for diagnostic purposes for testing preserved lung tissue obtained at biopsy [99]. The advantage of real-time PCR in detection of systemic infection was demonstrated in a study which found 15 of 29 (52%) patients with serological evidence of *M. pneumoniae* infection had a positive assay, while conventional PCR was uniformly negative [100].

For PCR-positive, culture-negative patients, it is important to ascertain whether clinically significant respiratory disease is actually present, since this may reflect asymptomatic carriage with a very low bacterial load, prior antibiotic therapy, persistence of DNA after resolution of infection has occurred, organisms residing in an intracellular location not amenable to culture, or perhaps a nonspecific PCR target. A positive PCR assay in a patient who is serologically negative may indicate that the specimen was obtained too early in the infection for measurable antibody to have developed, antimicrobial therapy that may have blunted the immune response, or an inadequate immune response due to any type of immunosuppressive condition. Negative PCR results in patients who are culturally and/or serologically positive could indicate technical problems with the PCR or inhibitors. Combining PCR with serology has been advocated as a possible means to distinguish colonization from active disease, but this also adds to the cost of testing and will not overcome the problem common in adults who do not mount an acute phase IgM response.

There is no universal consensus regarding what constitutes the best respiratory specimen to be tested by PCR. Combining nasopharyngeal and oropharyngeal specimens may provide the greatest diagnostic yield [101]. Another study reported that sputum was superior to oropharyngeal or nasopharyngeal specimens in young adults with serologically proven *M. pneumoniae* infection [102]. From a practical standpoint in sampling young children and many adults with fairly mild illness, sputum is not produced, so nasopharyngeal or oropharyngeal samples may be the only specimen types available.

A study from the Centers for Disease Control and Prevention [103] compared three real-time PCR assays to detect *M. pneumoniae* prospectively in an outbreak investigation utilizing the Applied Biosystems ABI 7500 system employing two different TaqMan primer-probe sets targeting the ATPase gene and a new assay targeting the CARDS toxin gene on 54 respiratory samples. Primers/probes for the CARDS toxin were M181-F: TTTGGTAGCTGGTTACGGGAAT; M181-R: GGTCGGCACGAATTTCCATATAAG; and M181-P: TGTACCAGAGCACCCCA GAAGGGCT. Eighteen cases were positive with all three assays. When dilutions of *M. pneumoniae* reference strains were tested, the CARDS toxin PCR assay consistently detected 1–5 CFU, while the other two assays targeting the ATPase genes detected 5–50 CFU. These findings support further study of the CARDS toxin gene as a PCR target. Two published studies describing multicenter comparisons of various NAATs for *M. pneumoniae* detection [104, 105] reported significant variations in test performance among participating laboratories, making a strong case for an organized proficiency test program, which has been used in Europe [106].

Multiplex PCR assays have been developed in a variety of formats for detection of *M. pneumoniae* along with other respiratory pathogens such as *Chlamydia pneumoniae* and *Legionella pneumophila*. Multiplex assays for detection of *M. pneumoniae* and other respiratory pathogens have also been combined with other techniques including reverse line blot assays and microarrays [107–109].

The LAMP technique amplifies DNA under isothermal conditions with high efficiency, specificity, and speed and has been applied to detection of *M. pneumoniae* in clinical specimens using the P1 gene sequences in direct comparison to real-time PCR with the P1 gene target [110]. They showed this assay to be specific with a detection limit of 200 copies and found 100% concordance with real-time PCR.

Real-time NASBA uses DNA hybridization probe molecular beacons that fluoresce only upon hybridization with their targets, and detection takes place in a fluorescence reader [111]. NASBA can provide rapid results with sensitivity as good as or better than PCR, with reports of a detection threshold as low as 5–50 CFU [111, 112]. The main advantage of NASBA is that it works at isothermal conditions. This assay has been described in monoplex and multiplex format and has been developed as a commercial kit (NucliSENS, bioMérieux). Multiplex NASBA has a slightly lower sensitivity than monoplex NASBA when applied to dilutions of wild-type in vitro-generated RNA [113].

Numerous commercial NAATs for detection of *M. pneumoniae*, including monoplex and multiplex systems, used in Europe for several years work in a comparable manner to noncommercial assays [23, 114–116]. There are now several commercial molecular-based systems that have received FDA clearance and are now sold in the United States. The BioFire Diagnostics FilmArray® Respiratory Panel (RP) (Salt Lake City, UT) detects nucleic acids in nasopharyngeal swabs for several viruses and bacteria, including *M. pneumoniae*. The newer FilmArray® Respiratory Panel (RP) EZ is a Clinical Laboratory Improvement Amendment (CLIA)-waived version that tests for 14 respiratory viruses and bacteria, including *M. pneumoniae*. The GenMark ePlex® Respiratory Panel (RP) (GenMarkDx, Carlsbad, CA), like the BioFire, combines extraction, amplification, and detection in one multiplex system for detection of respiratory viruses and bacteria, including *M. pneumoniae*, with results available in about an hour. The NxTAG Respiratory Pathogen Panel (Luminex Molecular Diagnostics, Inc., Toronto, ON, Canada) detects several viruses and bacteria, including *M. pneumoniae*. Up to 96 extracted samples are analyzed by multiplex PCR and bead hybridization and read on the MAGPIX instrument. The Meridian Bioscience (Cincinnati, OH) illumigene Mycoplasma Assay is a monoplex LAMP assay that can easily be incorporated into clinical microbiology laboratories that do not have extensive molecular diagnostic facilities. Another approach that has undergone preliminary evaluation to detect *M. pneumoniae* in a rapid and cost-effective manner is the utilization of a microfluidic real-time PCR platform in which it uses electrical fields to rapidly and precisely manipulate discrete nanoliter-sized droplets within an oil-filled chamber under the control of a software program. A summary of some of the more recently described NAAT assay formats for detection of *M. pneumoniae,* that have been included in published articles, including comparison studies, is provided in Table 2.6.

Table 2.6 Examples of commercial nucleic acid amplification tests for detection of *M. pneumoniae*[a]

Product	Technique/detection	Pathogens detected	Manufacturer	References
Loopamp *Mycoplasma pneumoniae* DNA amplification kit	LAMP[b], turbidity	*M. pneumoniae*	Eiken Chemical, Tokyo, Japan	[201–203]
Illumigene Mycoplasma	LAMP[b], turbidity	*M. pneumoniae*	Meridian Bioscience Inc., Cincinnati, OH	[117, 204]
FilmArray RP[c]	Multiplex PCR, microarray	*B. pertussis*, *M. pneumoniae*, *C. pneumoniae*, and several respiratory viruses	bioMérieux/BiFire Diagnostics, Inc., Salt Lake City, Utah	[117, 205, 206]
ePlex Respiratory Panel (RP)[c]	Multiplex PCR, hybridization, electrochemical detection	*M. pneumoniae*, *C. pneumoniae*, and several respiratory viruses	GenMarkDx, Carlsbad CA	[117, 207]
NxTAG Respiratory Pathogen Panel (RPP)[c]	Multiplex RT-PCR, hybridization		Luminex Molecular Diagnostics, Inc., Toronto, ON, Canada	[208, 117]
Chlamylege	Multiplex PCR, hybridization	*M. pneumoniae*, *C. pneumoniae*, *Legionella* spp.	Argene, Inc. Shirley, NY	[116]
ArgeneChla/Myco pneumo assay	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*	bioMérieux/Argene Marcy l'Etoile, France	[209]
Mycoplasma/Chlamydophila pneumoniae real-time PCR kit	Duplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*	Diagenode, Liege, Belgium	[210, 211]
Simplex *M. pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	Focus Diagnostics, Cypress, CA	[211]
Venor MP	Monoplex PCR, agarose gel, and real time	*M. pneumoniae*	Minerva Biolabs, Berlin, Germany	[106, 115, 211]
Mycoplasma pn. Q-PCR Alert	Monoplex PCR, real time	*M. pneumoniae*	Nanogen Advanced Diagnostics, Buttigliera Alta, Italy	[211, 106]

(continued)

Table 2.6 (continued)

Product	Technique/detection	Pathogens detected	Manufacturer	References
Genaco Resplex I	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *H. influenzae*, *N. meningitidis*	Qiagen, Venlo, Netherlands	[106]
RespiFinder 19	Multiplex PCR, capillary electrophoresis	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, and several viruses	Patho Finder, Maastricht, Netherlands	[106]
RespiFinder SMART 22	Multiplex PCR, real time	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, and several viruses	Patho Finder, Maastricht, Netherlands	[209]
Seeplex PneumoBacter ACE	Multiplex PCR, capillary electrophoresis	*S. pneumoniae*, *H. influenzae*, *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*, *B. pertussis*	Seegene, Seoul, Korea	[209]
GeneProof *M. pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	GeneProof, Brno, Czech Republic	[210]
BactoReal *Mycoplasma pneumoniae*	Monoplex PCR, real time	*M. pneumoniae*	Ingenetix GmbH Vienna, Austria	[210]
M. pneumoniae LightMix kit	Monoplex PCR, real time	*M. pneumoniae*	TIB MolBiol, GmbH, Berlin, Germany	[210]
BD Probe Tec ET	Strand displacement, fluorescence	*M. pneumoniae*	BD Diagnostics, Sparks, MD	[106]
NucliSENS Easy Q	Multiplex NASBA[d], molecular beacons	*M. pneumoniae*, *C. pneumoniae*	bioMérieux, Marcy l'Etoile, France	[34, 212]

[a]Table has been adapted from Waites et al. [23] in *Clinical Microbiology Reviews* with permission from the publisher. Examples are limited to commercial assays for which publications were available. Some products evaluated in previous years may no longer be available or may now be sold by different companies
[b]*LAMP* loop-mediated isothermal amplification
[c]FDA cleared and available in the United States
[d]*NASBA* nucleic acid sequence-based amplification

The genome of *M. pneumoniae* M129 (type P1-1) was sequenced in 1996 and reannotated in 2000 [119, 120]. The sequence of the FH strain (type P1-2) was subsequently published along with another strain designated 309 [121, 122]. Fifteen strains collected over a span of many years in the United States, China, and England have been sequenced and annotated using NGS technology, demonstrating an overall high degree of sequence similarity among them and conservation of most genes within the two major subtypes. The major variation was detected in the P1 and open reading frame (ORF) 6 of the adhesin complex with no evidence of horizontal gene transfer [123]. Study of another 23 clinical isolates, mainly from Europe along with transcriptomes and proteomes of two strains, one from each of the two main P1 subtypes, revealed new subclasses related to geographic origin of the strains [25]. Initial genotyping studies for *M. pneumoniae* established two P1 subtypes based on type-specific repetitive elements [124, 125]. Recent advances in WGS have enabled more regions in the genome with high discriminatory power to be identified and utilized to develop typing systems such as MLST, MLVA, and SNP techniques that do not require bacterial isolates. A summary of these methods is provided in Table 2.7. RFLP uses several enzymes to digest the amplicon and group them based on their band patterns [126]. Denaturing gradient gel electrophoresis (DGGE), which can detect single-base differences, can also be employed to distinguish P1 PCR products [127]. PCR high-resolution melt provides a rapid one-step method for typing *M. pneumoniae* isolates [128] that differentiates P1 subtypes without sequencing or hybridization procedures. To type *M. pneumoniae* directly in clinical specimens, a culture-independent amplification and sequencing method was developed by Dumke [129]. Random amplification of polymorphic DNA (RAPD) analysis has also been used to classify *M. pneumoniae* strains according to P1 subtypes with results similar to those obtained by PCR-RFLP [126, 130]. NASBA has been used to amplify RNA under isothermal conditions followed by hybridization using 16S rRNA to assess differences between the two P1 subtypes [131]. Another DNA amplification method for distinguishing the P1 subtypes is the amplified fragment length polymorphism (AFLP) technique. Although the sequence variation of the RepMp elements in the P1 gene was the initial basis on which to classify the two main *M. pneumoniae* subtypes, other sequence differences between the subtypes are observed throughout the genome [123, 132–134]. With the identification of more P1 subtype variants that are generated by homologous recombination, P1 typing itself appears to be ambiguous to classify *M. pneumoniae*. However, according to WGS analysis of clinical isolates collected from various times and geographic regions, the two subtypes appear to be evolutionary stable lineages [25, 123, 134].

Differences in the two main P1 subtypes may be operative in the development and cycling of epidemics. One or the other subtype may predominate in specific geographic areas and that there can be changes in the predominance of one or the other subtype over time [23]. However, this is not always the case according to other studies in which both subtypes may occur in about the same proportions [135]. This predominance of one or another subtype may be due to development of subtype-specific antibodies following initial infection that provide some degree of protection for that subtype but not the other. Dumke reported differences between the P1

Table 2.7 Summary of typing methods for *Mycoplasma pneumoniae*[a]

Typing scheme	Technique	Types	Advantages	Disadvantages	References
Single-gene/locus typing		Subtypes 1 and 2, plus variants	Separate clinical isolates into two major stable subtypes	Not able to track strains	
P1 typing	Restriction fragment length polymorphism (RFLP)				[126, 138, 213]
	Denaturing gradient gel electrophoresis (DGGE)				[127]
	Real-time PCR coupled with high-resolution melt (HRM) analysis		Fast		[214]
	PCR and sequencing		Culture-independent		[129]
16S rRNA gene	Nucleic acid sequence-based amplification (NASBA)				[131]
16S-23S rRNA gene spacer region	PCR and sequencing				[129]
SNPs in MPN528a and P1	Pyrosequencing				[132]
MPN459 and MPNA5864	Duplex real-time PCR		Fast, culture-independent		[140]
Multilocus variable tandem repeat analysis (MLVA)			More discriminative than P1 typing	No correlation with P1 types	[135]
Five-locus scheme	Multiplex PCR and capillary electrophoresis/sequencing	>26 MLVA types	Strain tracking	Locus 1 is not stable, too discriminative	[215, 216]
Four-locus scheme	Multiplex PCR and capillary electrophoresis/sequencing	Less than major MLVA types	Stable locus, correlation with macrolide-resistant phenotype	Less discriminative than five-locus scheme	[217–219]

(continued)

Table 2.7 (continued)

Typing scheme	Technique	Types	Advantages	Disadvantages	References
Multilocus sequence typing (MLST)			More discriminatory than four-locus MLVA typing and P1 typing methods		
Eight genes/ locus	Snapshot minisequencing	9 SNP types	Correlation with P1 types, fast, culture-independent	No correlation with MLVA types	[142]
Eight housekeeping genes	PCR and sequencing	12 sequence types	Correlation with P1 types and MLVA types, culture-independent	Laborious and expensive sequencing	[141]
Whole bacterial cells					
MALDI-TOF MS typing	Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS)	Subtype 1 and subtype 2, plus variants	Quickly identify and type in one step	Culture-dependent, cost of mass spectrometer	[189, 190]
NA-SERS typing	Nanorod array-surface-enhanced Raman spectroscopy (NA-SERS)	Subtype 1 and subtype 2, plus variants	Detect and type in one step, easy sample preparation		[193]

[a]Table has been reproduced from Waites et al. [23] in *Clinical Microbiology Reviews* with permission from the publisher

subtypes in their capacity to colonize and survive in a guinea pig model and that preinfection of the animals with the different subtypes induced subtype-specific immunity and affected the type of surviving bacteria [136]. However, Ursi found no relationship between subtype and degree of illness in 24 patients [130]. Subtype 1 and subtype 2 strains form different biofilms in vitro [137], and subtype 2 strains may have higher expression of CARDS toxin [25]. Clearly, additional work needs to be done in this area to ascertain whether there is a relationship between P1 subtypes and virulence.

Dorigo-Zetsma [138] carried the P1 subclassification further using RFLP analysis of PCR products of the P1 gene. They identified 5 subtypes within the P1 type 1 group and an additional 3 subtypes within the P1 type 2 group among a collection of 2 reference strains and 21 clinical isolates. The clinical or epidemiological significance of this extended typing scheme has not been determined.

NASBA has been used to detect differences between the subtypes using two type-specific probes [131]. Spuesens used pyrosequencing to discriminate the two subtypes based on an SNP in MPN528a and another SNP in the conserved region of the P1 coding gene [139]. Zhao developed a duplex real-time PCR assay targeting two subtype-specific genes to type *M. pneumoniae* directly from clinical specimens [140] and detected a subtype shift from type 1 to type 2 that occurred in 2013 in Beijing, China. PFGE of *M. pneumoniae* strains allows study of the whole genome as opposed to a single gene [126] and enables division of P1 subtype 2 into two additional subgroups, but it is a very time-consuming and tedious procedure to perform.

An early attempt to develop MLST for typing *M. pneumoniae* failed [134]. Using WGS analyses, a new MLST scheme was developed based on the polymorphisms of eight housekeeping genes [141]. Twelve sequence types (STs) were identified in 57 clinical isolates, and 2 distinct genetic clusters were formed, representing the 2 main subtypes. This scheme is more discriminatory than the four-locus MLVA typing and P1 typing methods. This method is PCR-based and does not require culturing the organism. The disadvantage is a large amount of laborious and expensive sequencing. This MLST scheme for strain typing is supported by a public web-based database (http://pubmlst.org/mpneumoniae). Another multilocus SNP typing method, using Snapshot technology, overcomes the sequencing disadvantage [142]. Similarly, eight genes were selected according to the extensive analysis of the whole-genome sequences of the clinical strains. The Snapshot minisequencing technology is based on the single-base extension of a specially designed minisequencing primer that anneals one base upstream of the SNP using a fluorochrome-labeled dideoxynucleotide (ddNTP). A strong correlation between the SNP types and P1 types was observed.

MLVA was developed for genotyping *M. pneumoniae* directly from clinical specimens by Dégrange [135]. Zhang reanalyzed the published NGS data of *M. pneumoniae* genomes and found 13 VNTRs displaying different levels of inter- and intra-strain copy number variations. Several new MLVA schemes were proposed for different purposes of strain typing [143].

Dumke applied a nested PCR-based assay to *M. pneumoniae* in which part of the repMp2/3 element of the P1 gene was amplified followed by sequencing. The product of the first amplification was then subjected to a semi-nested PCR to amplify the complete repetitive element repMp2/3 [129]. This new approach for molecular typing can theoretically expand epidemiological studies as well as identify emergence of new combinations of repetitive elements in the P1 gene that may occur through homologous recombination.

Macrolide resistance in *M. pneumoniae* is becoming a major problem in Asia and is now spreading to Europe and North America [23]. Real-time PCR assays have been developed to detect three major mutations in domain V of 23S rRNA that confer macrolide resistance directly in clinical specimens [23, 144–146]. This method of direct detection of resistance genes is based on the fact that nucleic acid will melt at a precise temperature that is related to the nucleotide base composition. A rapid and inexpensive method that combines nested PCR, single-strand conformation

polymorphisms (SSCPs), and capillary electrophoresis (CE) detects macrolide-resistant mutants directly from throat swabs [147]. Pyrosequencing has also been applied for detection of macrolide resistance as well as for molecular strain typing [150, 139]. Other direct molecular methods for detection of macrolide resistance are a cycleave PCR method [148], a combination of nested PCR, single-strand conformation polymorphisms (SSCPs) and capillary electrophoresis [149], simple probe PCR [150], and allele-specific PCR amplification [151].

The UAB Diagnostic Mycoplasma Laboratory performs a multiplex real-time PCR assay to detect point mutations in all three positions of the 23S rRNA gene as shown in Fig. 2.2. This assay, described in detail elsewhere [145], is performed reflexively whenever there is a positive real-time PCR assay for the presence of the organism.

For the detection of macrolide resistance mutations, primers MpnMR2063F and MpnMR2063R as shown in Table 2.8 define a 224 bp amplicon containing the

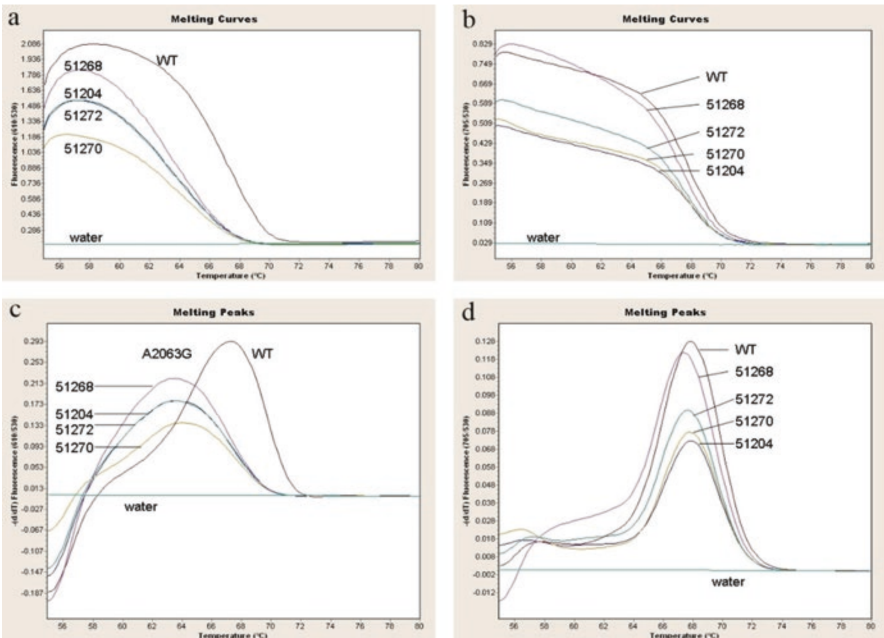

Fig. 2.2 Real-time PCR detection of macrolide-resistant M. pneumoniae in clinical specimens. Genomic DNA of two patients containing the A2063G mutation conferring macrolide resistance verified by sequencing were purified and tested together with a wild-type (WT) control (*M. pneumoniae* strain M129, ATCC #29342). Melting curves (**a**, **b**) and corresponding melting peaks (**c**, **d**) are shown. A2063/A2064 mutations were analyzed in channel 610 (**a**, **c**). The WT melting peak was 67.31 °C, while Tm of A2063G mutants were 63.25 ± 0.04 °C. Thus, a 4 °C difference between WT and mutant was observed. The C2617 assay is shown in channel 705 (**b**, **d**). Because all samples did not have mutations at this position, they showed similar WT Tms of about 68 °C as predicted

Table 2.8 Primers and probes used in real-time PCR for primary detection and identification of macrolide resistance in *M. pneumoniae*[a]

Primers	
MpLCrepF	5′- TCTTTACGCGTTACGTATTC-3′
MpLCrepR	5′- AGTGTGGAATTCTCTGGAA -3′
Probe	
MpLCrepS	5′- FAM-CTGGTATAACCGGTTTGTTAAG-TAMRA-3′
Secondary assay performed on M. pneumoniae-positive specimens to detect 23S rRNA mutations	
Primers	
MpnMR2063F	5′-ATCTCTTGACTGTCTCGGC-3′
MpnMR2063R	5′-CCAATTAGAACAGCACACAACC-3′
MpnMR2617F	5′-GGCTGTTCGCCGATTAAAG-3′
MpnMR2617R	5′-TACAACTGGAGCATAAGAGGTG-3′
Probes	
MpnMR2063P1	5′-GGCGCAACGGGACGGAAAGA-Fluorescein-3′
MpnMR2063P2	5′-LC Red 610-CCGTGAAGCTTTACTGTAGCTTAATATTGATCAGG-Phosphate-3′
MpnMR2617P1	5′-GTCCCTATCTATTGTGCCCGTAGGAAG-Fluorescein-3′
MpnMR2617P2	5′-LC Red 705-TGAAGAGTGTTGCTTCTAGTACGAGAGGACCGAA-Phosphate-3′

[a]Detection method uses Roche LightCycler with the *repMp1* target and procedure originally described by Dumke [98] for primary detection of *M. pneumoniae* and the method described by Li Xiao [145] for identification of 23S rRNA mutations associated with macrolide resistance [145]

2063/2064 position where mutations are likely to occur that is recognized by probes MpnMR2063P1 and MpnMR2063P2.

Several studies carried out by French [135], German [153], Japanese [154, 155], and Chinese [159–161] investigators have examined macrolide-resistant *M. pneumoniae* in an attempt to ascertain how these infections have evolved and spread geographically. No clear association was observed between the macrolide-resistant isolates and P1 or MLVA types. Using the modified four-locus MLVA scheme, investigators in China and the United States found that MLVA type 4-5-7-2 was associated with macrolide resistance [152, 159–161].

2.4.2 *Ureaplasma* spp.

Real-time PCR assays have targeted primarily the urease gene and its subunits [29, 162–164] and the *mba* gene [29, 165]. Yoshida [86] described a conventional PCR-based method that can be applied to urine specimens of patients with NGU. The PCR targets are the 16S rRNA genes of *M. genitalium*, *M. hominis*, *U. parvum*, and *U. urealyticum.* When compared to direct sequencing, this technique produced

similar results and showed no cross-reactivity. Its advantages include less cost and a 2- to 3-hour turnaround time.

The UAB Diagnostic Mycoplasma Laboratory PCR assay for ureaplasmas [29] is based on the *UU063* gene (NP_077893), which encodes a conserved hypothetical protein that is identical in all 4 U. parvum serovars and a 15,072 bp open reading frame (ORF), UUR10_0680, that is conserved (>99.97%) in all 10 U. urealyticum serovars. Primers and probes used in this procedure are provided in Table 2.9. This assay compares favorably with culture and will accurately detect and distinguish the two species. Several studies reported that *U. urealyticum* is more pathogenic than *U. parvum*, but conflicting results have been found by others [12]. Thus, the question is not firmly settled. At present, it is not necessary for clinical diagnostic purposes to identify ureaplasmas to the species level. Some molecular-based assays that include detection of *Ureaplasma* spp. in clinical specimens are commercially available in various European countries, but not in the United States at present. PCR can also be used in combination with other techniques including reverse line hybridization blotting combined with multiplex PCR (mPCR/RLB) and microarrays [107, 108, 166–168]. The mPCR/RLB technique has been used to develop multiplex assays that detect numerous respiratory and urogenital pathogens [107, 109, 168]. In situ hybridization (ISH) is a non-PCR-based molecular technique that has been specifically utilized for detection of ureaplasmas. ISH allows visualization of the localized gene expression within the context of tissue morphology [169].

PCR assays to characterize the *Ureaplasma* spp. at the serovar level have mainly focused on primers based on the *mba* gene and its 5′-end upstream regions [162, 165, 170, 171, 173]. Because of limited sequence variation in the *mba* genes, earlier PCR-based methods lacked the capacity for complete separation of all 14 serovars.

Table 2.9 Primers and probes used in real-time PCR for detection and differentiation of *U. parvum* and *U. urealyticum*[a]

U. parvum primers	
UP063#1F	5′-TGCGGTGTTTGTGAACT-3′
UP063#1R	5′- TGATCAAACTGATATCGCAATTATAGA -3′
Probes	
UP063#1 probe1	5′-TGG-TTT-AAC-GTG-TTT-TTG-AAG-TGC-TAC-AAA-AT--Fluorescein-3′
UP063#1 probe2	5′- LC Red 640-CCC-ATT-TCA-GCC-ATG-GTG-CCA-TCA-Phosphate -3′
U. urealyticum primers	
UU127#1F	5′- CAGTAGCAAATCGTGCTTACA-3′
UU127#R	5′- TCATTAAAATCATTTGCACTAGTCAAAATA-3′
Probes	
UU127#1 probe1	5′-GAT-AAT-AAC-ACT-TGG-ACA-ATT-TTT-AAC-CAA-AGC-GA-Fluorescein
UU127#1 probe2	5′-LC Red 705-AAG-GAT-TAG-AGT-TTT-GTT-GCC-ATG-GTA-GTC-AAA-Phosphate-3′

[a]Details of procedure are available in the publication by Xiao [29]

Moreover, whole-genome sequencing of all 14 serovars has shown *mba* to be part of a large gene family present in many variations in different serovars, and the gene is phase variable [29, 31]. In consideration of the limitations of earlier serotyping techniques, a 14 serovar-specific monoplex PCR assay was developed and shown to accurately distinguish between the 2 species and among all 14 serovars without cross-reactivities [29]. Data from examination of large numbers of clinical isolates using this serovar-specific PCR assay have shown that *Ureaplasma* pathogenicity is unlikely to be associated with specific individual serovars and that horizontal gene transfer among serovars results in many organisms expressing markers of multiple serovars simultaneously. These findings suggest that serotyping is impractical and of limited value for assessment of pathogenicity [174]. Primers and probes utilized to distinguish the 14 *Ureaplasma* serovar type strains are shown in Table 2.10.

PFGE is a widely accepted reference standard for genotyping. A simplified PFGE method has been used to differentiate U. parvum from *U. urealyticum*, to distinguish among the 14 serovars, and a limited number of clinical isolates of the same serovars, making it possible to separate readily the two *Ureaplasma* species from one another and most of the serovars, with the exception of some of the closely related serovars of *U. urealyticum* (Fig. 2.3). Using PFGE to evaluate all 14 serovar type strains and a large number of clinical isolates distributed among the 14 serovars, we were able to show a wide genotypic heterogeneity in *U. parvum* and *U. urealyticum* type strains of different serovars as well as great genetic variation of strains within the same serovar for most of the 14 serovars. This degree of discrimination to and within the serovar level achievable by PFGE cannot be completely achieved by other molecular typing methods currently available which include PCR-based assays targeting single genes and intergenic regions [172].

The RAPD PCR method has been applied to *Ureaplasma* spp. by several investigators [173, 175–178]. It is quicker and less technically demanding than PFGE and is more discriminatory than the single-gene *mba* typing method, but there is no published study comparing PFGE and RAPD-PCR typing of *Ureaplasma* spp.

Glass published the first genome sequence of *U. parvum*, serovar 3 in 2000 [179], providing early insights into virulence factors. WGS of all 14 serovars and 5 additional clinical isolates [31] showed the mba gene was part of a large superfamily, which is a phase variable gene system, and that some serovars have identical sets of mba genes. Most differences among serovars are hypothetical genes, and in general the 2 species and 14 serovars are extremely similar at the genome level. *U. urealyticum* appears to be more capable of acquiring genes horizontally, which may contribute to its greater virulence for some conditions. Our data indicate that ureaplasmas exist as quasi-species rather than as stable serovars in their native environment. Therefore, differential pathogenicity and clinical outcome of a ureaplasma infection are most likely not on the serovar level, but rather may be due to the presence or absence of potential pathogenicity factors in an individual ureaplasma clinical isolate and/or patient-to-patient differences in terms of autoimmunity and microbiome [31, 174].

Zhang and coworkers developed an MLST scheme for U*reaplasma* spp. based on four housekeeping genes [180]. Although it had the capacity to differentiate the

Table 2.10 Primers and probes used in real-time PCR to detect and differentiate *Ureaplasma* spp. serotypes[a]

Specificity	Target gene/region	Primer pair	Probe	Reference
Up and Uu	16S rRNA	Ure-1S, Ure-1A	Ure-P1	[220]
Up	Urease gene subunits and adjacent regions	UPure F-M, Upure R-M	UPure1MGB	[164]
Up	Urease gene subunits and adjacent regions	UU-1524R, UU-1613F	UU-parvo	[163]
Up	*UU063*	UU063#1F, UU063#1R	UP063#1 probe 1, UP063#1 probe 2	[29]
Uu	*mba* gene and upstream regions	UU F, UU R		[162]
Uu	Urease gene subunits and adjacent regions	Uuure F-C, Uuure R-C	Uuure FP	[162]
Uu	Urease gene subunits and adjacent regions	Uuure F-M, Uuure R-M	Uuure2MGB	[164]
Uu	Urease gene subunits and adjacent regions	UU-1524R, UU-1613F	UU-T960	[163]
Uu	*UUR10_0554*	UU127#1F,UU127#1R	UU127#1 probe 1, UU127#1 probe 2	[29]
Serovar 1	*mba* gene and upstream regions	UP F, UP R	UP FP1	[165]
Serovar 1	Member of *mba* gene family but not *mba*	UP1F1-2, UP1R1-2	UP1 probe 1	[29]
Serovar 2	Putative lipoprotein	UU2_F_1, UU2_R_1		[29]
Serovar 3	*mba* gene and upstream regions	UP F, UP R	UP FP3	[165]
Serovar 3	Conserved hypothetical protein	UP3F1-2, UP3R1-2		[29]
Serovar 4	Intergenic	Uu04_1F, Uu04_1R	Uu04_1 probe 1, Uu04_1 probe 2	[29]
Serovar 5	ATP-dependent RNA helicase	Uu05-3F, Uu05-3R	Uu05-3 probe	[29]
Serovar 6	*mba* gene and upstream regions	UP F, UP R	UP FP6	[165]
Serovar 6	*mba*	UP6F1, UP6R1		[29]
Serovar 7	*mba*	UU7_F_1, UU7_R_1		[29]
Serovar 8	Intergenic	UU8_F_1, UU8_R_1		[29]
Serovar 9	FtsK/spoIIIE family protein gene	UU9_F_1, UU9_R_1		[29]
Serovar 10	Member of *mba* gene family but not *mba*	UU10_F_4, UU10_R_4	UU10_P_4	[29]
Serovar 11	Intergenic	UU11_F_1, UU11_R_1	UU11_P_1	[29]

(continued)

Table 2.10 (continued)

Specificity	Target gene/region	Primer pair	Probe	Reference
Serovar 12	Conserved hypothetical protein	Uu12_1F, Uu12_1R	Uu12_1 probe 1, Uu12 _1 probe 2	[29]
Serovar 13	Conserved domain protein, intergenic region, and putative single-strand binding protein	UU13_F_1, UU13_R_1		[29]
Serovar 14	*mba* gene and upstream regions	UP F, UP R	UP FP14	[165]
Serovar 14	Intergenic	UP14F1, UP14R1		[29]

[a]Actual primer sequences are not included due to space limitations but they can be found in the individually cited publications

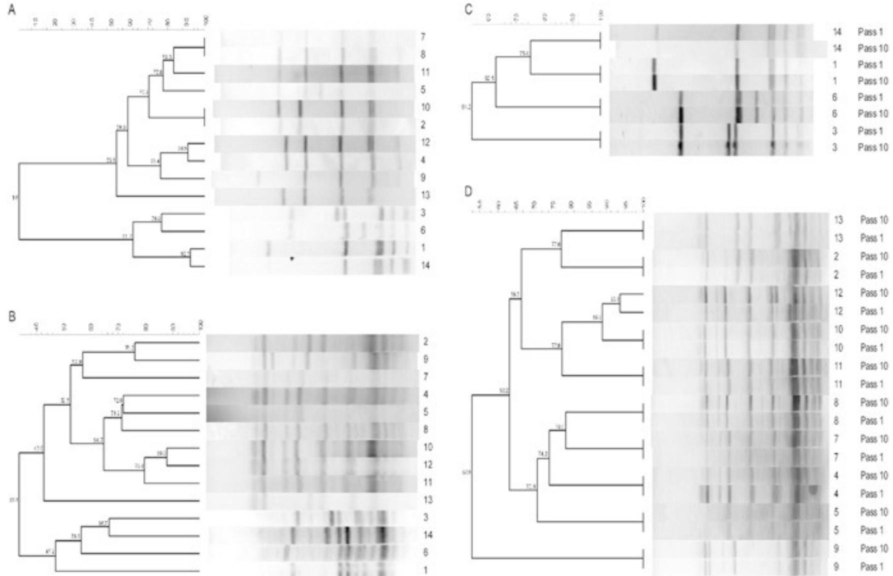

Fig. 2.3 PFGE banding patterns and dendrograms for 14 ATCC serovars of *Ureaplasma* spp. *BamH* I restriction pattern. The two species were separated. Except serovars 10 and 12, all of the serovars were also separated

2 species into cluster I (*U. parvum*) and cluster II (*U. urealyticum*), it was inadequate to separate the 14 serovars or associate sequence types with clinical outcomes. They subsequently developed an expanded MLST typing scheme [181] and performed further studies on *Ureaplasma* isolates from infertile couples [182, 183]. Isolates from cluster II were more likely to transmit between infertile couples and be associated with clinical manifestations including impaired sperm motility. Fernández [184] utilized MLST to reveal a high degree of diversity with 14 clones

among *U. parvum* and *U. urealyticum* isolates from the United States. These findings were similar to what was reported earlier in China [180] and Switzerland [185]. Most MLST clones found in the United States are the same as those from the other countries. Importantly, seven of the clones found in the United States study had been associated with fluoroquinolone resistance in the Swiss study. The MLVA typing method has not yet been developed and applied to *Ureaplasma* spp.

2.5 Real-Time PCR Procedures for Detection of *Mycoplasma* and *Ureaplasma* Species

Real-time PCRs for detection and characterization of mycoplasmas and ureaplasmas have utilized the ABI Prism 7900HT (Applied Biosystems, Carlsbad, CA), the iCycler iQ (Bio-Rad, Hercules, CA), and the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN).

2.5.1 Specimen Collection

Specimens should be collected in sterile tubes with caps secured tightly, frozen at −80 °C, transported on ice to the laboratory where the test will be performed and remain frozen until DNA isolation. Blood (minimum 0.5 ml) should be collected in a vacutainer tube containing acid citrate dextrose (ACD). PCR can be performed directly on undiluted fluids. Specimens can be inoculated into 0.3 ml to 0.7 ml of a commercial universal transport media or phosphate-buffered saline (PBS) at time of collection or as soon as possible thereafter. For swab specimens, use only Dacron, polyester, or eSwabs. Calcium alginate and cotton swabs can be inhibitory. Swab specimens should be rinsed in either transport media or PBS and the swab extracted. Excess fluid should be removed by pressing the swab against the inside of the tube or Cryovial before removing and discarding the swab. The 10B or SP4 broths used for mollicute culture do not have any deleterious effect on performance of the real-time PCR assays utilized with the Roche LightCycler, so they are suitable as a transport medium. It is mandatory to verify that culture broth or any other type of transport system is not inhibitory before using it for PCR transport. Each clinical specimen type must be validated separately before results can be used to direct patient care.

2.5.2 DNA Extraction

The simple lysis and proteinase K treatment usually yields PCR-detectable DNA unless the specimen is inhibitory [186]. Suitable specimens include body fluids (other than blood) and transport media containing material obtained from swabs. Potentially inhibitory specimens including blood, tissue samples, lower respiratory secretions, and subcultures should be purified by the QIAamp® DNA Blood Mini

Kit (Qiagen) or other commercial genomic DNA purification kits according to the manufacturer instructions.

Automated or semiautomated nucleic acid isolation methods, such as QIAGEN BioRobot EZ1 (Qiagen), NucliSENS® (bioMérieux), easyMAG (bioMérieux), or MagNA Pure LC (Roche Applied Science), can also be used to prepare samples. Each laboratory must perform an evaluation of every assay component from sample type, transport media, extraction method to final PCR amplification and detection procedures using the specific primers and probes, reaction conditions, and controls applicable to the assay to ensure the techniques are valid and there is no PCR inhibition at any step. The easyMAG nucleic acid extractor actually enabled superior amplification results for *M. pneumoniae* when compared to the QIAGEN blood mini kit and the NucliSENS miniMAG systems [34, 187]. Some of the newer automated commercial PCR systems are true sample-to-result instruments that incorporate DNA extraction and PCR amplification into their process so specimens do not have to be manipulated once they are loaded into the instrument.

2.5.3 PCR Programs and Operating Conditions

Many aspects of the real-time PCR procedures are instrument and protocol-specific, such that analytic sensitivity, specificity, primer selection, and all aspects of the operating program have to be validated separately for each method and instrument.

2.5.4 Quality Control

Careful attention to quality control procedures should limit the risk of false-positive and false-negative results of PCR assays. False-positive results from contamination are a major problem for conventional PCR but have been minimized with real-time PCR. In addition to human errors, reasons for false-negative results include the presence of PCR inhibitors in the clinical specimen, suboptimal reagent preparation and reaction conditions, and inefficient extraction of the target DNA. Many substances, such as hemoglobin, polysaccharides, and mucolytic agents, and certain compounds used for DNA extraction, such as ethanol or detergents, are potent amplification inhibitors and as are swabs such as calcium alginate and those with aluminum shafts [188]. These inhibitory factors and suboptimal PCR conditions can be detected by simply mixing a positive control DNA with the sample after purification. However, this external control strategy cannot reveal inefficient DNA extraction. The use of an internal control overcomes the limitations of the external control. The internal control can be a plasmid or oligonucleotide containing a sequence similar to or unrelated to the target but can be differentiated from the assay PCR. It is essential in the initial validation of a PCR assay that all aspects that can potentially impact the results must be optimized so that once the assay has been validated the quality control procedures for individual runs will not be overwhelming.

2.5.5 Determination of Assay Sensitivity and Specificity

PCR analytical sensitivity should be performed against serial dilutions of template DNA, either bacterial genomic DNA from a defined inoculum titer or a plasmid containing the target sequence, and can be expressed in terms of amount of DNA detected or numbers of organisms (CFU). The analytical specificity should also be tested against other pathogens that appear in the same body locations or show sequence similarities to the targets. Human genomic DNA should always be included because of its presence in clinical specimens and possible inhibitory effects. Real-time PCR assays for mollicutes such as *Ureaplasma* spp. may have detection limits of 5 to 10 copies/reaction mixture [29]. Assay reproducibility should be verified by testing the same samples multiple times.

The choice of which genes to use as PCR or other NAAT targets for optimum detection and characterization of the desired organism or component should be carefully considered. Too often the PCR targets used in early *Mycoplasma* and *Ureaplasma* assays were directed toward genes encoding antigens that upon additional study have proven inadequate due to sequence variation among different isolates. It is important to test the PCR on well-characterized clinical specimens that are also tested by other methods such as culture and/or serology in order to establish clinical sensitivity.

2.5.6 Multiplex Real-Time PCR for Detection of *Mycoplasma* and *Ureaplasma* Species

The ELITech Group (Bothell, WA) MDx ELITe InGenius platform is a fully automated sample-to-result system integrating nucleic acid extraction and real-time PCR amplification, allowing for detection of microorganisms directly from clinical samples. The UAB Diagnostic Mycoplasma Laboratory has evaluated the ELITech MGB Alert *Mycoplasma Ureaplasma* Real Time PCR for Research Use Only and determined it provides results comparable to our laboratory developed PCRs. Gene targets are *GADPH* for *M. genitalium* and *M. hominis* and *UreC* for *U. parvum* and *U. urealyticum*. Instrument ease-of-use and decrease in hands-on time make ELITe InGenius platform attractive for clinical detection of urogenital mollicute spp. in a clinical laboratory setting. There are also reagents for detection of *M. pneumoniae* based on *repMp4* that we have shown to perform in a comparable manner to the *repMp1* assay [98].

2.6 Other Detection and Genotyping Systems

Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) is a common method for bacterial identification that is both rapid and accurate. This technique analyzes whole bacterial cells instead of nucleic acid. Although the initial cost for instrumentation is significant, the cost per test is

negligible, and organism identification can be achieved within minutes once an isolated colony is available. Pereyre [189] used the Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) to correctly identify 96% of all mollicute isolates tested including 50 *M. pneumoniae*, which were distinguished from *M. genitalium* and *M. amphoriforme*, and clustered them into two groups, corresponding to their P1 subtypes. Xiao [190] used MALDI-TOF MS to subtype 38 *M. pneumoniae* isolates that had been identified by real-time PCR and classified as P1 subtype 1 or 2 by full-length sequencing of the P1 gene. The time-consuming and insensitive nature of the prerequisite culture in order to perform MALDI-TOF MS potentially limits its usefulness for *M. pneumoniae* and makes it unsuitable for *M. genitalium.*

Silver nanorod array-surface-enhanced Raman spectroscopy (NA-SERS) is based on the acquisition of unique SERS spectra from clinical specimens without the need for amplification by growth or PCR. Hennigan used NA-SERS to distinguish *M. pneumoniae* strains M129, FH, and 11-3 and to classify spectra for ten specimens as positive or negative for *M. pneumoniae* with >97% accuracy in concentrations as low as 82 CFU per sample [191]. NA-SERS provided a detection limit similar to quantitative PCR and correctly classified the two main subtypes of *M. pneumoniae* and variants in 32 strains [192]. Thirty *M. pneumoniae* isolates from diverse geographic origins were distinguished by NA-SERS from a panel of 12 other human mycoplasmal species with 100% accuracy and classified with 96% accuracy for type 1, 98% accuracy for type 2, and 90% accuracy for type 2V strains [193]. Another evaluation of Raman spectra that included 102 clinical isolates along with strains M-129 and FH identified 2 major clusters which together included 81% of the strains, but they did not correlate completely with P1 subtypes [194]. The major advantage of NA-SERS is its ability to detect and type *M. pneumoniae* in a single test.

2.7 Discussion and Summary

Development and application of molecular-based methods for mycoplasmas and ureaplasmas in human infections over the past three decades have significantly improved the ability to detect, identify, and genetically characterize these organisms resulting in expansion of knowledge about the diseases they cause and enabled more rapid and accurate diagnosis for diagnostic purposes. NAAT-based detection methods have lessened the reliance on serology for M. pneumoniae and have become the detection method of choice for *M. pneumoniae* and *M. genitalium*. When used for diagnostic or epidemiological purposes, there is justifiable concern over accuracy since most assays have never been sufficiently validated against other molecular or culture-based methods. The few comparative clinical studies of various NAATs and preliminary studies of interlaboratory proficiency testing from Europe have indicated there are considerable differences with these assays for detection of mycoplasmal infections, as well as the capabilities of the individual testing laboratories.

It is clear that the future of diagnostic mycoplasmology and epidemiological research rests with molecular-based technology, even though culture, phenotypic methods, and traditional antimicrobial susceptibility testing will continue to have an important role, especially for *M. hominis* and *Ureaplasma* spp. Therefore, it is very important that large-scale comparisons must be performed to compare reproducibility and accuracy of NAATs. This must include side-by-side comparisons of new assay formats and gene targets with existing assays using the same as well as different targets and with other established methods, including culture when feasible. Such comparisons should ideally include a broad selection of specimen sources from different geographic areas.

Within the past few years, several NAATs have been FDA cleared for detection of *M. pneumoniae* and *M. genitalium.* Testing for *M. genitalium* is expected to become much more common in persons with sexually transmitted infections as evidence for its role in disease becomes more widely appreciated and understood. It is anticipated that more specific recommendations for testing and treatment guidelines from regulatory organizations, including the US Centers for Disease Control and Prevention, will be forthcoming, particularly as new NAATs enter the commercial market in the United States and become more readily available. True point-of-care tests that are uncomplicated and inexpensive that can be used in an ambulatory setting for detection of *M. pneumoniae* and *M. genitalium* are urgently needed.

Another major advance in molecular-based testing in mycoplasmology has been use of genotyping of clinical isolates and original clinical specimens to track the epidemiology of antimicrobial resistance. The alarming increase in macrolide resistance in both *M. pneumoniae* and *M. genitalium* has influenced development of commercial real-time PCR assays that detect macrolide resistance mutations.

Next-generation sequencing (NGS) makes it possible to sequence microbial genomes inexpensively and accurately within a few hours. As the turnaround time and costs continue to decrease, NGS utilization is expected to rise. As part of a worldwide effort to make modern infectious disease diagnostics available to the developing world, more and more nucleic acid-based diagnostic microchip and microdevice technologies are becoming available to test for specific diseases in locations where a lack of electrical grid access make more conventional diagnostic assays and devices unavailable. These point-of-care devices typically employ isothermal amplification techniques, which require much less energy than other conventional NAATS and require samples of only a few microliters. Likely, in a very few years, handheld devices will be available to screen samples rapidly and cheaply for a broad range of pathogens, including mycoplasmas.

2.8 Summary

Several species of mycoplasmas and ureaplasmas are well-known human pathogens responsible for a broad array of conditions involving adults and infants, and they may cause severe systemic disease in susceptible hosts. Greater attention is now being given to these organisms as a result of improved methods for their laboratory

detection made possible by molecular-based techniques. PCR-based technology can be employed for primary detection in clinical specimens as well as to characterize the organisms genetically for epidemiological purposes. Complete genome sequences are now available for all of the important human pathogens in the *Mycoplasma* and *Ureaplasma* genera. Information gained from genome analyses and improvements in efficiency of DNA sequencing are continuing to advance the field of molecular detection and genotyping of these organisms. This chapter provides a summary and critical review of methods suitable for detection and characterization of mycoplasmas and ureaplasmas of humans with emphasis on molecular genotypic techniques.

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Neisseria meningitidis

3

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3.1 Introduction

Neisseria meningitidis infection is an ongoing cause of significant morbidity and mortality, despite prompt antibiotic use and medical support, worldwide across income settings. The organism is a human commensal and asymptomatic coloniser, primarily in the pharynx. Clinical manifestations vary ranging from a mild febrile illness to fulminant life-threatening bacteraemia with or without meningitis. Invasive meningococcal disease (IMD) occurs sporadically and in outbreaks.

There is significant value in typing invasive strains as this provides insight into ongoing epidemiological trends, facilitates clinical public health tracing, and strengthens the ability to inform public health strategies including vaccination schedules, development, and response, and as a result, there stands a long history of typing methods. Historically, typing was based on a stepwise confirmation of identification, often using biochemical testing, followed by serogrouping.

Progressively, methods of nucleic acid testing have been adopted, with the relatively recent adoption of next-generation sequencing methodologies and whole-genome sequencing. This chapter overviews the classification, clinical significance, and identification of *N. meningitidis*, before assessing serological, enzymatic, and nucleic acid approaches to typing.

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3.2 Classification and Clinical Significance

Named after microbiologist Albert Ludwig Sigesmund Neisser, the genera *Neisseria* is classified within order *Neisseriales*, family *Neisseriaceae*, and was originally described in 1885 [1]. Currently, 29 species are recognised [2]. Two species are recognised as common human pathogens: *N. gonorrhoeae* (an obligate pathogen) and *N. meningitidis* (an opportunistic pathogen). Other *Neisseria* species rarely opportunistically infect humans.

N. meningitidis is found only in humans, where it asymptomatically colonises the nasopharynx; an estimated 8–25% of the population is believed to be colonised [3]. Colonisation rates are highest in adolescents and young adults. It spreads from human to human via respiratory droplet. Virulent strains can invade the blood stream from the respiratory tract with bacteraemia ensuing, and meningitis results when the blood-brain barrier is crossed. The clinical presentation varies but can involve pyrexia, purpura, petechiae, and shock in septicaemic cases (1 in 5 cases), and additionally vomiting, neck stiffness, headache, and photophobia in meningitis [4]. Importantly, there are manifold clinical presentations of IMD, and gastrointestinal and musculoskeletal signs are also reported – such as diarrhoea, vomiting, and leg pains [5, 6]. Where the adrenal glands fail, Waterhouse-Friderichsen syndrome can result [7]. The incubation time of IMD is estimated at 2–10 days [8].

Meningococci are armed with an array of virulence factors, such as endotoxins, capsule preventing phagocytosis, pili, and adhesions which are likely associated with poorer outcomes. The case fatality rate of untreated meningococcal meningitis is around 50%; meningococcal septicaemia is associated with an 8–15% case fatality [9]. Surviving patients may suffer from long-term morbidity, including limb amputation and adverse neurological outcomes including deafness.

Globally, it has been estimated up to 1,200,000 cases of IMD occur annually, resulting in up to 135,000 deaths [3]. Challenges exist due to underreporting, and typing remains a key component of surveillance. The greatest burden of disease is in Africa, where the meningitis belt spans from Senegal to Ethiopia [10]. Historically, outbreaks in the meningitis belt have resulted in attack rates reaching 10–1000 cases per 100,000 population, with the majority of cases historically being serogroup A [11]. The extensive serogroup A vaccination program in this area has now resulted in serogroups C, X, and W being the most prevalent [12]. In Latin America, the incidence is up to 1.8 cases per 100,000, although underreporting presents challenges. In Brazil, Chile, and Argentina, serogroups C, W, and B prevail [13]. Rates in Asia are similarly difficult to estimate due to underreporting, although these are suspected to be significant particularly in resource-poorer countries [14]. Rates in Europe, North America, and Australia are less than 0.5 to 1 per 100,000 where serogroups B, C, and increasingly W and Y are prevalent [11, 15, 16]. To reduce mortality, rapid diagnosis, and prompt, aggressive treatment is required [17].

Vaccination remains a pivotal component of global control, with preventative immunisation used to prevent outbreaks and reactive immunisation used to respond to outbreaks. A range of classes of vaccine exist, including polysaccharide (often in

outbreak response), conjugate (in prevention and outbreak response), and protein-based (for prevention or outbreak response) [9].

3.3 Identification

3.3.1 Phenotypic

Traditionally testing relied on phenotypic methods. Isolates can be derived from a range of sample types, including blood, CSF, site-specific samples, skin lesions, and the throat [18]. When visualised using light microscopy, *N. meningitidis* appear as Gram-negative diplococci and are frequently intracellular occurring within polymorphonuclear leukocytes. Sample materials can be cultured on a range of solid or liquid media. Blood culture bottles typically contain enriched media and alert when growth is detected. Enriched nonselective solid media (e.g. chocolate agar, blood agar) are frequently used for sterile site samples, where overgrowth from other bacteria is not anticipated. Culture of non-sterile site material, if indicated, can be performed on selective media (e.g. modified New York City, Modified Thayer Martin) [18]. Isolates are typically incubated in 3–7% CO_2, at 35–37 °C for 48 hours. Isolates typically have consistent Gram stain and are oxidase positive. Carbohydrate utilisation tests should reveal acid production with glucose and maltose but not with lactose or sucrose [19]. Enzyme substrate testing is gamma-glutamylaminopeptidase-positive, nitrate reduction testing is negative, and isolates are colistin-resistant [20]. Supplementary testing is frequently performed using various methods, such as:

- Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF), although accuracy is imperfect [21] and confirmatory testing is required
- Agglutination serogroup testing, although performance varies with encapsulation and isolate growth [19]
- DNA detection methods, such as PCR, which frequently target ctrA and sodC [22]

Susceptibility testing may be performed using a standardised testing system. EUCAST (version 11) provides antimicrobial breakpoints for benzylpenicillin, ampicillin and amoxicillin, cefotaxime and ceftriaxone, meropenem, ciprofloxacin, chloramphenicol, rifampicin, minocycline, and tetracycline [23]. CLSI (M100 ED31:2021) provides breakpoints for penicillin, ampicillin, cefotaxime and ceftriaxone, meropenem, azithromycin, minocycline, ciprofloxacin, levofloxacin, sulfisoxazole, co-trimoxazole, chloramphenicol, and rifampicin [24]. Antimicrobial susceptibility data may provide supportive evidence interlinking with other typing data [25]. However, as a typing tool in itself, a range of considerations must be broached, including acquisition or loss of resistance, comparing categorical resistance data with quantitative MIC data, differences between testing systems over time, laboratory capabilities and quality, and the potential failure to capture information revealed using other typing modalities.

3.3.2 Serology

Serological testing is not commonly used but potentially provides a retrospective diagnosis and may have value where diagnosis is required post-initiation of antibiotic therapy. However, cross-reactivity with some assays may occur postvaccination. Serum is typically used. Assays may target human immunoglobulin response to outer membrane proteins on invasive *N. meningitidis* isolates [26]. A high IgM or significant rise in IgM or IgG titre suggests infection. Serological testing has a good negative predictive value [18].

3.4 Typing Methods

Meningococcal typing is done for a number of reasons: to determine information on local cases, if sporadic or part of an outbreak, to determine vaccine implementation and/or efficacy, and to monitor global epidemiology. The reasons for the typing influence the selection of method. Typing of *N. meningitidis* can be performed through a number of modalities, each with varying merits. Some approaches are now historical and rarely used. Broadly speaking, typing can be performed on isolate material or potentially on clinical samples dependant on the methodology. Methods may be phenotypic (e.g. based on antibiotic susceptibility data, discussed above), serological (i.e. assessing immunogenic characteristics of isolates, such as based on capsular materials), enzymatic, or based on nucleic acid methodologies (i.e. assessing for genetic variability between isolates). The following description is not exhaustive, but attempts to overview key methods of current or historical importance.

3.4.1 Serological

Neisseria meningitidis is an antigenically complex organism. Isolates may be encapsulated, permitting them to cause invasive disease by evading the immune system. However, different capsule types present different antigenic signatures, resulting in variable immune responses. This characteristic can be leveraged by assays that characterise variations in these signatures.

3.4.2 Serogrouping

Serogrouping provided one of the earliest and traditional ways of typing *N. meningitidis* and remains widely used. The approach is based on variable reactivity to groups of capsular polysaccharides, which can be ascertained using monoclonal antibodies [27]. Hierarchically, isolates are characterised by serogroup, serotype, subserogroup, and then immunotype. Currently, 12 serogroups are recognised, A, B, C, E, H, I, K, L, W, X, Y, and Z, with 6 serogroups A, B, C, W, X, and Y, accounting

for the majority of IMD worldwide [28]. A thirteenth group, D, is no longer recognised [19].

Specifically, serogroups are based on capsular polysaccharide. For example, Group A polysaccharide primarily has O-acetylated residues at position C3 [29], whereas Group B polysaccharide is a homopolymer of α (2 → 8) N-acetyl neuraminic acid [30]. Serotypes are based on epitopes present on Porin B class 2 and class 3 outer membrane proteins and sub-serotypes are based on epitopes based on Porin A class 1 outer membrane proteins (e.g. P1.1, P1.16 [31, 32]). Twelve immunotypes (L1–L12) are based on variations in the composition of lipopolysaccharide [33]. The historical type of an isolate was represented as serogroup/serotype/sub-serotype/immunotype (e.g. B:15:P1.16:L3) [31, 34].

Historically, serogrouping and serotyping methods have included co-agglutination, dot-blot, and ELISA [35]. Rapid diagnostic testing for serogroup level has relied on immunochromatographic testing or latex agglutination testing, with several kits demonstrating variable performance characteristics commercially available [36].

Advantages of serogrouping include rapid determination of isolates associated with invasive disease. Consequently, serogrouping forms a foundation of global knowledge of type distribution. Additional advantages include reduced technical complexity for serogroup-level typing versus other methods, standardisation between laboratories, accessibility, and a long-standing history of use. Disadvantages of serogrouping include lower ability to discriminate. Additional reagents are required for finer-level typing studies that include outer membrane proteins and lipopolysaccharides, and reagents may be limited. An additional challenge includes the existence of non-serotypable strains. Non-typability may be due to the absence of capsular material, but also potentially due to masking or low expression of surface antigens, and the potential establishment of new serological types requiring ongoing expansion of reagent panels [37].

3.4.3 Meningococcal Antigen Typing System (MATS)

The meningococcal antigen typing system (MATS) was relatively recently developed to aid in the determination of the expression and immunoreactivity of antigenic components in MenB strains that correlate with 4CMenB components (fHbp, NHBA, and NadA) and therefore provides an indication of vaccine coverage with the Bexsero® vaccine [38]. The approach utilises a modified sandwich enzyme-linked immunosorbent assay (ELISA) [39] to assess for the expression of antigen in bacterial culture material matching vaccine components (fHbp, NHBA, and NadA) [39]. MATS additionally includes information for the PorA serosubtype, determined by PCR for VR 2 and strain matching for PorA 1.4 [39]. For each target, the relative potency is compared against a positive bacterial threshold and scored as positive or negative, and the strain is determined to be PorA = P1.4 (positive or negative). The MATS method is quite labour-intensive, requires specialised reagents, and has been replaced in most laboratories doing sequencing by the Bexsero®

Antigen typing scheme which is implemented in the www.PubMLST.org/neisseria database [40]. This shows the presence of the genes but does not provide information about the expression by the genes.

3.4.4 Enzymatic

3.4.4.1 Multilocus Enzyme Electrophoresis (MLEE)

Multilocus enzyme electrophoresis permits organisms to be typed by assessing variations in how intracellular enzymes electrophorese on starch gel between strains, with unique profiles being referred to as electrophoretic types (ET). Electrophoretic types that are closely related are considered a clonal complex. The mobility of enzymes under electrophoresis varies based on amino acid substitutions which alter electrostatic charge and are reflective of genetic mutations. A broad array of metabolic housekeeping enzymes are used (e.g. see [41]). The approach has been used extensively for typing *N. meningitidis*, with an early use involving the analysis of the intercontinental spread of clones belonging to the ET-5 complex [42]. The methodology has a range of strengths, including its ability to better characterise hypervirulent complexes versus serotyping alone. For example, ET37 can contain B, C, Y, or W [43]. Disadvantages, however, include interlaboratory variation, including in the number and selection of enzymes analysed, handling potential variability in gene expression, labour intensitivity, being time-consuming, and technical challenges [44]. The disadvantages of MLEE were overcome with the development of multilocus sequence typing which sequences the housekeeping genes.

3.4.5 Nucleic Acid-Based Methods

3.4.5.1 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a gel-based approach which has historically been used extensively to type *N. meningitidis* [45]. The approach typically involves numerous steps, including culturing bacterial material, which is mixed with agarose and poured into a plug mould; lysed to release the DNA, which is cut with a restriction enzyme, typically Spe1 and Nhe1; loaded into a gel; and placed in an electric field that periodically changes direction to produce bands, which are then stained and read [44, 45]. Advantages of PFGE include its ability to detect clusters in the short term from specific sites with typically good levels of discrimination [18]. A key challenge includes difficulties in interlaboratory comparison due to differing methodologies and laboratory conditions [18].

3.4.5.2 Restriction Fragment Length Polymorphism (RFLP)

The use of RFLP for *N. meningitidis* is long-standing, with Fox et al. demonstrating its value in 1991 [37]. Historically, the process relies on the culture of *N. meningitidis*, followed by DNA extraction, DNA fragmentation using specified restriction

endonucleases, and then gel electrophoresis to visualise the resultant bands. An advancement involved the use of PCR amplification (i.e. PCR-RLFP), whereby specific genetic targets (e.g. porA, porB) are amplified using PCR, with enzymatic restriction applied to the targets [46]. A range of restriction endonucleases have been used. Historically, the advantage of traditional RFLP was that it permitted finer separation of strains from epidemics that would have been indistinguishable using serotyping and serosubtyping [47]. Additionally, it permitted typing of isolates that cannot be fully serotyped and serosubtyped [48]. However, several downsides existed, including non-transferability of results and the need to have culture materials. Given PCR-RFLP does not require viable culture material, it can be performed directly on clinical samples (e.g. in CSF from patients who have been treated with antibiotics) [49]. This method has been superseded in laboratories performing sequencing.

3.4.5.3 Ribotyping

Ribotyping constitutes a subset of RFLP and has had long-standing use in the typing of meningococcal isolates, with early use by Jorden et al. in 1991 [50]. The methodology relies in the restriction of ribosomal RNA. Traditionally, meningococcal culture material was lysed and DNA extracted and purified. Restriction enzymes were then applied (e.g. ClaI, EcoRI, HindIII), with restricted DNA electrophoresed and blotted with hybridisation with a DNA probe [51]. Historical examples of the use of this methodology include analysis of the 1994 epidemic in Sao Paulo [51]. Modifications to the approach have been proposed, such as PCR-RFLP ribotyping which broadly uses the same principles as PCR-RFLP, which relies on amplification of the intergenic spacer region lying between 16S and 23S rRNA, and then digesting the PCR product (e.g. with AluI and TaqI), although given the low rate of mutation may be unable to characterise short-term epidemiological events as well as standard ribotyping [52]. Overall, the key advantage of ribotyping is that it provides good discrimination of strains [52], although this was historically dependent on the restriction enzyme selected [50]. Historically, a potential challenge involved cost [53]. This method has been superseded in laboratories performing sequencing.

3.4.5.4 Random Amplified Polymorphic DNA (RAPD)

RAPD is a long-standing typing methodology [54] that has been historically applied to meningococci, such as typing of a meningococcal university outbreak in 1994 (e.g. [55]). The methodology involves culture of isolates, cell lysis, DNA extraction, application of predetermined arbitrary short primers, amplification using PCR, electrophoresis, and pattern analysis (e.g. see [55]). Advantages include discrimination, speed, being relatively inexpensive, reproducible between laboratories, not requiring prior sequence knowledge, and requiring less DNA versus some other methods (e.g. PFGE). Disadvantages include the inability to compare results between laboratories, making it suitable for local outbreak investigation but not suitable for national or international comparison of results. This method has been superseded in laboratories that perform sequencing.

3.4.5.5 Multilocus VNTR Analysis (MLVA)

MLVA examines repetitive DNA motifs that contain variable-number tandem repeats. Typically, the method involves lysing culture isolates, amplifying the nucleic acid using a series of primers (e.g. see [56]). The size of resultant PCR products is determined using capillary electrophoresis or other sequencing methods to produce a DNA fingerprint (e.g. see [57]). Given the high discriminatory ability, it has been suggested that MLVA is well suited to fine typing clonal complexes or performing rapid assessment of suspected outbreaks [58]; in particular, it can separate epidemiologically separated meningococcal isolates from epidemiologically linked isolates that share the same MLST or MLEE types [59]. Historically, it has also been considered technically straightforward, low cost, relatively quick to perform, and able to provide transmissible and unambiguous results [58, 60]. Conversely, however, it may not be suited to typing samples collected over an extended period or across extensive areas [59]. This method has been superseded in laboratories that perform sequencing.

3.4.5.6 PorA Sequencing

Sequencing-based approaches to typing are becoming increasingly relevant, with the development of faster, cheaper, and more accessible technologies. Targeted sequencing of key meningococcal genes has been utilised historically to explore relationships between meningococcal isolates. Gene targets vary, but considerable work has focussed on the porA gene (e.g. [61–63]), which encodes the outer membrane protein PorA and defines subtype; two variable regions (VR1, VR2) are of particular importance and consequently the focus of sequence analysis [64]. The porA protein carries epitopes (loop 1, VR1, and loop 4, VR2) which act as targets for monoclonal antibodies used in subtyping, and consequently the method acts as a molecular surrogate of serological typing analysis. Historically, the method has been PC-based, whereby primers specific to the target regions amplify the sequence of interest. Sequencing is performed and then typing performed by comparing the obtained sequence against known database. Advantages of the porA sequencing include it being rapid, portable, and highly discriminating and consequently suitable for the analysis of short-term clusters. An online database is available (https://pubmlst.org/neisseria/PorA/). Additional benefits include having a finer resolution versus serological subtyping and characterisation of non-typable isolates.

3.4.5.7 Multilocus Sequence Typing (MLST)

MLST was first introduced by Maiden et al. in 1998, who looked at 107 isolates of *N. meningitidis* and 11 housekeeping genes [44]. The technique has subsequently been applied to a broad number of other pathogenic organisms, including bacteria, fungi, and parasites. Effectively, MLST is built on similar concepts to MLEE, but rather than assessing enzymes, it relies on a PCR-based approach, whereby a number of stable targets are amplified (e.g. using seven PCRs) that code for housekeeping genes that mutate relatively slowly [65]. Target housekeeping genes frequently

include the housekeeping genes: putative ABC transporter (*abcZ*), adenylate kinase (*adk*), shikimate dehydrogenase (*aroE*), glucose-6-phosphate dehydrogenase (*gdh*), pyruvate dehydrogenase subunit (*pdhC*), and phosphoglucomutase (*pgm*) [65]. A range of modifications have also been proposed for MLST, including extended MLST (eMLST, [66]) and ribosomal MLST (rMLST, [67]). Whilst the method is often performed on culture material, methods of testing clinical samples directly have been proposed [68].

Traditional MLST has a range of key advantages regarding the typing of *N. meningitidis*. The technique is portable, providing unambiguous results that are easily compared between laboratories nationally and internationally and are suitable for long-term comparisons [18, 60]. These factors have made MLST suitable for assessing global epidemiology. Additional advantages include direct determination of genetic changes between isolates, versus, for example, MLEE. MLST may permit clonal relationships between organisms to be determined, which would otherwise be overlooked using serogrouping alone [68]. Widespread use has been facilitated by the development of a web-based comparison website https://pubmlst.org/neisseria/. Other advantages include portability, discrimination, reproducibility, and relative straightforward workflow [44, 65]. Cited disadvantages include cost and labour intensitivity [60].

Core genome MLST (cgMLST) is a more recent innovation that builds on the underlying premise of traditional MLST [69]. The typing methodology harnesses the power of next-generation sequencing and high-performance computing to analyse 1605 core meningococcal loci, with distance matrices visualised phylogenetically using bioinformatic tools (e.g. using Neighbour-Net [70]). Examples of the recent use of this approach include the characterisation of serogroup C and W outbreak strains in Ireland [71], spatiotemporal analysis of invasive isolates in Tuscany [72], and analysis of invasive serogroup W isolates in Sweden [73]. The key advantages of cgMLST include its level of discrimination, unambiguous typing, and reproducibility. Additionally, the presence of an online database facilitates analysis (https://pubmlst.org/neisseria/). Disadvantages include technical complexity and the requirement for specialised sequencing equipment and bioinformatics skills, which limit access and potentially increase costs.

The current internationally accepted typing nomenclature for meningococci, which utilises the typing results of a number of methods, is serogroup: PorA type: FetA type: multilocus sequence type (clonal complex), e.g. B: P1.19,15: F5-1: ST-33 (cc32) [74].

3.4.5.8 Next-Generation Sequencing

Next-generation sequencing, also known as massively parallel sequencing, has revolutionised the typing of *N. meningitidis* as it has made large-scale whole-genome sequencing (WGS) practical for laboratories that are suitably resourced for the expensive equipment, computational power, and bioinformatic analysis capability required [75]. In recent years, the cost of WGS has decreased

dramatically, and the increase in technology and availability of automated analysis tools has meant public health laboratories have been able to implement this technology for the investigation of meningococcal outbreaks and for surveillance. Once sequenced, the data is stored allowing analysis of historical isolates. As the whole meningococcal genome is sequenced, the analysis can provide the results for a range of previous typing methods such as serogroup porA, MLST, cgMLST, fetA, and Bexsero® Antigen typing scheme. Dedicated workflows have been developed (e.g. see [76, 77]). As WGS can distinguish the difference in single-nucleotide polymorphisms (SNPs), it can be used for cluster analysis both in localised outbreaks and in meningococcal population epidemiology. Disadvantages include expense, technical complexity, and the requirement for specialised sequencing equipment and bioinformatics skills. Advantages include the results being comparable nationally and internationally and the ability to share sequence data between laboratories, to use bioinformatics, to reanalyse isolates without resequencing, and to perform population cluster analysis.

3.5 Conclusion

Typing methodologies have advanced considerably since Dopter first reported a link between meningococcal isolates and antigenic responses in 1909 [78]. With the progressive development of more advanced serological, enzymatic, and nucleic acid-based schemes, facilitated by advancements such as EIA-based technologies, PCR, and sequencing technologies, the advancements in meningococcal typing have permitted greater characterisation of isolates and the ability to better discriminate and represent clonality. Broadly speaking, as the technologies have progressively improved over the past century, the general goals of typing have remained constant: to aid in diagnosis and treatment, support and advance public health investigation, strengthen preventative activities (such as vaccine development), and ultimately reduce the global rate of mortality associated with IMD. Undoubtedly, the advent and increased availability of next-generation sequencing will drive further advancements in meningococcal typing into the foreseeable future. Whilst providing a finer resolution understanding of organism genomics, when coupled with the appropriate bioinformatic tools, it also permits reverse compatibility with a range of historically important systems through in silico prediction and consequently will continue to revolutionise laboratory processes.

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Check for updates

Nontuberculous *Mycobacteria*

4

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4.1 Introduction on the Genus *Mycobacterium*

After the first description of *Bacillus leprae* in 1875 by Hansen and the following discovery of *Mycobacterium tuberculosis* by Robert Koch in 1882, the genus finally emerged as a taxonomic group of pathogens named *Mycobacterium* as proposed by Lehmann and Neumann in 1896 [1]. The genus is composed of aerobic rod-shaped Gram-positive acid-fast microorganisms, most of them exhibiting facultative intracellular growth and having varied environmental reservoirs. Some *Mycobacterium* spp. also are associated with important well-known historical human diseases such as leprosy and tuberculosis, among others, while also being pathogenic for animals, some of them with zoonotic potential.

The use of the term "atypical acid-fast microorganisms" was introduced in 1935 to designate a mycobacterial isolate that caused human disease but could not be differentiated from *M. tuberculosis* by morphology, pigmentation, and virulence in animals [2]. Three years later, Costa Cruz isolated a fast-growing *Mycobacterium* from a human abscess that he named *M. fortuitum* [3]. A series of mycobacteria different from the *tuberculosis bacillus* started to be recognized as etiologic agents of human diseases, including *M. marinum* 1926 [4], *M. ulcerans* (1950) [5], *M.*

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intracellulare (initially named as *Nocardia intracellularis* in 1949 and later on renamed as *M. intracellulare* in 1965) [6], *M. kansasii* (1953) [7], and *M. scrofulaceum* (1956) [8], as main examples [9, 10].

It is known for decades that natural habitats of NTM are aquatic and soil environments [11]. The majority of *Mycobacterium* species have no impact on human health and occasionally, as opportunists, are responsible for human infections. However, some species are both environmental and pathogenic, while some are obligatory pathogenic. Contamination probably occurs through aerosolization or aspiration of water and/or soil particles and through exposure to traumatized skin and is generally not transmitted person to person. Therefore, it is important to identify the species that cause an infection in cases where the symptoms are sufficient to support sample collection [12–14].

4.1.1 The Taxonomy of Mycobacteria

Considering the present version of the List of Prokaryotic Names with Standing in Nomenclature (LPSN) database (available at http://www.bacterio.net/m/mycobacterium.html), a total of 192 validly published taxa are included in the genus *Mycobacterium*, including species and subspecies that are distributed in three major groups: (i) the *Mycobacterium tuberculosis* complex (MTBC), (ii) the distinct specie *Mycobacterium leprae*, and (iii) the nontuberculous mycobacteria (NTM), also called mycobacteria other than the MTBC. Traditionally, mycobacteria have been divided into rapidly (RGM) and slowly growing mycobacteria (SGM), the former needing less than 7 days for visible colony formation on solid culture media, the latter more than 7.

Through the years, the systematic taxonomy of this genus has evolved considerably based on grouping of phenotypic properties, analysis of chemotaxonomic characteristics, and sequence comparison of the 16S rRNA; of the 65-kDa heat-shock protein; of the genes *recA*, *rpoB*, *gyrA*, *gyrB*, *secA1*, *sodA*, *tuf*, and *smpB*; of the tmRNA; and of the 16S-23S rRNA intergenic spacer (ITS) region, performing a multilocus sequence analysis approach of concatenating several gene sequences, by interspecific DNA-DNA hybridization technique and/or, most recently, by genomic comparison.

Recent studies have suggested a new taxonomic classification and phylogenomic structure for mycobacteria based on datasets of genes/proteins from the genomes of different species. In 2018, Gupta et al. [15] suggested the redefinition of mycobacterial taxa based on amino acid insertions or deletions of fixed lengths within a specific position at a conserved region, named conserved signature indels (CSIs). These clade-specific marker gene sequences were proposed as a better definition of relationships among mycobacteria for determining the vertical inheritance and phylogenetic tree building as performed on datasets of concatenated protein sequences and proposed to relocate the mycobacterial taxa into five distinct genera: *Mycobacterium*, *Mycobacteroides*, *Mycolicibacillus*, *Mycolicibacter*, and *Mycolicibacterium*. However, Tortoli et al. [16] and most of the researchers in this study field preferred

Table 4.1 Nomenclature for the main *Mycobacterium* species as summarized by Tortoli et al. [16]

Classical nomenclature	Nomenclature adjustment proposed by Tortoli et al. [16][a]	Nomenclature according to Gupta et al. [15]
Mycobacterium abscessus subsp. *abscessus*		*Mycobacteroides abscessus* subsp. *abscessus*
Mycobacterium abscessus subsp. *bolletii*		*Mycobacteroides abscessus subsp. bolletii*
Mycobacterium abscessus subsp. *massiliense*		*Mycobacteroides abscessus subsp. massiliense*
Mycobacterium avium subsp. *avium*	*Mycobacterium avium* subsp. *avium var. avium*	
Mycobacterium avium subsp. *paratuberculosis*		
Mycobacterium avium subsp. *silvaticum*	*Mycobacterium avium* subsp. *avium var. silvaticum*	
Mycobacterium chelonae subsp. *chelonae*		*Mycobacteroides chelonae*
Mycobacterium chelonae subsp. *bovis*		
Mycobacterium chelonae subsp. *gwanakae*	*Mycobacterium chelonae* subsp. *bovis*	
Mycobacterium fortuitum subsp. *fortuitum*	*Mycobacterium fortuitum*	*Mycolicibacterium fortuitum*
Mycobacterium fortuitum subsp. *acetamidolyticum*	*Mycobacterium fortuitum*	*Mycolicibacterium fortuitum*
Mycobacterium gordonae		
Mycobacterium immunogenum		*Mycobacteroides immunogenum*
Mycobacterium intracellulare subsp. *intracellular*		
Mycobacterium intracellulare subsp. *chimaera*		*Mycobacterium chimaera*
Mycobacterium intracellulare subsp. *yongonense*	*Mycobacterium intracellulare* subsp. *chimaera*	
Mycobacterium kansasii		
Mycobacterium parafortuitum		*Mycolicibacterium parafortuitum*
Mycobacterium smegmatis		*Mycolicibacterium smegmatis*
Mycobacterium tuberculosis complex		*Mycobacterium tuberculosis*
Mycobacterium ulcerans		

[a]Empty field: nomenclature identical to the one of the first column

to use the classical nomenclature and reinforced the use of genome comparison for taxonomic classification.

By using the average nucleotide identity (ANI) and genome-to-genome distance (GGD) to analyze all the *Mycobacterium* taxa, Tortoli et al. [16] performed a detailed review and suggested specific adjustments for this genus. We detached the main species citing the classical and previously proposed nomina in Table 4.1.

4.1.2 *Mycobacterium tuberculosis* and *Mycobacterium leprae*

The two major human mycobacterioses are tuberculosis and leprosy. In 2018, ten million people fell ill of tuberculosis worldwide, killing 1.5 million of these, and 1 in 6 coinfected with HIV [17]. Although the disease is curable, a major problem is resistance to rifampicin, evolving often to multidrug and sometimes extreme drug-resistant disease, difficult to cure with long, toxic, and expensive treatment schemes and high mortality rates.

Almost 210,000 new cases of leprosy were reported in the same year, and just like TB, these are curable with a multidrug therapy and fortunately still presenting relatively low drug resistance levels [18]. However, relapse is quite common, and the World Health Organization recommends vigilance for drug resistance. The major problems regarding this disease are late diagnosis causing physical disability and stigma.

Tuberculosis is caused by organisms belonging to the MTBC that was recently redefined as a single species [19], disease being mostly caused by *M. tuberculosis var. tuberculosis* and *M. tuberculosis* var. *bovis*. Leprosy is caused mostly by *M. leprae* although a second species called *M. lepromatosis* and described mainly in Mexico is causing a particular clinical form of leprosy called Lucio syndrome [20, 21]. However, both disease characteristics and geographic distribution of the latter pathogen are under active study.

During the last two decades, basically since the availability of the complete genome sequences of *M. tuberculosis* [22] and *M. leprae* [23], a large number on studies on genetic variability between strains in either species have been described. Procedures for detection of strain variability have been used in studies on definition of species, phylogeny, evolution, strain virulence, transmissibility, molecular epidemiology, drug resistance, and host response, and these topics have been covered in several good reviews. Because another review on this is beyond the objective of this chapter, we refer to some recent papers and chapter, respectively, for MTBC [24–26] and for leprosy [27].

4.2 Clinical Significance of Nontuberculous Mycobacteria

4.2.1 Disease Caused by Infection with Rapidly Growing Mycobacteria

The clinically most prevalent RGM species are *M. abscessus*, *M. chelonae*, and *M. fortuitum*. While *M. abscessus* is mostly isolated from clinical respiratory specimens, *M. fortuitum* is recovered more frequently from non-respiratory specimens. The spectrum of diseases varies among the main species of the group [28–32]:

- *M. abscessus* – Pulmonary infections, primarily associated with bronchiectasis associated with cystic fibrosis or other comorbidities, skin and soft tissue infections after cosmetic procedures or surgeries, prosthetic device infection, tenosynovitis, and osteomyelitis
- *M. chelonae* – Surgical wound infections, abscesses, keratitis, catheter-related bacteremia, and hematogenously disseminated disease in immunosupressed individuals
- *M. fortuitum* – Skin and soft tissue infections (surgical or other traumatic lesions), chronic discharging sinuses, pulmonary infections among individuals harboring underlying pulmonary diseases, superficial lymphadenitis, prosthetic device infection, catheter-related sepsis, prosthetic valve endocarditis, and others

4.2.2 Disease Caused by Infection with Slowly Growing Mycobacteria

The major clinical syndromes associated with SGM include progressive pulmonary disease, skin and soft tissue infection due to direct inoculation, lymphadenitis, and disseminated disease in severely immunocompromised individuals by *M. avium* complex (MAC) and other NTM [31, 33]. The major clinical syndromes caused by specific species are:

- MAC – Lung diseases in HIV-negative patients, commonly associated with cystic fibrosis or middle-aged or elderly men, alcoholics, and/or smokers presenting or not underlying chronic obstructive pulmonary disease (COPD), mainly nonsmoking women over 50. MAC also causes disseminated disease in severely immunocompromised patients (such as AIDS or other syndromes and upon use of immunosuppressive drugs), solitary pulmonary nodules, and hypersensitivity pneumonitis syndrome [34, 35].
- *M. avium* subsp. *paratuberculosis* (MAP) – One of the possible etiological agents of Crohn's disease (CD) due to the characteristic tuberculous-like gastroenteritis in humans and similarities to the clinical and histopathological findings to the Johne's disease in ruminants caused by MAP. Some studies have described the isolation of this pathogens from lymph nodes and blood of patients with CD [36].

- *M. kansasii* – Considered the second most common respiratory NTM and associated with pulmonary disease similar to tuberculosis in patients with COPD, malignancy, immunosuppressive drugs, pneumoconiosis, alcohol abuse, and/or HIV infection. This species has also been described causing disseminated disease, mainly in HIV-positive individuals [37].

Other human pathogenic SGM include *M. malmoense*, *M. marinum*, *M. simiae*, and *M. xenopi*, all associated with similar pathologies caused by other NTMs [38]. *Mycobacterium ulcerans* is particularly related to localized skin lesions progressing to extensive ulceration that may result in functional disabilities [39].

4.2.3 Considerations on Virulence and Drug Resistance

Because both virulence and drug resistance are important characteristics of NTM that can vary considerably both on a species and strain level, we mention these in this chapter as strain typing can be beneficial for the patient. Nonetheless, few data exist on direct correlation of these characteristics and characterization of NTM strains, and this in contrary to strains belonging to the MTBC (see part 1.2).

Virulence of NTM is related to their complex lipid-rich cell wall and cholesterol catabolism as a source of energy and material for the synthesis of the cell wall, proteins, and cell envelope lipoproteins responsible for bacterial adherence and their ability to form biofilms. Due to the hydrophobic nature of the cell wall, NTMs can adhere to a wide range of organic and inorganic materials, promoting as such colonization followed by either pseudo-infections or true infections. In the last few decades, there has been a report of an increase in outbreaks and diseases caused by NMT [40].

NTM are naturally resistant to a wide spectrum of antibiotics that include most TB drugs. The selective pressure imposed by other microorganisms in the soil and in the water, probably producing antimicrobials, may have led NMTs to develop innumerable resistance mechanisms to maintain their survival [41]. One of these is the thick hydrophobic double-membrane cell envelope of mycobacteria that also acts as a major permeability barrier. It was shown already in the 1990s that isolates of the then called *M. chelonae-M. abscessus* complex have a cell envelope about 10–20 times less permeable than that of *M. tuberculosis*. In addition, morphotypic antibiotic resistance, a phenomenon of varying degrees of drug resistance in *M. avium* which is associated with a reversible colony morphology switch (white/red on Congo red containing agar, transparent/opaque), is also attributed to changes in permeability owing to cell wall modifications [42]. Such morphologic changes might have a genetic basis and should therefore be traceable by genotyping.

Efflux pumps contribute to intrinsic drug resistance by preventing accumulation of antibiotics in the bacteria and have been mainly described for fluoroquinolones and macrolides [43]. The NTM species also induce the expression of certain genes resulting in the modification of the target binding site of the drug, the so-called inducible drug resistance, and in the case of macrolide resistance in *M. abscessus* which is mediated by the *erm(41)* gene, encoding a ribosomal methylase and

sequencing of this gene and *rrl* can predict susceptibility to clarithromycin in strains of the *M. abscessus* group [44], but this correlation does not seem to be absolute [45]. The use of strain typing for prediction of drug resistance in this group and more particular *M. abscessus* subsp. *massiliense* was demonstrated very recently by MLST and WGS [46]. The differentiation of the subspecies of the *M. abscessus* complex is indeed important because they differ in resistance to antibiotics and in treatment response.

Some genotyping tools allow simultaneous differentiation of NTM to the species and/or subspecies level and inform on drug susceptibility. GenoType NTM-DR (NTM-DR, Hain Lifescience, Nehren, Germany) line probe assay (LPA) is such a tool that enables identification of the MAC species (*M. avium*, *M. intracellulare*, and *M. chimera*), *M. chelonae*, and subspecies of the *M. abscessus* complex. The assay also allows for detection of antibiotic resistance to macrolides and aminoglycosides, including polymorphisms in the *erm (41)* gene.

4.3 Molecular Identification and Genotyping of Mycobacteria

Among the NTM species, only about one third is familiar to microbiologists and doctors, so their identification guides therapeutic treatment and provides clues regarding the source and route of exposure. Due to the presence of these mycobacteria in the environment, a laboratory control monitoring the growth of NMT is established following clinical and microbiological criteria known for decades. When dealing with sterile clinical specimens such as in pleural fluid, blood, cerebrospinal fluid, and tissues, among others, a single NTM is confirmative for infection, while for diagnosis of lung disease, positivity in two samples of spontaneous sputum or in one bronchoalveolar lavage sample is needed [47].

Traditional phenotypic identification procedures for NTM to the complex and sometimes species level are laborious and based on time-consuming biochemical and morphology-based tests, including their initial differentiation form the MTBC. For these tests, confluent growth is required, may take more than 20 days to achieve adequate growth, and has the limitation to be basically species-specific [48, 49]. Time for identification has much been reduced due to the development of molecular tools for NTM identification. Nevertheless, the combination of conventional and nucleic acid-based procedures is still used in many laboratories for precise diagnosis and eventual strain typing.

The molecular identification methods for diagnosis have expanded significantly, and among the most widely used are:

- The polymerase chain reaction restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65* method) [50]
- Direct PCR (partial) gene sequencing with the principal target genes 1*6S*, *hsp65*, and *rpoB* including single target or MLST analysis [51, 52]
- Commercial rapid test based on DNA-strip technologies: INNO-LiPA Mycobacteria v2 (Fujirebio, H.U. Group, Japan); Speed-oligo® (Vircell,

Granada, Spain); GenoType CMdirect VER 1.0 (Hain Lifescience GmbH, Nehren, Germany), detecting MTBC and more than 20 clinically relevant NTM from patient specimens; and GenoType *Mycobacterium* CM VER 2.0 (Hain Lifescience GmbH, Nehren, Germany), detecting MTBC and more than 20 clinically relevant NTM from cultures

Despite being useful for identification of the species level and thus for accurate diagnosis, (most of) these methods do not discriminate within the specie and subspecies levels, with an exception of sequencing.

Hence, molecular typing procedures that characterize below the species level have been developed almost simultaneously and almost exclusively based on nucleic acid analysis (Fig. 4.1). They have been used for improving the epidemiological vigilance of mycobacteriosis based on detection of strain variability, transmission, outbreak investigations, as well as differentiation of reinfection and persistence/resistance. Through genotyping, the general idea was created that infection with NTM normally occurs from environmental sources [53]. Nonetheless, patient-to-patient transmission has been demonstrated between cystic fibrosis (CF) patients [54], and more studies are needed to evaluate the extend of such transmission events. To illustrate the major typing procedures used for NTM and their main applications, we summarize literature according to publication data in Table 4.2.

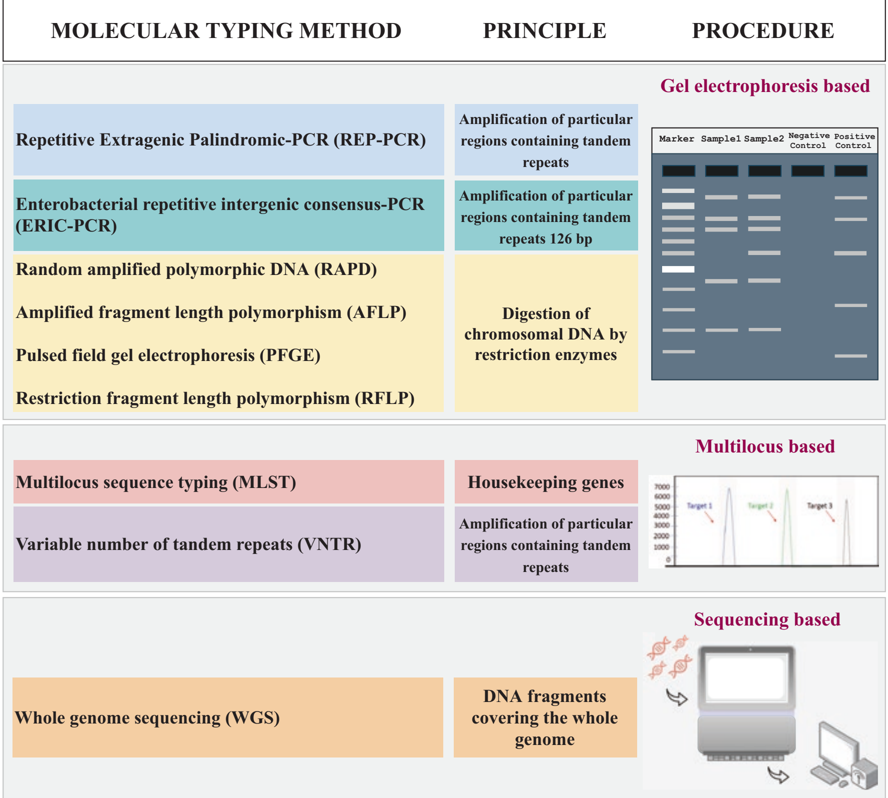

Fig. 4.1 The main molecular typing methods applied to Nontuberculous Mycobacteria

Table 4.2 Application and limitations of the main molecular genotyping methods applied to nontuberculous mycobacteria classified chronologically

Molecular typing method	Applications	Limitations
Repetitive extragenic palindromic-PCR (REP-PCR) [55]	Pseudo-outbreak [56], identification of source of infection [57], outbreak, and genetic diversity [58]	It is not an accurate tool for identifying organisms to the subspecies level. Low discriminatory power
Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) [99, 100]	Genetic diversity [53, 59], distinguish *M. paratuberculosis* from other mycobacteria (IS900/ERIC-PCR) [60], study of clonality [61], genetic diversity and infection control [62], outbreak [63–65]	It has necessarily a higher DNA quality. It has difficult reproducibility once it generates many bands, and thus, it is difficult to analyze
Random amplified polymorphic DNA (RAPD) [66]	Study of clonality [61, 67, 68], genotypic diversity and infection control [62, 69], outbreak [70], differentiation of infection and pseudo-infection/pseudoendemic [71], characterization of novel specie proposal [72], genetic diversity [73], identification of source of infection [74–77], clonality [78], strain discrimination [79], pseudo-outbreak [80, 81], outbreak [82]	Lacks inter-test and interlaboratory reproducibility; potential for misinterpretation is greater than that by PFGE. There is not a universal primer; we must test a set of primers. It generates many bands, and thus, it is difficult to analyze
Amplified fragment length polymorphism (AFLP) [83]	Identification of source of infection [84, 85], molecular epidemiology [86], genetic diversity [87, 88]	It is not an accurate tool for identifying organisms to the subspecies level. Low discriminatory power
Pulsed field gel electrophoresis (PFGE)	Outbreak [63–65, 89–94] and pseudo-outbreak [95, 96], molecular epidemiology [97], novel specie proposal [72], genetic diversity [58], identification of source of infection [98–104], study of clonality [59], differentiation of relapse from reinfection [105], specie differentiation [60, 106, 107]	PFGE depends on DNA quality, and the typing results can be influenced by a method of DNA isolation, electrophoresis/running conditions [108]. Inability to type *M. abscessus* due to DNA degradation [107]. High cost of reagents compared to ERIC and RAPD [62]
Restriction fragment length polymorphism (RFLP) [109]	Genetic diversity [59, 108, 110], specie differentiation [106, 111], identification of source of infection [98, 112–115], specie identification and differentiation [116–120]	It is labor-intensive and requires a high level of operator skill
Multilocus sequence typing (MLST)	Specie identification [97, 110, 121, 122], molecular epidemiology [123], differentiation of infection from reinfection [124], phylogeny [97] and characterization of novel specie proposal (*Mycobacterium paraintracellulare* sp. *nov*. [125])	High cost of reagents compared to ERIC and RAPD [62]

(continued)

Table 4.2 (continued)

Molecular typing method	Applications	Limitations
Variable number of tandem repeats (VNTR) [126]	Influence of genotype [127], transmission [128, 129], genetic diversity [108, 130–134], phylogeny and association of genotypes to drug susceptibility [135–137], genotypes associated with clinical aspects [138], phylogeny [139, 140], identification of coinfection, source of infection [141, 142]	The genetic diversity can be influenced by homoplasy [143]
Whole-genome sequencing (WGS)	Transmission assessment [144], novel mutation proposal [46], strain discrimination [145], identification of source of infection [146, 147], taxonomy/phylogeny [148–151].	Higher cost compared to others

Because methods are based on different procedures that might include enzymatic digestion, PCR amplification, agarose gel analysis, sequence or fragment analysis, and fragment size estimation or counting, among others, their applicability depends on the diagnostic or typing purpose, and choice is therefore based on a combination of characteristics such as simplicity and speed of execution, cost, and differentiating power. However, one of the most important characteristics of a genotyping technique for strain differentiation is the discriminatory power, and in the case of several NTM species, PFGE presents the highest value and could in some way be considered as the reference technique [152].

Alternative fragment analysis-based procedures such as REP, AFLP, RAPD, and ERIC-PCR might be easier to perform but have the limitation that patterns and interlaboratorial comparison can be more complex. Moreover, simple variations on the DNA extraction protocol can have serious impact on the result.

One interesting application of such procedures was a study on *M. fortuitum* isolates from mammaplasty patients belonging to ITS genotype V that had indistinguishable RAPD-PCR and ERIC-PCR patterns, confirming that infections at other hospitals were caused by different *M. fortuitum* genotypes and that there was no clonal dissemination between hospitals [65]. Another study using the same tools demonstrated that ERIC-PCR has the potential to be used as a screening tool and useful for rapid epidemiological typing tools for *M. fortuitum* infections [62].

When compared to ERIC, both PFGE and RFLP demonstrated a higher resolution [60]; however, ERIC is still valid as a complementary or alternative tool for outbreak investigation, especially when working with *M. abscessus*. Compared to RAPD, however, ERC demonstrates either a higher [62, 64, 65] or a similar discriminatory power [61].

In other studies, PFGE showed similar results as REP-PCR for *M. abscessus* typing [153, 154]. Combined with VNTR typing, PFGE demonstrated a nice tool for discrimination within *M. kansasii* [108], a species that was described as being

composed of seven subtypes [155]. Recently, six of these subspecies have been elevated to a species rank and named *M. kansasii* (former type I), *M. persicum* (II), *M. pseudokansasii* (III), *M. ostraviense* (IV), *M. innocens* (V), and *M. attenuatum* (VI) [150, 156, 157]. Even so, this organism is still presenting considerable variability as presented by division of *M. kansasii* (type I) into two *hsp*65 subtypes as observed also by the overall genome organization [158]. This was confirmed in a later study adding more genomes [159], so WGS seems WGS a promising tool for future strain typing studies.

Despite being time-consuming, labor-intensive, and resource- and expertise-demanding, turning PFGE difficult to perform on a large-scale basis, it is still considered by many as the pillar method for molecular typing of NTM because of its high discriminatory power [93]. However, for some species, particular caution needs to be taken such as the case for *M. abscessus* that may present DNA degradation [152]. The subjectivity part of comparison of PFGE restriction patterns can reduce guideline focused on interpretation and using rigid algorithms but not totally eliminated [160].

The MLST technique has shown the highest sensitivity and specificity for identification to the species level of NTM [51], including discrimination of *M. abscessus* from other NTM species [97, 121]. But for typing of isolates of this species, again, PFGE was superior [161]. In another study, as expected, WGS showed a clearly higher discriminatory power in comparison with VNTR and therefore in practice the only molecular tool suitable to effectively discriminate isolates of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, with clonal groups with different drug resistance patterns and suggesting transmission between patients [145]. Interestingly, a recent study compared a large amount of clinical strains completing a total of 175 NTM species by comparing whole-genome data and developing a new MLST algorithm based on 184 genes [122]. Their MLST-based identification showed higher accuracy than conventional MLST, and besides the potential to rapidly detect pathogens, the higher amount of data might, future wise, allow the use of this combined MLST-WGS approach for strain typing.

A sometimes very severe infection of subcutaneous tissue is observed during Buruli ulcer (BU), a neglected tropical skin disease caused by *M. ulcerans* [162], and molecular tools have contributed considerably to understanding the transmission and disease reservoirs [129, 151, 163]. Among these, VNTR has demonstrated a large genetic diversity [128] also adequate for phylogenetic assessment [132] of this species. Recently, the application of WGS through a phylogeographic analysis revealed a predominant sublineage of *M. ulcerans* that arose in Central Africa and proliferated in its different regions of endemicity during the Age of Discovery [151].

A recent excellent review by Shin et al. [164] focuses on genotyping of MAC/MAP and demonstrated that these species are mostly isolated from environmental sources such as in water and soil, therefore being the ecological niche for *M. avium* and *M. intracellulare*. Despite *M. avium* being excreted from infected animals and contaminates the environment, there seems to be no evidence for similar environmental contamination by *M. intracellulare*. Typing methods for strains from this complex can improve our understanding of estimating the infection pathway among

animals, humans, and the environment and evaluation of the treatment outcomes and the pattern of recurrence of MAC infection. The transmission of MAC species is not yet clearly defined, and together with the complex drug susceptibility pattern, more reliable and feasible genotyping methods of MAC are urgently needed,

The RGM *M. chelonae*, besides causing infections as related above, is commonly associated with skin and soft tissue infections and postsurgical infections after implants, transplants, and injections such as sclerotherapy and mesotherapy [165, 166]. Detection of source of infection is possible by molecular epidemiology studies on [56] and outbreaks by PFGE [90, 99] and/or ERIC-PCR [64]. Although considered a single species with *M. abscessus* until 1992, when *M. chelonae* was elevated to the species status, they share partial 16S rRNA signatures and are therefore still called the *M. chelonae-M. abscessus* group [167]. Among other phenotypical and molecular tools, RFLP was used to propose a division of this group [168] separating these species [111]. Genotyping by MLST to what were apparently particular stains of *M. chelonae* [169, 170] or of the *M. chelonae-M. abscessus* group [171] has also led to the identification of new (sub)species of these organisms.

Among the molecular tools, the only non-nucleic acid-based identification and typing technique for *Mycobacterium* isolates that we cover here is that based on matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS), a technique that during the last decade has turned into a timely and cost-effective identification procedure in routine microbiology laboratories [172]. In brief, a small amount of bacterial mass from a log phase culture is collected, heat inactivated, and treated with ethanol and the dried cell pellet vortexed or sonicated with beads in acetonitrile and formic acid before covering the dried extract with a special matrix. Identification is obtained both at the genus and species level between a range of 80% and 98% depending on the study [173]. The method has some limitations that have been only partly resolved. One is the impossibility to identify subspecies within the so-called *Mycobacterium* complexes that is still not possible for the MTBC. In the case of the *M. abscessus* complex, an algorithm for differentiation of the three subspecies was described [174], while the same was obtained by the use of principal component analysis [175]. Interesting also is that the formerly single species *M. kansasii* composed of seven genotypes resulted in reproducible and unique MALDI-TOF spectra that differentiated six of these [176], now separate species [150, 156, 157]. Another example of the promising evolution of this identification technique is the recently described algorithm for the differentiation of *M. intracellulare* from *M. chimaera* [177].

Two commercially systems for MALDI-TOF, each with their own *Mycobacterium* reference library, that of Bruker Biotyper with Mycobacterial Library v5.0.0 (164 species) and bioMérieux VITEK MS with v3.0 database, were recently compared and yielded similar results, although some problems were encountered in both systems for differentiation within complexes [178]. Because of the increasing number of *Mycobacterium* species and redefinition of their taxonomy, the constant need of updating of such databases to maintain accuracy of the identification is obvious [168]. Such databases have been constructed for MALDI-TOF users and can be accessed at https://microbenet.cdc.gov.

To our opinion, combined MALDI-TOF and genotyping analysis might be useful future wise, but the recent tentative to use the former technique alone for strain typing or lineage definition within the MTBC seems nothing but what the author's nicely called "a dream for the moment" [179].

4.4 Summary

Mycobacterium is a genus of *Actinobacteria* that are acid-fast bacilli closely related to *Corynebacteria*, *Rhodococcus*, and *Nocardia*. The genus now contains almost 200 recognized species with pure pathogenic species with best known examples *Mycobacterium tuberculosis* and *Mycobacterium leprae* and many environmental species that are sometimes also opportunistic pathogens. Mainly due to the evolution of genotyping techniques, many new species have been described during the last (two) decades, and many are to follow. Besides recognition of species, identification to the subspecies or strain level can teach us about disease transmission and bacterial population genetics and speeds up diagnosis, prediction of drug susceptibility, and evolution of disease and can therefore improve treatment. This chapter concentrates on current knowledge of strain typing of the main clinically important mycobacteria.

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Oral Streptococci

5

Marlise I. Klein

5.1 Introduction

The oral cavity is a complex environment because of its diverse habitats (soft and hard tissue surfaces, saliva), where distinct microorganisms preferentially colonize different ones. The oral microbiota has an abundance of bacteria species and harbors fungi, protozoa, archaebacteria, and viruses. Among bacteria, oral streptococci species are among the most abundant species found in the mouth. They are the primary colonizers of the oral cavity surfaces in microbial communities known as oral biofilms. Oral streptococci are early colonizers in the biofilm developmental process because of their surface adhesins that interact with glycoproteins (derived from the host and microorganisms) present on the salivary pellicle that covers all surfaces in the oral cavity. From 1990 to 2010, there was a rush to genotype the oral streptococci, especially Streptococcus mutans and Streptococcus sobrinus, species associated with cariogenic biofilms (biofilms that causes dental caries) to determine the transmissibility of strains (genotypes) from caregivers to children.

However, as our understanding of the oral microbiota and its dynamics in a homeostatic versus a dysbiotic microbiota evolved, more and more researchers are looking for species (or a combination of species—community) present in the oral cavity associated with a specific condition or conditions. Thus, besides knowing what species (who) is there, investigations are focusing on what the species are doing there. However, to know 'what', it is paramount to identify correctly 'who', and there are several methods for this purpose. Some oral streptococci species can also migrate from the mouth to other human body sites, where they can colonize and cause infections, such as infective endocarditis. Thus, this chapter focuses on the tools available for typing known oral streptococci.

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5.2 The Oral Microbiota Is Diverse, and Several Methods Are Employed to Investigate Oral Streptococci

Oral streptococci can colonize the oral cavity immediately after delivery [1, 2] and are pivotal for the arrangement and construction of the oral microbiota, especially biofilms on soft and hard tissues [3]. In the past, most studies on oral microorganisms used culturing combined with phenotyping methods, including serotyping. However, molecular methods are replacing them because of the technological advances and decrease in their costs. Nevertheless, culturing methods complement well the molecular strategies to characterize and identify the microorganisms present in the oral cavity, primarily for species characterization of clinical strains (i.e., clinical isolates that can be strains of the same species or distinct species). Therefore, the strategy to be employed will depend on the question being asked (Fig. 5.1).

The process for the identification and classification of microorganisms by culturing includes several steps. It initiates with sample collection and transport (using an appropriate solution, buffer, or medium—transport medium). Once in the lab, these samples need to be dispersed by agitation or sonication, followed by sample dilution and plating onto Petri dishes containing selective or nonselective agar medium. These plates are incubated at adequate conditions (known oral streptococci are facultative anaerobes). After colony growth, microorganisms can be isolated for growth in pure cultures by selecting and transferring a colony to a new plate for subculturing. The microorganisms can be characterized and classified (identified) by several

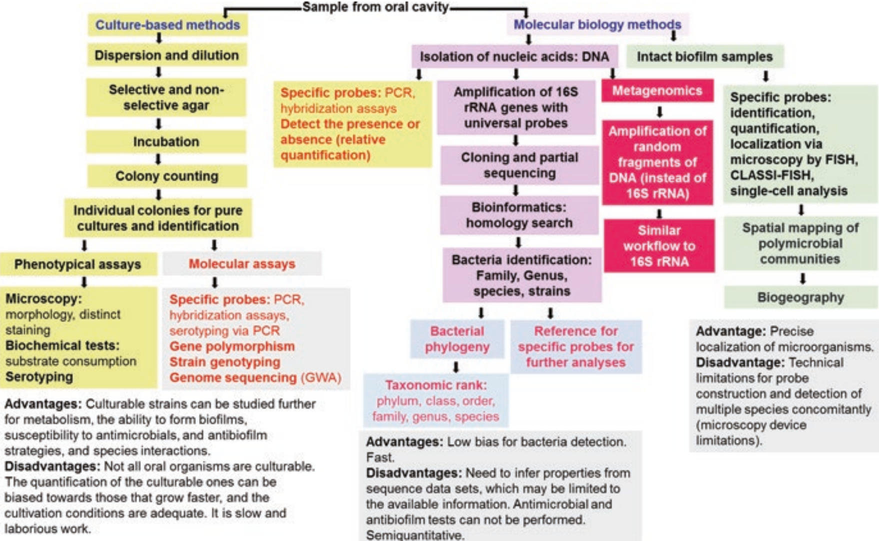

Fig. 5.1 Workflow to analyze samples from the oral cavity. Samples from the oral cavity can be saliva (stimulated or unstimulated), dental plaque (biofilm) from supragingival or subgingival areas and distinct teeth or teeth areas (also, root canal), and mucosa (tongue, palate, gingiva). Oral streptococci can be detected in all of them

methods, such as biochemical tests (e.g., substrate consumption like glucose metabolization—sugar metabolism, exoenzyme production), microscopy (morphology, distinct staining), serotyping, and molecular typing. The molecular typing includes serotyping by polymerase chain reaction (PCR), identifying species by species-specific gene by PCR, sequencing of 16S ribosomal RNA (16S rRNA) gene, DNA relatedness methodologies (genotyping via PCR using random primers, multilocus sequence typing (MLST)), and genome sequencing (e.g., that can be used for the genome-wide association (GWA) studies). Although these steps are time-consuming and laborious, they can provide clinical isolates (strains of the same species or distinct species) that can be characterized further via antimicrobial and antibiofilm testing, for example, among other assays.

5.3 Main Known Oral Streptococci Species Involved in Biofilm Formation and Disease Occurrence

Currently, the *Streptococcus* genus is clustered in eight groups (*mitis*, *sanguinis*, *anginosus*, *salivarius*, *downei*, *mutans*, *pyogenes*, and *bovis*), and oral species belong to six of them— *mitis*, *sanguinis*, *anginosus*, *salivarius*, *downei*, and *mutans*, as shown in Table 5.1 [4–6]. The phylogeny and taxonomy of oral streptococci were extensively analyzed and revised recently [4–6]. The species linked to dental caries development include species of the mutans group *S. mutans* and *S. sobrinus*, associated with species of distinct genus and kingdoms [7, 8]. In contrast, the species of the mitis and sanguinis groups are abundant in biofilms associated with dental surface health. The exception is Streptococcus gordonii that has been implicated as an accessory pathogen for periodontal disease development [9]. Thereby, here the focus will be on the assays available to study them. As more whole genomes of distinct strains become available, a reshuffle of groups and species may occur in the future. Also, potentially new species may be uncovered. For example, a *Streptococcus* strain, designated strain A12, was isolated from supragingival dental plaque of a caries-free individual and is highly arginolytic, antagonizing *S. mutans* [10].

In addition, its capability to produce ammonia can help neutralize acids that could demineralize teeth to cause cavities, promoting a health-associated microbiota, hindering dental caries development.

5.4 Typing Tools

Most studies evaluated *S. mutans* because of its association with the development of cariogenic biofilms. Thus, the following information on tools is mostly based on that species, with few exceptions. The most popular molecular typing tools are a form of PCR or have PCR (or its derivative) in its workflow. However, as sequencing cost decreases, it is becoming more popular, despite its drawbacks, as described below. In addition, additional methodology for identification and typing is being proposed, such as mass spectrometry.

Table 5.1 *Streptococcus* genus groups found in the oral cavity and the species in each group (in mid-2020)

Groups[a]	Species	Notes	References
Mitis	*S. pseudopneumoniae* *S. pneumoniae* *S. mitis* *S. oralis* *S. peroris* *S. infantis* *S. australis* *S. parasanguinis* *S. cristatus*	*S. cristatus* was classified as belonging to the *mitis* group by Jensen et al. [5], but it was classified as part of the *sanguinis* group by Richards et al. [4]. Also, the former *S. oligofermentans* is classified as *S. cristatus* by Jensen et al. [5] "*S. dentisani* is equivalent to '*S. mitis* biovar 2′, and together with *S. tigurinus* and *S. oralis* are part of the same phylogenetic clade and, thus, should be considered a single species, with *S. oralis* comprising three subspecies: *S. oralis* subsp. *oralis* subsp. nov., *S. oralis* subsp. *tigurinus* comb. nov. and *S. oralis* subsp. *dentisani* comb. nov" [5]	[4, 5]
Sanquinis	*S. gordonii* *S. sanguinis* *S. cristatus*	Same note as above for *S. cristatus.*	[4]
Anginosus	*S. intermedius* *S. constellatus* subsp. *pharyngis* *S. anginosus*		[4]
Salivarius	*S. salivarius* *S. vestibularis*	*S. thermophilus* is also in this group, but not as a common oral inhabitant	[4]
Downei	*S. downei* *S. criceti*		[4]
Mutans	*S. ratti* *S. mutans* *S. sobrinus* *S. macacae*	*S. ratti* and *S. macacae* are found in the mouth of hamsters/rats and monkeys, respectively. The study by Richards et al. [4] did not analyze *S. sobrinus* strains, which were previously classified in this group	[4]

[a]The groups names are based on the classification made by Richards et al. [4]

5.5 Endpoint PCR for Species Identification and Detection of Specific Genes

PCR can be used to identify a specific species by using species-specific primers and probes, detect the serotype of a species via serotype-specific primers, and verify the genotype of strains from the same species. Also, PCR products can be sequenced. Specifically, endpoint PCR followed by agarose gel to detect the PCR product is one of the simplest approaches to identify bacteria; an example is shown in Fig. 5.2. Several primers in the literature were developed to detect and type oral streptococci. The ones for *S. mutans* are extensively described in the first edition of this book [11]. However, some of them are mentioned here again, given their importance in the field, besides other species (Table 5.2).

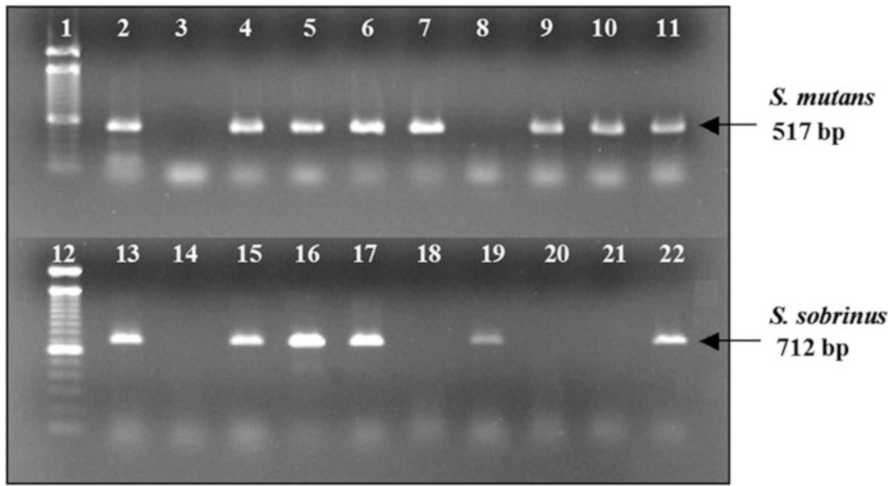

Fig. 5.2 An agarose gel showing the results of PCRs with primers for identification of *S. mutans* (amplification of *gtfB* gene with primers GTFB-F and GTFB-R, product size of 517 bp) and *S. sobrinus* (amplification of *gtfI* gene with primers GTFI-F and GTFI-R, product size of 712 bp) ([12], Table 5.2). Lines 1 and 12: molecular size markers. Lines 2 and 13: positive controls (DNA template from *S. mutans* and *S. sobrinus*, respectively). Lines 3 and 14: negative controls (absence of DNA template). Lines 4–7 and 9–11: DNA from clinical isolates identified as *S. mutans*. Lines 15–17, 19, and 22: DNA from clinical isolates identified as *S. sobrinus*. Line 8: clinical isolate that was presumptive of mutans streptococci but was not confirmed as *S. mutans* by PCR. Lines 18, 20, and 21: clinical isolates that were presumptive of mutans streptococci but were not confirmed as *S. sobrinus*. Of note, all clinical isolates that were presumptive of mutans streptococci (total of 1181 isolates via colony morphology on mitis salivarius bacitracin agar, [13, 14]) were first tested with primers for *S. mutans* because of its higher prevalence, and those that were negative were then tested using primers for *S. sobrinus* (less prevalent than *S. mutans*)—among the 1181 clinical isolates, 102 were not *S. mutans* or *S. sobrinus*, while 968 were *S. mutans* and 111 were *S. sobrinus* [15]

Several studies used PCR with primers designed for specific genes to detect their presence in a collection of strains of the same species or from the same genus. For example, the clinical isolates, as identified via PCR in Fig. 5.2, were subjected to fingerprinting via AP-PCR [15], and later, representative isolates of a specific genotype were selected to investigate whether the competence-associated genes known at that time were present via PCR in clinical isolates and whether they were polymorphic via Southern blot [19].

5.6 Quantitative PCR

Quantitative PCR (qPCR) is a PCR that uses, in addition to specific primers, monitoring technology via fluorescence to quantify the levels of a specific species or genus in a clinical sample, for example. There are several fluorescence approaches.

Table 5.2 Example of primers used to identify and serotype oral streptococci. The sensitivity of each pair of primers can vary (see references for details)

Species	Target (gene)	Name	Sequence (5′ to 3′)	Product size (bp)	References
S. mutans	*gtfB*	GTFB-F	ACT ACA CTT TCG GGT GGC TTG G	517	[12]
		GTFB-R	CAG TAT AAG CGC CAG TTT CAT C		
S. sobrinus	*gtfI*	GTFI-F	GAT AAC TAC CTG ACA GCT GAC T	712	
		GTFI-R	AAG CTG CCT TAA GGT AAT CAC T		
S. mutans	*htrA* locus and an intergenic locus	Sm479F	TCG CGA AAA AGA TAA ACA AAC A	479	[16]
		Sm479R	GCC CCT TCA CAG TTG GTT AG		
S. mutans[a]	Serotype *c*	SC-F	CGG AGT GCT TTT TAC AAG TGC TGG	727	[17]
		SC-R	AAC CAC GGC CAG CAA ACC CTT TAT		
	Serotype *e*	SE-F	CCT GCT TTT CAA GTA CCT TTC GCC	517	
		SE-R	CTG CTT GCC AAG CCC TAC TAG AAA		
	Serotype *f*	SF-F	CCC ACA ATT GGC TTC AAG AGG AGA	316	
		SF-R	TGC GAA ACC ATA AGC ATA GCG AGG		
	Serotype *k*	CEFK-F	ATT CCC GCC GTT GGA CCA TTC C	296	[18]
		K-R	CCA ATG TGA TTC ATC CCA TCA C		

[a]Among *S. mutans* serotypes, c is the most prevalent, while f and k are the least frequent and are associated with extraoral infections, including infective endocarditis [11]

The common ones are the TaqMan system (which uses an oligonucleotide probe beside the pair of primers needed for PCR) or a dye that fluoresces only when bound to double-stranded DNA (e.g., SYBR green). Both approaches can compare samples with unknown bacterium cell numbers to a standard curve to determine the quantity of the target sequence in a clinical sample. There are several reports in the literature. For example, a recent study used the primer pair Sm479F/Sm479R (Table 5.2) with SYBR to quantify *S. mutans* in clinical samples [20]. qPCR can also be used to detect variation in nucleotide sequences via single-nucleotide polymorphism (SNPs) analysis [21].

5.7 Genetic Fingerprinting Studies: Determination of Clonal Diversity and Genotypes

Several methodologies have been employed to determine DNA relatedness to uncover clonal diversity and genotypes. The initial studies were performed with PCR-RFLP (restriction fragment length polymorphism), chromosomal DNA fingerprint (CDF), and arbitrarily primed (AP)-PCR methods, as detailed before [11].

In addition, PCR-based DNA suppressive subtractive hybridization (SSH) [22] and comparative genomic hybridization (CGH) [23, 24] based on the *S. mutans* UA159 genome published in 2002 [25] were used to demonstrate that some isolates did not have some of the genes present in that reference strain. Furthermore, two MLST strategies were developed to evaluate clonal relationships among strains of *S. mutans*. The first strategy included eight conserved housekeeping genes [26], while the second comprised six housekeeping genes and two putative virulence genes [27]. Later, a study compared both approaches to validate data from repetitive extragenic palindromic polymerase chain reaction (rep-PCR) genotypes [28]. Both approaches can discriminate clonal or genotype complexes.

An indirect way of testing species diversity was to detect the presence of specific genes and the polymorphism of specific genes in a collection of clinical isolates. For example, a PCR strategy to study the TnSmu2 region identified important variations among distinct strains, suggesting a core genome and a dispensable genome [29]. Another study investigated whether the competence-associated genes known at that time were present via PCR in clinical isolates of *S. mutans* isolated from children with and without caries and whether they were polymorphic via Southern blot [19]. Later, a similar strategy combining PCR and Southern blot was used to evaluate 14 sensor kinases in distinct isolates [30]. These are only some examples of studies performed over the years.

5.8 Sequencing

The development of next-generation sequencing (NGS) of the 16S rRNA gene provided the most robust and powerful technique for bacterial identification. The curated databases of 16 rRNA sequences [e.g., the Ribosomal Database Project [31], GenBank at https://www.ncbi.nlm.nih.gov/refseq/targetedloci/16S_process/, Greengenes, Silva, EzBioCloud], obtained early on by chain termination method (Sanger sequencing) and chemical degradation method, were of utmost importance to the methodology. The 16S rRNA gene encodes the RNA found in the small subunit of the ribosome, responsible for translating DNA sequence (RNA from genes) into proteins using the universal genetic code. The fidelity and maintenance of this translation property are paramount; thereby, some regions of the rRNA gene are highly conserved. These sequences can be used to align genes from distinct species. However, some regions present variations so that each species has a unique sequence, allowing the distinction between similar species.

Thus, an unknown bacterium can be identified via the DNA sequence of its 16S rRNA gene. It can be compared to the sequences curated in the databases using tools like the Basic Local Alignment Search Tool for nucleotide (BLASTn). Moreover, the conserved and variable regions of the 16S rRNA gene sequence can be used as targets for PCR, to detect either a specific-species by using species-specific primers or total bacteria via a universal primer that detects all bacteria species or even a primer for genus identification. Furthermore, the comparison of 16S rRNA gene sequences can provide information on the species evolution or bacterial phylogeny. In this analysis, a tree diagram of evolutionary relationships can be constructed based on the conserved regions and differences in the variable regions. This approach also allows the determination of the relationship of bacteria at the genus and species level. The phylogenetic information can help to design primers that are homologous to all known oral streptococci. Of note, the ribosomal 16S phylogeny is limited because it does not account for the lateral transfer of genes, including virulence genes. As most known oral streptococci are naturally competent [32], ribosomal 16S phylogeny has its limitations. Hence, as more complete genomes become available, genomic comparisons via GWA analyses can be better tools for phylogenic classification. One example is the reclassification of some oral streptococci performed recently [5].

Another drawback of 16S rRNA gene sequencing in oral microbiology is that although it allows identification of bacteria at the level of species, it does not usually provide enough information to resolve oral communities at the subspecies level, nor it can detect eukaryotic microorganisms (fungi, protozoa) and/or viruses. Nevertheless, 16S rRNA gene sequencing has helped uncover essential information in the oral communities [33].

Furthermore, while the sequencing of 16S rRNA is used to identify the species (either from clinical isolates or in a community), the sequencing of the whole genome of a collection of strains from the same species can be used to pinpoint the pangenome and the core genome and variable or accessory genome ("dispensable" genome). Several reports have demonstrated that the variable part may contain significant strain-specific virulence determinants, including antimicrobial resistance. Before the decrease in the cost for sequencing, to verify polymorphism of a specific gene in clinical isolates strains, strategies included PCR associated with Southern blot (e.g., *S. mutans* competence-related genes [19]). Currently, sequencing has replaced it, but because of the drawback of sequence coverage, quantitative PCR of SNPs is employed to complement the sequencing information [21]. As mentioned above, before the advent of whole-genome sequences, several genetic fingerprinting strategies were used to evaluate clonal relationships among strains. Also, the metagenomics of a microbial community can provide information on antimicrobial resistance genes present [34].

The sequencing of the whole genome of a collection of strains from the same species compared to other species of the same genus can reveal important traits of a species. For example, the sequencing of 57 isolates of *S. mutans* [via whole-genome shotgun (WGS) sequencing] from individuals of known dental caries status, besides some closely related species (*Streptococcus ratti*, *Streptococcus macaccae*, and

Streptococcus criceti), demonstrated several features that explain the successful adaptation of *S. mutans* to the oral cavity [35]. One of them was a set of genes present in all isolates of *S. mutans* but absent in other species evaluated; among those genes were those associated with metabolic processes that may have evolved with human dietary changes over time. Next, 15 of these strains that presented a high degree of gene content diversity were subjected to further phenotypic characterization and genetic analyses [36]. The outcomes were the species phenotypic diversity, where *S. mutans* evolved several adaptive strategies to persist in the mouth and participate in the disease process when conditions are favorable [36]. Of note was the confirmation of the absence of some specific genes performed via PCR, which corroborated previous findings of competence-associated genes in clinical isolates that were evaluated using PCR and Southern blot [19].

Recent studies investigated health-associated oral streptococci species that present promising potential as probiotic candidates to prevent dental caries [37, 38]. The whole genomes of several streptococci isolates (encompassing ten species: *S. australis*; A12-like; *S. cristatus*; *S. gordonii*; *S. intermedius*; *S. mitis*; *S. oralis*, including *S. oralis* subsp. *dentisani*; *S. parasanguinis*; *S. salivarius*; and *S. sanguinis*) were sequenced. The sequencing information was used to verify the relationship with ammonia production via the arginine deiminase system (ADS). However, the study found that similar genotypes or specific oral streptococci species can present distinct phenotypes [37]. Next, bioinformatics was used to select genes for deletion, which then impaired the isolates/strains' ability to compete with the pathogen *S. mutans* [38]. Thus, a combination of methodological tools can help determine whether a specific strain could be selected to promote oral health.

5.9 DNA Hybridization Assays

The methodologies checkerboard analysis and microarray hybridization required specific probes (known sequences of a specific species, e.g., 16S rRNA) and were once widely used to detect and quantify specific species in DNA isolated from clinical samples but have now been replaced. For example, checkerboard analysis was employed to map the microbial complexes in subgingival [39] and supragingival [40] biofilms and associate those complexes with health and disease. Moreover, the quantitative Human Oral Microbe Identification Microarray (HOMIM) based on 16S rRNA was used to assess several clinical samples [41, 42]. The HOMIM was replaced by the Human Oral Microbe Identification using Next Generation Sequencing (HOMI*NGS*) technology, also based on 16S rRNA [43].

Moreover, as mentioned above, among the strategies of genetic fingerprinting studies, the screening of clinical isolate libraries or laboratory strains was performed using DNA hybridization approaches. For example, earlier efforts via PCR-based DNA suppressive subtractive hybridization (SSH) [22] and later via comparative genomic hybridization (CGH) [23, 24] were made based on the *S. mutans* UA159 genome published in 2002 [25]. These studies demonstrated that some isolates did not have some of the genes in that reference strain.

Another category of hybridization assay relies on undisturbed/intact samples where specific probes with fluorescence markers are hybridized in situ. The most popular is fluorescence in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes, followed by its variant *PNA-FISH* (peptide nucleic acid fluorescence in situ hybridization). The use of fluorescent markers in loco is advantageous for the precise localization of microorganisms [44]. However, there are technical limitations for probe construction and detection of multiple species concomitantly (microscopy device restrictions). Some of these limitations are being overcome by a derivative method known as combinatorial labeling and spectral imaging FISH (CLASI-FISH), which is a strategy that uses genus- and family-specific probes to visualize simultaneously and differentiate microorganisms in intact samples [45–47], but still may be further improved.

Besides these hybridization assays, strategies can introduce intracellular or extracellular fluorescence markers to investigate how strains interact with host components or other microorganisms in vitro and/or in vivo (e.g., animal models) by quantifying them using flow cytometry (less common due to limitations of analyzing fluorescent signals in complex samples, such as biofilms, [48, 49]) or microscopy (more feasible, [50]).

5.10 Summary

The continued development of strategies to improve the understanding of oral streptococci roles in oral microbiota will occur. Therefore, it is germane to keep in mind the research and/or diagnostic questions being asked to select the best approach to answer them. There will be a combination of classical microbiological, biochemical, molecular, and imaging approaches in most cases. For example, one study performed whole-genome shotgun (WGS) sequencing of two *S. mutans* strains [21], one fluoride-sensitive and the other fluoride-resistant [51]. Next, it analyzed the sequence of genes identified as involved in fluoride resistance using PCR, Sanger sequencing, and qPCR to identify single-nucleotide polymorphisms (SNPs) responsible for the resistant phenotype [21]. There are also efforts to use other approaches, such as matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), to identify streptococci species [52]. Using tools available will also help devise better approaches to prevent and/or treat diseases and maintain host health.

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6 Streptococcus pneumoniae

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6.1 Introduction

Streptococcus pneumoniae, also known as pneumococcus, is phylogenetically allocated into the *mitis* group, within the viridans streptococci. *Streptococcus pneumoniae* is a highly important bacterial pathogen that frequently colonizes the human upper airways, especially in young children. It can spread from the nasopharynx or oropharynx to different body sites or, via droplets, to other individuals and lead to the development of invasive and noninvasive diseases in all age groups. Noninvasive pneumococcal diseases such as acute otitis media and sinusitis are more common and usually milder conditions [1]. Pneumococcal conjunctivitis, although less frequent, can also occur [2].

This pathogen is a major cause of community-acquired pneumonia. Pneumococcal pneumonia can be a noninvasive (non-bacteremic) or an invasive (bacteremic) disease. Up to 25–30% of patients with pneumococcal pneumonia develop bacteremia. Pneumococcal bacteremia can also occur without pneumonia. Sepsis is a condition frequently associated with pneumococcal bacteremia. Meningitis is another major life-threatening invasive pneumococcal disease (IPD). Other uncommon IPDs include septic arthritis, endocarditis, pericarditis, peritonitis, cellulitis, osteomyelitis, and brain abscess [1, 3, 4].

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6.2 Molecular Methods for Pneumococcal Typing

Molecular typing methods are crucial to understand the changes in the epidemiology of pneumococcal infections and the dynamics of the evolution of pneumococcal population. Different selective factors have been driving pneumococcal epidemiology and evolution, including antibiotic use and the introduction of pneumococcal vaccines.

Serotype determination has been the primary focus to understand the epidemiology of pneumococcal strains for decades since vaccines have targeted their capsular polysaccharides [5]. Surveillance studies allowed the detection of the "serotype replacement" phenomenon, characterized by the reduction or elimination of vaccine serotypes and the emergence of non-vaccine types associated with both colonization and disease, after the advent of pneumococcal conjugate vaccines (PCV) [6].

Motivated by the emergence of antimicrobial resistance in the 1990s and the global spread of drug-resistant organisms, various subtyping methods have been evaluated to differentiate pneumococcal strains [5]. Several genotyping methods such as ribotyping, BOX fingerprinting, pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST) have been widely used to subtype pneumococcal isolates [7, 8] and to identify "serotype switch" events, which consist in a change of serotype of a single clone by alteration or exchange of its capsule polysaccharide synthesis (*cps*) locus [9, 10].

The use of these typing methods combined with serotyping and antimicrobial resistance patterns has allowed isolates from different epidemiological regions to be examined for potential relationships and the identification of persistent local and global clones [5]. In this context, the Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 to guide global surveillance of drug-resistant *S. pneumoniae* and to standardize laboratory methods and epidemiological definitions for identifying internationally disseminated clones ([11]; https://www.pneumogen.net/pmen/criteria.html). Later, the PMEN has also decided to include major invasive drug-susceptible clones that are globally disseminated.

Data from numerous surveillance projects performed in various countries over the past 30 years show that although there is considerable diversity among resistant strains, a small number of highly successful clones have emerged within countries and in some cases have achieved massive geographical spread across both national and continental boundaries [5]. To date, the PMEN has recognized 43 clones. See https://www.pneumogen.net/pmen/criteria.html for nomenclature and criteria for inclusion as a global clone. Pneumococci belonging to some of these clones not only are geographically widespread but also represent a significant proportion of resistant strains in a given epidemiological setting. Isolates belonging or closely related to many of these clones have been associated with both disease and colonization in children and adults, even after widespread PCV use.

New candidates to PMEN clones need to be subjected to three different molecular typing methods to confirm they are unique: PFGE, MLST, and PBP fingerprinting. PFGE and MLST will be discussed below. PBP fingerprinting is performed since alterations in penicillin-binding proteins (PBPs) are the major mechanism of

resistance to beta-lactams in *S. pneumoniae*. Most of the high-level penicillin resistance is due to alterations in PBP 1a, 2b, and 2x, and methods for PBP fingerprinting include restriction fragment length polymorphism (RFLP) analysis or sequence comparisons of PCR products amplified from *pbp1a*, *pbp2b*, or *pbp2x* [12].

6.3 Serotyping

Serotype determination is an essential step to characterize pneumococcal strains. Serotypes are determined by biochemical and antigenic differences in the polysaccharide capsule, the pneumococcal dominant surface structure that plays a critical role in virulence. Currently, there are around 100 recognized serotypes, which are grouped into more than 40 serogroups based on their antigenic similarities [13]. Serotype distribution varies greatly according to clinical source, patient's age, geographical location, and over time. The pneumococcal capsule is the target of current pneumococcal vaccines, which provide serotype-specific protection. Widespread use of these vaccines has driven changes in serotype prevalence among both carriage and disease isolates. Thus, accurate identification of pneumococcal serotypes is of paramount importance for pathogen surveillance and evaluation of PCV impact. Two main approaches have been widely used for determining pneumococcal capsular types: serologically by detecting serogroup- and serotype-specific capsule epitopes and genetically by detecting nucleotide sequence of capsule genes.

6.3.1 Neufeld Test (Quellung Reaction)

The Quellung reaction was first described in 1902 and is still considered the gold standard method for pneumococcal serotyping [14, 15]. It is highly sensitive and specific and is based on the reaction of specific antibodies against the capsule polysaccharides. If the antibodies recognize a specific capsule epitope, they bind and produce a change in the refractive index of light passing through the capsule, which then appears "swollen" when visualized under a microscope [15]. Reaction can be

Table 6.1 Streptococcus pneumoniae antisera pools used in the Quellung reaction

Pool	Serotypes/serogroups included
A	1, 3, 4, 6, 8
B	14, 15, 18, 23, 28
C	7, 19, 20, 24, 40
D	5, 9, 11, 16
E	12, 13, 33, 44, 46
F	2, 10, 17, 22, 31
G	29, 34, 35, 39, 42, 43, 47
H	25, 36, 37, 38, 41
I	21, 27, 32, 45, 48

viewed using oil immersion at a 1000× magnification in a light microscope. However, a counterstain (methylene blue) or phase-contrast microscopy can be used to further enhance visibility.

For Quellung-based serotyping, each pneumococcal isolate is sequentially tested against antisera pools, each one combining different mixtures of serotype/serogroup antisera (Table 6.1). When a positive reaction is observed with a certain pool, the isolate is then tested against individual serotype and serogroup antisera present in that pool. Serotypes within serogroups are further differentiated using panels of factor antisera, which give different profiles of positivity for each serotype in a certain serogroup. Thus, the Quellung reaction can be laborious and costly and requires technical expertise to generate accurate results. An example of how to differentiate serotypes within serogroup 19 with factor sera is shown in Table 6.2.

6.3.2 Latex

Serological determination of capsular types became faster and easier with the development of latex agglutination tests, which use anti-rabbit IgG-coated latex particles sensitized to pooled and selected individual serotype-specific antisera. The agglutination reaction that occurs between the pneumococcal cell and the type-specific antibodies results in visible clumping, eliminating the requirement for microscopy [16]. However, the latex agglutination method is intended to narrow the identification down to a group or pool of serotypes, and then Quellung can be done using specific antisera for each serotype in the group or pool. In addition, commercially available latex agglutination kits for pneumococcal serotyping are still of high cost and, thus, unfeasible for use in laboratories of low- and middle-income countries (LMIC).

6.3.3 Serotype Deduction

Due to the complexity and high costs to obtain and store antisera to perform direct pneumococcal serotyping, several alternative strategies have been developed to deduce pneumococcal capsular types. Such approaches do not characterize the polysaccharide capsule itself, but are usually simple and easy to perform. Consequently, they have greatly reduced reliance upon conventional phenotypic serotyping, providing capsular type-determining potential to laboratories that lack

Table 6.2 Differentiation of serotypes comprised by serogroup 19 in the Quellung reaction

Serotype	Factor 19b	Factor 19c	Factor 19e	Factor 19f
19A	–	+	–	–
19B	–	–	+	–
19C	–	–	–	+
19F	+	–	–	–

type-specific antisera and other reagents needed for conventional serotyping [5]. Since the 2000s, genotype-based approaches have been increasingly applied, permitting the development of epidemiological surveillance in studies involving carriage and disease isolates, especially focusing on the impact of PCV introduction. The most widely used approaches involve PCR or sequencing, although DNA microarrays have also been developed [17, 18].

More recently, proteomic-based methods such as matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and Fourier-transform infrared spectroscopy (FTIR) have been tested to determine pneumococcal capsular types, consisting in promising alternatives [19, 20].

6.3.3.1 PCR-Based Approaches

Initially, some PCR-based approaches to deduce pneumococcal serotypes relied on RFLP assays, based on the amplification of partial or whole *cps* operon followed by digestion with one to three restriction enzymes [21, 22]. However, these assays have not been commonly used.

One widely used method for serotype deduction was developed by the US Centers for Disease Control and Prevention (CDC) and involves a sequential multiplex PCR-based scheme easily adjustable to different serotype distributions [23–25]. Currently, this approach includes 41 oligonucleotide primer pairs used for conventional multiplex PCR assays capable of deducing 70 serotypes. Schemes of eight sequential multiplex PCR assays adapted to Africa, Latin America, and the United States are already available (https://www.cdc.gov/streplab/pneumococcus/resources.html).

This approach has great application for deducing serotypes from clinical specimens when causal pneumococcal strains cannot be recovered [26] and has been extended to deducing pneumococcal serotypes present in nasopharyngeal secretions [27]. However, some inaccuracy may occur when using this PCR assay for carriage isolates, since non-pneumococcal *mitis* group streptococci can carry *cps* locus homologs of pneumococcal strains. This is more likely when analyzing oropharyngeal specimens, especially those recovered from adult populations [28, 29].

More recently, real-time quantitative PCR (qPCR) assays have been explored to determine pneumococcal capsular types [30, 31]. Usually, qPCR methods are faster, more sensitive, and more specific than conventional PCR; however, they are more expensive and have limited capacity for multiplexing.

CDC has adapted the conventional sequential multiplex PCR assays to a triplex sequential real-time PCR format. This approach currently uses 21 oligonucleotide primer pairs distributed over 7 reactions to target 37 serogroups/serotypes. Schemes adjusted to four geographic areas are already available: Africa, Asia, Latin America, and the United States (https://www.cdc.gov/streplab/pneumococcus/resources.html).

A different multiplex real-time PCR protocol was also described. Eleven multiplex qPCR assays arranged in duplex, triplex, or quadriplex formats were designed to identify and quantify 40 prevalent pneumococcal serotypes directly from nasopharyngeal and blood specimens [32].

It is important to realize that these methodologies and schemes are subjected to be continuously refined as additional serotypes are added and primer sets updated to improve specificity [5].

6.3.3.2 Sequencing-Based Approaches

The *cpsB* (*wzh*) gene is the main target of such approaches. One strategy involves the polymorphism analysis of partial *cpsA* (*wzg*)-*cpsB* sequence along with a serotype-specific PCR targeting the *wzy* gene [33].

Another approach, termed sequetyping, uses the whole *cpsB* gene sequence to increase resolution [34, 35]. A third approach, named capsular sequence typing (CST), relies on the partial *cpsB* sequencing [36]. Usually, these methodologies are followed by BLAST analysis (https://blast.ncbi.nlm.nih.gov/) against GenBank database. In a slightly modified version of the CST approach, isolates sharing 99.8% identity (maximum divergence in one single nucleotide) in partial *cpsB* gene sequences queried against GenBank were considered to belong to the same capsular type. Still, closely related serotypes such as serotypes 6C and 6D may not be distinguished from each other [37].

Next-generation sequencing (NGS) methodologies have already been used to determine pneumococcal serotypes. A target enrichment-based NGS method was developed to detect and predict all pneumococcal serogroups, but only 32 to the serotype level, directly from nasopharyngeal specimens and primary culture [38].

As costs for NGS continue to decrease, whole-genome sequencing (WGS) has been increasingly used, and serotype information can be extracted from WGS data. Some bioinformatics tools to predict pneumococcal serotype from genomic data such as PneumoCaT (*pneumo*coccal *ca*psular *t*yping [39]) and SeroBA [40] have already been developed. These two approaches use WGS data generated with Illumina NGS technology and are freely available at https://github.com/.

PneumoCaT is a fully automated pipeline that uses a two-step approach for predicting capsular types from *pneumococcal* genomic data. By annealing and comparing WGS data to *cps* operon reference sequences of 92 capsular types and 2 subtypes (new molecular variant patterns), a serotype is successfully assigned if a single capsular locus matches >90%. If more than one capsular locus sequence matches, PneumoCaT then uses a capsular type variant database to differentiate serotypes within a serogroup. PneumoCaT requires a mean depth of 20 reads across the mapped sequence and minimum depth of 5 reads for mapping (https://github.com/phe-bioinformatics/PneumoCaT) and demands significant computational and memory resources.

SeroBA is a *k*-mer-based pipeline that uses a database adapted from PneumoCaT. *k-mers* are subsequences of a specific length (*k*) within a nucleotide sequence. This approach can accurately predict serotypes by identifying the *cps* locus directly from raw WGS data at low depth of coverage, using computational resources more efficiently (https://github.com/sanger-pathogens/seroba).

CDC has also develop an in silico method to predict serotypes and additional characteristics, such as antimicrobial resistance, pilus type, and MLST, from Illumina paired-end WGS data [41]. The CDC pneumococcal typing pipeline is an

in-house tool, but scripts, database, and reference files can be accessed at https://github.com/BenJamesMetcalf/Spn_Scripts_Reference.

6.4 Pulsed-Field Gel Electrophoresis

PFGE is a genotyping method for evaluation of total chromosomal DNA digested by restriction enzymes based on the pulsed-periodic reorientation of electrophoretic fields on an agarose gel, allowing a clear separation of DNA fragments of various sizes (from kb to Mb) [42, 43]. Clonal assignment of bacteria evaluated by PFGE is based on fingerprinting or banding patterns, and the number of different bands between two isolates can be correlated to the number of genetic events that led to their differentiation over time. Interpretation of results can be done visually (for instance, using the criteria established by [43]) or by using dedicated software such as BioNumerics (Applied Maths).

PFGE has long been considered the gold standard method for subtyping several microorganisms [42] and is especially useful to address short-term evolutionary questions. For pneumococcus, the most commonly used restriction enzyme is *Sma*I, and PFGE was one of the first widely used subtyping methods, revealing that certain drug-resistant pneumococcal clones were distributed worldwide [44]. Along with other characteristics, these findings culminated in the establishment of the PMEN as mentioned above.

PFGE, however, is laborious and time-consuming. Over time, faster and more practical approaches such as MLST have replaced PFGE as the method of choice for molecular typing of pneumococcal isolates.

6.5 Multilocus Sequence Typing

MLST is a genotyping method that has been consistently applied in molecular epidemiology and population structure and evolution of different organisms, especially pathogenic bacteria. MLST usually employs sequencing of seven genomically unlinked housekeeping genes that are then compared to previously identified sequences (or alleles). Identical sequences correspond to the same allele, designated by a number. The seven-number (or allelic) profile derived from the alleles assigned for each of the seven loci is then used to designate the sequence type (ST). MLST data are portable and available on online databases (https://pubmlst.org/). STs can be grouped into clonal complexes (CC), which typically include clones that share five or six alleles [45]. An MLST scheme for *S. pneumoniae* was developed in 1998 using internal fragments (about 500 bp) of seven housekeeping genes [46]. Alternative MLST primers that provide slightly larger fragments for five of the seven loci can be found at the CDC website (https://www.cdc.gov/streplab/pneumococcus/resources.html). Over 15,000 STs have already been described and are deposited at the *S. pneumoniae* MLST database (https://pubmlst.org/spneumoniae/). MLST can also be predicted from WGS data directly at https://pubmlst.org/ or in

websites and pipelines that use allele sequence and profile data obtained from the PubMLST database, such as the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/) and the CDC pneumococcal typing pipeline.

Different algorithms such as eBURST and minimum spanning tree have been widely used to analyze MLST data. eBURST is an online version of the based upon related sequence types (BURST) clustering algorithm [47]. The original BURST algorithm has been adapted for use as a plug-in for the BIGSdb database software [48]. An optimized version termed global optimal eBURST (goeBURST) was developed to identify alternative patterns of descent [49] and is available at http://www.phyloviz.net/goeburst/.

By eBURST, strains related to each other by sharing at least six of seven loci, designated single-locus variant, can be easily connected to create a clonal group. In addition, eBURST is able to predict the founding genotype within a group. In turn, goeBURST allows for the creation of groups with double-locus variant (DLV) and triple-locus variant STs. This algorithm has proved to be a powerful tool for pneumococcal surveillance purposes, allowing for simple resolution of epidemiologically important CCs [5].

MLST combined with capsular type determination has allowed the research community to understand the dynamics of pneumococcal population genetic structure, especially following PCV implementation. It is also an effective means by which serotype switching can be identified and traced [50].

Several MLST-based studies have allowed for insightful analysis of population genetic structure, revealing clonal emergence and expansion within individual serotypes, associated with carriage and disease. In addition, carriage surveillance studies are frequently conducted to measure PCV effects in a given population, since invasive pneumococcal isolates are not easily recovered from clinical specimens and colonization is an important step for the development of pneumococcal diseases and transmission of the microorganism.

For example, the emergence of multidrug-resistant (MDR) serotype 19A strains as a major IPD cause in all age groups has been largely documented in countries following seven-valent PCV (PCV7) or ten-valent PCV (PCV10) introduction [51]. MLST analyses have revealed that the major lineage associated with the emerging MDR serotype 19A strains in Brazil, Colombia, and the United States was CC320/271^{19A} [8, 52, 53]. ST320 is a DLV of ST236, known as the PMEN clone Taiwan^{19F}-14, suggesting the occurrence of capsular switching. In Europe, however, CC230, which includes the PMEN clone Denmark^{14}–32-ST230, had already been identified as the major MDR serotype 19A lineage causing IPD before vaccine introduction but increased after PCV7 use [54]. These data reinforce the importance of surveillance programs in different geographical settings.

In some countries where the 13-valent PCV (PCV13) replaced PCV7, frequency of serotype 19A IPD has already been declining. However, other serotypes are likely to continue emerging, as additional serotypes are included in new vaccine formulations targeting the polysaccharide capsule. Ongoing surveillance is, therefore, warranted, and MLST or ideally WGS analyses will be important to understand the dynamics of the ongoing changes.

6.6 Whole-Genome Sequencing

The size of *S. pneumoniae* genome is approximately 2.0 Mb [55]. As mentioned above, WGS provide information that can be used to deduce serotype and MLST. The CDC pneumococcal typing pipeline also provides WGS-based identification of pilus islet type (PI-1 and PI-2) and resistance to beta-lactam and 11 non-beta-lactam antimicrobial agents. Through PBP 1a, 2b, and 2x sequence analysis, it can also predict minimum inhibitory concentration (MIC) to six beta-lactam agents, including penicillin, amoxicillin, meropenem, cefotaxime, ceftriaxone, and cefuroxime [41].

Furthermore, advances in NGS have been allowing researchers to perform comparative genomics and investigate the pneumococcal population dynamics in greater detail. The identification of single-nucleotide polymorphisms (SNPs) across the isolates can be highly informative and reveal evolutionary relationships. Two different strategies have been used: whole-genome SNP (wgSNP) and core genome SNP (cgSNP). Analysis of core genome appears to be more suitable for surveillance and phylogenetic purposes since it compares single-copy genes present in all genomes of a given species.

SNP analysis, however, considers each point mutation as a single evolutionary event. To overcome this issue, the allelic variation concept of MLST, in which insertion, deletion, and recombination in multiple loci are considered unique evolutionary events, has been applied to the whole-genome level, which seems biologically more relevant. MLST-based approaches for comparative genomics include, for instance, whole-genome MLST (wgMLST) and core genome MLST (cgMLST). Both analyses provide higher resolution for strain characterization than the classical MLST scheme since more than a thousand loci are examined. The wgMLST approach analyzes a greater number of genes and maximizes the resolution but may also include highly variable elements such as repetitive genes and pseudogenes [56]. In contrast, cgMLST possesses higher epidemiological relevance and stability. A cgMLST scheme for *S. pneumoniae*, consisting of alleles from 1360 loci, was recently developed and is available in the PubMLST database. In addition, this database also hosts a collection of over 9000 published pneumococcal genomes within the PubMLST Pneumococcal Genome Library (https://pubmlst.org/spneumoniae/; [48]).

Core genome analysis is the most widely used approach to assess the phylogenetic relationship and population structure of pneumococcal strains. An interesting approach to evaluate population genomics of post-PCV changes in pneumococcal carriage epidemiology in the United States involved phylogeny and population clustering using maximum likelihood and Bayesian analyses based on SNPs in 1194 core genes. Isolates were classified into 22 sequence clusters (SCs), which had significantly different recombination rates. In addition, the evolution of the pneumococcal population following PCV7 introduction was primarily driven by changes in the frequency of distinct genotypes extant before vaccine implementation [55, 57].

Another group used a set of 1160 core genes to generate a cgSNP-based tree and demonstrated that unencapsulated strains isolated from epidemic conjunctivitis

outbreaks belong to a diverse pneumococcal lineage (SC12), designated as the epidemic conjunctivitis cluster [2].

Recently, the Global Pneumococcal Sequencing (GPS) project was created to assist worldwide comparisons focusing on the pneumococcal evolution in the PCV era. The GPS project is the largest pneumococcal genomic surveillance study, and over 13,000 genomes are publicly available (http://www.pneumogen.net/gps/). To standardize genomic definition of pneumococcal lineages and provide a global understanding of pneumococcal population, 621 Global Pneumococcal Sequence Clusters (GPSCs) have been defined and can be assigned to any pneumococcal genomic dataset, providing important information on serotype distribution, antimicrobial resistance, and invasiveness [58].

6.7 Concluding Remarks and Future Perspectives

Molecular typing of bacterial pathogens is essential for studying the epidemiology of diseases associated, and many of such typing schemes have been applied for pneumococcus over the years. In addition to providing insights into clonal spread, infection transmission, and evolutionary changes, molecular typing of pneumococcal isolates has been crucial for assessing the impact of pneumococcal vaccines. However, many of the most recent techniques are still inaccessible for many laboratories in LMIC due to the high cost associated and the requirement of technical expertise, hindering the gather of robust global information on pneumococci. Thus, the current challenge in molecular typing of pneumococcus is to develop a fast, accurate, reproducible, and widely accessible method. Limitations and disadvantages of current typing methods have been fostering the development of many promising alternative proposals, such as those based on proteomic-based methodologies. In addition, the advent of more cost-effective WGS platforms has significantly expanded our knowledge on pneumococcal epidemiology, and may be in a near future the new “gold standard” molecular typing approach for pneumococcus.

6.8 Summary

Streptococcus pneumoniae is a major pathogen associated with several invasive and noninvasive diseases, especially community-acquired pneumonia. Different selective factors have been driving pneumococcal epidemiology and evolution, including antibiotic use and the introduction of pneumococcal conjugate vaccines. In this chapter, we will approach molecular typing methods that have been widely used to characterize this species and to understand the changes in the epidemiology of pneumococcal infections and the dynamics of the evolution of pneumococcal population.

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Part II

Urogenital Pathogens

Neisseria gonorrhoeae

7

Raphael Cavalcante de Medeiros, Sergio Eduardo Longo Fracalanzza, and Raquel Regina Bonelli

7.1 Introduction

Neisseria gonorrhoeae is a human intracellular pathogen, the etiological agent of sexually transmitted infection (STI) gonorrhea. This microorganism infects the host mucous membranes, such as the oropharynx, conjunctiva, urethra, cervix, and rectum. In male patients, gonorrhea symptoms are associated with urethritis and purulent discharge; in contrast, in female patients, most cases are asymptomatic, which may lead to complications such as pelvic inflammatory disease (PID), infertility, and disseminated gonococcal infection (DGI) [1, 2].

Neisseria gonorrhoeae has become a worldwide health problem due to its ability to accumulate resistance mechanisms, mostly chromosomal. Currently, the World Health Organization (WHO) recommends dual therapy with ceftriaxone and azithromycin for the treatment of genital, anorectal, and oropharyngeal gonococcal infections [3]. However, the emergence of resistance to these drugs raises concerns. *N. gonorrhoeae* presently integrates the list of urgent threat pathogens of the CDC, which estimates 1.14 million infections per year in the United States, being 550,000 of these infections caused by resistant gonococci [4–6].

Diagnosis of infections caused by *N. gonorrhoeae* is often clinical, based on symptomatology, but laboratory diagnosis may be necessary to confirm the etiological agent, since *Chlamydia trachomatis* may cause similar diseases. Classical phenotyping techniques such as culture and antibiogram contribute to the detection of the microorganism and correct use of antimicrobial therapy, which is crucial to prevent the selection of new resistant strains.

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7.2 Laboratory Diagnosis of *N. gonorrhoeae*

Culture and isolation of the microorganism is the most common strategy to diagnose gonorrhea. Specimen collection varies according to the site of infection, patient age, and gender, making use of specific swabs or, in some cases, urine. Collected swabs must be placed in a swab transport medium or inoculated in culture medium immediately after sampling. *N. gonorrhoeae* is a fastidious bacterium; thus, enriched media such as chocolate agar or modified Thayer Martin (MTM) agar must be used. MTM is particularly recommended for the isolation of *N. gonorrhoeae* because it contains not only the nutrients to support the growth of the isolates but also antibiotics (vancomycin, colistin, trimethoprim, and nystatin) that inhibit the growth of commensal bacteria and fungi. Optimal growth conditions are 35–37 °C in a moist 3–7% CO_2-enriched atmosphere. Better recovery rates are achieved by early incubation in this environment, but this time depends on the transport methodology adopted. Other methods used to identify gonococci in clinical specimens are nucleic acid amplification tests (NAATs) [7–9].

Many different NAATs to detect *N. gonorrhoeae* have been developed, including amplification methods such as PCR, quantitative PCR (qPCR), real-time quantitative PCR (RT-qPCR), ligase chain reaction (LCR), transcription-mediated amplification (TMA), and strand displacement amplification (SDA), in conventional protocols or commercial rapid tests. These tests also differ in the nucleic acid sequences used as a target; sequence part of the *opa* genes, 16S rRNA, a chromosomal pilin gene-inverting protein homolog, and a direct repeat region called DR-9 have already been used. Most commercial NAATs are able to identify the occurrence of *N. gonorrhoeae* in vaginal and endocervical swabs from women, urethral swabs from men, and first catch urine from both men and women [8, 10]. Moreover, some NAATs were designed to detect resistance mechanisms [11–13].

The application of NAATs has reduced the use of culture to identify *N. gonorrhoeae* of clinical samples. NAATs can detect the bacteria even when the cells are no longer viable and are frequently used to investigate the disease in non-symptomatic women. In this sense, according to CDC, its application is cost-effective, preventing sequelae due to these infections [8, 10, 14].

7.3 Evaluation of the Susceptibility to Antimicrobials

The antimicrobial susceptibility test for *N. gonorrhoeae* is performed in agar base GC with 1% defined growth supplement (supplement VX) [15]. Breakpoints for penicillin (PEN), tetracycline (TET), ciprofloxacin (CIP), spectinomycin (SPT), azithromycin (AZM), and ceftriaxone (CRO), among other drugs, are available for disk-diffusion and minimum inhibitory concentration (MIC), the last possibly executed by gradient methods such as *Etest*® and MICE®, or agar dilution. Besides being used as a reference to evaluate susceptibility, MIC values may also indicate the occurrence of highly impacting resistance mechanisms. Currently, EUCAST and CLSI recognize isolates with MIC >1 μg/mL as non-wild-type (WT) or

non-susceptible (S) to azithromycin, respectively. Regarding ceftriaxone, the cutoff value adopted varies slightly; EUCAST considers isolates with MIC >0.125 μg/mL as resistant, and CLSI considers isolates with MIC ≤0.25 μg/L as susceptible [15, 16].

The antimicrobial resistance mechanisms in gonococcal strains are mostly chromosomal encoded, such as alterations in the porin B structure or overexpression of the efflux pump MtrCDE (reduces the susceptibility to PEN, TET, AZM, and CRO), mutations in GyrA and ParC (confers resistance to CIP), mutations in 23S rRNA (confers resistance to AZM), and mosaic structure in the PenA peptidyl transferase, with or without additional point mutations (confers resistance to CRO). Moreover, plasmids that carry the beta-lactamase *bla*-$_{TEM1}$ gene (Asia, Africa, Nimes, New Zealand, Rio/Toronto, Johannesburg, and Australia) or the gene *tetM*, encoding a ribosome protective protein (American and Dutch), increase MICs of PEN and TET to resistance levels, respectively [5, 6, 17]. Molecular resistance mechanisms in gonococcus may be identified by nucleic acid amplification tests (NAATs) or genome sequencing.

7.4 Molecular Typing of *N. gonorrhoeae*

Surveillance studies for *N. gonorrhoeae* are conducted in several countries around the world, mainly because the therapy is usually prescribed based on protocols, and it is necessary to monitor the emergence and dissemination of multidrug-resistant strains. In this context, typing methods are highly relevant, providing data that can discriminate the isolates, enabling the recognizing of antimicrobial-resistant or well-succeeded lineages. For these analyses, it is possible to use gel-based typing techniques, such as pulsed-field gel electrophoresis (PFGE), opa typing, and multilocus variable-number tandem repeat analysis (MLVA); strategies based on gene sequencing, as multilocus sequence typing (MLST), *Neisseria gonorrhoeae* multiantigen sequence typing (NG-MAST), and *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR); or sequencing of the complete genome. Generally, for surveillance purposes, molecular typing is associated with epidemiological and phenotypic data.

7.4.1 Pulsed-Field Gel Electrophoresis (PFGE)

The PFGE method is based on the use of rare cutting endonucleases (such as *XbaI*, *NheI*, *BgIII*, and *SpeI*), which produces larger fragments of the genome [18]. These enzymes are used to treat an agarose plug containing lysed whole cells of the microorganisms. After this preparation, the plugs are inserted into an agarose gel, which is placed in particular electrophoresis equipment that emits electrical pulses in different orientations throughout the running process (PFGE equipment). The band profiles generated on the gel are called pulsotypes and may be analyzed by comparison. PFGE is appropriate for analysis of isolates prepared strictly in the same way,

which makes the comparison of results from different studies or workgroups challenging [19]. For gonococci, PFGE presents good data congruence, especially when combined with other techniques, such as NG-MAST [20, 21].

7.4.2 Opa Test

Opa proteins consist of a family of outer membrane proteins that interact with the host cells and undergo phase variation during infection [22]. Opa test is based on the amplification of 11 *opa* genes (*opaA*–*opaK*), followed by digestion with a restriction enzyme (TaqI) resulting in different band profiles on a polyacrylamide gel [23, 24]. This method is highly discriminatory when applied to gonococci, and results do not suffer alterations with the phase variation process; however, a significant disadvantage is the difficulty of analyzing the electrophoretic profiles and correlating data with other laboratories [25]. Overall, it is more applicable for microepidemiology purposes, such as characterization of clusters in a specific geographic region, identification of transmissions by sexual networks, and studies of cases suspicious of reinfection or mixed infections [26].

7.4.3 Multilocus Variable-Number Tandem Repeat Analysis (MLVA)

MLVA is a molecular typing technique based on the analysis of variable-number tandem repeat (VNTR) of multiple loci in the genome, using amplification and electrophoresis as methods. The comparison of MLVA profiles can be visualized in minimum spanning trees, which enables this method for epidemiological purposes. This technique is considered very discriminatory for *Neisseria meningitidis*, which motivated its adaptation to gonococci, with five loci of VNTR [27, 28]. MLVA has not been extensively used, but offers high discriminatory power also for *N. gonorrhoeae*, and can be used as a sub-typing methodology for isolates that have similar PFGE profiles in a strategy that does not require sequencing of alleles. Similar to opa test, it is applicable for typing a defined, somehow correlated, collection of isolates.

7.4.4 Multilocus Sequence Typing (MLST)

The MLST method for genus *Neisseria* was first developed for *Neisseria meningitidis*, applying seven housekeeping genes present in the chromosome of this species (*abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdhC*, and *pgm*) selected due to their low variation throughout the evolutionary process. Later, the same scheme was adopted for *N. gonorrhoeae*. Target sequences of approximately 500 bp of the selected genes are sequenced, and based on their identity, alleles receive arbitrary numbers. After analyzing the entire multilocus profile, the combinations of the arbitrary number attributed to each of the seven alleles give origin to a sequence type (ST) [29].

The MLST database (https://pubmlst.org/neisseria) reiterates that STs can be grouped into clonal complexes, according to their similarity to a central genotype. In the genus *Neisseria*, STs are arranged in a clonal complex when four or more allele sequences are similar to those of the central genotype [30]. MLST website also provides the Bacterial Isolate Genome Sequence database (BIGSdb) software, which allows comparison studies based on whole-genome sequences with reference strains (FA1090 and NCCP11945) [31].

MLST is widely used in surveillance studies in *Neisseria gonorrhoeae* with focus on antimicrobial resistance. Typing through this technique has allowed the detection of worldwide relevant STs. For instance, ST-1901 is related to critical resistance profiles to third-generation cephalosporins, due to the frequent occurrence of the *penA* mosaic gene in these strains. Ceftriaxone-resistant *N. gonorrhoeae* strains belonging to ST-1901 were described in France and Spain [32, 33], and reduced susceptibility to third-generation cephalosporins was reported in isolates of the same ST in different parts of the world, including European countries, Japan, the United States, and Brazil [34–37]. Other STs identified associated with ceftriaxone resistance were ST-7363 in Japan and Australia [38, 39] and ST-1903 in Japan and Canada [40, 41].

Thus, MLST for *N. gonorrhoeae* is useful to correlate phenotypic and genotypic data from strains, allowing better traceability in surveillance studies. Since it is based on conserved genes, it is also a reliable strategy to evaluate the evolution of STs.

7.4.5 *Neisseria gonorrhoeae* Multiantigen Sequence Typing (NG-MAST)

NG-MAST is based on the analysis of internal and hyper-variant regions of genes *porB* and *tbpB*, which respectively encode an outer membrane porin and the beta subunit of a transferrin-binding protein. Forward and reverse reactions are performed for each gene, resulting in fragments of 737 bp and 589 bp for *porB* and *tbpB*, respectively. After sequencing, sequences must undergo quality control, with trimming and alignment of the sequences with a preserved region pre-established by the technique (*por*, TTGAA, 490 bp, and *tbpB*, CGTCTGAA, 390 bp) [42, 43].

NG-MAST has been integrated to the online platform of PubMLST for analysis and attribution of NG-MAST-STs to uploaded sequences (https://pubmlst.org/). Currently, more than 19,000 NG-MAST STs have been assigned, which represents the approximate data of 11,403 alleles of *porB* and 2895 of *tbpB*, that keep increasing. *porB* is generally more variable than *tbpB* and is considered to be responsible for the high discriminatory power of NG-MAST [35, 43].

Indeed, NG-MAST may be used to subtyping isolates assigned in the same MLST-ST for a more precise epidemiologic analysis [35, 44–46]. NG-MAST is also a simple sequencing-based method to be combined with PFGE, improving the identification of pulsotypes [26]. However, it is noteworthy that when compared with whole-genome sequence (WGS) data, the same NG-MAST-ST may be identified in not correlated isolates [45, 46].

7.4.6 *Neisseria gonorrhoeae* Sequence Typing for Antimicrobial Resistance (NG-STAR)

Neisseria gonorrhoeae sequence typing for antimicrobial resistance (NG-STAR) is a molecular typing technique based on the molecular resistance profile of *N. gonorrhoeae*. Considering that antimicrobial resistance in *N. gonorrhoeae* is determined mostly by chromosomal mutations and is strongly associated with specific clones, NG-STAR can be an excellent tool for typing isolates and screening of relevant clones from an epidemiological perspective.

NG-STAR performs molecular typing based on the alleles of chromosomal genes related to antimicrobial resistance (*penA*, *mtrR*, *porB*, *ponA*, *gyrA*, *parC*, 23S rRNA), through analysis of the presence of specific mutations. The database and NG-STAR website (https://ngstar.canada.ca) is hosted by the Public Health Agency of Canada, National Microbiology Laboratory [47].

The inference of an NG-STAR-ST follows the same logic of MLST and NG-MAST assignments, after submission of the sequences of the seven selected genes. It is possible to send the data in a multi-FASTA file or to upload the complete genome. NG-STAR can also be accessed by PubMLST, through BIGSdb (https://pubmlst.org/bigsdb?db=pubmlst_neisseria_seqdef), which allows more complete analysis of the isolates.

If after a preliminary analysis an NG-STAR type is not assigned due to the identification of alleles not previously reported, by the submission of a new allele, the user may enter additional information such as date and country of isolation of the bacteria, age and gender of the patient, presence or absence of a beta-lactamase gene, and antimicrobial resistance profile of the isolate, including MIC values for penicillin, cefixime, ceftriaxone, ciprofloxacin, azithromycin, spectinomycin, and tetracycline. It is also possible to add epidemiological information regarding the isolate. NG-STAR database has increased considerably in the last 2 years. When it was released in 2017, there were 215 STs, contrasting with the current 3893 STs.

The correlation of NG-STAR with other typing methodologies such as MLST and NG-MAST can be of great value for surveillance studies, allowing the screening of resistant clones circulating in several countries with higher reliability. In this sense, NG-MAST contributes to a more comprehensive epidemiological study, adding to other typing methods information associated with phenotypic aspects of gonococci.

7.4.7 Whole-Genome Sequencing Typing

Whole-genome sequencing (WGS) typing offers higher resolution than other molecular typing techniques based on selected genes such as MLST, NG-MAST, and NG-STAR. Complex studies related to phylogeny can be performed with WGS, using the analysis of single-nucleotide polymorphism (SNPs) or Bayesian analysis strategies, which can be performed with softwares like Parsnp, RAxML, and BEAST. Finally, it is also possible to apply genome mapping methodologies adopting *N. gonorrhoeae* reference strains as a basis for comparison. WGS data

may be used to typify isolates by MLST, NG-MAST, and NG-STAR, as well as to identify chromosomal and plasmidial resistance mechanisms [48–52].

7.5 Conclusion

Neisseria gonorrhoeae is a microorganism with high genetic plasticity, mainly due to the presence of DNA uptake sequences (DUS) in its genome, which allows the exchange of genetic material during all phases of its growth [53], and also due to the absence of a CRISPR-Cas9 system, which helps to remove exogenous genetic material received [54]. Such plasticity makes molecular typing of this species challenging and raises attention to the fact that typing methods must be continuously evaluated and, if necessary, updated.

MLST, NG-MAST, and NG-STAR are very efficient in discriminating isolates based on housekeeping genes, hyper-variant regions of selected genes, or genes associated with antimicrobial resistance, respectively, and may be used complementarily. However, especially in studies aimed at surveillance of significant collections of isolates, the use of WGS can be of great value. From the analysis of sequenced genomes, it is possible not only to compare the strains in detailed phylogenetic trees but also to typify them by such methodologies based on alleles. Moreover, sequences uploaded in open-access databases enable the comparison of isolates obtained in different studies. Thus, WGS data aids in better discrimination of strains, as well as improves the monitoring of multidrug-resistant lineages circulating in the world. However, the high costs of WGS and the necessity of specialized professionals to perform the data analysis required may represent drawbacks for the adoption of WGS as a typing method in many studies.

Molecular methods based on electrophoretic profiles or gene sequencing are available for *N. gonorrhoeae* typing. Laboratories may elect a typing method considering the infrastructure and resources available. All typing strategies are valuable when results are correctly interpreted, respecting the limits of the applied technique. Still, in all and any study resembling typing of *N. gonorrhoeae*, composing a representative collection of isolates is frequently more challenging than performing typing itself. Thus, efforts to facilitate the collection of strains in remote regions or low-income countries, ideally associated with epidemiological data, are necessary to improve the *N. gonorrhoeae* global surveillance.

7.6 Summary

Neisseria gonorrhoeae is the etiological agent of gonorrhea, a sexually transmitted infection. This microorganism is considered a worldwide health problem due to its ability to accumulate resistance mechanisms to different antimicrobial agents, including those currently recommended by the WHO to treat gonorrhea. Molecular typing of *N. gonorrhoeae* makes possible surveillance projects focused on epidemiological aspects or antimicrobial resistance. Whereas some techniques are useful

to characterize well-defined collections of isolates, like the *opa* test and PFGE, others, such as MLST and WGS analysis, can recognize internationally distributed clones, which are usually involved with the spread of resistance. This chapter presents the rationale, applicability, and limitations of several methods developed for *N. gonorrhoeae* typing, providing bases for choosing strategies according to the purpose of each study and leading proper interpretation of the results obtained.

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Streptococcus agalactiae (Group B Streptococcus)

8

Sarah Shabayek

8.1 Introduction

Streptococcus agalactiae or group B streptococcus (GBS) is a gram-positive pathobiont carrying the Lancefield group B polysaccharide antigen. It is an asymptomatic colonizer of the gastrointestinal and genitourinary tracts of up to 30% of healthy pregnant women [1]. GBS has emerged in the early 1930s as a veterinary pathogen and a frequent cause of bovine mastitis [2]. In 1938, the first human cases due to GBS were reported [3]. In the 1960s [4], GBS has been reported to cause severe perinatal infections, and since then, GBS remained a leading neonatal pathogen [5–7]. GBS diseases are divided into early-onset infections (EOD) which develop in the first week of life and late-onset infections which appear between 7 days after birth and 3 months of age (LOD). EOD usually manifest as sepsis and pneumonia and LOD as meningitis. Maternal carriage is a principal risk factor for GBS vertical transmission during birth [1, 8].

The current guidelines released by the Centers for Disease Control and Prevention (CDC) recommend intrapartum antibiotic prophylaxis (IAP) for positive carriers to prevent perinatal GBS disease [1]. The guidelines include a universal culture-based screening approach for pregnant women at 35–37 weeks of gestation in order to limit unnecessary IAP. The active implementation of the IAP program has resulted in a significant decline in neonatal mortality and morbidity [1, 9]. Despite being harmless to healthy women, GBS is also responsible for serious invasive and noninvasive infections in pregnant women such as meningitis, endocarditis, osteomyelitis, amnionitis, bacteriuria, endometritis, cellulitis, and fasciitis. In addition, GBS can cause severe invasive infections in elderly and immunocompromised patients with underlying disease such as HIV, cancer, and diabetes [7, 10–14].

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Proper IAP to the targeted risk group and successful treatment of GBS disease require carefully adapted detection protocols for GBS in clinical samples. The basic protocol released by CDC [1] includes selective enrichment broth cultures for clinical samples prior to detection using different microbiological techniques. Several microbiological options are available for GBS detection and identification. These include blood agar, Granada-type media, chromogenic media, CAMP test, serological testing, identification with soft ionization and mass spectrometry (MALDI-TOF MS), hybridization methods, and molecular-based methods.

8.2 Laboratory Detection of GBS

8.2.1 Timing of GBS Screening and Specimen Collection

The timing of specimen collection is of great importance as GBS colonization status is dynamic and can change during pregnancy. GBS colonization status early in pregnancy cannot be used as a proxy for intrapartum colonization. Hence, prenatal GBS culture in the late third trimester (≤5 weeks before delivery) is preferable to earlier trimesters [1, 15, 16]. Swabbing both the vagina and rectum increases GBS culture yield compared to the vagina or cervix alone without the rectum [1, 17–21]. Therefore, the current CDC guidelines recommend vaginal-rectal screening specimens at 35–37 weeks of pregnancy [1]. When immediate laboratory processing is not possible, swabs should be kept in an appropriate transport medium, such as Amies or Stuart medium at 4 °C for up to 4 days. The GBS culture yield declines significantly at room temperature over time [1, 22–24].

8.2.2 Selective Enrichment

Direct agar plating alone results in missing about 50% of positive GBS colonizers, and their cultures are reported as false-negative carriers. Selective enrichment of collected swabs leads to a substantial increase in GBS detection [6, 25–27]. The commercially available selective culture broths are Lim broth (Todd-Hewitt broth supplemented with 10 μg/ml colistin and 15 μg/ml nalidixic acid) and TransVag broth (Todd-Hewitt broth supplemented with 8 μg/ml gentamicin and 15 μg/ml nalidixic acid) [6]. These broths are usually available without blood. However, the recovery of GBS from selective enrichment broth can be increased by the addition of 5% sheep blood, e.g., Baker broth (Todd-Hewitt broth with 8 μg/ml gentamicin, 15 μg/ml nalidixic acid, and 5% sheep blood) [26]. It is noteworthy that Lim broth is preferable for selective enrichment. In contrary to TransVag broth where the absence of blood was reported to inhibit GBS growth, Lim broth has superior ability to inhibit gram-negative bacteria and promote GBS growth even without blood [27].

On the other hand, false-negative GBS carriage due to competitive growth inhibition by *Enterococcus faecalis* in selective broth [28] can be avoided by inoculating swabs on an adequate plating medium in addition to the selective enrichment

broth such as blood agar or selective blood agar (neomycin-nalidixic acid agar [NNA] or colistin-nalidixic acid agar [CNA]), Granada agar, or chromogenic agar. Once GBS colonies are detected on the agar, parallel selective broth cultures can be discarded [6, 29].

8.2.3 Presumptive Identification

GBS strains are gram-positive cocci arranged in pairs or chains and show large colonies (3–4 mm in diameter) on defibrinated sheep blood agar. Most isolates are beta-hemolytic with a narrow zone of hemolysis. Separated beta-hemolytic colonies demonstrate a characteristic ring-like zone of hemolysis [30–32]. A small percent of human GBS (2–5%) are nonhemolytic [1, 6].

Almost all GBS [6, 33, 34] are positive for CAMP (Christie, Atkins, Munch-Petersen) test [35]. Up to 98% of GBS are CAMP-positive [30]. CAMP is a cytolytic toxin which has the ability to lyse sheep erythrocytes, producing a unique synergistic zone of hemolysis when treated with sphingomyelinase (the staphylococcal beta-lysin). A characteristic arrowhead zone of hemolysis is formed when streaking a GBS strain perpendicular to a streak of a strain of beta- lysin-producing *Staphylococcus aureus* on sheep blood agar. The arrowhead zone appears adjacent to the point where the two streaks come into proximity [34, 36]. Both beta-hemolytic and nonhemolytic strains are positive for the CAMP factor encoded by the *cfp* gene which is mainly used for the molecular detection of GBS [33].

GBS has the ability to produce carotenoid pigmented colonies when grown anaerobically on Granada-type media. These are starch-rich media where starch initiates the production of granadaene (red-orange pigment) with the aid of methotrexate and Bacto Proteose Peptone N3 (BD) [6, 37–39]. All beta-hemolytic strains of GBS produce pigmented colonies on Granada-type media [40]. The formulation was developed by de la Rosa et al. in 1992 [37]. Anaerobic incubation is necessary when culturing GBS on Granada agar and not in Granada broth (ChromID Granada Biphasic from bioMérieux or Strep B Carrot broth from Hardy Diagnostics) [6]. GBS also produce orange pigmented colonies under anaerobic conditions on GBS agar (Islam) [41] marketed by Oxoid and described by Islam in 1977 [42]. This medium lacks methotrexate, and pigmentation can be enhanced with trimethoprim and sulfonamides. No other streptococcal species produce granadaene; hence, it can be considered as specific single-step identification of beta-hemolytic GBS [6, 40] whereas nonhemolytic GBS do not produce the orange pigment [43]. However, nonhemolytic and nonpigmented GBS strains may not be potentially pathogenic and mostly have a bovine origin. That is why Granada-type media cannot be used in veterinary diagnostic settings for GBS [40].

GBS detection can also be achieved using commercially available chromogenic media such as Brilliance GBS (Oxoid), ChromID Strepto B (bioMérieux), StrepB Select (Bio-Rad), and CHROMagar StrepB (CHROMagar). These media are incubated in the dark under normal atmospheric conditions. Anaerobic incubation hampers the development of the colored GBS colonies. However, chromogenic media

are neither 100% specific nor 100% sensitive, and further confirmatory tests are required for reliable identification of GBS [6].

Furthermore, GBS can hydrolyze hippurate producing glycine which is easily detected by ninhydrin [44]. Benzoic acid is a second by-product due to hippurate hydrolysis and can be detected by ferric chloride [41]. Hippurate hydrolysis test is fast and easy to perform. However, due to low specificity, it is unreliable in GBS detection [41]. In addition, GBS can be distinguished from other beta-hemolytic streptococci such as *S. pyogenes*, *S. porcinus*, and beta-hemolytic enterococci by a negative pyrrolidonyl arylamidase (PYR) test, while the formers are PYR-positive [6, 45, 46]. Alternatively, commercially available biochemical profiling kits such as the RapID STR system and API Rapid Strep identification system manufactured by bioMérieux and Remel, respectively, can be used for GBS identification with high accuracy [6].

8.2.4 Serological Methods

Identification of GBS by agglutination with the Lancefield group B antigen is widely used for routine diagnostic purposes. It was first established by Lancefield and Hare in the 1930s for the identification and grouping of beta-hemolytic streptococci [47]. It is based on group-specific serological reactions with the carbohydrate antigens of the cell wall of streptococci. Commercial streptococcal latex grouping kits or coagglutination tests (containing nonviable staphylococci coated with a group-specific hyperimmune antiserum) are available where positive agglutination reactions show clumping of the latex particles or staphylococcal cells [6]. Direct latex agglutination testing on selective enrichment broths rather than pure cultures has shown acceptable sensitivity and was sometimes preferred for fast turnaround times and less workload [28, 48].

8.2.5 Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

More recently matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has become available for GBS identification [49]. It is an emerging technique for bacterial identification to the species level within few minutes [50]. It is based on the analysis of the protein profiles using soft ionization methods. Results are visualized as spectra in the region between 2 and 20 kDa which are then matched with a database of known spectra. For automated analysis, raw data are processed with special software (MALDI Biotyper software, Bruker Daltonique, France) [49, 51, 52]. The maximum matching score value is 3. Scores ≥2.0 are reliable for bacterial identification to the species level [49]. It should be noted that the accuracy of this method is mainly dependent on the reference database which is carefully designed using strains representing the major phylogenetic lineages that represent the species under test [49]. The main advantages of

MALDI-TOF MS are speed and accuracy [49, 51, 52]. The MALDI-TOF MS technology has been adopted in many laboratories as a fast and powerful tool for bacterial identification [53]. It is expected to replace traditional phenotypic methods in the near future.

Lartigue et al. (2009) [49] correctly identified 110 GBS isolates using MALDI-TOF MS showing 100% accuracy. The isolates were further characterized by serotyping and multilocus sequence typing (MLST). Interestingly, the authors observed significant variations in the score values according to serotypes and MLST types. The highest scores (≥ 2.3) were significantly obtained for serotype III compared to other serotypes and for MLST type 23 (ST23) compared to other STs. Binghuai et al. (2014) [51] used MALDI-TOF MS to confirm the identification of putative GBS colonies obtained on the chromogenic ChromID Strepto B agar (STRB). They recommended STRB combined with MALDI-TOF MS as a fast, sensitive, and accurate method for the identification of GBS carriers among pregnant women. However, one of the major limitations of the MALDI-TOF MS technology is that it still cannot be used for direct detection of GBS in clinical samples. Mixed cultures are also inappropriate for MALDI-TOF MS analysis, and identification is mostly restricted to pure isolates where an adequate number of pure colonies under test is required for a satisfactory mass spectrum [50].

8.2.6 Hybridization-Based Methods

The most commonly used hybridization kit for GBS identification is the Gen-Probe Accuprobe Group B Streptococcus culture identification kit [1, 30]. It is a non-amplified nucleic acid amplification test. It is based on a hybridization reaction of a chemiluminescent-labeled single-stranded DNA probe with a complementary rRNA strand of GBS for positive GBS cultures. The chemiluminescence of labeled DNA-rRNA hybrids is measured in a Gen-PROBE laminator [30]. The test has an acceptable sensitivity with a shorter turnaround time than traditional culture [54].

8.2.7 Nucleic Acid Amplification Tests (NAAT)

The CAMP factor gene *cfb* [33] and the *scpB* gene [55] were the first amplification candidates for GBS PCRs. Today, several gene targets have been developed and evaluated for GBS identification [6]. The US Food and Drug Administration (FDA) has approved a number of nucleic acid-based tests, which are commercially available, for GBS detection directly from clinical specimens or after selective broth enrichment, and three of these are available as fully automated real-time PCR systems for intrapartum GBS detection such as the SmartCycler and Xpert Technology (Cepheid), the Illumigene system (Meridian Bioscience), and the BD MAX system (Becton, Dickinson) [6]. Nucleic acid amplification tests (NAATs) demonstrate both higher sensitivities and rapid turnaround times compared to culture methods [56]. However, NAATs lack antibiogram results which are required in case of

penicillin allergy. According to the CDC guidelines, the usefulness of NAATs may be restricted for women at term with unknown GBS colonization status. Besides, when used for antenatal GBS screening, NAATs are recommended to be preceded with selective enrichment cultures for better detection results [1]. Couturier and coworkers investigated the effect of the period of selective broth enrichment on the sensitivities of three FDA-cleared NAATs. A selective enrichment incubation period of 18–24 hours provided optimal sensitivities for GBS detection in comparison to periods of 4–8 hours [56].

8.3 Molecular Typing Methods

Several techniques have been described for characterizing GBS and classifying strains for epidemiological purposes, and each tool provides a certain level of data in the context of epidemiological surveillance. These include capsular polysaccharide serotyping, surface protein-based typing, pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), multilocus variable-number tandem-repeat assay (MLVA), and clustered regularly interspaced short palindromic repeats (CRISPR).

8.3.1 Capsular Polysaccharide Serotyping

GBS strains can be classified into ten distinct serotypes (Ia, Ib, II–IX) based on the sialic acid-rich capsular polysaccharide (CPS) [57, 58]. The most common phenotypic methods for capsular typing are serological methods based on Lancefield capillary precipitation or latex agglutination using commercially available kits [59, 60]. However, such methods are not reliable enough and always result in a percentage of non-typeability (NT) or serotyping errors due to variable or poor capsule expression which differs between GBS strains particularly bovine isolates which usually lack CPS [60]. Various genotypic protocols have been described for molecular capsular serotyping such as PCR and sequencing [61], PCR and DNA dot blot hybridization [62], multiplex PCR plus reverse line blot hybridization (mPCR/RLB) [63, 64], PCR and subsequent restriction enzyme digestion of amplified capsular polysaccharide genes [65], serotype-specific DNA microarray [66], and paired sets of multiplex PCRs [67]. Despite reliable molecular serotyping techniques, all these methods are multistep techniques. Besides, all, except mPCR/RLB developed by Kong et al. in 2008 [63], were introduced prior to inclusion of the most recent discovered serotype IX [58]. In 2010, Imperi and colleagues [68] published a one-step multiplex PCR for molecular capsular typing in GBS. Such protocol accurately classifies strains into all known GBS CPS from Ia to IX. In comparison to former protocols, this method seems to be reliable, faster, and feasible to perform with basic molecular biology tools.

Distribution of GBS serotypes is geographically distinct and changes over time [69–71]. While serotypes Ia, Ib, II, III, and V are predominant colonizers in the

United States and Europe [8, 72–76], serotypes VI and VIII are dominant colonizers among pregnant women in Japan [77, 78], serotype IV in the United Arab Emirates [79], serotype V in Egypt [80], and serotype VI in Malaysia [81, 82]. The new GBS serotype IX was first reported in Denmark in 2007 [58]. Although rarely reported, a recent study in Ghana [83] demonstrated serotypes VII and IX as the most common colonizers in pregnant women.

Invasive GBS isolates show a different serotype distribution. Serotype III is strongly associated with late-onset meningitis in neonates [11, 76, 84–86], and serotypes Ia and V are associated with invasive diseases in nonpregnant adults [11, 87–89]. Interestingly, the emergence of serotypes IV, VI, VII, and VIII has been recently demonstrated among both invasive and colonizing isolates [83, 85, 90–98] pointing out the effect of time on the serotype distribution map and highlighting the importance of continuous monitoring of the circulating GBS serotypes worldwide.

8.3.2 Surface Proteins

In addition to CPS-based typing, GBS surface proteins have been found to be useful epidemiological markers. Similar to CPS, they are principle virulence factors and confer protection against GBS infection. Combined CPS and surface protein typing allows grouping of GBS into lots of serovariants [99]. The C protein (Ibc) was the first surface protein antigen identified in GBS [99], whereas the main surface-anchored proteins of GBS belong to the alpha-like protein (Alp) family proteins which include alpha-C protein, Rib, Alp2, Alp3, Alp4, and epsilon protein (alpha-protein-like proteins, Alp1). These are encoded by the *bca*, *rib*, *alp2*, *alp3*, *alp4*, and *epsilon/alp1* genes, respectively [100]. The Alps are dominantly expressed among GBS strains, while negative Alps carriers are rarely reported representing up to 10% of the total isolates [101].

Protein typing of GBS was originally done using C and R antisera against the alpha-C protein and Rib protein. Identification was done using monoclonal antibodies, polyclonal antibodies, or genes by hybridization with probes. However, the antisera are not specific enough for surface protein identification, and cross-reactivity was frequently reported [100, 102]. Molecular protocols characterizing GBS surface proteins were later published. These are PCR-based including PCR and DNA blot hybridization, parallel PCRs for different proteins, or multiplex PCR. The PCR-based protein typing is preferred over serology due to reproducibility and better discriminatory power [100, 102].

Correlations of serotypes with surface proteins have been reported. It was observed that the clinically important type III strains do not express the C protein which is also rarely found in serotype V strains although expressed by the common serotypes Ia, Ib, and II [99, 100, 103]. On the other hand, significant associations between serotype III and Rib [99, 100] and between serotype V with Alp3 were reported [80, 99, 100, 102]. In general, the Rib protein was reported to be strongly associated with the majority of serotype III and rarely with serotype V. Other notable associations were serotype VI strains expressing the epsilon surface protein [80]

and serotype VIII expressing Alp3 [100]. Of course, none of the associations was exclusive. The same surface protein was often expressed by different serotypes, and vice versa, various serotypes carried the same surface protein.

8.3.3 Pulsed-Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a powerful typing tool that provides increased discrimination in resolving bacterial strains compared to capsular typing. The technique is based on the analysis of chromosomal DNA restriction endonuclease profiles on an agarose gel [104]. A standard protocol for performing PFGE typing of GBS isolates was first described by Fasola et al. in 1993 [105]. It involved the evaluation of nucleic acid digestion fragment profiles with both conventional and pulsed-field electrophoresis to examine the genetic relatedness and diversity in GBS. A modified PFGE protocol was published in 2001 by Benson and Ferrieri [106]. The updated method was faster reducing the workflow time from 6 to 8 days to 3 days. Besides, it improved the resolution power both visually and quantitatively.

PFGE was proposed as an alternative to capsular typing. It was assumed that isolates sharing similar PFGE profiles may belong to the same serotype [105, 106]. However, no clear correlation between PFGE patterns and the assigned serotypes was found. A study on 78 epidemiological unrelated invasive GBS strains reported different PFGE patterns within each serotype where the greatest heterogeneity was observed among type IV, Ia, and II strains, while serotypes Ib, III, and V were more homogeneous [107]. Gherardi et al. (2007) [108] demonstrated identical serotypes shared by isolates of different PFGE groups, and conversely, isolates within the same PFGE groups belonged to different serotypes. Pillai et al. (2009) [109] conducted a large PFGE investigation cross-capsular patterns using 872 GBS strains from 152 individuals. The investigators concluded that identical PFGE patterns not necessarily correspond to the same serotype. They found within the same individual that similar PFGE profiles corresponded to the same serotypes; however, isolates from different individuals sharing similar PFGE profiles sometimes belonged to different serotypes. Hence, PFGE cannot be used as a serotype predictor of GBS isolates across different subjects.

Although PFGE types are difficult to compare between studies [69], the method is of great importance in investigating the genetic relatedness, evolutionary divergence, modes of transmission, and outbreaks. Thomas-Bories and colleagues [110] suggested a clonal relationship between the American and French serotype V GBS strains as they showed indistinguishable fragment patterns from the prevalent American clone. Bovine GBS strains sharing the same PFGE patterns with human isolates were proposed to have a close relation to the human isolates as well as their ability to infect humans or vice versa [111]. Common fragment profiles for GBS strains from mother-infant pairs suggested vertical transmission and maternal colonization as a principal risk factor [112, 113]. Dissimilar PFGE patterns of the same

serotype for GBS isolates from paired specimens of mother and infants [113] proposed contamination or mixed culture infection or horizontal gene transfer. Likewise, indistinguishable PFGE profiles with different serotypes from the same individuals [114] inferred capsular switching by horizontal gene transfer of capsular genes [113, 114]. Investigating a mastitis outbreak in ewes, which resulted in high morbidity and economic losses due to diminished milk production, revealed identical PFGE profiles of the isolated GBS strains [115].

8.3.4 Multilocus Sequence Typing (MLST)

Multilocus sequence typing (MLST) was first proposed by Maiden and coworkers in 1998 [116] as a powerful typing tool for bacterial pathogens. MLST is similar, in principle, to multilocus enzyme electrophoresis (MLEE) except that allele profiles are defined according to multilocus sequences of housekeeping genes (usually seven) instead of the electrophoretic migration of their amplified gene products [116, 117]. MLST is an unambiguous method as it classifies bacterial strains according to the internal fragment sequences of defined housekeeping genes. Distinct allele numbers are assigned for every different sequence within a bacterial species. Then each isolate is defined by an allelic profile or sequence type (ST) [116, 117]. A seven-gene MLST scheme for GBS was developed by Jones and colleagues in 2003 [118]. The scheme was based on seven housekeeping genes *adhP*, *atr*, *glcK*, *glnA*, *pheS*, *sdhA*, and *tkt* where each isolate is designated by a seven-digit allelic profile. The scheme was validated using a global capsule-typed strain collection. Isolates with the same allelic profile are assigned to the same sequence type (ST), and then STs are grouped into lineages or clonal complexes (CC). A particular CC is named after its ancestor ST or the most prevalent ST [119]. Sequences are submitted to a public MLST data base at *http://www.mlst.net*. Hence, the most attractive about MLST compared to other typing methods is that MLST is a portable epidemiological approach where electronic data are easily compared between laboratories [117].

Almost all human isolates belong to six CCs, CC1, CC10, CC17, CC19, CC23, and CC26, whereas the majority of the bovine GBS strains are exclusively grouped into CC61 and CC67 that were never demonstrated for any human isolate before [120, 121]. It is noteworthy that a particular ST is not restricted to a certain capsular serotype and GBS strains with similar ST may demonstrate different serotypes [118]. However, a remarkable association between certain STs and defined serotypes was found. STs 23, 24, and 7 are dominated by serotype Ia, and STs 8, 10, and 12 are dominated by serotype Ib [69]. Serotype II strains predominately belonged to ST28, and strains of ST1 are mostly presented as serotype V [69, 71]. Interestingly, the hypervirulent invasive serotype III mostly belongs to ST17, while the colonizing isolates of the same serotype correspond to STs 19 and 182 [69, 71]. Moreover, several reports characterized the emerging serotype IV belonging to ST196 [49, 71, 122–125].

8.3.5 Multilocus Variable-Number Tandem-Repeat Assay (MLVA)

Multilocus variable-number tandem-repeat analysis (MLVA) is similar in principle to MLST; however, MLVA is a molecular typing method that is based on the differences in variable number tandem repeats (VNTRs). Clonal-specific profiles correspond to a combination of VNTR loci instead of housekeeping genes [126]. According to MLVA, GBS clonal-specific profiles correspond to a pattern of five MLVA loci SATR1 to SATR5 which are the most diverse VNTR loci in GBS as described by Radtke and colleagues in 2010 [127]. The five loci were selected for a multiplex PCR protocol followed by capillary electrophoresis for size estimation. The allele number corresponds to the number of repeats at each locus. MLVA was found to provide higher strain resolution than other typing techniques such as MLST, PFGE, and combined CPS-protein types. The above study conducted by Radtke et al. [127] involved 126 GBS strains that were classified into 70 MLVA types (MTs) versus 36 STs by MLST and 19 combined CPS-protein types. In addition, a four-locus MLVA using STAR1 to STAR4 still provide higher discrimination power than MLST and combined CPS-protein typing. The four-locus MLVA was proposed by the authors as a simpler method for MLVA typing as it was able to resolve the 126 GBS strains into 34 MLST types. Furthermore, single MLVA loci were found to cluster some of the MLST or CPS types as well. A number of 54 or 55 repeats in SATR3 corresponded to ST17, 0 repeat in SATR5 corresponded to ST19, and 15 repeats in SATR2 corresponded to serotype IX.

Later, an updated MLVA scheme for GBS genotyping was developed by Haguenoer and coworkers in 2011 [128]. This used six VNTR loci (including three VNTR loci from those described by Radtke et al. [127]): SAG2, SAG3, SAG4 (SATR1), SAG7 (SATR2), SAG21 (SATR5), and SAG22. The VNTRs are amplified and visualized on agarose gel electrophoresis. The number of repeats for each VNTR is deduced from amplicon size, by comparison with the reference strain, for which the number of repeats is known. The allele number corresponds to the number of repeats. The allelic profile of a strain corresponding to the number of repeats at each VNTR is listed in the order SAG2, SAG3, SAG4, SAG7, SAG21, and SAG22. The updated scheme was found to be rapid, cheap, and easy providing results suitable for exchange and comparison between different laboratories worldwide. Similar to the original protocol proposed by Radtke et al. [127], the updated MLVA Scheme [128] has better discriminatory power than MLST. It could resolve 186 isolates from human and cattle into 98 MLVA types versus 51 MLST types. The updated scheme also correlates well with MLST in GBS [128]. The generated MLVA clusters were represented by major clonal complexes in MLST. The MLVA cluster 9 corresponded to all human strains of the CC17, and GBS isolates belonging to the CC23 were clustered in two groups: CC23-type III and CC23-type Ia.

Recent epidemiological studies started the implementation of MLVA for GBS genotyping instead of MLST. An investigation in Brazil including 83 commensal GBS isolates obtained from nonpregnant women utilized MLVA in order to assess the genetic relatedness between strains [129]. A total of 15 MTs were found. The authors observed that all isolates belonging to the capsular type II were clustered in

MT1 and all isolates except one had an identical MT profile. Another study in pregnant women in Brazil resolved 41 GBS strains into 30 MTs in comparison to only 4 capsular types, Ib, II, III, and V [130]. The most predominant MTs were MT1 and MT2 representing 12.2% and 9.75%, respectively. The study suggested a highly diverse population structure and confirmed the MLVA discriminatory power. Furthermore, MLVA was reported as a useful high-resolution genotyping tool for bovine GBS strains within and between farms where most of the GBS isolates demonstrated similar MLVA patterns specific for each farm [131].

8.3.6 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins, which comprise the CRISPR-Cas system, are promising targets which can be used as unique epidemiological markers for genotypic and phylogenetic characterization of GBS strains [132]. The CRISPR-Cas system has a crucial role in the adaptive immunity of bacteria against exogenic DNA elements invading the bacterial genome such as bacteriophages, plasmids, and mobile genetic elements (MGEs). The CRISPR-based defense confers specific immunization based on the invading nucleic acid sequences. The CRISPR-Cas system consists of highly conserved 25–40 bp short direct repeats (DRs) interspaced by spacer sequences which are non-repetitive and similar in size. Most CRISPR arrays are sided by a leader sequence upstream and a trailer-end sequence downstream of the terminal direct repeat (TDR). Spacer acquisition is polarized. New spacer sequences derived from foreign DNA are inserted at the leader-end, and the leader-end DR is concomitantly duplicated. This initiates the expression of the CRISPR-Cas system to produce specific proteins which target foreign DNA sequences for cleavage within the sequence corresponding to the spacers. Hence, trailer-end sequences represent past encounters and can be considered as ancestral DNA sequences [132, 133].

Up to date, two CRISPR-Cas systems have been described in GBS, type II-A system which is associated with the CRISPR1 locus and type I-C system that is associated with the CRISPR2 locus [134]. The type II-A system is found to be ubiquitous and functional, whereas the type I-C system is rare and most often incomplete [134, 135]. Lopez-Sanchez et al. [134] reported similarities between the majority of the spacer sequences of CRISPR1 and MGEs commonly found in the GBS genomes. Accordingly, they proposed CRISPR1 to play a role in modulating the GBS mobilome through maintaining and controlling the diversity of the spacer sequences which may contribute to the fitness of GBS in diverse environments. In addition, they showed CRISPR1 to be a dynamic system with extensive spacer polymorphism. They described CRISPR1 as a powerful means for typing, subtyping, and tracing of GBS strains circulating worldwide overtime with higher strain discrimination ability than other systems such as MLST. Beauruelle and coworkers [133] used CRISPR1 to follow the vaginal carriage of GBS among 100 women over an 11-year period. An investigation of 126 GBS isolates belonging to 31 distinctive

STs by MLST classification revealed 115 unique spacer sequences according to the CRISPR1 locus typing system [136]. Moreover, an interesting correlation between CRISPR and MLST has been reported. An investigation classifying 351 GBS isolates based on CRISPR1 found clones sharing the spacer 8 and spacers 45, 89, and 373 discriminate ST1 or ST196, ST19, and ST28, respectively [134]. Furthermore, the hypervirulent ST17 clone was found to harbor lower number of spacers in comparison with other lineages using the CRISPR1 system [136].

8.3.7 Other Genotyping Techniques

PCR fingerprinting techniques such as random amplified polymorphic DNA PCR (RAPD-PCR), repetitive extragenic palindromic PCR (REP-PCR), and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) have also been described for GBS [137–140]. RAPD-PCR is based on amplifying arbitrary DNA fragments using short (9–10 bp) random primers with low stringency annealing conditions [141]. REP-PCR involves the use of repetitive sequence target primers that are complementary to highly conserved 33 bp inverted repeat sequences in the bacterial genome [142]. ERIC-PCR is similar to REP-PCR; however, ERIC elements are 126 bp long [142]. All these PCR techniques result in fingerprinting strain-specific band patterns by gel electrophoresis. However, as with PFGE, results of fingerprinting PCRs are difficult to compare between laboratories especially if different primers are used [69].

RAPD-PCR has been used to determine vertical and horizontal GBS transmission in neonates. Brzychczy-Wloch et al. (2008) [143] demonstrated similar mother-infant RAPD profiles for newborns born to colonized mothers suggesting vertical transmission, whereas Strus et al. [144] proposed nosocomial GBS transmission for colonized newborns of noncolonized mothers due to identical RAPD patterns with isolates in other hospital wards. Moreover, Brandolini and coworkers [145] used RAPD assay to investigate GBS transmission through maternal breast milk. Others utilized RAPD to cluster GBS isolates from different anatomical sites [146]. In addition, GBS isolates with resistant phenotypes were successfully clustered by RAPD [147–151]. Furthermore, RAPD assay was also used to reveal genetic relatedness of unrelated GBS clones associated with some invasive infections [138, 152]. Although having less discriminatory power than MLST and PFGE, RAPD results are still acceptable for genotyping GBS strains. A major advantage of RAPD is that it is rapid, simple, and inexpensive with satisfactory discrimination level between clones and good reproducibility [69].

Studies assessing REP-PCR for GBS are limited. Most of the available literature demonstrates REP technique for genotyping GBS from fish. Besides, Al Nakib and others [153] assessed the discriminatory power of the semiautomated repetitive sequence-based PCR DiversiLab® system (DL) rather than the original REP protocol to reveal the genetic relatedness of GBS clones. They compared DL with MLST

and PFGE in classifying invasive GBS isolates. Weak clustering coincidence of DL with MLST and PFGE was found suggesting DL less favorable than MLST and PFGE to investigate the genetic diversity in GBS. Generally, a major drawback of REP-PCR is its poor reproducibility and sensitivity to variations in laboratory conditions [153, 154].

Similar to REP-PCR, literature utilizing ERIC-PCR for GBS genotyping is scarce. The usefulness of ERIC-PCR in GBS typing was first investigated by Dabrowska-Szponar and Galiński in 2003 [155]. Later, the genetic diversity of 86 GBS strains was investigated by ERIC-PCR in a Chennai-based hospital in 2008 [137]. The authors reported ERIC-PCR to be a simple, fast, and inexpensive genotyping method with acceptable discriminatory power when characterizing a large number of GBS strains.

8.4 Conclusion

Precise detection of GBS carriages is largely dependent on the sampling procedure with respect to timing and source. Rectovaginal screening at 35–37 weeks of pregnancy is highly recommended for successful implementation of the IAP program and prevention of invasive neonatal infections. Despite the availability of advanced detection techniques, traditional phenotypic methods are still preferable and being used worldwide as the former are expensive and require special laboratory infrastructure. Until now no vaccine is available to protect against GBS disease; however, the running clinical trials are expected to release a vaccine that is serotype-specific. Hence, continuous mentoring of circulating serotype strains is of great importance. Serotype predominance is geographically distinct, and prevalent types in one area are not in another. Currently, MLST represents the most attractive epidemiological tool for classifying GBS strains due to the presence of a portable MLST database where electronic data are easily compared between laboratories.

8.5 Summary

Streptococcus agalactiae or group B streptococcus (GBS) is a leading cause of neonatal morbidity and mortality worldwide. It is an asymptomatic pathobiont of gastrointestinal and genitourinary tracts of women, and maternal colonization is a major risk factor for vertical transmission. The current guidelines released by the Centers for Disease Control and Prevention (CDC) recommend intrapartum antibiotic prophylaxis (IAP) for positive carriers to prevent perinatal GBS disease. GBS is also responsible for serious invasive and noninvasive infections in pregnant women and can cause severe invasive infections in elderly and immunocompromised patients with underlying disease such as HIV, cancer, and diabetes. Precise detection of GBS carriages is largely dependent on the sampling procedure with

respect to timing and source. Further investigation of the circulating strains in a given population is crucial in tracing pathogenic clones and important in providing a clear epidemiology picture that helps in GBS treatment and future implementation of prevention strategies. This chapter presents the most important tools for GBS detection and identification. Traditional and modern epidemiological techniques are also discussed in detail.

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Treponema

9

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9.1 Introduction

Treponemes that are pathogenic for humans include the causative agents of venereal syphilis (Treponema pallidum subspecies *pallidum*) (TPA), yaws (*T. pallidum* subsp. *pertenue*) (TPE), bejel (*T. pallidum* subsp. *endemicum*) (TEN), and pinta (*Treponema carateum*). In addition, some treponemal species commonly found in the oral cavity are highly associated with periodontal diseases [1]. However, in terms of economic, health, and social impact, venereal syphilis (hereafter referred to as syphilis) outweighs any of the conditions caused by other pathogenic treponemes. Syphilis is endemic in many countries, and there's a resurgence among industrialized nations. In the United States, syphilis disproportionately affects minority populations, and recent data from the Centers for Disease Control and Prevention (CDC) shows a surge in primary and secondary syphilis cases among gay, bisexual, and men who have sex with men (MSM) [2].

Penicillin remains effective for the treatment of infections caused by the *T. pallidum* subspecies. Azithromycin has been used as an alternative to penicillin therapy, but treatment failures associated with mutations in the 23S rRNA gene have been documented in TPA strains [3–5], and resistant strains have been reported in many countries [6–8]. Resistance to azithromycin has also been noted in TPE strains from Papua New Guinea [9].

In the mid-twentieth century, yaws was prevalent in the tropics; syphilis was endemic in Afghanistan, North Africa, southern Africa, Southwest Asia, China, and Europe; and pinta was reported in Mexico and Central and South America. Yaws

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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control campaigns using penicillin aluminum monostearate and benzathine penicillin were very effective in reducing the prevalence of the active form of the disease in countries such as Haiti, Indonesia, and Jamaica. This led to the epidemiological concept that penicillin treatment was necessary for asymptomatic household contacts and presumed latent cases in order to eliminate the reservoir of infection and reduce further transmission [10]. Soon after its establishment in 1948, the World Health Organization (WHO) initiated a worldwide campaign together with the United Nations Children's Fund to control yaws in 46 countries. Consequently, 50 million people were treated over two decades, resulting in a decrease in the prevalence of the disease by about 95%, but foci of the disease still remain.

Compared to the endemic treponematoses, syphilis has garnered more attention because of its mode of transmission, morbidity in adults, and sequelae in infants born to infected mothers. Syphilitic ulcers have been associated with an increased risk of acquisition or transmission of the human immunodeficiency virus (HIV), and high rates of coinfection have been documented among MSM [11–14]. Syphilis rates peaked in western countries around World War II with a subsequent sharp decline in rates that coincided with the widespread use of penicillin for treatment. Syphilis rates have been on the rise in the past two decades, and novel tools are still needed to diagnose, treat, manage, and curb the rising numbers of syphilis cases in the United States and globally [15].

The lack of an in vitro cultivation system for *T. pallidum*, until very recently when a culture method was developed by Edmondson et al. [16], and the inability to genetically manipulate the bacterium are obstacles to elucidation of virulence factors involved in the pathogenesis of *T. pallidum*. In addition, the complex natural history of syphilis and the stigma associated with the infection have made epidemiological studies of this pathogen challenging. Most studies have focused on syphilis, so this chapter places emphasis on genotyping or genetic characterization of TPA, but advances made with the strain typing of the causative agent of yaws are also discussed.

9.2 Characteristics and Classification of *Treponema*

Members of the genus *Treponema* belong to the family *Spirochaetaceae* and have a unique cell structure, with the cell envelope consisting of an outer membrane, a peptidoglycan layer, an inner membrane, and a characteristic number of flagellar filaments located in the periplasmic space. The shape of the organism is often described as helical, coiled, or serpentine and exhibits a characteristic "corkscrew" motility in liquid media. Treponemal cells typically range in length from 5 to 20 μm and between 0.1 and 0.5 μm in diameter and are visualized best by dark-field microscopy. Treponemes are poorly stained by the amine dyes and, given their relatively narrow cell dimensions, are a challenge to detect using standard light microscopes.

The genus Treponema has a diverse group of organisms which include pathogenic or commensal microbial flora of humans, animals, and insects. These

organisms are also found in environmental sites such as contaminated aquifers. Bacterial species belonging to this genus are typically anaerobic and are host-associated, and a few are known to be pathogenic for humans. Of the pathogenic treponemes, the etiologic agent of syphilis (TPA) is the most frequently studied and comprises of approximately 70% proteins, 20% lipids, and 5% carbohydrates. The lipid composition of *T. pallidum* is complex and is comprised mainly of phospholipids (cardiolipin) and a poorly characterized glycolipid [17]. Another two very closely related human pathogens, TPE and TEN are responsible for yaws and bejel, respectively. *T. carateum* causes pinta, and while related to *T. pallidum*, it was placed in a separate species owing to the lack of genetic information. Treponema paraluiscuniculi causes venereal syphilis in rabbits and is closely related to the etiologic agents of yaws, bejel, and venereal syphilis in humans [18, 19]. Due to similarities among the pathogenic *T. pallidum* subsp. in terms of disease manifestation and progression (typically painless skin lesions, spontaneous healing, asymptomatic periods, chronicity) and inability to differentiate them on the basis of morphologic, antigenic, biochemical, or serological criteria, diagnosis has relied on a combination of geographic location, transmission route, clinical presentation, and serological tests [17]. However, molecular methods or tests are now available that can differentiate among the subspecies [20–22].

Treponemes found in oral cavities include *Treponema denticola*, *Treponema vincentii*, *Treponema pectinovorum*, *Treponema medium*, *Treponema amylovorum*, *Treponema maltophilum*, and *Treponema socranski* and have been shown to be associated with gingivitis and eventual progression to periodontitis or with persistent periodontitis [19, 23]. *Treponema refringens* and *Treponema phagedenis* are commensals found in the genital tract of humans. Other species belonging to the genus *Treponema*, such as *Treponema succinifaciens*, *Treponema bryantii*, and *Treponema primitia*, have been isolated from the intestine of horses, the rumen of cows, and the hindgut of termites, respectively. Of the human-associated spirochetes, *Treponema refringens*, *Treponema phagedenis*, *Treponema denticola*, and several other oral spirochetes can be cultured on artificial culture media. In contrast, TPA is primarily propagated through intratesticular inoculation of rabbit testes due to reproducibility, viability, and contamination issues noted for in vitro culture methods over decades of research. Recent studies show promise for sustained propagation of laboratory and clinical TPA isolates using a defined culture medium [16].

9.3 Epidemiology and Clinical Significance

9.3.1 Epidemiology

Current epidemiological data on the endemic treponematoses is difficult to obtain since most countries no longer collect data as a part of routine surveillance, owing in part to the stigma of underdevelopment associated with these diseases [24, 25]. The prevalence of yaws is common in Western or Central Africa, Southeast Asia, and Pacific Islands. Bejel is reported in Sahelian Africa and Saudi Arabia, and pinta

is found in Central and South America [26]. Data from the WHO spanning 1950 to 2016 showed 99 countries and territories are endemic for yaws, 22 for bejel, and 19 for pinta [25]. Only 15 countries have current data on yaws derived from routine surveillance systems. A WHO initiative to eliminate yaws globally by 2030 is currently underway [27].

Based on global sexually transmitted infection (STI) surveillance data from 2018, there were an estimated six million new cases of syphilis reported worldwide, of which 1.6–1.7 cases per 1000 population were recorded for syphilis (men and women) and 473 cases per 100,000 live births for congenital syphilis suggesting a huge burden of TPA relative to other STIs [28]. Syphilis is endemic in many developing countries, while industrialized nations are characterized by low-level transmission and sporadic outbreaks. In the United States, syphilis rates were on the decline from 1990, when the crack cocaine epidemic reached a nadir; however, since 2000 and 2001, the trend has reversed. The majority of cases have been reported among men, suggesting that the spread of the disease was occurring mainly among MSM. This increase in syphilis is characterized by high rates of HIV coinfection, high-risk sexual behaviors, and recreational drug use. Recent CDC surveillance data for syphilis showed an increase in syphilis cases among men and women between 2017 and 2018 in the United States, with MSM accounting for the majority of primary and secondary syphilis cases [29]. During 2018, the total number of syphilis cases for all stages including congenital syphilis was 115,045. The highest rates of primary and secondary syphilis were noted among black men aged 25–29 in the United States. The rate of congenital syphilis in the United States has also increased significantly, reflecting a 185% increase relative to 2014. Syphilis is also on the rise in Australia, Canada, China, and Western European countries [30].

9.3.2 Clinical Significance

Endemic treponematoses are transmitted through skin-to-skin contact, affecting the skin and bone, and often occurs among children under 15 years of age or infrequently in adults [26]. In addition, bejel can also be transmitted via mucous membranes and using contaminated utensils or drinking vessels, and the disease affects the oral and nasal mucosae and bone. Primary lesions normally appear on extremities (lower extremities in yaws) or, on rare occasion, on the oral mucosa as in the case of bejel. Untreated endemic treponematoses leads to a variety of late complications including destructive osteitis, saddle nose, and deterioration of the palate and nasal septum as evident in yaws and bejel and depigmented lesions over hands, wrists, elbows, ankles, and feet in pinta cases [26].

Syphilis exhibits a variety of clinical manifestations and may affect multiple body organs. An invasive infection of syphilis can be divided into distinct stages: primary, secondary, latent, and tertiary syphilis. Primary syphilis is usually characterized by the presence of a solitary chancre, a painless ulcer that begins in most instances as a papule at the site of inoculation; however, some patients do present with multiple lesions. Extragenital lesions have been reported to occur but are less

frequent and tend to be painful compared to lesions in genital areas. The primary lesion usually appears 10–90 days postinfection with an average of 3 weeks. Typical primary syphilitic lesions appear in the genital area with bilateral inguinal lymphadenopathy observed in most individuals. The disease is systemic following initial infection, and 30% of untreated primary syphilis cases present with multiple secondary lesions of the skin or mucous membranes.

Secondary syphilis typically appears 6–8 weeks after the appearance of a primary chancre. It is characterized by evanescent macular rash followed by symmetric papular eruption, which occurs on the entire trunk and the extremities including the palms of the hand and soles of the feet. Condylomata lata, which are raised, whitish, or gray lesions, are frequently observed in warm, moist areas such as the vulva or perianal region. Other symptoms include generalized lymphadenopathy, fever or malaise, sore throat, headache, and weight loss [31]. In about 30% of patients, primary lesions are still present when secondary manifestations appear. *TPA* invades the central nervous system (CNS) in at least 40% of secondary syphilis cases [32].

The secondary and tertiary stages are separated by a prolonged period of latency, which is characterized by a lack of clinical signs and symptoms of disease and positive serological test results. A presumptive diagnosis can be made based on reactive nontreponemal and treponemal tests with no history of prior treatment or, in the case of treatment, a fourfold increase in nontreponemal test titer [33]. The latent stage is separated into early or late latent phases depending on their approximate duration postinfection. The symptoms of late syphilis usually appear 10–20 years after initial infection, affecting any organ in the body and occurring in approximately a third of patient cases that fail to receive appropriate treatment [33]. Late or tertiary syphilis is also divided into neurosyphilis, cardiovascular syphilis, and late benign syphilis. Except for neurosyphilis, late benign syphilis is relatively uncommon these days most probably as a result of inadvertent treatment of syphilis with treponemocidal antibiotics. In the pre-antibiotic era, about a third of patients with untreated syphilis developed tertiary disease, which manifested as neurosyphilis, gummas, or cardiovascular disease. Late neurosyphilis, which usually occurs in 15 to 20% of patients, presents as paresis, tabes dorsalis, or meningovascular syphilis; however, some patients may have asymptomatic disease with cerebrospinal fluid (CSF) abnormalities such as pleocytosis, elevated protein levels, and a positive Venereal Diseases Research Laboratory (VDRL)-CSF test. Diagnosis of tertiary syphilis is based on a combination of clinical history, serological tests, examination of chest radiographs for suspected cardiovascular syphilis, and long bone radiographs to detect bony gummas. Concomitant HIV infection has an impact on neurologic involvement in syphilis, and several studies have documented the rapid progression from early syphilis to neurosyphilis characterized by meningitis or cranial nerve defects. In settings where both endemic treponematoses and venereal syphilis are prevalent, disease history, clinical presentation, and serological test results are important considerations for patient management.

Congenital syphilis occurs when *TPA* enters the fetal circulation by transplacental passage from an infected mother and can lead to stillbirth, early fetal death,

premature delivery, low birthweight, neonatal death, and infection in infants [34]. Clinical signs of congenital syphilis include hepatosplenomegaly, cutaneous lesions, osteochondritis, and snuffles. About 50% of infants with congenital syphilis are asymptomatic at birth; however, teeth and bone malformation, deafness, blindness, and learning disabilities may develop later. In order to ensure effective prevention and detection of congenital syphilis, the CDC recommends that women are screened for syphilis during early pregnancy, beginning with the first prenatal visit, subsequent testing at 28 weeks of gestation and again at delivery for women who are at increased risk or live in communities with a high syphilis prevalence rate [35].

9.4 Laboratory Diagnosis

The diagnosis of treponematoses is dependent on clinical presentation, knowledge of geographic and epidemiological factors, and results of diagnostic tests. Laboratory tests are essential for confirmation of clinical findings and can be performed using direct detection methods or more commonly serological tests. The advances made with molecular techniques allow *T. pallidum* to be differentiated at the subspecies level using specific genetic signatures. Molecular methods such as PCR are also useful in identifying early infection when a lesion is present but antibodies are not yet detectable. Details on the laboratory methods used for the diagnosis of treponematoses with an emphasis on syphilis are described in this section, together with the utility and limitations of currently available diagnostic procedures.

9.4.1 Serological Methods

The laboratory diagnosis of syphilis is challenging, owing to the varied clinical manifestations and, in some cases, the difficulty in obtaining adequate or appropriate specimens for testing. Serological tests are the most commonly used diagnostic methods for syphilis, though venereal syphilis cannot be differentiated from nonvenereal treponematoses based on serology alone. Serological methods can be grouped into nontreponemal and treponemal test types. The VDRL slide test, rapid plasma reagin (RPR), unheated serum reagin (USR), and toluidine red unheated serum test (TRUST) represent the nontreponemal group of tests that detect all classes of antibodies to cardiolipin, lecithin, and cholesterol in serum or plasma. One of the limitations of nontreponemal test is that it may yield a reactive result due to autoimmune conditions, HIV, malaria, injection drug use, or old age, thus requiring a need for reflex testing with a treponemal test, which detects antibodies specific to *T. pallidum*. Treponemal assays include the fluorescent treponemal antibody absorption (FTA-ABS), *T. pallidum* hemagglutination assay (TPHA), *T. pallidum* particle agglutination assay (TPPA), enzyme immunosorbent assays (EIAs), chemiluminescent immunoassays (CIA), and line immunoassays (LIA) that detect antibody to whole bacterium or surface-exposed *T. pallidum* proteins. Automated EIAs and CIAs are being increasingly used in high-volume laboratories due to their

potential to improve workflow efficiency. In addition, treponemal tests have been described to show higher sensitivity for treponemal antibody detection especially for primary and latent stage syphilis cases [36]. The FTA-ABS was commonly used in the past but less common these days because of its poor sensitivity for primary syphilis [36], requirement for fluorescence microscopy equipment and reagents, and complexity of testing and result interpretation which require highly trained personnel. The TPHA has been largely replaced by TP-PA. However, the use of treponemal tests as a diagnostic method also has limitations. Treponemal tests cannot distinguish among the pathogenic treponemal species [37], and treponemal antibodies typically remain detectable for life even in successful treatment, limiting its use for treatment monitoring. Follow-up testing with a quantitative nontreponemal test is a well-established approach for measuring syphilis treatment efficacy.

In the United States, traditional and reverse sequence screening algorithms are frequently used for syphilis diagnosis. The traditional algorithm begins by screening with a nontreponemal test, and if reactive, followed by testing with a treponemal test. The reverse sequence algorithm begins with a treponemal test, and if reactive, followed by nontreponemal testing. A reflex testing with TPPA is recommended when nontreponemal test results are discordant with the treponemal test results. The reverse algorithm is being increasingly used in current testing practices [38]. Of note, the European Centre for Disease Prevention and Control (ECDC) uses a different approach that begins with a treponemal test, and if reactive, followed by another treponemal assay (different than the first treponemal test used in this screening algorithm). The reactive result of the second treponemal tests is reflexed with a quantitative RPR to monitor treatment response. Both reverse and ECDC algorithms were evaluated in low syphilis prevalence populations and were described to have comparable performance for syphilis screening [39]. An analysis of all three algorithms by Tong et al. showed lower sensitivity of the traditional algorithm and higher diagnostic efficacy for reverse and ECDC algorithms in high-prevalence populations [40].

Delayed seroreactivity and false-negative serological results have been reported in patients coinfected with HIV, but it is relatively uncommon, and the vast majority of patients can be accurately and reliably diagnosed with serological tests in conjunction with clinical evaluation [41].

In addition to serum antibody detection, serological assays are also used to aid in the diagnosis of neurosyphilis with the CSF-VDRL test most commonly used on CSF specimens [37, 42]. False-negative results may occur with the CSF-VDRL test, in which case an FTA-ABS test can be used since the latter test is highly sensitive although it lacks specificity. CSF-TPPA test has also been described for neurosyphilis with a titer ≥1:640 shown to identify patients with neurosyphilis when CSF-VDRL was nonreactive [42, 43]. Additional studies are needed to determine the efficacy of treponemal tests for the diagnosis of neurosyphilis using CSF. The performance characteristics of various serology tests have been described [44].

In contrast to standard serology assays that must be performed in a laboratory setting, rapid point-of-care tests that detect either treponemal or nontreponemal antibodies or both are currently in use for syphilis and endemic treponematoses at

the point of care or settings with limited resources [45–47]. The Syphilis Health Check is the only FDA-cleared rapid test in the United States and shows comparable performance to traditional serological assays [48]. Several other rapid syphilis tests are available and have gone through quality evaluations such as CE Mark, WHO Prequalification, or Brazilian National Health Surveillance Agency (ANVISA). Some rapid tests are also available with an automated reader or can be paired with smartphones, which eliminates the subjectivity of test interpretation [49]. Rapid tests are beneficial in outreach and point-of-care settings and will improve access to syphilis testing throughout the world.

9.4.2 Direct Detection Methods

Treponemes cannot be observed with an ordinary light microscope due to their narrow width; therefore, a microscope equipped with a dark-field condenser is required. Dark-field microscopy (DF) is useful to detect *TPA* in moist lesions of primary and secondary (e.g., ulcerative lesions and condyloma lata). The sensitivity of DF compared to clinical findings and PCR or serology varies between 75% and 100% for primary syphilis and 58% and 71% for secondary syphilis [50–55]. DF is not recommended for oral lesions since the morphology of other treponemes such as *T. denticola* is indistinguishable from *T. pallidum*. The test cannot differentiate among the three *T. pallidum* subspecies. It is also not recommended for testing lymph node aspirate, cerebrospinal fluid (CSF), or other body fluids. A major advantage of DF is that it can be performed in a peripheral clinic with a very short wait time. However, the DF technique has some limitations in that it must be performed within 20 minutes of specimen collection because it relies on observation of motile treponemes. Immunostaining is based on the direct fluorescent antibody test for *T. pallidum* (DFA-TP) and is used on the same specimen as DF except that the material on the slide is left to air dry for 15 minutes and fixative applied prior to staining [56]. The H9–1 monoclonal antibody is specific for pathogenic *T. pallidum*; therefore, the test can be used on oral lesions. Immunofluorescent detection is no longer available in the United States since monoclonal and polyclonal antibodies are not FDA-cleared and have not been validated for clinical diagnostic testing or quality control is not performed routinely. Immunohistochemistry (IHC) and silver staining are used to stain and examine tissue biopsies from the brain, placenta, umbilical cord, or skin from secondary, tertiary, or congenital syphilis; however, the sensitivity of silver staining is poor due to the difficulty with distinguishing spirochetes from reticulum fibers and artifacts in tissue samples.

9.4.3 Molecular-Based Methods

A number of PCR assays have been described in the literature for the detection of *TPA*; however, the majority of studies use either *polA* or *tpn47* as target genes [53, 57–59]. Multiplex PCR (MPCR) assays that detect causative agents of genital ulcer

disease (GUD), which include *TPA*, *H. ducreyi*, and Herpes simplex virus 1 and 2, have also been developed [44]. PCR has not been widely adopted for clinical use, and serological tests remain the mainstay for diagnosis of syphilis. There are no FDA-cleared nucleic acid amplification tests (NAATs) for *TPA* in the United States. Quest Diagnostics (Secaucus, New Jersey) offers a laboratory-developed, CLIA (Clinical Laboratory Improvement Amendments)-regulated real-time PCR test for *TPA* for use on CSF, whole blood, serum, or a genital swab. Two commercial companies offer CE Mark MPCR tests for use in Europe, Seegene (Seoul, South Korea) markets the Allplex Genital Ulcer Assay that detects seven pathogens [HSV-1 and HSV-2, *H. ducreyi*, *TPA*, lymphogranuloma venereum (LGV), cytomegalovirus (CMV), varicella-zoster virus (VZV)], and SpeeDx (Sydney, Australia) markets the PlexPCR® VHS assay that detects HSV-1 and HSV-2, *TPA*, varicella-zoster virus (VZV). PCR is most useful for exudative lesions of primary syphilis with a sensitivity ranging from 72% to 95% [53, 57–62]. The sensitivity of PCR on secondary lesions ranges from 20% to 86% with the higher sensitivity reflecting detection of *TPA* in moist lesions (i.e., mucous patches, condylomata lata) [53, 57–62]. PCR can be used on tissue biopsies from secondary syphilis provided samples are tested soon after collection or frozen immediately if testing will be performed at a later time [51, 63]. Yang and colleagues found that 40% of MSM patients with secondary syphilis did not present with oral ulcers but tested positive by PCR on oral swabs [59]. PCR can be used on CSF as an adjunct test to confirm the diagnosis of neurosyphilis in symptomatic seropositive adults and amniotic fluid, neonatal CSF, or neonatal blood in cases of suspected congenital infection; however, a negative result in any of these specimens does not rule out infection [64–68]. PCR is not recommended for whole blood or blood fractions due to low sensitivity. A real-time PCR that detects and distinguishes among the three subspecies has been developed; however, the test has missed yaws cases in the Solomon Islands due to sequence variation in the PCR primer binding sites [69]. Currently, a pan treponemal PCR based on either *polA* or *tpn47* is being used for lab confirmation of yaws, but modifications can be made to the real-time PCR assay to allow reliable detection of TPE, or alternate species-specific assays can be used.

9.5 Typing Methods

9.5.1 CDC and Enhanced CDC Typing Schemes

Prior to development of the first typing system for syphilis [70], attempts at intrastrain and interstrain differentiation of the three *T. pallidum* subsp. (*pallidum*, pertenue, and *endemicum*) using phenotypic methods such as protein profiles [71], lectin agglutination patterns [72], or serotyping system using monoclonal antibodies raised against TPA proved unsuccessful [73]. In addition, attempts at molecular differentiation identified point mutations in several genetic loci, but these differences were insufficient for strain typing [18, 74]. The first typing system was based on PCR-restriction fragment length polymorphism (RFLP) analysis of the

treponemal repeat protein (*tpr*) genes and determination of the number of 60 bp repeats within the acidic repeat protein (*arp*) gene [70, 75] and is known as the CDC typing (CDCT) method. The typing system has since been enhanced by inclusion of a subtyping method which incorporates sequence analysis of a variable region in a hypothetical protein gene (*tp0548*) and is referred to as the enhanced CDC typing (ECDCT) method [76]. A second subtyping method which involves determining the number of guanine mononucleotide repeats (MNRs) in the ribosomal protein S1 gene (*rpsA*) gene (*tp0279*) has since been added to ECDCT method to create a four-component typing system (Fig. 9.1).

The first component of the typing system involves nested PCR amplification of an approximately 1.8 kb region of subfamily II (*E*, *G*, *J*) of the *tpr* genes using primers B1 FP, 5'ACTGGCTCTGCCACACTTGA3'/A2 RP, 5'CTACCAGGAGAGG GTGACGC3', and IP6 FP, 5'CAGGTTTTGCCGTTAAGC3'/IP7 RP, 5'AATC AAGGGAGAATACCGTC3', followed by restriction digestion with *Mse*I and analysis of fragment sizes by agarose gel electrophoresis or on an automated instrument such as a TapeStation 4200 (Agilent Technologies, Palo Alto, CA). The second component is PCR amplification of a 60 bp repeat region of the *arp* gene with primers N1 FP (5'ATCTTTGCCGTCCCGTGTGC3') and N2 RP (5'CCGAGT GGGATGGCTGCTTC3') and determining the number of repeats, which have been shown to vary from 3 to 25, except 21 and 24 repeats have not been reported [63, 70, 75, 77–83]. The third component of the typing system entails PCR amplification of an 84 bp variable region of a hypothetical protein gene (*tp0548*) using primers FP2 (5' GGTCCCTATGATATCGTGTTCG3') and RP2 (5'GTCATGGATCTGCGA GTGG3'), and subtypes are assigned a lowercase letter of the alphabet [76, 84]. The

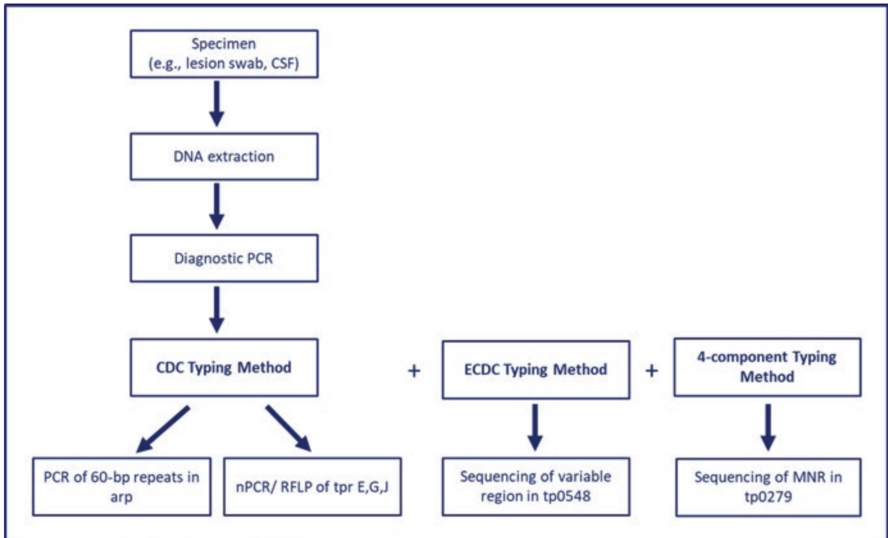

Fig. 9.1 Schematic diagram of the *Treponema pallidum* typing system showing the CDC, ECDC, and four-component typing methods

fourth component of the typing system involves PCR amplification and sequencing of an MNR within the *rpsA* gene using primers 220I (5'GCGCCCCAGACCCGCTCT3') and 220 J (5'GAGCGATGATCACGGTCCCCAT3'). Five repeat sizes (8, 9, 10, 11, 12) have been reported to date [78]. A strain type is designated based on a combination of the number of 60 bp repeats, the RFLP pattern of the *tpr* genes, number of G residues within *rpsA*, and sequence pattern within *tp0548*. For example, a *TPA* strain with type 14*d*9g has 14 60 bp repeats, the *d* RFLP pattern, and 9 G repeats, and sequence pattern g.

The ECDCT method which incorporates *tpr*/*arp*/*tp0548* has been applied in a number of studies, and more than 30 strain types have been described worldwide with 14*d*/f, 14*d*/g, and 14*f*/f being the predominate types [76, 79, 84–92]. Because the majority of strains fall into a few types worldwide with 14*d* being the most common, the *rpsA* subtyping method was introduced in an attempt to further characterize strains. Adding this component to ECDCT appears to have marginally increased the discrimination of strains, but additional studies are needed to determine its usefulness in different settings. Although different specimen types have been used for typing, samples from moist primary and secondary lesions and earlobe scrapings have thus far produced the best results [93].

There have been many molecular epidemiological studies on syphilis using the CDCT or ECDCT methods, and some studies report associations between strain type and patient characteristics or macrolide mutations. Sutton and colleagues found an association between the 14*f* strain type and white race in Arizona [94]. A study in China reported differences in strain type distribution among geographical regions [79]. Using the SBMT method, Grillova and colleagues analyzed samples over a 3-year period in Czech Republic and found associations between strains types and patient characteristics; the SU2R8 genotype was more common among individuals 35 years and older and in samples from Prague; the SU5R8 genotype was identified only in samples from the Brno area; and the SU2R8 genotype was more common among MSM; and macrolide resistance strains were prevalent among MSM patients [95]. Marra and colleagues reported an association between 14*d*/f and neurosyphilis where 50% (21/42) of patients with this strain type had neurosyphilis versus 10 (24%) patients infected with any other type [76]. Zhang and colleagues showed that strains with the 14*i*/a strain type correlated with serofast status versus patients who were serologically cured [96]. Martin et al. found 100% (38/38) of strains were resistant to macrolides with most strains being type 14*f* [97]. The 14*d*/g strain type was the dominant strain in specimens analyzed in Sydney Australia with 99% (91/92) harboring the macrolide-resistant mutations versus 67% (68/99) of non-14d/g strains [89]. A study in Japan showed an association between heterosexuals and 14*d*/f strains and macrolide resistance [88]. In 2015, two clusters of ocular syphilis were reported in California and Washington raising concerns about a potential outbreak; however, typing revealed that multiple strain types were involved [98]. Because typing relies on a few target genes, there may be other unidentified genetic markers that may correlate with increase in ocular syphilis cases or other clinical phenotypes which needs further investigation. Ocular syphilis

cases have also been reported in other countries even prior to these reported clusters [99–101].

The four-component typing scheme has been applied to TPE strains collected from yaws patients in Ghana and Vanuatu and laboratory strains isolated from yaws cases in Ghana, Indonesia, and the Republic of the Congo with 22 strain types found among 44 fully typed strains [102]. Interestingly, only two *tpr* RFLP patterns were observed among strains from multiple geographic origins, and *arp* repeats varied from 2 to 12, whereas in TPA the vast majority of strains have 14 repeats. In addition, for MNRs in *tp0279*, most strains had 11 or 12 repeats, whereas 9 repeats are common in TPA strains. Bejel is usually found in semiarid regions, but there have been two reports in Cuba and Japan suggesting sexual transmission of *TEN* [103, 104] and other reports of imported cases from endemic areas [105–107]. Although the ECDC typing system has been widely used for over two decades, it is evident that the method is labor-intensive and difficulties have been reported with amplification or stability of the *arp* and *tpr E*/*G*/*J* loci in clinical specimens resulting in inconsistent results with the number of *arp* repeats or RFLP patterns of the *tpr E*/*G*/*J* loci in samples from the same patient [81, 108]. These difficulties may arise from a combination of low abundance of genomic DNA in specimens resulting in either nontypeable samples or possible preferential amplification of the *tpr* genes resulting in aberrant RFLP patterns or differences in genotypes between lesion swabs and blood possible due to immunological differences between skin and blood compartments.

9.5.2 Sequence-Based Molecular Typing

The first sequence-based molecular typing (SBMT) method was introduced in 2006 and is based on three targets: *tp0136*, *tp0548*, and 23S rRNA for strain typing [109]. The SBMT typing method was shown to further discriminate strains especially the 14*d* strain type identified by the CDCT method. Antibiotic resistance mutations may not be stable targets for strain typing because mutations may appear due to antibiotic pressure, but resistance markers may not correlate with the background genome [84].

9.5.3 Multilocus Sequence Typing

Multilocus sequence typing (MLST) is usually based on PCR amplification and sequencing of 450–500 bp internal fragments of 7 housekeeping genes by the Sanger method. An MLST method for *TPA* was recently described by Grillova and colleagues, which targets three genetic loci (*tp0136*, *tp0548*, *tp0705*) [110]. Two of the three genes were first used in the SBMT method, while *tp0548* sequencing is common to all typing methods (SBMT, CDCT, ECDCT, MLST), but MLST includes a much larger fragment (1095 bp) for *tp0548* compared to the ECDCT method. The PCR primers used for MLST are shown in Table 9.1. The *TPA* MLST

Table 9.1 PCR primers used for MLST of *T. pallidum* subsp. *pallidum*

Gene	External primer (5′-3′)	Coordinates[a]	PCR product size	Internal primer (5′-3′)	Coordinates[a]	PCR product size	Source
tp0136	AACCCGTTAGCGCCCAACAT	157,804–157,823	1789 bp	AGTGTCTTCCTCGTCCGTTC	158,206–158,225	1206 bp	[127]
	TCCCAGCTCAGCCGAATCTC	159,570–159,589		CACGTGGTGGTGTCAAACTT	159,392–159,411		
tp0548	TGGGGCACTAAACCGGAAGA	593,136–593,155	1567 bp	GCGGTCCCTATGATATCGTGT	593,285–593,305	1065 bp	[127]
	TACGGGCATTTGCGGATAGG	594,683–594,702		GAGCCACTTCAGCCCTACTG	594,330–594,349		
tp0705	GGTCTATATGCAGCCCTTCTTC	772,663–772,684	1181 bp	TGCGGCTTATCCTGATGAATAG	772,917–772,938	803 bp	[110]
	GCTTGAGAACGATACCGGATAC	773,822–773,843		TATTCTGCGGCGTTGGATAG	773,700–773,719		

[a]Cognate to *T. pallidum* Nichols genome (CP004010.2)

Table 9.2 PCR primers used for MLST of *T. pallidum* subsp. *pertenue*

Gene	Primer	Sequence (5′-3′)	PCR product size	ORF region (nt)	Source
tp0548	Sense	5'-GGTCCCTATGATATCGTGTTCG-3'	300	130–212	[76]
	Antisense	5'-CGTTTCGGTGTGTGAGTCAT-3′			
	Sequencing	5'-GTCATGGATCTGCGAGTGG-3′			[115]
tp0136	Sense	5'-CCATCCAGTCGGAAGTGC-3′	563	223–675	[115]
	Antisense[a]	5'-CATATCGAGAAACTGTTCGCC-3'			
	Antisense[b]	5'-CGTGCAGGCAGAACTCATT-3'	464		
	Sequencing	5'-CCATCCAGTCGGAAGTGC-3'			
tp0326	Sense	5'-AAGAGCATTCGTTTCGCTCC-3'	441	2031–2342	[115]
	Antisense	5'-CCGGACCGTAGCTCATTTTG-3'			
	Sequencing	5'-GACACCAAGGCCGAGTTCTA-3'			

[a]Antisense primer for *tp0136* subtypes A–F
[b]Antisense primer for *tp0136* subtype G

scheme is based on allelic profiles that are assigned a three-letter code (e.g., 1.2.1), where the first number corresponds to the *tp0136* allele, the second number to *tp0548*, and the third to *tp0705*. Allelic profiles 1.1.1 and 1.3.1 have been identified as the predominant MLST profiles in strains from Cuba, Czech Republic, France, and Switzerland [110–112], and based on previous sequence analysis of *tp0136* and *tp0548* [113], these strains belong to the SS14 clade. The allelic profile 1.3.1 [110, 111, 114] and 1.26.1 [111] were shown to be associated with the A2058G mutation that confers macrolide resistance, while the A2059G mutation and macrolide susceptibility were associated with allelic variants 1.1.3 and 1.1.8, respectively [111]. Overall, MLST was shown to be more discriminatory than the previously described typing methods, and the 14*d*/g strain type was differentiated into 5 allelic profiles; however, more studies are needed to show its utility for studying the molecular epidemiology of syphilis. The *TPA* MLST database can be found at https://pubmlst.org/tpallidum/.

An MLST method has been described for TPE that utilizes *tp0136*, *tp0548*, and *tp0326* for strain characterization [115]. The PCR primers used for typing are shown in Table 9.2. Using this typing scheme, 7 MLST types (JG8, SE7, TG8, TD6, TG6, SD6, JD8) were identified among 194 specimens from children with yaws on Lihir Island in Papua New Guinea. JG8 was the predominant type observed over the 3.5-year study period, accounting for 82% of specimens.

9.6 Whole Genome Sequencing

Next-generation sequencing (NGS) technology continues to advance at a rapid pace making it possible to perform whole genome sequencing (WGS) at a fraction of the cost compared to two decades ago. Currently, there are about 122 published TPA

genomes [116–120], 14 for TPE [69, 121–123], and 2 for TEN [124, 125] available in the National Center for Biotechnology Information (NCBI) database. Published genomes include rabbit-passaged strains and direct sequencing of strains from clinical specimens. All NGS studies for *TPA* used Agilent SureSelect target enrichment combined with Illumina-based sequencing with the exception of one study that used an anti-TP antibody enrichment method to describe the first whole genome sequence of TPA directly from a clinical specimen [126]. The first global phylogenetic analysis of *TPA* directly from clinical specimens was described by Aurora and colleagues who showed that TPA strains belong to either an SS14 lineage or Nichols lineage and TEN and TPE strains formed a separate cluster [117]. The Nichols-like strains had a greater nucleotide diversity and were found mainly in North America, while SS14-like strains formed a genetically homogenous group (SS14-Ω cluster) and had a more wider geographic distribution with samples from Europe and North and South America. A study by Beale et al. showed that the SS14-Ω cluster could be further separated into two lineages composed of European and North American samples (SS14Ω-A) and Chinese and North American samples (SS14Ω-B) [119]. The SS14Ω-B lineage could be further separated based on origin of the samples. Marks and colleagues performed WGS on TPE strains obtained from yaws patients in the Solomon Islands located in the South Pacific and found that strains fell into two distinct clades and had a distinct lineage compared to previously sequenced laboratory strains. In addition, the TEN genomes from clinical specimens were highly conserved and were separated by a maximum pairwise distance of <20 SNPs. As WGS and target enrichment technology improve, it should provide better tools to study *T. pallidum* from clinical specimens, thus permitting a better understanding of the evolution and spread of the three subspecies and development of improved diagnostics and typing tools.

9.7 Conclusion and Future Perspectives

Although PCR has been proven for use on lesion specimens of early syphilis, serology tests remain the mainstay for laboratory diagnosis. Unless there is a paradigm shift in the diagnostic approach for syphilis and clinical laboratories start to implement NAATs, particularly in the United States, these tests will continue to be underutilized. As a result of this, access to specimens for molecular surveillance or outbreak investigation will be challenging and reliant on research studies. The rising rates of syphilis in the United States and worldwide highlight the ongoing need for genotyping and WGS tools to better understand the phylogenetic and epidemiological basis for the epidemic. While WGS will prove more useful for epidemiological studies of circulating strains, conventional genotyping remains important until WGS methodologies are more practical and widely adopted. The MLST methods described for TPA and TPE will be useful for future genotyping studies.

9.8 Summary

The genus *Treponema* comprises a diverse group of bacteria with some well-known human pathogens including the causative agents of venereal syphilis (*Treponema pallidum* subspecies *pallidum*), yaws (*T. pallidum* subsp. *pertenue*), bejel or endemic syphilis (*T. pallidum* subsp. *endemicum*), and pinta (*T. carateum*). This chapter provides an update on genotyping and next-generation sequencing tools being used to study the molecular epidemiology and phylogenetics of *T. pallidum*. Diagnostic tests in use for syphilis and endemic treponematoses are also described. The enhanced Centers for Disease Control and Prevention typing (ECDCT) method has been applied to syphilis over the past two decades and more recently shown to be applicable to yaws; however, newer methods such as multilocus sequence typing (MLST) and whole genome sequencing (WGS) are being implemented for molecular epidemiological studies.

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