Iqbal Ahmad · Shamim Ahmad
Kendra P. Rumbaugh *Editors*

Antibacterial Drug Discovery to Combat MDR

Natural Compounds, Nanotechnology and Novel Synthetic Sources

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Preface

Considering the rapid emergence and spread of multidrug-resistant bacterial pathogens across the globe and the slow discovery of new antibiotics, there is a serious threat to the current availability of bacterial infection chemotherapy. Considerable efforts from academia and industry have been made to develop new and alternative ways to overcome this problem, but the problem remains very serious and warrants immediate attention. In addition to classical approaches, improved and new technologies, including nanomaterials, antipathogenic drugs, and the reemergence of natural products as potential new therapeutics leads, are encouraging developments. There have been several books published covering specific applications; however, this book aims to provide a holistic and comprehensive view of the subject starting with the problem and then discussing classical to modern approaches of drug discovery and alternative ways of combating bacterial infection especially by MDR bacteria. The main areas of interest include various aspects of drug discovery strategies, natural products from various sources (microbes, marine, and lower to higher plants) as novel antibacterials, and the prospects of nanotechnology and nonmaterials in drug discovery and drug delivery.

The concept of this book was developed in India-UK Antimicrobial Resistance Sandpit Meeting 2017, organized by the UK Research Council and DBT, India. The concept was then shared by all the authors and among editors of the book and finally with the Springer Nature publishing team. The editors would like to thank all those who contributed to the discussion, planning, writing, and publishing of this book. They hope that this compilation will provide the most important aspects of antibacterial drug discovery of newer molecules from natural to synthetic sources.

The book chapters are divided into four sections. **Part I: The Challenge of Antibiotic Resistance and Tolerance** covers mechanisms of bacterial resistance and biofilm-related tolerance, impediments to the discovery of new antimicrobials, and developing models with which to test the efficacy of new compounds. **Part II: New Antibiotic Drug Discovery Approaches and Progress** and **Part III: Alternative Antibiotic Resistance Treatment Strategies** discuss a wide array of approaches being explored for anti-infective drugs, from targeting virulence factors, biofilm production, and quorum sensing to using medicinal plants, essential oils, pre- and probiotics, and bacteriophages anti-infective compounds. In silico molecular modelling and computational approaches to rational drug design are also discussed. **Part IV: Prospects of Nanomaterials: Antibacterials and Drug Delivery Agents**

discusses current advances in nanomaterials and nanoparticles for combating MDR bacteria.

We hope that students, teachers, researchers, and companies involved in drug discovery will find ***Antibacterial Drug Discovery to Combat MDR: Natural Compounds, Nanotechnology and Novel Synthetic Sources*** to be a useful resource. With great pleasure, we extend our sincere thanks to all the contributors for their timely response, excellent contributions, and consistent support and cooperation. **We are also grateful to Prof. Tariq Mansoor,** the Vice Chancellor of the Aligarh Muslim University, former Principal of Jawaharlal Nehru Medical College, and Head of the Department of Surgery in the Faculty of Medicine, AMU, for his encouragement and support to the faculty members for Research and Innovation. The cooperation received from postdoctoral researchers, Dr. Mohd. Shavez Khan and Dr. Meenu Maheshwari, and research students, Mr. Faizan Abul Qais and Miss Samreen, in the Department of Agricultural Microbiology, AMU, India, in the book preparation is gratefully acknowledged. We greatly appreciate and thank Dr. Derek Fleming, TTUHSC, Lubbock, USA, for his extensive help in the editing process.

We welcome any suggestions and comments from readers for future improvement of the book in new edition.

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Professor Iqbal Ahmad is currently a Full Professor and Ex-Chairman in the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. He is also the Coordinator at the Faculty of Agricultural Sciences, AMU, for BSc (Hons) Agriculture course. He has extensive teaching and research experience in applied and interdisciplinary microbiology and medicinal plant-derived natural products. His present research interest is in the field of AMR in pathogenic bacteria of environmental and clinical origin and conducting research on interference of quorum sensing-linked virulence factors and biofilms by natural products including medicinal plants as possible alternative strategy to combat MDR problem. He has guided several PhDs and MSc students; published more than 150 original research papers, 10 edited books, and 50 book chapters; and completed four extramural research projects. The research work is fairly cited by scientific community. He is an Active Reviewer/Member of editorial board of many high-ranked research journals.

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Antibacterial Drug Discovery: Perspective Insights

Iqbal Ahmad, Faizan Abul Qais, Samreen, Hussein Hasan Abulreesh, Shamim Ahmad, and Kendra P. Rumbaugh

Abstract

Over the last two decades, the development of new antibacterial drugs has been very limited due to many reasons. In light of the alarming situation of antimicrobial resistance (AMR), it is now vital to act promptly to develop new ways to combat the resistance problem through an integrated approach. Despite the slow progress of drug discovery by pharmaceutical companies, natural products have definitely provided an abundant source of new antibacterial leads. On the other hand, genomics- and proteomics-based drug discovery approaches have been more disappointing when it comes to the discovery of new antibacterials with novel modes of action. In the recent past, improved screening strategies and developments in target identification and validation, combinatorial chemistry, and the use of biochemical synthetic-based approaches have provided hope for the development of new antibacterial leads. Other approaches like novel anti-infective and anti-virulence target-based strategies such as quorum sensing, biofilm, virulence, and pathogenicity inhibitors are gaining popularity among drug discovery researchers. Similarly, nanotechnology-based drug delivery has seemingly unlimited application for improving the efficacy of antibiotics, where metallic and natural nanomaterials with antibacterial efficacy are under scrutiny

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for their possible therapeutic application. In this chapter, we aim to provide a brief overview and discussion of the potential for the various strategies mentioned above to combat drug-resistant bacterial infections.

Keywords
Antimicrobial resistance · AMR · Natural products · Antibacterials · Screening strategies · Target identification · Combinatorial approaches · Efflux pump inhibitors · Anti-infective approaches · Nanoparticles · Drug delivery

1 Introduction

Antimicrobial resistance among clinical and environmental bacteria has become a widespread phenomenon, which has been recognized by most international, national, and local health regulatory agencies (Arya 2002; Smith and Coast 2002). Since their introduction, antibiotics have saved countless lives. However, the development of resistant strains of bacteria was reported soon after the introduction of the first antibiotic, and the rise in resistance has reached a point where medical experts are now warning of a return to the pre-antibiotic era. Many of the pathogenic bacteria associated with human diseases are now multidrug-resistant (MDR) (Perron et al. 2012; Zarrilli et al. 2013), and many Gram-positive and Gram-negative nosocomial pathogens have attained the status of problematic MDR, or "superbug" (Zhang 2010). These MDR pathogens possess a variety of mechanisms that convey drug resistance and the capacity to acquire new genes and/or disseminate resistance genes through various gene exchange mechanisms (Dzidic and Bedeković 2003; Davies and Davies 2010).

Due to the lack of discovery of new antibacterial drugs and the rising AMR problem, scientific and healthcare regulatory bodies have prioritized efforts to immediately address this problem both locally and globally (Projan 2003; Singh and Barrett 2006; Brown and Wright 2016). Various approaches to address antibiotic resistance are discussed by many authors in this book. Here, we aim to provide some perspective insights into these antibacterial drug discovery efforts.

2 Antimicrobial Resistance (AMR): A Global Problem and Threat to Human Health

In the last two decades, the world has witnessed a threatening increase in the absolute number of MDR bacterial pathogens. Major world organizations including the World Health Organization (WHO), European Centre for Disease Prevention and Control (ECDC), and US Centers for Disease Control and Prevention (CDC) now consider antimicrobial resistance as a major and emerging threat to global public health problem (Roca et al. 2015). In the twenty-first century, AMR has become an alarming concern on the forefront of public healthcare problems. In Europe only,

nearly 400,000 people are known to be infected with multidrug-resistant bacteria that cause approximately 25,000 deaths (Prestinaci et al. 2015). Similarly, as per the CDC report in 2013, about 2 million people in the United States were infected with bacterial pathogens that were resistant to at least one conventionally used antibiotic, and nearly 23,000 people died due to infections caused by MDR bacteria (USCDC 2013). Similarly, the emergence of MDR also increased substantially in Asia, Africa, Latin America, the Middle East, and other parts of the world between 2002 and 2011, but exact data is not available (Laxminarayan et al. 2013). This growing, global AMR issue has also considerably contributed to the world's economic health-care burden. It is difficult to assess the total cost of antibiotic resistance worldwide, but undoubtedly, the economic burden due to AMR is substantial (Kaier et al. 2008; Taylor et al. 2014; Tillotson and Zinner 2017).

The development of AMR is due to exposure of pathogens to antimicrobial drugs, which induce a selective pressure resulting in drug-resistant pathogens. The emergence of resistant microorganisms, either by mutations or the acquisition of mobile genetic elements carrying resistance genes, may also occur irrespective of the presence of antibacterial agents (Roca et al. 2015). Hence, the main driving force underlying the prevalence and emergence of AMR is the aggressive and persistent use of antimicrobials both in patients and livestock or release into the environment by other means (Michael et al. 2014). The major drivers of AMR have now been identified to a large extent and are recognized globally (Castro-Sánchez et al. 2016). It is also clear that their management should follow a "one health approach" (Collignon 2012).

3 Approaches for Antimicrobial Drug Discovery

The decade between 1950 and 1960 was considered the golden era of antibiotic discovery, but it was abruptly followed by a gap of almost four decades during which no new antibiotics with novel mechanisms of action were discovered (Fig. 1). This led researchers and pharmaceutical industries to attempt innovative drug discovery approaches. A revolution in computing technology made it possible to combine and analyze larger sets of data, and many new strategies such as genomics- and proteomics-based, high-throughput screening, and synthetic approaches were attempted, albeit without major success (Brown and Wright 2016). Some of the approaches for the discovery of antibiotics, which have been used over the last 80 years, are discussed below:

3.1 Classical Approach for Screening of Antibacterial Drugs

Most of the currently used antibacterial drugs were discovered through the classical approach, used from 1940 to the late 1960s, by which natural products, synthetic or semisynthetic compounds from innumerable sources (mainly microbes), were directly screened for their promising antibacterial activity against a spectrum of

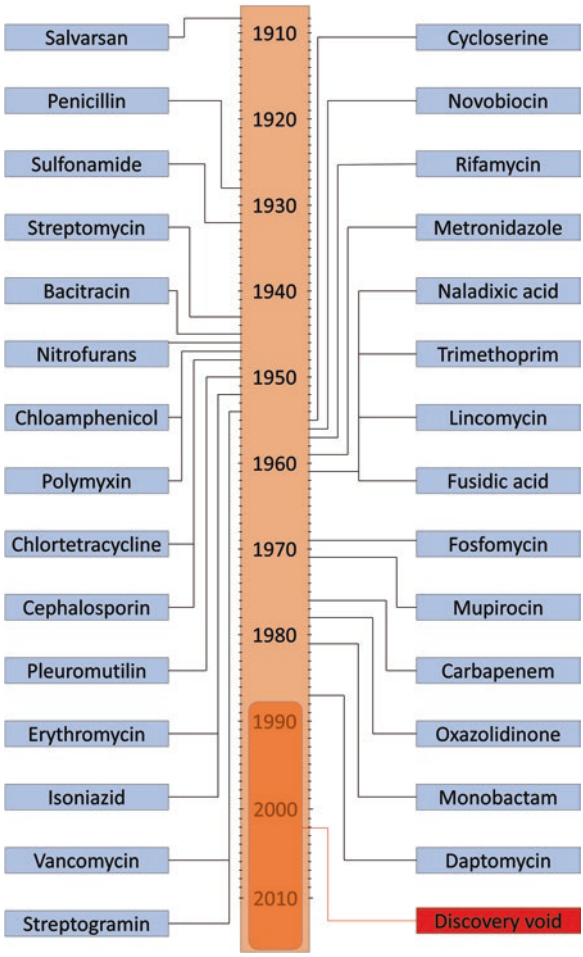

Fig. 1 Timeline of antibiotics discovered or patented (Silver 2011)

bacteria. After this period of "classical antibiotic discovery," there was gap of almost 40 years until the first representative of a new class of antibiotic was released in the market in 2000. One of the reasons for such a prolonged gap in antibiotic discovery is that most of the pharmaceutical industries were engaged in optimizing the already discovered antibiotics to develop their efficacy, spectrum, tolerability, and dosing interval. Moreover, a perception that the problem of bacterial infections had been solved also stalled efforts to develop new drugs. Nevertheless, the availability of scientific literature on antibacterial natural products during that lag period indicates the investment of continuous effort by academic researchers toward the discovery of new antibacterial lead compounds (Newman et al. 2000; Harvey et al. 2015). Such drug development efforts did not prove to be very productive as the compounds discovered were either inferior in their efficacy profile, too complex to be chemically modified, or belonged to already discovered classes of antibiotic (Brötz-Oesterhelt and Sass 2010). The regulations on the safety and efficacy of antibiotics

have substantially increased over time with a parallel improvement in therapeutic standards and technical advancements. Subsequently, the regulatory requirements needed for the approval of a newly discovered antibiotic are much higher today. Many antibiotics that were approved during the golden age of antibiotic discovery might not be able to clear today's regulations (Bax and Green 2015).

3.2 Poor Progress on Genomics- and Proteomics-Based Antibacterial Drug Discovery

The slow progress in the discovery of new antibacterials from microbial extracts and the discovery of a new synthetic quinolone class of antibacterials encouraged researchers to focus on screening novel compounds from natural product libraries and low-molecular-weight synthetic compounds. The availability of sufficient bacterial genomic information in the mid-1990s prompted the development of new screening strategies of antibacterials that led to the beginning of the "genomics era" (Brötz-Oesterhelt and Sass 2010; Lewis 2013). During this time, screening inhibitors against preselected targets were considered more relevant than phenotypic screening. To date, more than one thousand eubacterial genomes have been sequenced that can be exploited for comparative analyses for new antibacterial drug discovery (NCBI 2019). The availability of genomic data supported the idea that there were numerous unidentified targets that could be exploited for antibiotic therapy. The genomes of important bacterial pathogens were compared with available eukaryotic genomes to identify the targets which were conserved among the desired bacterial genera but evolutionary distant in eukaryotes. Using this approach, approximately 150–350 potential targets were assembled by pharmaceutical companies (Freiberg et al. 2004; Payne et al. 2007). The validation of targets crucial for bacterial survival were performed by knockout analyses, mutation studies, and inducible gene expression experiments under in vitro conditions. Such experiments were usually conducted against one Gram-positive and one Gram-negative model species of bacteria only, as it would create massive workload to mutate the target in every species of interest (Brötz-Oesterhelt and Sass 2010). Target proteins were expressed, purified, and screened by high-throughput assays against libraries consisting of a million of synthetic compounds. Extensive effort was put into this approach of antibacterial drug discovery to evaluate the quality of novel targets, and the investment did prove fruitful in identifying suitable leads that were further optimized as potential antibacterial candidates (Fernandes 2006).

For example, GlaxoSmithKline (British pharmaceutical company) selected >350 genes/targets by comparative genome analyses of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae*. Among them, 127 were identified as essential targets that were present in at least one of these test organisms, and finally 67 targets were screened as purified proteins. The high-throughput screening of 260,000–530,000 compounds against these 67 targets only produced 16 hits, in which 5 of them resulted in leads, and ultimately only 1 lead series progressed to development (Brötz-Oesterhelt and Sass 2010). The target only proved to be

suitable for a narrow spectrum, and therefore, the resulting inhibitor was out licensed to Affinium (biotech company). Likewise, Pfizer (American pharmaceutical corporation) found only four leads that were screened from 65 high-throughput screening campaigns in which none of them even reached clinical trials (Miller 2008). Cubist (United States biopharmaceutical company) also tried a somewhat different approach and concentrated on a specific target class, i.e., aminoacyl-tRNA synthetases. All 20 representatives of this target family were essential for bacterial survival. Cubist screened 17 enzymes against a smaller library of 50,000 compounds, with no success (Gallant et al. 2000). Many other pharmaceutical companies such as Bristol Meyers Squibb and Wyeth had similar experiences using high-throughput screening approaches, with many concluding that there was negligible economic or scientific benefit to this method of antibacterial drug discovery (Brötz-Oesterhelt and Sass 2010; Lewis 2013).

3.3 Structure-Based Synthetic Approaches

Recent advancements in nuclear magnetic resonance spectroscopy, X-ray crystallography, and computational tools have created a new direction in the progress of antibacterial drug discovery. Apart from genes, the structures of numerous antibacterial targets have become available, facilitating modeling studies as a screening strategy. Structure-based strategies include virtual screening of new compounds, target-based de novo compound design, fragment-based screening, or determining reaction intermediates. Promising structures that have been studied for antibacterial drug discovery are some topoisomerases, aminoacyl-tRNA synthetases, RNA polymerase, peptide deformylase, certain membrane-bound enzymes required for peptidoglycan biosynthesis, and other diverse groups of metabolic enzymes (Kohanski et al. 2010; Brötz-Oesterhelt and Sass 2010). The structures of substrates, inhibitors, or reaction intermediates have also been solved to generate useful information regarding active site topology, which is employed for the discovery of antibacterial drugs. Recently there have been many examples of structure-based design being used for the identification of new lead structures and the optimization of already discovered antibiotics (Barker 2006; Wimberly 2009).

Iclaprim, a successor of trimethoprim, a diaminopyrimidine antibiotic, reached phase III clinical trials to treat staphylococcal skin infections; however, it did not clear the regulatory standards of the US FDA (Peppard and Schuenke 2008). Trimethoprim competitively inhibits dihydrofolate reductase, an enzyme required for the biosynthesis of tetrahydrofolate. Mutation of one amino acid in the active site of *S. aureus* dihydrofolate reductase alters the trimethoprim-enzyme interaction, creating resistance to the drug (Dale et al. 1997). The mechanism of resistance was understood from the crystal structure of trimethoprim–*S. aureus* dihydrofolate reductase complex. This information was used in modeling studies to design new diaminopyrimidines with enhanced antibacterial activity against the dihydrofolate reductase of Gram-positive bacteria. Iclaprim resulted from such an approach for which the trimethoxyphenyl side chain was replaced by a dimethoxychromene

substituent. This modification increased the hydrophobic interactions in the target protein, resulting in a 20-fold higher affinity compared with unmodified trimethoprim (Schneider et al. 2003).

In another rational design program, high-resolution crystal structures of bacterial ribosome-inhibitor complexes paved the way for the discovery of a new series of m-terphenyls including RX-B72. RX-B72 binds to the A-site of the bacterial ribosome overlapping with the oxazolidinone binding site. The best compounds discovered in this series exhibited very good MIC values against Gram-negative pathogens (Ippolito et al. 2009).

3.4 Revisiting Natural Products for Antimicrobial Drug Discovery

The failure of high-throughput screening assays and small synthetic molecule approaches resulted in an interest among scientists to return to natural products in the search for antimicrobials (Butler and Buss 2006; Baltz 2008; Nicolaou et al. 2009). This is not surprising considering that almost 3/4 of all antibiotic classes are from natural products. Natural antimicrobial products are advantageous over synthetic compounds as natural products have greater structural diversity, unique molecular architectures, and functional complexity (von Nussbaum et al. 2006). Moreover, the antibacterial activity of natural compounds is better due to the fact that antibiotic-producing strains have evolved over longer periods of time in order to compete for ecological niches (Brötz-Oesterhelt and Sass 2010). Researchers agree that only a fraction of the antibacterial agents produced by microbial communities globally have been discovered (Baltz 2006; Clardy et al. 2006). While the majority of antibiotics known today are produced by *Streptomyces* species, it is expected that even more streptomycetes antibiotics are waiting to be discovered (Clardy et al. 2006). Hence, a new strategy for future development of antibiotic drugs is to search for novel natural products with modern technologies. Unexplored natural habitats are being explored to search for new antimicrobials, and improved culture conditions are making previously unculturable microorganisms cultivatable (Nett and König 2007; Muscholl-Silberhorn et al. 2008). For example, a pilot study indicated that previously unculturable microbes could be grown by growing them along with other species from their natural habitat (Kaeberlein 2002). In addition, modern molecular biology tools have made it possible to express foreign biosynthetic gene clusters, and pools of DNA from different environments can be probed by metagenomic techniques (Clardy et al. 2006).

Due to the gap in the discovery of new antimicrobials in the late twentieth century, many pharmaceutical companies decided to revisit already available natural product libraries. Wyeth (pharmaceutical company) initiated a project to reinvestigate fractions of their natural product collection they had previously discarded due to their narrow spectrums of activity. For example, a glycopeptide class of mannopeptimycins was obtained from a fraction of *Streptomyces hygroscopicus* LL-AC98. This antibiotic complex was known to Wyeth since the 1950s, but they didn't

perform structural studies until the beginning of the twenty-first century (CORDWINDER 1862; He et al. 2002). To date, natural product complexes have shown antibacterial activity against penicillin-resistant streptococci, methicillin-resistant *S. aureus*, and vancomycin-resistant *Enterococcus* (Singh et al. 2003). Mannopeptimycins also inhibit peptidoglycan synthesis, but they have other binding sites than that of vancomycin, which explains their activity against vancomycin-resistant *Enterococcus* (Ruzin et al. 2004). Another novel antibiotic obtained from natural products is plectasin (a peptide antibiotic) that was isolated from *Pseudoplectania nigrella* by Novozymes (global biotechnology company) (Mygind et al. 2005). Plectasin is a 40-amino-acid-long oligopeptide that closely resembles the defensins of invertebrates (Mygind et al. 2005). NZ2114, a new derivative of plectasin, exhibited enhanced activity against staphylococci and streptococci (Andes et al. 2009) in comparison to naturally occurring plectasin.

Similarly, Merck (American pharmaceutical company) ventured to rescreen its culture extract collection for novel inhibitors of selected targets. They discovered platensimycin by expressing FabF (the ketoacyl-ACP synthase II) in *S. aureus* (Young et al. 2006; Wang et al. 2006). Platensimycin inhibited FabF with an IC50 of 48 nM. The MICs were in the μg/ml range for streptococci, staphylococci, and enterococci. In vivo efficacy against disseminating *S. aureus* infection was also demonstrated in mice (Lee et al. 2006). For Gram-negative bacteria, Cubist has developed a lipopeptide (CB-182804) exhibiting bactericidal activity against *Acinetobacter*, *Pseudomonas*, *Escherichia*, and *Klebsiella*. The peptide is currently in phase I clinical trials against MDR Gram-negative bacteria (Brötz-Oesterhelt and Sass 2010). The company has not yet disclosed the structural details and profile of this antibiotic.

Simultaneously, academic researches continue to screen microbes from various extreme environments including the deep ocean. These academic efforts have resulted in the discovery of novel compounds, which might be developed into new antibiotics in the future (Butler and Buss 2006). These efforts have successfully demonstrated that exploring microbial diversity from culturable and nonculturable microbes can result in the discovery of new compounds that can refill the dry pipeline of drug candidates.

Scientists at pharmaceutical companies and universities have invested innumerable efforts to screen and identify potent broad-spectrum antibiotics from plants but have failed. One possible reason for this failure is that plants may use different chemical strategies to manage microbial infections that aim to reduce the selective pressure for the development of resistance (Lewis and Ausubel 2006). For instance, certain plant-derived antibacterials show potent activity in combinations while exhibiting limited efficacy alone. A classic example is the combination of berberine and 5′-methoxyhydnocarpin. Berberine, commonly present in barberry plants, is a DNA intercalator and increases membrane permeability (Amin et al. 1969). Additionally, the positive charge on berberine enables it to accumulate in bacterial cells (Severina et al. 2001). Considering it has such a broad target, berberine should be a perfect antibacterial (Lewis and Ausubel 2006). However, berberine alone is ineffective because it is easily pumped out by pathogen-encoded multidrug resistance pumps (Hsieh et al. 1998). In barberry plants, another compound, 5′-methoxyhydnocarpin, was isolated that is

potent in blocking the efflux pumps that expel berberine. The combination of berberine and 5′-methoxyhydnocarpin acts as an effective antibacterial; however, neither compound is very effective alone (Stermitz et al. 2000). Similar is the case with many other phytocompounds such as rhein, plumbagin, resveratrol, gossypol, and coumestrol where the antibacterial activity is enhanced up to 100-fold by disabling efflux pumps (Lewis and Ausubel 2006).

Another chemical strategy that plants use to overcome bacterial infections is the production of compounds that selectively target bacterial virulence but not bacterial growth. Although there is a lack of abundant literature on the specific mechanisms, many plant extracts and phytocompounds, such as *Hibiscus sabdariffa*, *Momordica charantia*, *Forsythia suspense*, and green tea, have been reported to inhibit bacterial virulence (Kalia 2013; Khan et al. 2018; Qais et al. 2019). Research is still ongoing to find plant-based novel antibacterials with multiple targets and broad-spectrum activity.

4 Alternative Approaches for Targeting Bacterial Pathogens

Apart from conventional antibiotics, there are other approaches that may reduce the selective pressure of developing AMR. The most useful replacements for antibiotics are bacteriocins, bacteriophages, and predatory bacteria or other natural compounds that inhibit bacterial growth. Each of these approaches has its own pros and cons in terms of efficacy, benefits, health risks, and costs (Allen et al. 2013). One common pro is that these alternative strategies can be used to target a specific group of bacteria, a desirable trait to reduce the selection of resistance among nontargeted bacteria (Allen et al. 2014). Some of these approaches are discussed below:

4.1 Bacteriocins

Antimicrobial peptides are alternative agents for conventional antimicrobials. A group of antimicrobial peptides, which are nontoxic to mammalian cells, are bacteriocins (Allen et al. 2014). These are small ribosomally synthesized peptides, secreted by bacteria to inhibit the growth of other closely related bacterial species. Bacteriocins form pores by inserting into the plasma membrane of target bacteria, causing lysis of the cell. It has been found that almost all major lineages of bacteria produce bacteriocins. According to some estimates, approximately 99% of all bacteria secrete at least one bacteriocin. Thus, there is an immense diversity of such compounds that can be potentially exploited for therapeutic purposes (Snyder and Worobo 2014). Many commensal bacteria produce bacteriocins that could potentially be exploited (Cotter et al. 2013). For instance, lactic acid bacteria produce a bacteriocin called nisin A; it is currently used as a food preservative in many countries due to its bacteriodical activity. Bacteriocins produced from other food-grade

microbes could also be adapted considering their long historical use in food products such as cheese or yogurt (Vidhyasagar and Jeevaratnam 2013).

Bacteriocins can also be used to treat bacterial infections, including those that are MDR. A bacteriocin produced by *Enterococcus faecium* was active against 29 different vancomycin-resistant *Enterococcus* strains; however, it did not inhibit the growth of other pathogens such as *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus*, *Klebsiella pneumoniae*, or *E. coli* (Shokri et al. 2014). Another bacteriocin, thuricin CD, killed *Clostridioides difficile* without disturbing the normal microbiota (Zendo 2013). Low-molecular-weight bacteriocins are documented to be more resilient; however, high-molecular-weight bacteriocins are more prone to degradation by intestinal proteases or heat (Bastos et al. 2010; Allen et al. 2014; Shokri et al. 2014). Due to their narrow spectrum of antibacterial action, bacteriocins should exert selective pressure only on the species they target. For example, nisin A has been used extensively but no resistance has been reported (Zendo 2013). On the contrary, *E. coli* and *Listeria monocytogenes* have developed resistance to bacteriocins under in vitro conditions by long-term exposure at progressively increasing concentrations (Naghmouchi et al. 2011).

4.2 Phage Therapy

Phage are viruses that infect bacteria and cause lysis. Therapeutic application of phage to kill pathogenic bacteria is called phage therapy, and it has been used to treat infections in humans as well as animals (Johnson et al. 2008; Abedon et al. 2011). Phage therapy was developed to be used topically to treat infections such as skin infections or paranasal sinus infections (Chan et al. 2013). However, there is evidence suggesting phage are effective against systemic infections as well (Smith and Huggins 1982; Biswas 2002; Międzybrodzki et al. 2012). Phage have a very narrow spectrum of bacteria they can infect. So, unlike antibiotics, they do not harm nontarget bacteria (Allen et al. 2014). Studies have suggested that phage specificity depends on the phage titer that may either be narrow or broad (Koskella and Meaden 2013). Currently, the use of phage therapy for human infections is mainly limited to Eastern European countries (Międzybrodzki et al. 2012). In the United States, phage therapy is used for biocontrol of plant pathogens and foodborne pathogens in animals (Brussow 2007; Balogh et al. 2010; Goodridge and Bisha 2011). The application of phage therapy to treat human infections in Western countries is significantly restricted by regulatory agencies.

4.3 Predatory Bacteria

The use of predatory bacteria for treatment of infections is unconventional compared to phages and bacteriocins, but it presents a fascinating possibility as an alternative for antibiotics. Different types of predatory bacteria have been isolated, but the *Bdellovibrio* and like organisms (BALOs) exhibit particular potential. BALOs mainly prey on

Gram-negative bacteria for nutrients and energy (Dwidar et al. 2012). The genomes of BALOs encode numerous hydrolases, DNases, and proteases presumably used to digest prey or to attack bacterial biofilms (Lambert and Sockett 2013; Pasternak et al. 2013). BALOs can potentially be useful against complex microbial communities dwelling in biofilms where antibiotics have limited access (Sockett and Lambert 2004; Van Essche et al. 2011). Predatory bacteria have been investigated in clinical settings to target multidrug-resistant pathogens including *Acinetobacter baumannii*, *K. pneumonia*, *E. coli*, *Pseudomonas putida*, and *P. aeruginosa* (Kadouri et al. 2013). There are reports that BALOs can colonize the host's intestinal tract and serve as both a probiotic and antibiotic (Dwidar et al. 2012). In one study, BALOs were orally administered to *Salmonella enterica*-challenged chickens, resulting in a reduction of inflammation (Atterbury et al. 2011). The collateral effects of BALO administration to nontarget bacteria have yet to be explored in detail; however, some evidence suggests that BALOs do not colonize in vivo (Allen et al. 2014). Thus, despite some promising preliminary data, more extensive research is needed to validate the safety of BALOs.

4.4 Combinatorial/Synergistic Approaches

Another approach to manage MDR pathogens is to use a combination of drugs. In doing so, the toxic effects of antibiotics can be reduced and their potency enhanced (Khameneh et al. 2016). A combination of antibiotics and non-antibiotics can also be exploited to target resistance mechanisms and interfere with bacterial signaling pathways (Worthington and Melander 2013a). One such well-known strategy is to combine β-lactam antibiotics with β-lactamase inhibitors (Worthington and Melander 2013b). Plant extracts, phytocompounds, essential oils, as well as nanoparticles have exhibited synergistic interactions with different classes of antibiotics against microorganisms, including drug-resistant strains (Wolska et al. 2012; Langeveld et al. 2014). In clinical settings, the combination of two or more antimicrobial drugs is used to treat MDR infections, including those caused by bacteria and fungi. For instance, the combination of four drugs is used to treat *Mycobacterium tuberculosis* infections (Mitchison and Davies 2012). The widespread emergence of MDR pathogens has demonstrated that the use of single antibiotics often poses more selective pressure, and hence combination therapy should be utilized to reduce the further emergence of drug-resistant pathogens (Tamma et al. 2012). For example, the combination of amoxicillin and clavulanic acid is used; whereby, β-lactamase production is inhibited by clavulanic acid, and amoxicillin inhibits cell wall biosynthesis. This combination has allowed the continued use of amoxicillin to treat infections caused by pathogens that may have developed resistance to β-lactam antibiotics (Ball 2007). Reserpine, a MDR pump inhibitor, is used in combination with ciprofloxacin to suppress resistance in *S. aureus* and *Streptococcus pneumoniae* strains (Lomovskaya et al. 2001). Likewise, celecoxib, another MDR efflux pump inhibitor, improves the sensitivity of *S. aureus* to many antibiotics such as ampicillin, chloramphenicol, kanamycin, and ciprofloxacin (Kalle and Rizvi 2011). In addition, phytocompounds

and biosurfactants are considered safe and have been approved by the FDA for use in pharmaceuticals and food (Joshi-Navare and Prabhune 2013).

Combination approaches can be subcategorized into three categories based on the drug target (Worthington and Melander 2013b; Hamoud et al. 2014): combining antibiotics that target different pathways, combining antibiotics that target different parts of the same pathway, and combining antibiotics that attack the same target by multiple mechanisms. The success of combination therapy against infection depends on the ability to kill bacteria, avoid resistance, minimize host toxicity, and not disturb the natural microflora. To further boost the efficacy of combination therapy, drug delivery is also important. Overall, the key features of a combination treatment include (Hagihara et al. 2012) enhancement of antibiotic activity by synergistic effect(s), prevention of resistance emergence, possession of anti-biofilm activity, improvement of antibiotic penetration to cells and tissues, and inhibition of virulence factors, such as toxin or enzyme production in pathogens.

4.5 Use of Nanoparticles as Nanomedicine

Many alternative strategies that have been proposed to combat MDR bacteria use nanotechnology to develop novel nanomaterials that possess broad-spectrum antimicrobial action (Baptista et al. 2018). Nanoparticles (NPs) are promising because they possess bactericidal action and also have the capacity to deliver conventional antibiotics (Wang et al. 2017). A wide range of nanomaterials have been developed and tested, including liposomes, metallic vectors, polymer-based nano-drug carriers, and gold NPs (Burygin et al. 2009). An important aspect of nanomedicine is the delivery of drugs to the site of infection by either attaching the drugs to the large NP surface area or by encapsulating antibiotics within a nanostructure (Gholipourmalekabadi et al. 2017). Nanomaterials typically range from 0.2 to 100 nm in at least one dimension and exhibit high surface-to-volume ratios. Nanomaterials can have different chemical, mechanical, electrical, optical, magnetic, and electro-potential properties compared to their bulk materials (Hajipour et al. 2012; Rudramurthy et al. 2016). NPs can enhance the solubility, stability, and biocompatibility of drugs, giving them an advantage over conventional therapies for the treatment of infections caused by drug-resistant bacteria (Rudramurthy et al. 2016; Gholipourmalekabadi et al. 2017). Among metal nanoparticles, silver nanoparticles are the most studied and effective nanomaterial against pathogenic bacteria; however, other metal and metal oxide nanoparticles such as zinc, copper, titanium, tin, and iron also exhibit antibacterial potential (Hemeg 2017; Qais et al. 2018).

While conventional antibiotics have limited membrane permeability, thereby reducing their potency (Andrade et al. 2013), NPs can penetrate the bacterial membrane either by endocytosis or through interactions with surface lipids (Huang et al. 2010; Wang et al. 2017). Moreover, multiple drug combinations can be loaded into or onto NPs to reduce the possibility of developing bacterial resistance (Huh and Kwon 2011). Some NPs also demonstrate broad-spectrum bactericidal activity against Gram-positive and Gram-negative pathogens (Rai et al. 2016; Zaidi et al. 2017). When used as drug carriers, NPs can protect

antimicrobial agents from degrading or inactivating enzymes while effectively delivering the drug to the target site (Huh and Kwon 2011; Wang et al. 2017). Clearly, NPs have significant potential to improve antibiotic therapy, but the systemic use of nanomedicine against drug-resistant bacteria is under scrutiny.

4.6 Anti-virulence Strategies Against MDR Pathogens

In 2014, the WHO declared the beginning of a post-antibiotic era and considered AMR a public health priority demanding global action. It is expected that by 2050 AMR will become a major killer, surpassing cancer, if no action is taken. New antibiotic discovery has been essentially nonexistent over the last several decades, with the exception of teixobactin, and new strategies to combat MDR bacteria must be developed (Ahmad et al. 2009; Totsika 2016). Targeting the virulence and pathogenicity of bacteria has been considered a promising strategy (Ahmad and Husain 2014). In theory, anti-virulence drugs should inhibit bacterial virulence, but not kill bacteria, thus lessening the emergence of resistance. One major anti-virulence strategy that has been pursued is to neutralize or inactivate bacterial toxins, which has been successful to prevent or relieve acute disease symptoms (Adalja and Kellum 2010; Lopez et al. 2010; Chen et al. 2011; Bender et al. 2015). In addition to bacterial toxins, other virulence mechanisms have been identified as potential drug targets (Rasko and Sperandio 2010; Ahmad and Husain 2014; Anthouard and DiRita 2015; Heras et al. 2015) such as bacterial adhesion and colonization (Steadman et al. 2014; Cascioferro et al. 2014), cell-to-cell communication (quorum sensing), secretion systems, and biofilm formation.

Despite their potential, the development of anti-virulence agents has primarily been pursued within the confines of academia and a few small biotech companies. The lack of interest by big pharmaceutical companies is likely due to an increase in development costs, with poor projected profits. However, with the increasing AMR problem and lack of available new antibiotics, the exploration and development anti-infective/anti-virulence drugs is becoming more attractive. Quorum sensing inhibitors or quorum quenching compounds are being developed as anti-virulence drugs against specific MDR bacteria such as *P. aeruginosa* (Kalia et al. 2014). Similarly, inhibition of fimbrial adhesion, a well-known virulence factor in *E. coli*, has shown promise against urinary tract infection in vivo (Guiton et al. 2012), and inhibition of type three secretion system (TTSS) (e.g., salicylidene acylhydrazides) has shown promising results against several pathogenic species (Baron 2010).

Biofilm formation by pathogenic bacteria is a common strategy used to establish infection and persist in the harsh host environment. Biofilm inhibition or eradication is an effective strategy to prevent and treat infection. Anti-biofilm drugs hold significant potential to enhance the efficacy of antibiotics, increase drug penetration, and reduce tolerance to antibiotics. While there are many types of anti-biofilm agents being explored, biofilm-degrading enzymes have shown particular efficacy in vitro and in vivo (Fleming et al. 2016; Fleming and Rumbaugh 2017, 2018). Although there are several challenges in evaluating and developing anti-virulence drugs

(Totsika 2016), these efforts are critical and may hopefully result in the discovery of new approaches (Allen et al. 2014).

5 Conclusions

The discovery of new antibacterial drugs with new modes of action has stalled, and AMR has now become a global problem and major threat to mankind. Developing new strategies to combat the MDR problem is now a priority. A number of conventional and modern approaches have been identified to reduce the emergence of drug resistance and develop new drugs. In consideration of the progress made so far on various fronts, we can conclude that:

(a) Natural products are still a major source for the discovery of new antibacterial leads.
(b) New molecular and bioinformatics approaches can be useful in obtaining new compounds.
(c) Alternative strategies such as combination drugs and antimicrobial peptides have potential in combating the MDR problem.
(d) Nanotechnological advances can be effectively harnessed to improve antibiotic performance.
(e) Anti-infective approaches should be more thoroughly explored and integrated into therapy when possible.

Lastly, concerted efforts by academia, industry, government, and the public are greatly needed to develop new antibiotics that will protect human and animal health in the future.

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Part I

The Challenge of Antibiotic Resistance and Tolerance

Problematic Groups of Multidrug-Resistant Bacteria and Their Resistance Mechanisms

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Abstract

The occurrence of multidrug-resistant pathogenic bacteria is steadily increasing, not only in medical centers but also in food, animals and the environment, which is of primordial concern for health authorities worldwide. The World Health Organization (WHO) published a global pathogen priority list to encourage international interdisciplinary research initiatives on the occurrence, dissemination, and epidemiology of the most dangerous multiresistant pathogens with the aim to develop effective prevention strategies against the spread of these bugs and new therapeutic approaches to treat infections in agreement with the One Health concept. According to the WHO global pathogen priority list, the most critical resistant pathogens include carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and carbapenem-resistant as well as third-generation cephalosporin-resistant *Enterobacteriaceae.* This critical group is followed by pathogens of high priority including vancomycin-resistant *Enterococcus faecium*, methicillin- and vancomycin-resistant *Staphylococcus aureus*, and clarithromycin-resistant *Helicobacter pylori*. Here, we summarize recent data on the occurrence and spread of these and other harmful resistant pathogens, on their resistance mechanisms as well as on the modes of resistance spread, as far as is known. We finish the chapter with an outlook on promising innovative strategies to treat infectious diseases caused by multiresistant pathogens – in combination with antibiotic therapy – as well as on approaches to combat the antibiotic resistance spread.

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Keywords
Antibiotic resistance · Bacterial pathogen · Biofilm · Horizontal gene transfer · Multidrug resistance · WHO pathogen priority list

Abbreviations

Agr	accessory gene regulator
BLNAR	ß-lactamase-negative ampicillin resistant
CDC	Centers for Disease Control and Prevention
COPD	chronic obstructive pulmonary disease
CRAb	carbapenem-resistant *Acinetobacter baumannii*
CRE	carbapenem-resistant *Enterobacteriaceae*
CRPa	carbapenem-resistant *Pseudomonas aeruginosa*
ESBL	extended spectrum ß-lactamase
EU	European Union
FDA	Food and Drug Administration
G-	Gram-negative
HGT	horizontal gene transfer
ICU	intensive care unit
IMP	active on imipenem
IS	insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	metallo-ß-lactamase
MDR	multidrug resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *S. aureus*
NDM	New Delhi MBL
OMP	outer membrane protein
OXA	oxacillinase
PBP	penicillin-binding protein
PMQR	plasmid-mediated quinolone resistance
PNSP	penicillin-non-susceptible *Streptococcus pneumoniae*
RND	resistance-nodulation-cell division
SCC*mec*	staphylococcal chromosome cassette *mec*
VIM	Verona integron-encoded MBL
VRE	vancomycin-resistant *Enterococci*
VREfm	vancomycin-resistant *E. faecium*
VRSA	vancomycin-resistant *S. aureus*
WHO	World Health Organization
XDR	extremely multidrug resistant

1 Introduction

Antibiotic drugs are unquestionably the most successful form of chemotherapy, and since people started to use them commercially, antibiotics have increased life expectancy in recent history by up to two decades (Shallcross 2014; Martens and Demain 2017). Nevertheless, modern mankind is facing the so-called antimicrobial resistance crisis (Barriere 2015; Martens and Demain 2017), annually accounting for an estimated two million antibiotic-resistant infections worldwide. It is proposed that, by 2050, 10 million deaths worldwide will be attributed to this issue (Robinson et al. 2016). In past times, the arsenal of new antibiotic drugs was satisfactory to manage the observed resistance in bacteria, but in recent years, overconsumption combined with the inappropriate prescription of antibiotics has resulted in the elevated occurrence of multidrug-resistant (MDR) and extremely multidrug-resistant (XDR) bacteria (Davies and Davies 2010; Banin et al. 2017). Beyond the abusive and not indicated use of antibiotics, poor infection control and substandard sanitation contribute to the resistance crisis. Widespread use of antibiotics in the agricultural industry has further accelerated this problem (Srinivas et al. 2017). For livestock applications, 50–80% of antibiotic drugs are administered (Cully 2014; Chang et al. 2015b), with a large fraction used at sub-therapeutic concentrations, aiming to promote growth and prevent diseases of livestock in several countries (Ter Kuile et al. 2016). Nevertheless, the European Union (EU) has banned the use of antibiotics as growth promotors. Further, countries outside the EU (such as the USA and Australia) have restricted the application of antibiotics in agriculture (Cogliani et al. 2011; Maron et al. 2013). Major mechanisms of how bacteria exert antibiotic resistance is, in addition to biofilm formation, also by acquiring new determinants via horizontal gene transfer (HGT) and mutations leading to suppressed antibiotic susceptibility (Blair et al. 2015). Bacterial biofilms in general show increased resistance to exsiccation, clearance by the immune system and lower susceptibility to antibiotics (Høiby et al. 2011). The increase in international mobility in the twenty-first century has had further strong effects on the spread of pathogenic bacteria throughout the world (Harvey et al. 2013; Laxminarayan et al. 2013; Shallcross 2014). The observed increasing rates of global antibiotic resistance has been accompanied with a decline in the number of companies developing new antibiotic drugs. Further, the number of approvals for new agents has significantly decreased (Chaudhary 2016; Sciarretta et al. 2016). This evolves as a major threat, as within a few years after the commercial introduction of new antibiotic drug, resistant strains are reported (Davies and Davies 2010; Smith et al. 2015). Since 1998, only two antibacterial agents that were approved by the Food and Drug Administration (FDA) have had a novel mechanism of action (Spellberg et al. 2004; Luepke et al. 2017). The problem of modified agents of known drug classes is, when widely applied, antibiotic-resistant bacterial strains might evolve more rapidly (World Health Organization 2001; Jensen et al. 2010). Thus, there is an urgent need for discovering new targets and designing new compounds. Recently, alternative therapeutics, such as phage therapy or antibodies, for the treatment of infections have been discussed

(Natan and Banin 2017; Pachón-Ibáñez et al. 2017; Tracanna et al. 2017; van der Meij et al. 2017).

Taking the alarming development and the imminence of antibiotic resistance into account, the WHO was asked to create a priority list of bacteria other than multiresistant *Mycobacterium tuberculosis*, in the hope it would support and focus research on the development of new antibiotic drugs effective against these pathogens. The introduced WHO global priority pathogen list aims to take a step forward in addressing this global crisis of antimicrobial resistance (World Health Organization 2017; Willyard 2017; Tacconelli et al. 2017). Thus, a multi-criteria decision analysis method was applied to prioritize resistant bacteria. Twenty bacterial species were selected and organized into three groups based on ten criteria. These three groups divided bacteria into critical, high-, and medium-priority pathogens (Fig. 1) (Tacconelli et al. 2017).

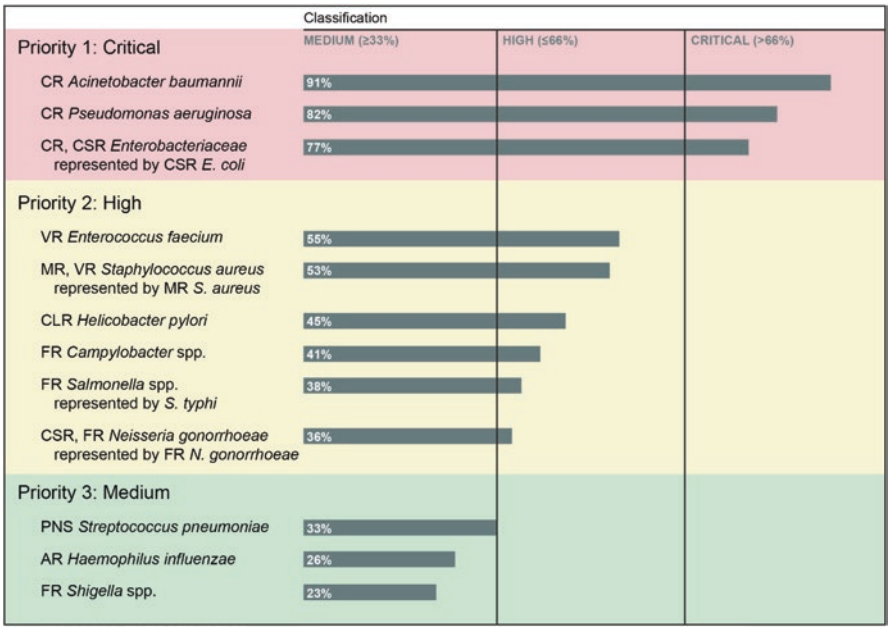

Fig. 1 Ranking of antibiotic-resistant bacteria according to the 10-criteria catalogue**.** Antibiotic-resistant bacteria were categorized according to ten criteria: treatability, mortality, healthcare burden, trend of resistance, prevalence of resistance, transmissibility, community burden, preventability in the healthcare setting, pipeline, and preventability in the community setting. 20 strains of drug-resistant bacteria were ranked and grouped according to the highest representative. Pathogens exhibiting more than 66% of the final weight were assigned to the priority 1 (critical) group, those between 33% and 66% were assigned to priority 2 (high), and bacteria less or equal 33% of final weight were ascribed to priority 3 (medium). *CR* carbapenem resistant, *CSR* 3rd-generation cephalosporin resistant, *VR* vancomycin resistant, *MR* methicillin resistant, *CLR* clarithromycin resistant, *FR* fluoroquinolone resistant, *PNS* penicillin non-susceptible, *AR* ampicillin resistant. (Figure adapted from Tacconelli et al. (2017))

In this review, we will give detailed information on bacterial species that, according to the WHO's global priority pathogen list, represent the most imminent dangers, further fueling the antibiotic resistance crisis. In addition to providing statistical information about their distribution, we will focus on the underlying mechanisms that have ultimately led to their emergence as antibiotic-resistant pathogens.

2 The Global Priority Pathogen List

2.1 Priority 1: Critical

2.1.1 Carbapenem-Resistant *Acinetobacter baumannii*

Acinetobacter are non-glucose-fermenting Gram-negative (G-) coccobacilli, primarily related with healthcare-associated infections. These bacteria harbor extensive intrinsic resistance determinants and have the capability to acquire new resistance factors (Peleg et al. 2008). *Acinetobacter baumannii*, an opportunistic pathogen, is associated with hospital-acquired infections and outbreaks worldwide, affecting particularly critically ill patients (Runnegar et al. 2010; Correa et al. 2017). The first reported *Acinetobacter* infections within an intensive care unit (ICU) date back to the 1960s (Stirland et al. 1969). Early *Acinetobacter*-mediated infections were easily treatable with ß-lactams and sulfonamides (Stirland et al. 1969; Abrutyn et al. 1978), but these treatment strategies shortly evolved to be inefficient due to the rising resistance rates (Lecocq and Linz 1975). In the 1980s, carbapenems were used as therapeutics to treat infections caused by MDR bacteria, but resistances to these antibiotics in *Acinetobacter* were reported shortly after their commercial introduction (Paton et al. 1993; López-Hernández et al. 1998; Gonzalez-Villoria and Valverde-Garduno 2016). Carbapenems are broad-spectrum ß-lactam antibiotics, widely used as last-line antibiotics, especially for the treatment of critically ill patients and infections induced by antibiotic-resistant G- bacteria (Papp-Wallace et al. 2011).

A. baumannii colonization rates in healthy humans are low (about 1%) but higher in some Asian populations. Community-acquired infections caused by carbapenem-resistant *A. baumannii* (CRAb) are uncommon and most likely occur in patients with underlying pulmonary disease, renal failure, diabetes, or excessive alcohol abuse (Falagas et al. 2007a). Nosocomial outbreaks of *A. baumannii* are generally difficult to control, as this bacterium is able to survive on abiotic surfaces for extended periods of time. The hands of the hospital staff are a common mode of transmission, but the spread can also be caused by exposure to contaminated equipment and aerosolized water droplets (Dijkshoorn et al. 2007). Elderly people, especially those in long-term care facilities, were shown to be an important reservoir of MDR *A. baumannii* (Denkinger et al. 2013).

In the 1990s, multiresistant strains were first detected in Asia, where they developed as a great public health challenge (Kuah et al. 1994; Siau et al. 1996). In South and Southeast Asian hospitals, high rates of carbapenem resistance among G- pathogens, especially in *A. baumannii* isolates, were observed (Hsu et al. 2017). In some

Asian countries, including Malaysia, Thailand, Pakistan, India, and Taiwan, *A. baumannii* belongs to the group of most abundant nosocomial pathogens (Chawla 2008). In Korea, the resistance rate of *A. baumannii* to imipenem, a representative of carbapenems, had increased to 85% by 2015, thus representing an enormous health threat (Kim et al. 2017). A combination of factors involving non-indicated prescription of antibiotic drugs and international travel, including medical tourism, contributed to the accelerated rise and spread of *A. baumannii* in South and Southeast Asia (Hsu et al. 2017). Interestingly, the increased frequency of *A. baumannii* isolated in the clinical setting showed a high correlation with the observed rise in antibiotic resistance (Carlquist et al. 1982). In the USA, it was observed that when *A. baumannii* causes healthcare-associated infections, more than 60% of the isolates showed resistance to carbapenems (Sievert et al. 2013). Even though the occurrence of *A. baumannii* changed only marginally from 2000 to 2009 in the USA, an ongoing decrease concerning the susceptibility to most classes of antibiotic drugs was observed. Further, a threatening third of all isolates manifested combined resistances to carbapenems, aminoglycosides, and fluoroquinolones (Landman et al. 2007).

While uncomplicated urinary tract infections and other minor infections have low mortality, patients with bloodstream infections from CRAb showed mortality rates of more than 40% (Wisplinghoff et al. 2004; Munoz-Price et al. 2010). Between 2010 and 2014, 60 cases of bacteremia caused by CRAb from 7 states in the USA were studied. Catheter-related bloodstream infections were the most abundant infections observed, and nearly half of the patients died within 30 days of diagnosis (Olesky et al. 2017). *Acinetobacter* infections are generally associated with several risk factors, including the use of mechanical ventilation and previous antimicrobial therapy. Prior hospitalization, longer duration of hospital stay, especially in ICUs, but also preceding the prescription of carbapenems, and the use of invasive procedures were identified as potential risk factors (Sheng et al. 2010).

The ability of *A. baumannii* to form biofilms most probably contributes to the observed prolonged survival on abiotic surfaces leading to subsequent transmission (de Breij et al. 2010). Further, biofilm formation on urinary catheters, central venous catheters, and endotracheal tubes may also prompt infection (Longo et al. 2014).

Differing but complementary mechanisms leading to reduced carbapenem susceptibility have been described for *A. baumannii* (Vila et al. 2007; Tang et al. 2014). The mechanisms of resistance include various carbapenemases (most commonly oxacillinases, OXA, and metallo-ß-lactamases, MBLs), AdeABC efflux systems, modification of penicillin-binding proteins (PBPs), and modification of outer membrane proteins (porins) (Yoon et al. 2015). A major intrinsic resistance mechanism is facilitated by the reduced number and size of certain outer membrane proteins (OMPs), leading to a compromised bacterial permeability to antibiotics than when compared to other G- organisms (Vila et al. 2007). Three OMPs have been associated with carbapenem non-susceptibility (Poirel et al. 2011). Intrinsic resistance-nodulation-cell division (RND)-type efflux pumps such as AdeABC, AdeFGH, and AdeIJK further play a role in carbapenem non-susceptibility (Yoon et al. 2015). The main way for resistance is hydrolysis of the drugs by an arsenal of intrinsic and

acquired carbapenem-hydrolyzing ß-lactamases (carbapenemases). The acquirement of carbapenemases, such as Ambler class A carbapenemases, class B MBLs, and class D oxacillinases, leads to the observed increased emergence of carbapenem resistance. Molecular classes A, C, and D comprise ß-lactamases characterized by a serine in their active site, while class B ß-lactamases are metalloenzymes containing zinc in their active center. While rare-chromosomally encoded cephalosporinases (Ambler class C enzymes) may possess a slightly extended activity on carbapenems, they most likely play a minor role in the clinics. Carbapenemases with catalytic efficiency on carbapenems are mostly grouped into Ambler classes A, B, and D (Queenan and Bush 2007). Ambler class B carbapenemases comprise a broader spectrum than the other enzyme classes. They show a strong hydrolytic activity against most ß-lactam antibiotics and are not inhibited by ß-lactam inhibitors (Palzkill 2013). Ambler class D OXA-type ß-lactamases are native chromosomal oxacillinases and are encoded by several bla$_{OXA}$ genes, the most common are *bla*$_{OXA\text{-}23\text{-like}}$, *bla*$_{OXA\text{-}24\text{-like}}$, *bla*$_{OXA\text{-}51\text{-like}}$, and *bla*$_{OXA\text{-}58\text{-like}}$ genes. These enzymes and the presence of insertion sequences (IS), like IS*Aba1*, IS*Aba3,* IS*Aba4*, and IS*Aba9,* play an important role in the development of CRAb. While native chromosomal oxacillinases are generally expressed in low abundance, IS contribute to the mobilization and expression of the OXA-type-ß-lactamases, thus conferring carbapenem resistance. The IS*Aba1* sequence is the most prevalent and was described in *A. baumannii* isolates for the first time in 2001. This IS has been found to be associated with a number of OXA-type ß-lactamases (Evans and Amyes 2014). Today OXA-23 belongs to the most prevalent subgroup of oxacillinases worldwide (Mugnier et al. 2009; Poirel et al. 2011).

Many CRAb isolates were further shown to be MDR, carrying additional resistance determinants for several other groups of antibiotics like aminoglycosides, fluoroquinolones, and tetracycline (Doi et al. 2015), thus leading to a major threat to modern healthcare and significantly fueling the global resistance crisis.

2.1.2 Carbapenem-Resistant *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen frequently responsible for nosocomial infections (Rossi Gonçalves et al. 2017), especially in ICUs or in patients with predisposing conditions (Pirnay et al. 2009). This bacterium can be found ubiquitously in the hospital, not only associated with patients or hospital staff but also on abiotic surfaces (Tsao et al. 2017). *P. aeruginosa* is the causative agent of pneumonia, urinary tract infections, and infections of skin and soft tissue but is especially implicated in pneumonia of critically ill and/or immunocompromised patients. The pathogen is prevalently isolated from the respiratory tracts of patients with chronic lung disease, such as cystic fibrosis (Aloush et al. 2006; Gellatly and Hancock 2013). Delayed detection and treatment lead to rapid progression to respiratory failure, sepsis, and multi-organ failure, which are all associated with high mortality rates (Kang et al. 2003). *P. aeruginosa* is also often isolated from lakes, sewage, soil, animals, plants, and plant detritus (Pirnay et al. 2009), and resistant strains are detected in swimming pools and hot tubs in the USA (Lutz and Lee 2011). Carbapenem-resistant *P. aeruginosa* (CRPa) was also detected in wastewater

treatment plant effluent and in downstream rivers in Switzerland (Czekalski et al. 2012; Slekovec et al. 2012). These strains act as a potential reservoir for determinants of carbapenem resistance (Pappa et al. 2016).

The highest rates of carbapenem resistance in *P. aeruginosa* were observed in Eastern Europe, with Hungary, Slovakia, Poland, Lithuania, Croatia, Romania, Bulgaria, and Greece presenting resistance rates of >25% (European Centre for Disease Prevention and Control 2015). An extensive spread of carbapenemase-producing clones was observed in Belarus, Kazakhstan, and Russia, thus showing a gradient of resistance in Europe that rises from Northwest to Southeast (Edelstein et al. 2013). In Brazil, 43.9% of the isolates from patients with *P. aeruginosa* bacteremia, most of them from ICU residents, were carbapenem resistant. Among these patients, 31.2% received inadequate therapy, and the mortality rate was as high as 58.6% (Rossi Gonçalves et al. 2017). In Brazil, the high prescription rate of antibiotics, particularly of ß-lactams, carbapenems, and fluoroquinolones (Rodrigues Moreira et al. 2013) was described to be instrumental in *P. aeruginosa* developing resistance to various antibiotic agents during therapy. This was shown to occur either by mutation in chromosomal genes or by HGT (Zavascki et al. 2005; Xavier et al. 2010). The carbapenem resistance of *P. aeruginosa* in Brazil is mostly due to the production of MBLs (Rossi Gonçalves et al. 2017). In some hospitals, the resistance rates can be up to 60% (Kiffer et al. 2005; Baumgart et al. 2010). In Taiwan, 15.9% of the *P. aeruginosa* isolates from infected patients were carbapenem resistant. This study stated that the risk of infection with CRPa increased by 1% with each day in hospital (Tsao et al. 2017); thus, prolonged stays in healthcare settings were identified as a major risk factor leading to *P. aeruginosa*-mediated infections. Further risk factors include the preceding use of antibiotics, invasive procedures, comorbidities, and antecedent surgery. Mechanical ventilation, enteral/nasogastric tubes and inappropriate therapy are also associated with bacteremia by CRPa (Rossi Gonçalves et al. 2017). Infections caused by resistant *P. aeruginosa* are further frequently related with age, cancer, heart disease, diabetes, and invasive procedures like hemodialysis and tracheostomy (Aloush et al. 2006; Buehrle et al. 2017). The presence of a central venous catheter as a significant risk factor is a matter of debate, as some studies suggest that catheter exchange helps to prevent *P. aeruginosa* biofilm formation and thus significantly reduced infection risk (Jamal et al. 2014), whereas others did not identify these as priority risk factors (Rossi Gonçalves et al. 2017).

The capability of *P. aeruginosa* to form biofilms (Suárez et al. 2010) has enabled the bacterium to proliferate in water distribution systems and colonize central venous catheters (Fig. 2) (Wang et al. 2012; Jamal et al. 2014). As example, all strains analyzed in the Brazil study mentioned above were identified as strong biofilm producers (Rossi Gonçalves et al. 2017). Additionally, all MBL-positive *P. aeruginosa* isolates from Brazil showed the ability to form biofilms in vivo (Perez and Bonomo 2018). The severity of infections, especially associated with invasive procedures, might be more pronounced due to biofilm formation, as the antibiotic is inhibited from penetrating the cells by the surrounding polymeric matrix composed of polysaccharides, proteins, and DNA (Costerton et al. 1999; Hoiby et al. 2010).

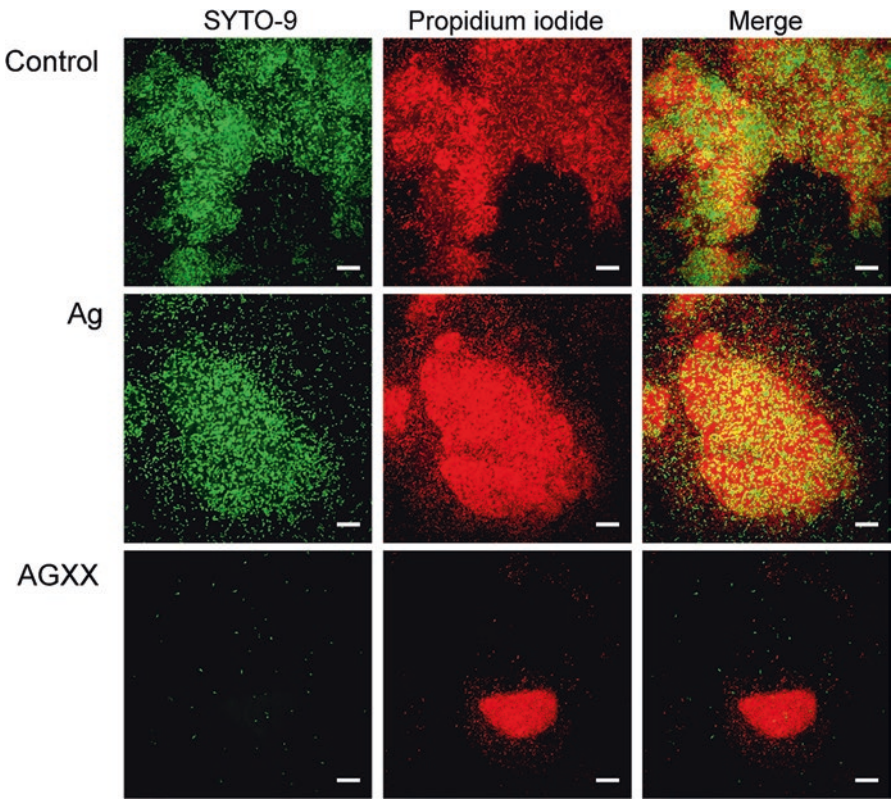

Fig. 2 Biofilm formation in *Pseudomonas aeruginosa*
Confocal images of biofilm formation by *Pseudomonas aeruginosa*. *P. aeruginosa* was grown on sterile coverslips for 24 h. The 24-h-old biofilms were exposed to silver (Ag) sheet or AGXX® sheet. The control panel refers to biofilm without any metal sheet. Biofilms were stained with SYTO9 (green) and Propidium Iodide (red) to visualize live and dead cells. Images show an average of Z-projections (500 nm spacing). Scale bars are 10 μm

The production of different enzymes, the lack of the outer membrane porin OprD, and the RND efflux pump systems MexAB-OprM and MexCD-OprJ, encoded on the genome, lead to the intrinsic resistance of *P. aeruginosa* to several classes of antibiotics. Resistance determinants, such as carbapenemase production, can also be acquired by HGT (Pirnay et al. 2009; Breidenstein et al. 2011; Poole 2011). Thus, *P. aeruginosa* has a great potential for developing a MDR phenotype (Schwartz et al. 2015).

Mutations in or lack of the porin OprD was shown to contribute to carbapenem resistance in clinical isolates of *P. aeruginosa* in Spain. OprD is a substrate-specific porin responsible for diffusion of amino acids (and also carbapenems) into the bacterial cell (Rojo-Bezares et al. 2014). A direct association between imipenem (a carbapenem) susceptibility and the levels of OprD expression was shown. Expression of OprD was not detected in imipenem-resistant isolates, whereas susceptible

bacteria showed close to normal expression levels (Dib et al. 1995). During imipenem treatment of *P. aeruginosa* infections in French hospitals, the most common mechanism of resistance was shown to be mutations in or loss of the porin OprD, with more than 85% of the isolates having lost the *oprD* gene (Fournier et al. 2013). Overproduction of chromosomally encoded AmpC ß-lactamases (also called cephalosporinase) and efflux pumps are further implicated in meropenem (a carbapenem) resistance in *P. aeruginosa* (Rodríguez-Martínez et al. 2009).

Expression/overproduction of RND efflux pumps further reduces carbapenem efficiency in *P. aeruginosa* (Choudhury et al. 2015; Pan et al. 2016). The MexAB-OprM efflux pump system plays a significant role in the intrinsic non-susceptibility of *P. aeruginosa* toward meropenem, quinolones, tetracycline, and chloramphenicol.

An important resistance mechanism of strains non-susceptible to ß-lactams is the expression of acquired carbapenemases. These isolates are usually resistant to all ß-lactams (Breidenstein et al. 2011; Poole 2011). Especially class B carbapenemases or MBLs are primarily encountered, with IMP-type (active on imipenem) enzymes predominantly encountered in Asia and VIM-type (Verona integron-encoded MBL) enzymes mostly found in Europe. Nevertheless, both enzymes are increasingly spreading globally (Walsh et al. 2005; Poole 2011). The most abundant carbapenemase is VIM; it can be plasmid-mediated and multiple copies lead to high-level meropenem resistance (San Millan et al. 2015), but it is usually integron-associated. IMP-6, another MBL, was demonstrated to be acquired from environmental bacteria by HGT (Xiong et al. 2013). Generally, MBLs occur as part of an integron structure on large genomic islands on the bacterial chromosome, but it was shown that they can also be encoded on transferable plasmids (Wright et al. 2015).

2.1.3 Carbapenem- and Third-Generation Cephalosporin-Resistant *Enterobacteriaceae*

Several representatives of G- *Enterobacteriaceae* are human pathogens, including *E. coli*, *Klebsiella* ssp., *Proteus* spp., *Enterobacter* spp., and *Serratia* spp. *Enterobacteriaceae* represent 50% of bacteremia cases, which are usually caused by redistribution of bacteria from their primary sites (Wilson et al. 2011). Infections with *Enterobacteriaceae,* most commonly arising from the gastrointestinal tract, involve high morbidity and mortality (Patel et al. 2008; Yamamoto and Pop-Vicas 2014). Even though infections caused by G+ pathogens are more common in healthcare settings, the highest mortality rate is associated with *Enterobacteriaceae* and other G- organisms (Wilson et al. 2011). *E. coli* and *Klebsiella pneumoniae* are the most abundant community – as well as hospital-acquired pathogens. These bacteria typically cause intra-abdominal infections, urinary tract infections, and primary bacteremia (Alhashem et al. 2017). Patient-to-patient transmission is comparably low, however, *K. pneumoniae* shows a higher rate of transmission than *E. coli* (Harris et al. 2007; Hilty et al. 2012).

Enterobacteriaceae are getting increasingly resistant to first- and second-line antibiotic drugs. Carbapenems are usually the treatment strategy for life-threatening infections by MDR *Enterobacteriaceae*, some of which produce extended spectrum

ß-lactamases (ESBLs). Infections with ESBL-producing G- bacteria and carbapenem-resistant *Enterobacteriaceae* (CRE) are increasing worldwide. Different geographical regions reveal carriage rates varying over time, but ESBL-producing *Enterobacteriaceae* occur globally nowadays, and carriage rates ranging from 8 to 28.8% have been reported in ICUs in Jerusalem and Korea, respectively (Friedmann et al. 2009; Kim et al. 2014). ESBL and AmpC enzymes together are responsible for the majority of the observed third-generation cephalosporin resistances in clinical isolates worldwide (Molton et al. 2013). In North American and European hospitals, those rates are around 10% for both *E. coli* and *K. pneumoniae*, while nosocomial ESBL rates as high as 80% and 60% were found in India and China, respectively (Livermore 2012). In the Indian community, *E. coli* resistance rates were as high as in the hospital environment. This might be due to the unregulated use of antibiotic drugs in agriculture and lower sanitation standards (Chaudhuri et al. 2011). In China, the rate of ESBL-positive strains among *E. coli* increased severely from 36.1% in 2002/2003 to 68.1% in 2010/2011 (Lai et al. 2014). For about three decades, a spreading of plasmid-mediated ß-lactamases in *Enterobacteriaceae* has been reported in Brazil. ESBL-producing strains, especially *K. pneumoniae* as the predominant pathogen, are widely distributed in healthcare settings (Sampaio and Gales 2016). In the USA, 18% of healthcare-associated infections in acute care hospitals and acute rehabilitation facilities can be attributed to ESBL-producing *Enterobacteriaceae* (Weiner et al. 2016).

The inadequate antibiotic prescription and inappropriate use of antibiotic drugs accelerated the spreading of CRE, leading to public concern. Selection pressure by the prescription of carbapenem antibiotics has been proposed to fuel the rapid spread of CRE (Yigit et al. 2001; Potter et al. 2016). In Europe, 17 countries reported increased dissemination or occurrence of CRE between 2010 and 2013 (Glasner et al. 2013). Infections caused by CRE especially affect severely ill patients with multiple comorbidities. ICU-resident patients revealed a notably high burden of infections with CRE and increased mortality when compared to non-ICU patients (Debby et al. 2012; Tischendorf et al. 2016; Papadimitriou-Olivgeris et al. 2017). Among ICU-resident patients in Israel, colonization with CRE was associated with at least a two-fold increase in the risk of infection by the colonizing strain (Dickstein et al. 2016). Recently, *E. coli, K. oxytoca*, and *Enterobacter cloacae* were frequently reported to harbor carbapenem resistance (Tzouvelekis et al. 2012; Gomez-Simmonds et al. 2016). Among the hospitalized patients, 3–7% are colonized by CRE in endemic areas, but these rates can vary between 0.3% and 50% depending on the healthcare setting, with the highest rates achieved in a Greek hospital (Banach et al. 2014; Bhargava et al. 2014; Papadimitriou-Olivgeris et al. 2012; Swaminathan et al. 2013; Vatopoulos 2008; Vidal-Navarro et al. 2010; Wiener-Well et al. 2010; Zhao et al. 2014). Greece has one of the highest rates of carbapenem-resistant G-bacteria globally. By 2008, carbapenem resistance had increased to 30% in hospitals and to 60% in ICUs (Walsh et al. 2005). A study in a tertiary hospital in China revealed that *K. pneumoniae* and *E. coli* were the most prevalent species. More than 70% of all nosocomial isolates exhibited high levels of resistance against ß-lactam antibiotics, while 64.9% of the strains harbored carbapenemase genes (Yang et al.

2017). CRE have also become widely distributed in the USA with 140,000 cases of nosocomial infections annually that show mortality rates between 26 and 44% (Centers for Disease Control and Prevention 2013; Falagas et al. 2014). Furthermore, *K. pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* became prevalent in Brazil in the last 10 years. KPC production is reported to be the most common resistance mechanism in carbapenem-resistant *K. pneumoniae* (Sampaio and Gales 2016).

Colonization of ICU patients with CRE is a massive risk factor for subsequent infection with *K. pneumoniae*. Almost 50% of the patients in a hospital in the USA developed an infection within 30 days after having been tested positive for colonization with the pathogens. Endoscopy and colonoscopy were shown to be risk factors for these infections (McConville et al. 2017). Further risk factors for increased susceptibility to CRE were the prescription of ß-lactam antibiotics within 30 days and receiving trimethoprim-sulfamethoxazole or glucocorticoids concomitant with an onset of bloodstream infection, as observed in a hospital environment in the USA (Bratu et al. 2005). Other risk factors were described to be comorbid conditions, prolonged hospital stay, critical illness, invasive medical devices, and mechanical ventilation (Falagas et al. 2007b; Gupta et al. 2011; Munoz-Price et al. 2013; Temkin et al. 2014). Long-term acute care hospital-resident patients experienced additional risk. For example, in Chicago 30.4% of patients in long-term facilities were colonized with KPC-producing *Enterobacteriaceae*, while only 3.3% of ICU patients from short-stay hospitals tested positive for colonization (Lin et al. 2013).

Genes encoding ß-lactamases on mobile genetic elements are one major mechanism contributing to the rapid dissemination of MDR G- bacteria worldwide. The most abundant mechanisms of ß-lactam resistance in *Enterobacteriaceae* were indeed described to be caused by the production of ESBLs, and a smaller proportion was due to altered efflux pump levels/activities or porin expression. ESBLs are mostly plasmid-encoded and can hydrolyze penicillins, broad-spectrum cephalosporins, and oxyimino-monobactams. These enzymes alone are not effective against cephamycins or carbapenems (Bradford 2001; Paterson and Bonomo 2005).

Enterobacteriaceae, harboring transmissible carbapenem resistance, have emerged as a big issue within the last two decades, and ß-lactamases present in these pathogens are a further driving force of resistance (Logan and Weinstein 2017). Major resistance mechanisms observed in CRE are the expression of high-level ESBLs or AmpC enzymes combined with mutations of porins, leading to decreased permeability to carbapenems or the acquisition of carbapenemase genes (Dai et al. 2013).

One resistance mechanism is mainly facilitated by plasmid-encoded ESBLs and AmpC cephalosporinases. AmpC activity in *Enterobacteriaceae* is mostly related with overproduction or derepression of chromosomal genes. Both enzyme types, when combined with mutations of porins, are described to confer resistance to carbapenems. Altered or completely lost porins can reduce diffusion into bacterial cells to rates that enable the action of ESBLs and AmpC enzymes (Paterson and Bonomo 2005; Bush and Fisher 2011). Further, drug efflux pumps and alterations in PBPs are associated with carbapenem non-susceptibility (Patel and Bonomo 2013).

KPC-producing *K. pneumoniae* was isolated in 1996 in the USA for the first time (Yigit et al. 2001). By 2015, KPC had spread globally and has become endemic in the Northeastern USA, Puerto Rico, China, Israel, England, Italy, Romania, Greece, Brazil, Argentina, and Colombia (Denisuik et al. 2013; Glasner et al. 2013; Rodríguez-Zulueta et al. 2013; Saito et al. 2014; Tängdén and Giske 2015; Chang et al. 2015a). KPC-producing *Enterobacteriaceae* can harbor variants of this gene; the most common are bla_{KPC-2} or bla_{KPC-3} on a Tn*3*-based transposon, Tn*4401* (Kitchel et al. 2009; Cuzon et al. 2011). The resistance level to carbapenem in KPC-producing strains can vary. This depends either on increased bla_{KPC} gene copy number, deletions upstream of the bla_{KPC} gene, and/or outer membrane porin loss (OmpK35 and/or OmpK36) (Kitchel et al. 2010; Patel and Bonomo 2013).

MBLs are categorized as class B enzymes, and VIM, NDM-1 (New Delhi MBL), and IMP are the most abundant representatives. The Indian subcontinent is the major reservoir for NDM-1-positive *Enterobacteriaceae* (Lascols et al. 2011), and low sanitation and hygiene levels lead to their wide occurrence in healthcare settings and in the community (Tängdén and Giske 2015). Most often, VIM and IMP MBLs are embedded in class I integrons on transposons or plasmids that lead to the spread. NMD-type MBLs are harbored on different plasmid incompatibility types. It has been proposed that the most abundant variant in *Enterobacteriaceae*, NDM-1, originated from *A. baumannii* (Dortet et al. 2012, 2014). More than two decades ago, the first transmissible carbapenemase gene, *IMP-1* MBL, was detected on an integron in *Serratia marcescens* in Japan. Shortly after the first description, a plasmid-mediated outbreak was observed in seven Japanese hospitals. Subsequently, dissemination of *Enterobacteriaceae* harboring the bla_{IMP-1} gene occurred throughout Japan (Ito et al. 1995). Further, Greece has been shown to be a hotspot for VIM-type *Enterobacteriaceae* and *K. pneumoniae* (Vatopoulos 2008; Logan and Weinstein 2017). In lower-income countries, NMD-1-type MBLs can spread via environmental sources in the community. In India, 4% of drinking water samples and 30% of seepage samples (water pools in streets or rivulets) contained bla_{NMD-1}-positive bacteria in 2011 (Walsh et al. 2011). Class D OXA ß-lactamases are a large group of oxacillinases and are frequently found in *Enterobacteriaceae* (Poirel et al. 2010; Carrër et al. 2010). A transferable plasmid harboring the bla_{OXA-48} gene is often associated with the spread of OXA-48-producing *Enterobacteriaceae*. The integration of the bla_{OXA-48} gene is facilitated by the acquisition of a Tn*199* transposon (Poirel et al. 2010, 2012a, 2012b; Carrër et al. 2010). OXA-48 enzymes reveal high activity on penicillins but low-level activity on carbapenems.

Intestinal carriage of *Enterobacteriaceae* harboring transmissible MDR also presents a major threat, as the intestine provides an environment where resistance determinants can be easily exchanged between bacterial strains. Strains encoding these genes often show additional acquired resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazoles, which have evolved as major threat to human healthcare.

2.2 Priority 2: High

2.2.1 Vancomycin-Resistant *Enterococcus faecium*

Enterococcus faecium is a G+ facultative anaerobic bacterium. *Enterococci* are capable of growing at hypotonic, hypertonic, acidic, and alkaline conditions. They hydrolyze bile esculin and pyrrolidonyl-B-naphthylamide, which inhibit the growth of most microorganisms (Huycke et al. 1998; Hollenbeck and Rice 2012). *Enterococci* are part of the normal gut flora and often used as indicators of fecal contamination (Boehm and Sassoubre 2014). They are found in human stool at up to 10^8 colony-forming units/g (Huycke et al. 1998; Mundy et al. 2000). *Enterococci* cause urinary tract infections, intra-abdominal and pelvic infections, surgical wound infections, bacteremia, neonatal sepsis, endocarditis, and rarely meningitis (Marothi et al. 2005). *Enterococci,* which are nosocomial pathogens, form biofilms, most likely contributing to their virulence and antibiotic resistance (Hollenbeck and Rice 2012; Hashem et al. 2017). These bacteria are responsible for about 12% of hospital-acquired infections (Hollenbeck and Rice 2012). *E. faecalis* and *E. faecium*, colonizing the gastrointestinal tract, can cause severe infections in immunocompromised patients (Miller et al. 2014). *Enterococci* are intrinsically resistant to cephalosporins, lincosamides, and nalidixic acid and are further not susceptible to low levels of aminoglycosides and clindamycin. They show acquired resistance to penicillin, vancomycin (a glycopeptide antibiotic), chloramphenicol, erythromycin, tetracycline, and fluoroquinolones and high-level resistance to aminoglycosides and clindamycin (Marothi et al. 2005).

The antibiotic resistance mechanisms of *E. faecium* include modification/inactivation of drug targets, overexpression of efflux pumps and a cell envelope adaptive response, assisting it to survive in the human host and in the nosocomial environment (Miller et al. 2014). *E. faecium* leads to biofilm-mediated infections in patients with medical devices. Atl_{Efm}, a major autolysin in *E. faecium,* contributes to stabilization of biofilms and surface localization of the virulence factor Acm, facilitating binding of Acm to collagen types I and IV. This presents Atl_{Efm} as potential target for treatment of *E. faecium* biofilm-mediated infections (Paganelli et al. 2013).

Nowadays, the majority of *E. faecium* isolates are resistant to ampicillin, vancomycin, and aminoglycosides (Arias et al. 2010). The emergence of vancomycin-resistant *Enterococci* (VRE) was first reported in 1986 in Europe, in 1993 in the USA, and in 1994 in Asia (Uttley et al. 1988; O'Driscoll and Crank 2015; Akpaka et al. 2017). Since then, the prevalence of vancomycin-resistant *E. faecium* (VREfm) has increased worldwide. VREfm causes 4% of healthcare-associated infections as per the reports from the National Healthcare Safety Network in America (Miu et al. 2016). The prevalence of VREfm has increased worldwide since 1986. A study on healthcare-associated infections in the USA reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention (CDC) found 80% of *E. faecium* isolates analyzed in 2006/2007 to be non-susceptible to vancomycin (Arias et al. 2010). In US hospitals, VREfm incidence had risen to 0.3% in 1989 and to 7.9% in 1993 (Schouten et al. 2000; Arias and Murray 2012). By 2002, 60% and in 2007 more than 80% of the *E. faecium* isolates in US hospitals revealed

vancomycin resistance (Arias and Murray 2012; Molton et al. 2013). By 2007, the prevalence of VREfm in Europe was higher than 30% in countries like Greece and Ireland, whereas Scandinavian countries reported very low rates (<1%) (Arias and Murray 2012). In Malaysia, the VREfm rate was 25.7% in 2006 (Getachew et al. 2009). In Canadian hospitals, the prevalence of VREfm increased from 1.8% in 2007 to 6.0% in 2013. Ninety percent of vancomycin-resistant isolates harbored the gene *vanA*. Interestingly, the prevalence of *vanB* vancomycin-resistant VRE in these medical centers decreased from 37.5% in 2007 to 0% in 2013 (Simner et al. 2015). A study conducted on hospitalized patients between 2009 and 2014 from seven Caribbean countries showed 90.9% of bacterial isolates to be *E. faecium*, and all of them were vancomycin resistant (Akpaka et al. 2017). In a study conducted in 30 hospitals in Argentina between 1997 and 2000, all *Enterococci* isolates were found to be non-susceptible to vancomycin. The incidence of *vanA*-positive VREfm was 98%, with minimal inhibitory concentrations (MICs) to vancomycin of 32–512 mg/l, while *vanB*-harboring strains revealed MICs to vancomycin of 16–32 mg/l (Corso et al. 2007).

Glycopeptides, like vancomycin, which interfere with the synthesis of peptidoglycan and thus inhibit bacterial growth, are commonly used in the treatment of enterococcal infections (Kristich et al. 2014). These antibiotics form complexes with C-terminal D-Ala-D-Ala peptide termini of peptidoglycan precursors on the outer surface of the cell. This prevents the cell wall biosynthetic enzymes (i.e., PBPs) from using them as substrates for transglycosylation and transpeptidation and hence leads to impairment of cell wall integrity (Kristich et al. 2014). In VRE, the C-termini of peptidoglycan precursors are exchanged to D-Ala-D-Lac or D-Ala-D-Ser, thus reducing the binding affinity of glycopeptides (such as vancomycin) to peptidoglycan by 1000-fold and sevenfold, respectively (Kristich et al. 2014; Ahmed and Baptiste 2017). This phenomenon disables glycopeptides to inhibit cell wall biosynthesis in bacteria (Kristich et al. 2014). Glycopeptide resistance is generally encoded on mobile genetic elements. However, some types of glycopeptide resistance are also chromosomally encoded (Kristich et al. 2014).

Genetic mechanisms of vancomycin resistance in *Enterococci* involve nine gene clusters conferring resistance to glycopeptides. The *van* gene cluster has components with various functions. A two-component signal transduction system consisting of VanRS (VanR is a response regulator/activator of vancomycin resistance and VanS a sensor kinase) recognizes glycopeptides and activates the expression of resistance genes in inducible *van* types. In the presence of vancomycin, the two-component system VanRS activates a promoter responsible for co-transcription of *vanA*, *vanH*, and *vanX* to regulate vancomycin resistance (Arthur and Courvalin 1993). VanH (a dehydrogenase converting cellular pyruvate to D-lactate) and VanA (a ligase forming D-Ala-D-Lac) produce modified peptidoglycan precursors, while VanX (cleaves D-Ala-D-Ala) and VanY (D,D-carboxypeptidases) remove unaltered peptidoglycan precursors (Kristich et al. 2014). Among the *van* gene clusters, *vanA* and *vanB* types of resistances are most common in hospitals and are found in enterococcal isolates from food, clinical, and veterinary samples (Hammerum 2012). *vanA* is generally carried on the transposon Tn*1546* and was first reported on

plasmid pIP816 in *E. faecium* BM4147 (Arthur and Courvalin 1993). *vanB* is harbored by Tn*5382-*/Tn*1549*-type transposons. These transposons are either plasmid- or chromosomally encoded (Kristich et al. 2014).

Infection control and antibiotic stewardship programs are important to prevent further development of antibiotic resistance and dissemination (Hollenbeck and Rice 2012). Control measures should include identification of patients colonized and infected by resistant *Enterococci*, strict adherence to hand hygiene, and active screening of high-risk patients (Faron et al. 2016).

2.2.2 Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is a G+ facultative anaerobic bacterium. It is part of the normal human microflora and is frequently found on the skin, in the respiratory tract, and in the nose. It is an opportunistic pathogen, accounting for about 80% of prosthetic infections. *S. aureus* forms strong biofilms and attaches firmly to medical devices and host tissues, causing chronic, difficult-to-treat infections (Kawada-Matsuo and Komatsuzawan 2012; Vaishampayan et al. 2018). *S. aureus* harbors a two-component regulatory quorum-sensing system, the accessory gene regulator (Agr), which plays an important role in biofilm-related infections (Qin et al. 2014).

Methicillin-resistant *S. aureus* (MRSA) is a leading cause of nosocomial infections. According to the reports from the National Healthcare Safety Network in America, MRSA is responsible for 8% of healthcare-associated infections (Miu et al. 2016). As per the recent US CDC report, among the 23,000 documented infections caused by antibiotic-resistant pathogens, almost half the cases were caused by MRSA (Hagras et al. 2017). MRSA lead to skin and soft tissue infections, respiratory tract infections, food poisoning, endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, suppurative diseases, and fatal sepsis. Immunocompromised patients, patients with implants or diabetes or patients undergoing surgery, elderly people, and newborns are high-risk groups for MRSA infections (Ohlsen 2009).

In a study conducted in the USA, Canada, Latin America, Europe, and the West Pacific region from 1997 to 1999, 32 to 47% of skin and soft tissue infections were found to be caused by *S. aureus* (Schito 2006). The CDC reported 80,461 infections and 11,285 deaths caused by MRSA in 2011 (CDC 2013). The prevalence of MRSA is increasing globally, especially in developing countries. The occurrence of MRSA was reported to be 75% among hospital specimens in Hong Kong from 1997 to 1999, 53.1% in Bangladesh in 2004, 80% in Chile in 2006, 26% in Malaysia from 2006 to 2008, 92.4% in Columbia in 2009, 44.1% in Ethiopia in 2010 and 2011, and 43% in Indonesia in 2014 (Pandey 2017). However, the prevalence of MRSA in livestock is lower in some Asian countries compared to European countries, like in Japan 0.9%, Malaysia 1.4%, Korea 3.2%, China 11.4%, Sri Lanka 13.8%, and Taiwan 14.4% as compared to Poland 20.6% and Germany and the Netherlands with more than 35% (Jayaweera and Kumbukgolla 2017).

Methicillin is a β-lactam antibiotic belonging to the penicillin class. Methicillin resistance can be transferred via HGT (New et al. 2016). The penicillin-binding protein, PBP2, is a key molecule conferring resistance to β-lactams. Methicillin-sensitive *S. aureus* (MSSA) harbors four PBPs (PBP 1–4), and all of them are

inactivated by β-lactam antibiotics. In contrast, MRSA strains encode an extra PBP2', with low affinity to β-lactams, thus facilitating cell wall biosynthesis even in the presence of β-lactam antibiotics. The expression of PBP2' is controlled by the MecR1-MecI regulatory system (Kawada-Matsuo and Komatsuzawan 2012). In addition, three factors responsible for methicillin resistance in the presence of Triton X-100 have been recognized, namely, *fmtA*, *fmtB*, and *fmtC/mprF*. *fmtA* has been identified as a new PBP. Inactivation of *fmtA* reduces methicillin resistance, while mutation of *fmtB* reduces methicillin and oxacillin resistance (Kawada-Matsuo and Komatsuzawan 2012). FmtC/MprF is a membrane-associated protein and its inactivation diminishes methicillin resistance by decreased modification of phosphatidylglycerol with L-lysine. FmtC/MprF determines resistance against host defensive peptides and thus plays a role in virulence and pathogenicity of *S. aureus*. Its inactivation leads to increased negative charge of the membrane surface and increased binding of antibacterial peptides to the surface (Berger-Bächi and Rohrer 2002). Mutations in *fmtC/mprF* in *S. aureus* were shown to further cause a decrease in vancomycin and daptomycin resistance (Bayer et al. 2015; Lin et al. 2018a). Another methicillin-resistant mechanism involves the mobile cassette element SCC*mec* (staphylococcal chromosome cassette *mec*) that is integrated into a *S. aureus* gene of unknown function, *orfX* (Chambers and DeLeo 2009). This cassette carries both the *mecA* and *mecC* genes that encode a novel specific penicillin-binding protein (PBP2a) and the site-specific recombinase genes *ccrAB* and/or *ccrC*. The SSC*mec* cassette was first described in 1999 (Ito et al. 1999). SCC*mec* elements are divided into type I to XI based on the *mec* and *ccr* gene complexes and further classified into different subtypes (Liu et al. 2016).

Vancomycin, a last resort antibiotic, has been widely used in the treatment of MRSA. However, excessive use of the drug has led to the development of vancomycin-resistant *S. aureus* (VRSA) (Appelbaum 2006). In 2002, the first VRSA isolate with a MIC of higher than 100 μg/ml was reported in Michigan, USA (Gardete and Tomasz 2014). Until 2008, 11 VRSA clinical isolates, which were also resistant to methicillin, had been reported, out of which 9 cases were identified in the USA, 1 in Iran, and 1 in India. Out of the nine from the USA, seven were clinical isolates from Michigan (Périchon and Courvalin 2009). The US strains harbor a plasmid-borne Tn*1546* element, most probably acquired by conjugation from glycopeptide-resistant *E. faecalis* (Périchon and Courvalin 2009). The mechanism of resistance observed in VRSA is similar to that in *Enterococci* by alteration of peptidoglycan precursors. The C-terminal D-Ala-D-Ala is substituted by D-Ala-D-Lac, diminishing the binding of vancomycin, thus no longer inhibiting the cell wall synthesis in the bacterium (Schito 2006).

2.2.3 Clarithromycin-Resistant *Helicobacter pylori*

H. pylori is a G- microaerophilic, spiral organism (Yonezawa et al. 2013). It is a human gastric pathogen that causes peptic ulcers, gastritis, gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma, chronic immune thrombocytopenic purpura in adults, and vitamin B12 deficiency (Shmuely et al. 2016; Alba et al.

2017). The route of transmission is commonly from person to person (Shmuely et al. 2016).

H. pylori forms biofilms, even on human gastric mucosa, reducing the susceptibility of the bacterium to different antibiotics including clarithromycin (but also metronidazole, erythromycin, amoxicillin, and tetracycline) (Yonezawa et al. 2015; Attaran et al. 2017). The incidence of clarithromycin resistance, and also the expression of efflux pump systems, is higher in biofilms compared to planktonic cells. Interestingly, the MIC of clarithromycin was increased by up to four-fold in 2-day-old biofilms and up to 16-fold in 3-day-old *H. pylori* biofilms (Yonezawa et al. 2013).

Clarithromycin is a macrolide, containing a 14-membered lactone ring with L-cladinose and D-desosamine groups of sugars (Alba et al. 2017). It binds to the 50S subunit of the bacterial ribosome and blocks the translation of peptides, thereby inhibiting bacterial growth (Yonezawa et al. 2013). The precise site of action of clarithromycin is the peptidyl transferase loop of domain V of 23S rRNA.

While clarithromycin is the first drug of choice to treat *H. pylori* infections, clarithromycin resistance in *H. pylori* has been linked to treatment failures, including poor compliance, resistance to antibiotics, and reinfection (Chey and Wong 2007; Shmuely et al. 2016). The incidence of clarithromycin-resistant *H. pylori* is higher in previously treated than in untreated patients (Shmuely et al. 2016). In developing countries, the annual occurrence of clarithromycin-resistant *H. pylori* is 4–15% higher than in industrialized countries, revealing rates of 0.5% (Gold 2001; Duck et al. 2004). A consistent increase in clarithromycin resistance has been reported in most countries. In Bulgaria, the resistance increased from 10% in 1996–1999 to 19% in 2003/2004. In the USA, the resistance was 6.2% in 1993 and the rate doubled in 9 years, to 12.9% in 2002. In Belgium, the rates increased from 6% in 1990 to 56% in 2009. In Japan, the resistance was 18.9% in 2002 and reached 27.7% in 2005. In a hospital in the USA, the resistance rate of *H. pylori* infections in patients between the ages of 3 and 19 years was as high as 50% (Shmuely et al. 2016). A meta-study compiling 87 studies on 52,008 *H. pylori* isolates from 2009 to 2014 gives a good overview of the prevalence of *H. pylori*. It included 43 Asian, 10 American, 5 African, and 29 European studies. There were 5.46% to 30.8% of *H. pylori* isolates resistant to clarithromycin, with the lowest rate in African and the highest rate observed in North American isolates. Among European countries, Norway showed the lowest resistance rate (5.9%), while Portugal showed the highest (42.4%). In Asian countries, the lowest resistance rates were observed in Malaysia (2.4%), while the highest rates were found in India (58.8%) (Ghotaslou et al. 2015). Recently, an increase in clarithromycin resistance among treatment failures showed 17.5% (primary resistance) to 63.2% after one eradication treatment failure (secondary resistance) and 75.4% after two eradication treatment failures (tertiary resistance) (Megraud et al. 2013; Selgrad et al. 2013).

Point mutations of the *23S* rRNA gene, mostly an adenine-to-guanine transition at positions 2142 and 2143, are the common mechanism of clarithromycin resistance, as they reduce the affinity of the drug to the ribosome (Megraud 1998; Yonezawa et al. 2013; Alba et al. 2017). Sporadic mutations in the translation

initiation factor IF-2, the ribosomal protein L-22, as well as in the efflux pumps, are other mechanisms of resistance (Alba et al. 2017). Excessive use of clarithromycin has led to the development of resistant strains of *H. pylori,* with the predominant mutations occurring in A2143G, A2142G, and A2142C in the *23S* rRNA gene, but T2182C, G2224A, T2215C, and C2694A in the V region of the *23S* rRNA gene have also been observed (Vianna et al. 2016; Alba et al. 2017). A2143G is the most frequently encountered mutation among the resistant strains in most European and Latin American countries (Vianna et al. 2016).

The latest Maastricht Guidelines recommend clarithromycin containing treatments against *H. pylori* infections in regions with low incidence of clarithromycin resistance. In regions with high levels of clarithromycin resistance, quadruple therapy with bismuth or the sequential therapy with 5 days of proton pump inhibitors and amoxicillin followed by 5 more days of proton pump inhibitors plus metronidazole and clarithromycin is recommended as the first-line treatment (Ghotaslou et al. 2015; Malfertheiner et al. 2012; Shmuely et al. 2016). In addition to the combinational use of antibiotics to treat infections, judicious use of antibiotics with the help of culture and antibiotic susceptibility testing of *H. pylori* and empiric eradication are essential to control further spread of antibiotic resistance (Boltin et al. 2015; Shmuely et al. 2016).

2.2.4 Fluoroquinolone-Resistant *Campylobacter* spp.

Campylobacter jejuni is a G- curve-shaped, thermophilic, and microaerophilic bacterium (Fernández and Pérez-Pérez 2016). It is a zoonotic, foodborne pathogen and causes about 500 million human infections worldwide annually (Bae and Jeon 2013; Bae et al. 2014). It is responsible for about 90% of the *Campylobacter* infections in humans (Iovine 2013) and is a leading cause of gastroenteritis since the late 1970s (Luangtongkum et al. 2009; Fernández and Pérez-Pérez 2016). *C. jejuni* has the ability to form biofilms on abiotic surfaces (Reuter et al. 2010; Bae et al. 2014) and can acquire antibiotic resistance genes in biofilms by natural transformation (Bae et al. 2014). The formation of biofilms likely increases the fluoroquinolone resistance among *Campylobacter* spp. (Bae and Jeon 2013). Gastroenteritis caused by *Campylobacter* is generally regarded as self-limiting. However, treatment is recommended in cases of a severe infection or infections in the immunocompromised elderly patients or in newborns and pregnant women (Fernández and Pérez-Pérez 2016). Fluoroquinolones such as ciprofloxacin are often used to treat *Campylobacter* infections. Spread of the bacteria from animals to humans often occurs via contaminated food. Poultry animals are especially seen as crucial reservoirs involved in this dissemination (Bae and Jeon 2013; Fernández and Pérez-Pérez 2016). The emergence of fluoroquinolone resistance in *Campylobacter* from food animals has evolved as a public health issue (Tang et al. 2017).

A study conducted among travelers returning to Finland from 1995 to 2000 showed that countries with especially high rates of ciprofloxacin-resistant *C. jejuni* were Spain with 22%, followed by Thailand and India, with 14%, and 6% of the isolates, respectively. The isolates were collected during two study periods (1995–1997 and 1998–2000). The study reported an increase in the incidence of resistance

among the investigated travelers between the two study periods from 40% to 60% within the study period (Hakanen et al. 2003). In 2000, the occurrence of ciprofloxacin-resistant *Campylobacter* spp. in clinical isolates (mostly *C. jejuni*) was 50% in Chile, 59.6% in Argentina, and 78% in Peru. In Argentina, 49.1% of the *Campylobacter coli* from a pediatric hospital were reported to be resistant to ciprofloxacin as well as to norfloxacin, another fluoroquinolone (Fernández and Pérez-Pérez 2016).

In Peru, an increase in ciprofloxacin resistance among *C. jejuni* and *C. coli* from 2001 to 2010 was reported. The highest rates of ciprofloxacin-resistant *C. jejuni* at the beginning and the end of the study were observed in Lima, with 73.1% and 89.8%, respectively, similar to resistance in *C. coli* (48.1% in 2001 and 88.4% in 2010) (Fernández and Pérez-Pérez 2016). A study conducted from 2003–2006 in Mexico reported ciprofloxacin-resistant *C. jejuni* isolates in chickens (85.8%), pigs (62.5%), cattle (39.8%), and humans (58.2%) (Zaidi et al. 2012). In Southern Ecuador, 90.9% of *C. jejuni* and 100% of *C. coli* strains, isolated from chicken liver for human consumption, were reported to be ciprofloxacin resistant (Fernández and Pérez-Pérez 2016). A recent study in the USA among feedlot cattle in 2012/2013 showed 35.4% of *C. jejuni* and 74.4% of *C. coli* to be fluoroquinolone resistant, a significant increase when compared to the 1.8% *C. jejuni* and 9% *C. coli* being non-susceptible to ciprofloxacin as reported earlier (Englen et al. 2005; Tang et al. 2017).

All fluoroquinolone resistance determinants reported in *Campylobacter* are chromosomally encoded. The frequency of emergence of fluoroquinolone-resistant mutants ranges from 10^{-6} to 10^{-8} per cell and generation (Luangtongkum et al. 2009).

The mechanisms of fluoroquinolone resistance in *Campylobacter* spp. are mainly due to mutations in *gyrA* and *parC* genes, encoding DNA gyrase and topoisomerase IV, respectively. Frequently, amino acid positions Thr-86, Asp-90, and Ala-70 of *gyrA* are mutated. Thr-86 mutations confer higher levels of resistance to ciprofloxacin as compared to Asp-90 and Ala-70. High-level ciprofloxacin-resistant *C. jejuni* isolates (MIC = 125 μg/ml) possess two mutations, in *gyrA* Thr-86 and in *parC* at Arg-139 (Engberg et al. 2001). Another mechanism of fluoroquinolone resistance in *Campylobacter* is the multidrug efflux pump CmeABC, consisting of a periplasmic protein acting as a bridge (encoded by *cmeA*) (Iovine 2013), an inner membrane drug transporter (encoded by *cmeB*), and an outer membrane protein (encoded by *cmeC*). CmeABC reduces the accumulation of the drug in the bacterial cell (Luangtongkum et al. 2009).

Regular and methodical surveillance of antibiotic resistance in *Campylobacter* spp. is an essential step in controlling the further spread of antibiotic resistance (Fernández and Pérez-Pérez 2016).

2.2.5 Fluoroquinolone-Resistant *Salmonella* spp.

Salmonella are G-, motile, zoonotic pathogenes that cause diseases like gastroenteritis, typhoid, paratyphoid, and bacteremia (Rushdy et al. 2013; Pribul et al. 2017). *S. enterica* is a human-restricted pathogen causing typhoid (González et al. 2018), a disease that is typically transmitted by the fecal-oral route (Schellack et al. 2018).

The bacterium resides in the gall bladder as the primary reservoir. Further, it forms biofilms on the gall bladder, which are recalcitrant to ciprofloxacin treatment (González et al. 2018). In 2010, 26.9 million new cases of typhoid fever and 200,000 deaths were determined worldwide (Abd-elfarag 2015; Adhikari et al. 2017; Ugboko and De 2014). A community-based prospective *Salmonella* surveillance study, conducted in Asia from 2001 to 2003, showed occurrence of *S. typhi*, namely, 37% in China, 65% in India, 84% in Pakistan, 85% in Indonesia, and 100% in Vietnam. In the same study, the prevalence of *S. paratyphi* was observed to be 63% in China, 34% in India, 14% in Indonesia and in Pakistan, and 0% in Vietnam (Khan et al. 2010). In the USA, 1.2 million cases of infection are reported annually (Boore et al. 2015). In 2016, 94,530 cases of salmonellosis were reported in the EU (European Food Safety Authority 2017).

Fluoroquinolones, specifically ciprofloxacin, are the drugs of choice to treat *Salmonella* infections. However, overuse of ciprofloxacin has resulted in increased resistance. Ciprofloxacin-resistant *Salmonella* was first reported in 1990 (Menezes et al. 2010). A study in Brazil conducted from 2009 to 2013 on isolates from food of animal sources and from environmental samples screened for fluoroquinolone resistance among the isolates. The most prevalent serotype obtained was *S. typhimurium* followed by *S. enteritidis*. The occurrence of resistance was highest for enrofloxacin (48%), followed by ciprofloxacin (43%) and ofloxacin (40%), and the lowest resistance was observed for levofloxacin (30%) (Pribul et al. 2017). Despite emerging ciprofloxacin resistance, this drug is recommended as the first-line therapy in children and adults (González et al. 2018).

The fluoroquinolone resistance in *Salmonella* is predominantly due to mutations in *gyrA* and *parC* genes, as also described for *Campylobacter* (Sjölund-Karlsson et al. 2014). The second mechanism of resistance is overexpression of the efflux system AcrAB-TolC (Rushdy et al. 2013). AcrAB-TolC belongs to the resistance-nodulation-division family and has three domains, a membrane fusion protein (AcrA), a drug efflux transporter (AcrB), and an outer membrane channel protein (TolC) (Kim et al. 2016). Overexpression increases the efflux of the antibiotic that acts synergistically with the alterations in outer membrane proteins which includes absence of some/all of these proteins, namely, Omp-A, Omp-C, Omp-D, and Omp-F (Rushdy et al. 2013).

Mechanisms of fluoroquinolone resistance in *Salmonella* food isolates were identified. Either the investigated isolates had only a single mutation in *gyrA* with S83T, S83F, and D87N being the most common amino acid substitutions or a pair of novel double mutations in *gyrA* resulting in H80N and S83T substitutions and a single *parC* mutation causing a Q91H substitution were identified (Lin et al. 2015). Another mechanism used by *Salmonella* is alteration of porin expression, thus reducing the penetration of fluoroquinolones into the bacteria (Rushdy et al. 2013). In addition to the mutations in *gyrA* and *parC* genes, and chromosomally encoded efflux pumps, a plasmid-mediated resistance mechanism encoded by *qnrA* has also been observed in *Salmonella* spp. (Sjölund-Karlsson et al. 2014).

It was recently suggested that fluoroquinolone-resistant *S. typhi* strains would occur in the future, even if the use of these drugs were diminished, as these

resistance mechanisms are not linked with fitness costs (Baker et al. 2013). This poses a great challenge to the public health. Surveillance of infections and epidemiology, as well as studying the genes responsible for antibiotic resistance in *Salmonella* spp., are imperative measures to control the spread of antibiotic resistance and to effectively treat infections (Nabi 2017).

2.2.6 Cephalosporin- and Fluoroquinolone-Resistant *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a G- pathogenic diplococcus with a special feature of antigenic variability, strengthening its survival in the human host (Patel et al. 2011). It inhabits mucosal surfaces of the urethra in male and the cervix in female (Patel et al. 2011) but can also be found in the rectal and the oropharyngeal mucosa (Costa-Lourenço et al. 2017). *N. gonorrhoeae* causes symptomatic and asymptomatic infections of the genital and extragenital tract (Patel et al. 2011). It is an etiological agent of gonorrhea and the second leading cause of sexually transmitted diseases (Costa-Lourenço et al. 2017). In men, it causes urethritis. Untreated infections may lead to epididymitis, reduced fertility, and urethral stricture. In women, the symptoms include abnormal vaginal discharge, dysuria, lower abdominal discomfort, and dyspareunia (Alirol et al. 2017). The risk of gonococcal infection is lowering with increasing age, as most cases occur in individuals under the age of 24 (Costa-Lourenço et al. 2017). *Gonococci* form biofilms in vitro and likely in vivo (Unemo and Shafer 2014). Approximately 62 million cases of *N. gonorrhoeae* infections occur every year worldwide (Patel et al. 2011).

Fluoroquinolones and cephalosporins are the drugs of choice to treat *N. gonorrhoeae* infections. Cephalosporins inhibit the growth of bacteria by inhibiting the cross-links of peptidoglycan in the bacterial cell wall by binding to PBPs. The cephalosporins, ceftriaxone and cefixime, are the most effective recommended treatment options against *N. gonorrhoeae* infections. However, resistance to these drugs has emerged in the past two decades.

Ciprofloxacin-resistant *N. gonorrhoeae* isolates were reported in the 1980s from many countries (Patel et al. 2011). By the end of 1992, the resistance rates in Japan were 40% (Patel et al. 2011). In India, the use of ciprofloxacin started in the 1990s, and by the end of 2000, most isolates were resistant (Patel et al. 2011). The resistance to ceftriaxone and cefixime was first reported in Japan and then spread all over the world (Unemo and Shafer 2014). Resistance to ceftriaxone in *N. gonorrhoeae* has been reported in several American countries since 2007 (Pan American Health Organization/World Health Organization 2018). In South Africa, among men attending healthcare clinics, the incidence of ciprofloxacin resistance in *N. gonorrhoeae* increased from 7% in 2004 to 32% in 2007. In Kenya, quinolone resistance increased since it emerged in 2007 from 9.5% to 50% in 2009 (Mehta et al. 2011). In Europe, 50,001 *N. gonorrhoeae* cases were reported in 2013, and 53% of the clinical isolates were resistant to ciprofloxacin and 4.7% to cefixime (Spiteri et al. 2014). In a report published by WHO-GASP-LAC in 2013, ciprofloxacin resistance rates in clinical *N. gonorrhoeae* isolates in Latin American countries stayed below 5% until 2004, increased to >15% in 2006, and reached >40% in 2010 (Dillon et al.

2013). The spread of these resistances is thought to occur through HGT (Hess et al. 2012). In 2014, the prevalence of gonorrhea disease in the southern part of the USA was 131 cases per 100,000 individuals (CDC 2014), and the CDC estimated 820,000 new cases annually. Thirty percent of the isolates were ciprofloxacin resistant in cases of men having sex with men and 12% in case of men having sex with women (CDC 2015).

The use of fluoroquinolones as a drug of choice to treat gonococcal infections was recommended in 1993. Already in 1997, the first strains resistant to fluoroquinolone were reported in Hong Kong and the Philippines. In 2004, fluoroquinolone was no longer recommended for treatment, but cephalosporins came into use as a treatment against gonococcal infections. In 2007, cephalosporin resistance was reported in Japan and Australia. A year later, reduced susceptibility to cephalosporins was identified in the USA. In 2011, the WHO and CDC revised the treatment guidelines, and ceftriaxone was included in the combination therapy to treat gonococcal infections. However, in 2012 the first cases of ceftriaxone resistance were reported from Japan (Buono et al. 2015).

Fluoroquinolone resistance in *N. gonorrhoeae* can be chromosomally as well as plasmid-mediated (Patel et al. 2011). As already stated for *Campylobacter* spp. and *Salmonella*, in cases of high-level fluoroquinolone resistance, mutations take place at positions 91 and 95 in *gyrA* and at positions 87 and 91 in *parC* (Kubanov et al. 2016) but also in genes associated with NorM efflux pumps that export fluoroquinolones (Golparian et al. 2014). The mechanism of cephalosporin resistance is primarily due to alteration of the structure and function of key proteins, such as PBP2, encoded by *penA*, and PorB1b showing porin activity (Ross and Lewis 2012; Golparian et al. 2014). Another strategy used by *N. gonorrhoeae* to combat cephalosporins is mutations in the MtrC-MtrD-MtrE efflux pump system, a member of the resistance-nodulation-division pump family (Golparian et al. 2014).

Gonococcal resistance to cephalosporins is severe due to limited alternatives to treat gonococcal infections. Thus, it is imperative to fill the gaps in the surveillance and MDR data to understand the epidemiology of gonococcal MDR (Wi et al. 2017). Additionally, strengthening of diagnosis of *N. gonorrhoeae* infections is recommended by the Pan American Health Organization and the WHO as a control measure (Pan American Health Organization/World Health Organization 2018).

2.3 Priority 3: Medium

2.3.1 Penicillin-Non-susceptible *Streptococcus pneumoniae*

S. pneumoniae is a G+ facultative anaerobic organism. It causes pneumonia, sinusitis, otitis media, upper respiratory tract infections, and bacteremia, resulting in morbidity and mortality in infants and children (Bogaert et al. 2000; Ahmadi et al. 2015; Diawara et al. 2017). *S. pneumoniae* also triggers meningitis, which is the most dangerous disease of the central nervous system (Ahmadi et al. 2015). The bacterium forms robust biofilms to survive in the human nasopharynx (Talekar et al.

2014) and is responsible for 11% of deaths worldwide (Ahmadi et al. 2015) with the highest mortality rates reported in Africa and Asia (Diawara et al. 2017).

The prevalence of penicillin-non-susceptible *S. pneumoniae* (PNSP) is increasing rapidly. The first PNSP was reported in Australia in 1967 (Hansman and Bullen 1967; Liñares et al. 2010). A study conducted in 11 pediatric tertiary care centers in Canada from 1991 to 1998 showed the emergence of two international clones of PNSP, serotype 9V and 14 related to the Spanish-French clone, and the 23-F Spanish-US clone (Greenberg et al. 2002). In the USA, an invasive PNSP clone 35B, which caused invasive infections in patients in ten different states from 1995 to 2001, was identified by the CDC and Prevention's Active Core Surveillance (Beall et al. 2002). The prevalence of PNSP in Canada increased from 2.5% in 1991 to 11.3% in 1998 (Greenberg et al. 2002). The occurrence of PNSP in hospitals was >70% in Korea, 45% in South Africa, 44% in Spain, and 21.8% in Brazil (Greenberg et al. 2002; Levin et al. 2003).

The prevalence of PNSP in some European countries was shown to be very high, 25–50% in Spain, France, and Greece; 10–25% in Portugal, Ireland, Finland, and Turkey, and 5–10% in Italy, and relatively low with 1–5% in the UK, Germany, Sweden, Austria, and Norway (EARSS Annual Report 2006; Reinert 2009). In Poland, the prevalence of PNSP among children (age 2 to 5 years) in 2011–2012 was 44.8% (Korona-Glowniak et al. 2016). The prevalence of PNSP in Argentina increased significantly from 15.8% in 1993 to 67.3% in 2002 (Bonofiglio et al. 2011), and in Morocco it was 22% in samples collected from 2007 to 2014 (Diawara et al. 2017).

The dissemination of antibiotic resistance in pneumococci is mainly clonal (Sjostrom et al. 2007). *S. pneumoniae* expresses six types of PBPs, namely, 1a, 1b, 2a, 2b, 2x, and 3. The mechanism of penicillin resistance involves modification within or in flanking regions of the amino acid motifs which form the active catalytic center of the PBPs. This alters the PBPs, namely, PBP2x, PBP2b, and PBP1a. These modified variants display a reduced affinity to ß-lactam antibiotics, while their enzymatic function is apparently unaffected (Hakenbeck et al. 2012; Reinert 2009; Schweizer et al. 2017; Zhou et al. 2016).

Detection of PNSP is crucial to prevent and treat infections caused by penicillin-resistant *S. pneumoniae*. Surveillance of the clonal distribution of PNSP in combination with epidemiological analyses will help in understanding the risk factors associated with them. Use of conjugate vaccines might also help in reducing non-susceptibility toward the antibiotic (Ahmadi et al. 2015; Hampton et al. 2018).

2.3.2 Ampicillin-Resistant *Haemophilus influenzae*

Haemophilus influenzae is a G- facultative anaerobic coccobacillus that can cause various diseases, with symptoms ranging from mild to severe (Baba et al. 2017). The bacterium is associated with a significant number of respiratory tract infections as well as serious invasive infections, like meningitis and sepsis (Kiedrowska et al. 2017). Further, community-acquired pneumonia, acute otitis media, acute epiglottitis, and sinusitis can be caused by *H. influenzae*. The bacterium is often part of the physiological bacterial flora of the upper respiratory tract but is frequently isolated

from the respiratory tract of COPD (chronic obstructive pulmonary disease) patients, where it can lead to severe symptoms (Finney et al. 2014; Garmendia et al. 2014).

Antibiotic treatment can give rise to the occurrence of resistant *H. influenzae* strains that are frequently non-susceptible to ampicillins, including ß-lactamase-negative ampicillin-resistant (BLNAR) strains. The highest rate of ß-lactamase production in strains of *H. influenzae* was observed in South Korea and Japan, where more than half of all isolates were tested positive (Tristram et al. 2007). High prevalence of BLNAR strains has evolved into major clinical concern. Over the last few years, a significant increase in the occurrence of BLNAR strains has been observed in many European countries and throughout the world (Sanbongi et al. 2006; Jansen et al. 2006; Tristram et al. 2007). In European countries, the prevalence of nosocomial BLNAR strains was reported to range between 15% and 30% (Jansen et al. 2006; Witherden et al. 2014).

Resistance of *H. influenzae* to ß-lactams can be either enzyme- (facilitated by ß-lactamases) or non-enzyme-mediated. Traditionally, the most commonly occurring ß-lactam resistance mechanism in *H. influenzae* is ß-lactamase production, with the gene encoded on plasmids (Tristram et al. 2007). Non-enzyme-mediated resistance (BLNAR) can be facilitated by increased expression of the AcrAB efflux pump (Kaczmarek et al. 2004). Further, in BLNAR strains, alterations in PBP3, encoded by the *fts1* gene, have been attributed to elevated resistance to ß-lactam antibiotics (Kaczmarek et al. 2004; Wienholtz et al. 2017). Distinct mutations in *fts1* led to decreased affinity for penicillins as well as cephalosporins (Thornsberry and Kirven 1974; Ubukata et al. 2001; Hasegawa et al. 2003). This has been proposed to be the main molecular mechanism of non-ß-lactamase-mediated resistance among BLNAR strains (Mendelman et al. 1984; Tristram et al. 2007; Skaare et al. 2014).

2.3.3 Fluoroquinolone-Resistant *Shigella* spp.

Shigella are G- facultative anaerobic, rod-shaped bacteria and are an important cause of acute diarrheal disease worldwide. The majority of cases occur among children under the age of five in developing countries (Kotloff et al. 2013; Khaghani et al. 2014). Generally, *Shigella* infections are restricted to the gastrointestinal tract, while extraintestinal infections, such as bloodstream infections, reactive arthritis, and neurological complications, are rare (Bhattacharya et al. 1988; Muthuirulandi Sethuvel et al. 2017). Infections caused by *Shigella* spp. in humans are easily transmittable from person-to-person or by contaminated food/water (Muthuirulandi Sethuvel et al. 2017). Shigellosis is endemic among poor populations in African and Asian countries. *Shigella* epidemics have been reported from Bangladesh, Sri Lanka, Maldives, Nepal, Bhutan, Myanmar, and the Indian subcontinent (Emerging and other Communicable Diseases and Control Organization 1994). Nowadays, global occurrence of multidrug-resistant *Shigella* spp. that reveal increased non-susceptibility to third-generation cephalosporins and fluoroquinolones has emerged as a critical health issue. This trend has been predominantly observed in Asia (Wang et al. 2006; Gu et al. 2012; Taneja and Mewara 2016). Nevertheless, reports of MDR lineages or strains with increased resistance to fluoroquinolones are piling up globally (Aggarwal et al. 2016; Nüesch-Inderbinen et al. 2016).

Resistance to fluoroquinolones in *Shigella* is based on two mechanisms occurring either singly or in combination: Alterations in the targets of these antibiotics and non-permeability of the membrane and/or overexpression of drug efflux pumps that lead to decreased drug concentrations inside the cell reduce antibiotic susceptibility. Mutations in *gyrA, a* subunit of the bacterial DNA gyrase complex, and *parC*, a subunit of the bacterial topoisomerase, have been identified as important determinants for fluoroquinolone resistance (Chu et al. 1998). Chromosomal mutations in these genes were shown to participate in the dissemination of fluoroquinolone-resistant *S. sonnei* isolates (Ma et al. 2018). Plasmid-mediated quinolone resistance (PMQR) factors seem to fulfill a minor but additive role in the reduction of the susceptibility to fluoroquinolones (Vinothkumar et al. 2017). The presence of PMQR genes can promote mutations within the quinolone resistance determining region, leading to fluoroquinolone resistance, but spread to other *Enterobacteriaceae* may occur (Nüesch-Inderbinen et al. 2016). Further, *qnr* genes on mobile genetic elements are also able to confer low-level resistance to fluoroquinolones (Ruiz 2003; Hooper and Jacoby 2015). These genes encode proteins protecting the bacterial DNA gyrase and topoisomerase from quinolone/fluoroquinolone inhibition, thus leading to low-level resistance (Tran and Jacoby 2002; Tran et al. 2005a, 2005b; Redgrave et al. 2014). These plasmids often also harbor other antibiotic resistance genes that can be transferred to other species by conjugation (Martínez-Martínez et al. 1998).

3 Conclusions and Perspectives

The occurrence of multidrug-resistant bacterial pathogens presents a global threat. Especially alarming is the increasing incidence of multiresistant pathogenic bacteria outside medical centers. For example, there is a rising incidence of multiresistant opportunistic or nosocomial pathogens in the population, in food animals, and also in wild animals, e.g., vancomycin-resistant *Enterococci* and carbapenem-resistant *P. aeruginosa* have been recently detected in migratory birds (Martins et al. 2018; Yahia et al. 2018). Thus, implementation of efficient antibiotic stewardship programs is urgently needed all over the world. In addition, alternative treatment options to cure and/or prevent severe infectious diseases caused by multiresistant pathogens are imperatively required to prevent the advent of the post-antibiotic era. Alternative options include among others antibacterial vaccines, herbal products, bacteriophages, and improved biosecurity measures, as summarized by Bragg et al. (2018). Our group has been working on the development of antibacterial vaccines targeting surface-exposed proteins involved in conjugative spread of antibiotic resistance genes among pathogens. One of these vaccine candidates directed to staphylococcal and enterococcal pathogens has been successfully tested in a mouse infection model (Laverde et al. 2017).

Treatment of infections by MDR bacteria is often aggravated by the formation of thick, robust biofilms on infected tissues. Therefore, alternative treatment approaches should include biofilm inhibitors, such as natural or engineered antimicrobial

peptides (Lin et al. 2018a; b) or extracts from medicinal plants (Mehta and Das 2018). It is well-known that biofilm formation is controlled by second messenger molecules, such as cyclic di-guanosine monophosphate (c-di-GMP), and by interbacterial cell-cell communication via quorum sensing systems. Recently, some progress has been made by detecting small-molecule inhibitors of c-di-GMP signaling (Opoku-Temeng and Sintim 2017). Another promising approach to successfully attack strong biofilm forming pathogens should be based on the continuous discovery of novel quorum sensing inhibitors which are often plant-based natural compounds (Defoirdt 2018; Mehta and Das 2018).

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Emergence and Spread of Multidrug Resistance in Ocular Bacterial Pathogens: A Current Update

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Abstract

The tremendous increase of multidrug-resistant bacterial pathogens has posed a serious threat in the management of infectious diseases. The human eye is known to commensally host the normal flora, including the opportunistic pathogens. The researchers have isolated and characterized numerous microbes belonging to different genera from healthy eye, including *Pseudomonas*, *Propionibacterium*, *Acinetobacter*, *Corynebacterium*, *Brevundimonas*, *Staphylococcus*, *Sphyngomonas*, *Streptococcus*, and many others. The human eye is virtually impermeable to microbes, despite being exposed to an array of microorganisms. Ocular infections usually occur through invasion of microbes that may come either from bloodstream or by breaching the ocular barriers. Both gram-negative and gram-positive bacteria are known to be responsible for ocular infections, with the major causative gram-positive bacteria being *S. pneumoniae*, coagulase-negative staphylococci, *S. aureus*, and *S. pyogenes*, with *N. gonorrhoeae*, *Moraxella* spp., *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *Proteus* spp. also being commonly isolated. Apart from the unchecked use of antibiotics and the dissemination of multidrug-resistant bacteria, development of biofilms on ocular surfaces are also a major concern for antimicrobial resistance. In biofilms, the antibiotics are less likely to penetrate, due to reduced rates of diffusions making some of the cells in biofilms more resistant, and

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eventually increasing the effective antibiotic dose by many folds in comparison to planktonic mode cells. In this chapter, a survey on the emergence and spread of MDR ocular bacterial pathogens has been made.

Keywords
Ocular pathogens · Ocular infections · Multidrug resistance · MDR · Biofilm

1 Introduction

In the recent past, especially in the last two decades, there has been a tremendous increase in the emergence of antimicrobial resistance (AMR) in bacterial pathogens. Multidrug-resistant pathogens have become an epidemiological concern, as they may spread through poor sanitation, individual contact, travel, and even the food chain (Sharma 2011a). Long-distance dissemination and spread of multidrug-resistant (MDR) bacteria, such as methicillin-resistant *Staphylococcus aureus*, extended-spectrum-β-lactamase (ESβL)-producing Enterobacteriaceae, vancomycin-resistant enterococci, and hypervirulent *Clostridium difficile*, is mainly implicated in international travel (Kennedy and Collignon 2010; Rogers et al. 2011). The flourishing industry of medical tourism and low-cost private medical care offers ample opportunity for clinically multidrug-resistant pathogens to be disseminated across geographical borders. Plasmid-encoded New Delhi metallo-β-lactamases (NDM) and several types of cefotaximases (CTX-M) are the two most resistant classes of carbapenamase and ESβLs that have been spread globally (Senok et al. 2012). A classic example NDM-1 dissemination was first reported in a Swedish patient who had been hospitalized in New Delhi, India (Yong et al. 2009). After that, NDM-1-producing bacteria have been subsequently isolated in many countries with a shared history of their citizens obtaining medical care in India (Nordmann et al. 2011). NDM1, an enzyme produced by the gene, *bla* $_{\text{NDM-1}}$, present on plasmids, was first reported in 2009 in New Delhi. This gene may be transferred to many other bacterial species, such as *E. coli* and *K. pneumoniae*, conferring multiple antibiotic resistance to multiple drug classes, including carbapenems (Ganguly et al. 2011).

The human eye is almost impermeable to external infectious agents, despite it being constantly exposed to an array of microorganisms. Ocular infections occur through external sources by invasion of microbes that come either from bloodstream, or by breaching the ocular barriers (Maneesh et al. 2016). Alteration in the normal flora of eye also contributes to variety of ocular diseases, such as conjunctivitis, blepharitis, canaliculitis, dacryocystitis, keratitis, orbital cellulitis, and panophthalmitis. Ocular infections are caused by bacteria, viruses, fungi, and parasites; however, bacteria are the most frequent causative pathogens (Amsalu et al. 2015). The most prevalent ocular infection-causing microorganisms are coagulase-negative staphylococci, *S. aureus*, streptococci, *Bacillus*, *Corynebacterium*, *Nocardia*, Enterobacteriaceae, and *Pseudomonas aeruginosa*.

Since 1990, fluoroquinolones have been the most widely used topical treatment against ocular infections. In patients with bacterial keratitis from Pittsburgh, there has been an increase (5.8% in to 35.0%) in ciprofloxacin resistance among *S. aureus* isolates between 1993 and 1997 (Goldstein et al. 1999). A survey on 1312 bacterial isolates from keratitis patients, isolated from 1984 to 1999, has reported the steady rate ofloxacin resistance in 33.4% of *S. aureus* isolates, while a substantial increase in chloramphenicol resistance was found in gram-negative bacteria (Tuft 2000). Similarly, in a study conducted in European countries between 2001 and 2002, there was a 5.5% resistance rate to a new fluoroquinolone, gatifloxacin, an 11.7% resistance rate to ofloxacin, and a 12.4% resistance rate to ciprofloxacin ocular bacterial pathogens (Morrissey et al. 2004). Indeed, the introduction of new fluoroquinolones, such as gatifloxacin, has only delayed the development of resistance, but not prevented it (Brown 2007). The incidence of ciprofloxacin resistance in methicillin-sensitive *S. aureus* (MSSA) isolates from ocular infections increased from 8% to 20.7% between 1996 and 2001. Moreover, resistance to ciprofloxacin in methicillin-resistant *S. aureus* (MRSA) increased from 55.8% to 83.7% during same time span (Marangon et al. 2004). There are also reports of chloramphenicol resistance from Brazil and Europe (Chalita et al. 2004; Morrissey et al. 2004).

In general, there is an alarming rise in antibiotic resistance among ocular pathogens. Therefore, in this chapter we have provided an overview on the microbiology of the human eye, its pathogens, and the current status of MDR in eye infection.

2 The Microbes of the Human Eye

The human eye is host to a large number of commensal and pathogenic microbes, as the ocular surface is constantly exposed to environment. This fact is now established from numerous studies that have routinely isolated and characterized the microbes from different parts of the ocular surface. *Staphylococci* are the most prevalent among human eye microflora and has been isolated from conjunctiva, lids, and tears. Relative abundance of microbiota by phylum and genus in the normal conjunctiva determined using 16S rRNA gene reads is represented in Fig. 3.1 (Dong et al. 2011). In studies involving the isolation of bacteria from the various surfaces of the eye, approximately 50% or more showed the presence of coagulase-negative staphylococci (Willcox 2013). The different species of staphylococci isolated from ocular surface swabs include *Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus capitis*, *Staphylococcus lugdunensis*, *Staphylococcus warneri*, *Staphylococcus hyicus*, *Staphylococcus lentus*, *Staphylococcus intermedius*, and *Staphylococcus schleiferi* (Larkin and Leeming 1991; Leitch et al. 1998; Graham et al. 2007; Høvding 2009; Shin et al. 2016). The next most widespread ocular bacteria isolated are *Propionibacterium* sp., followed by *Corynebacterium* sp. It should be noted that the growth of *Propionibacterium* sp. is dependent on the culture conditions, as it is an obligate anaerobe, and

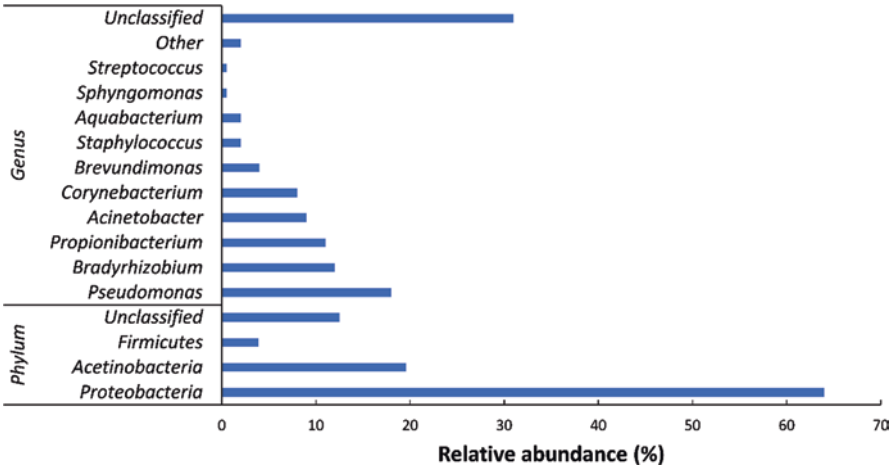

Fig. 3.1 Relative abundance of microbiota by phylum and genus in the normal conjunctiva determined using 16S rRNA gene reads Dong et al. (2011)

hence studies involving aerobic culture conditions are less likely to isolate such microbes (Willcox 2013). Conventionally, the detection and characterization of ocular bacteria are studied using culture-based methods (Locatelli et al. 2003; Graham et al. 2007). Recently, however, molecular-based methods, such as 16S rRNA gene sequencing, on samples from healthy and diseased eyes have revealed a wider range of microbes than previously thought. Indeed, these sequencing-based methods have discovered additional genera of bacteria, such as *Pseudomonas*, *Acinetobacter*, *Bradyrhizobium*, *Brevundimonas*, *Aquabacterium*, *Streptococcus*, *Sphingomonas*, *Streptophyta*, *Enhydrobacter*, *Methylobacterium*, *Bacillus*, and *Ralstonia* spp., that are present in either normal or diseased conditions (Dong et al. 2011; Lee et al. 2012; Zhou et al. 2014).

The diversity of eye microflora is still a debated topic, as there are discrepancies among different studies. Such inconsistencies are expected to be influenced by culture, collection, and transportation conditions. For instance, conjunctival swabs showing no growth ranged from 9% to 87% (Perkins et al. 1975; de Caro et al. 2008), and lid swabs exhibiting no growth were between 0% and 48% (Stapleton et al. 1997; Ozkan et al. 2012). For precision, bacterial growth from conjunctival swabs cultured in thioglycolate broth is expected to show the presence of coagulase-negative staphylococci. Likewise, culture on blood agar plates is favourable for growth of *Corynebacterium* sp. (Miño de Kaspar et al. 2008), and under anaerobic conditions, *Propionibacterium* sp. will flourish. Sleeping may also affect the population density of eye microflora, as a survey-based study from India involving the culture of conjunctiva found that the frequency of *S. aureus* and *S. epidermidis* isolation doubled immediately after 8 h of sleep (Ramachandran et al. 1995).

3 Pathogenic Bacteria of the Human Eye and Their Prevalence

According to World Health Organization (WHO) estimates, more than 285 million people were visually impaired globally, among which 39 million are blind and 246 million are with low vision. Surprisingly, more than 80% of the visual impairment, including compete loss of vision, was avoidable (Namitha et al. 2014). The major causes for visual impairments are uncorrected refractive errors (42%), followed by cataracts (33%). About, 90% of world's visually impaired subjects are from developing countries (Getahun et al. 2017).

The human eye is a complex sensory organ which is nearly impermeable to environmental agents under normal circumstances. Nevertheless, microbes attain access into the eye in certain circumstances via different routes and may cause infection (Mulla et al. 2012). Common routes of pathogen entry are surgery, trauma, and systemic diseases (Hemavathi et al. 2014). Conjunctivitis, dacryocystitis, blepharitis, and periorbital and orbital cellulitis are the most common ocular infections of external eye. Conjunctivitis is inflammation of conjunctiva, and bacterial conjunctivitis is characterized by conjunctival hyperaemia and mucopurulent discharge (Bertino 2009). Keratitis is inflammation of cornea that may cause corneal ulceration and, in severe cases, may lead to corneal blindness (Sharma 2011b). Inflammation of the eyelids is called blepharitis, and common symptoms are redness and itching along with and crusty or greasy eyelashes (Durand 2013). Endophthalmitis, an interior eye infection, is caused by entrance of exogenic organisms either through surgery, trauma, or an infected cornea (Azari and Barney 2013).

The prevalence of microbes involved in eye infection vary from region to region. The occurrence of common bacterial isolates in ocular infections is presented in Table 3.1. Gram-positive species were found in most of the case studies of subjects with external ocular infections. In a Nigerian study, *S. aureus* was most prevalent (27.7%), followed by coagulase-negative staphylococci (22.6%) (Olatunji et al. 2007). Similarly, the occurrence of gram-positive cocci in patients with eye infections from Jimma, Ethiopia, was reported as 52% (Chaudhary et al. 2010). In another study conducted in Hawassa, a city in southern Ethiopia, there was a 61.5% prevalence of gram-positive bacteria, in which 21% of the isolates were *S. aureus*, 18.2% were coagulase-negative staphylococci, and 14% were *S. pneumoniae* (Iwalokun et al. 2011). Further, a report from Libya found that *S. epidermidis* and *S. aureus* were the most common pathogens responsible for infections causing anterior blepharitis (Shiferaw et al. 2013). Similarly, another survey-based report on the prevalence of pathogens responsible for dacryocystitis in patients from Gondar, Ethiopia, it was reported that the prevalence of coagulase-negative staphylococci and *S. aureus* was 29% and 19.4%, respectively (Nazeerullah et al. 2014).

Table 3.1 Occurrence of common bacterial isolates in ocular infections

S. no.	Bacteria		Study location	Study duration	References
	Gram-positive	Gram-negative			
1.	*S. aureus* (23.7%)	*P. aeruginosa* (10.1%)	Aba, Nigeria	Jan 2005–Dec 2006	Ubani (2009)
	Coagulase-negative staphylococci (19.2%)	*H. influenzae* (7.7%)			
	S. pneumoniae (8.6%)	*K. pneumoniae* (6.2%)			
	S. pyogens (6.2%)	*E. coli* (4.4%)			
		N. gonorrhoeae (3.9%)			
		Moraxella spp. (3.0%)			
		Proteus spp. (1.5%)			
2.	Coagulase-negative staphylococci (37%)	*P. aeruginosa* (21%)	India	Mar 2010–Feb 2011	Mulla et al. (2012)
	S. aureus (13%)	*K. pneumoniae* (7%)			
		E. coli (3%)			
3.	*S. aureus* (28.1%)	*P. aeruginosa* (20.9%)	Southwest Ethiopia	Feb 2012–Oct 2012	Shiferaw et al. (2013)
	S. pneumoniae (13.5%)	*H. influenzae* (8.8%)			
	Coagulase-negative staphylococci (10.1%)	*E. coli* (5.4%)			
		Moraxella spp. (4.0%)			
		N. gonorrhoeae (2.7%)			
4.	*S. aureus* (26.6%)	*P. aeruginosa* (8.23%)	Tamil Nadu, India	Jan 2002–Dec 2007	Bharathi et al. (2010b)
	S. pneumoniae (22.14%)	*Moraxella* spp. (5.4%)			
	Coagulase-negative staphylococci (6.1%)	*H. influenzae* (3.45%)			
	S. pyogens (1.6%)	*E. coli* (0.9%)			
		Proteus spp. (0.6%)			
		N. gonorrhoeae (0.42%)			

(continued)

Table 3.1 (continued)

S. no.	Bacteria		Study location	Study duration	References
	Gram-positive	Gram-negative			
5.	Coagulase-negative staphylococci (31.4%)	*P. aeruginosa* (9.7%)	Shizuoka, Japan	Jan 2006–Dec 2009	Shimizu et al. (2013)
	S. aureus (21%)	*H. influenzae* (2.4%)			
	S. pneumoniae (3.2%)	*K. pneumoniae* (2.4%)			
		Proteus spp. (1.6%)			
		Moraxella spp. (0.8%)			
6.	*S. aureus* (24.6%)	*P. aeruginosa* (16.0%)	Bangalore, India	NA∗	Hemavathi et al. (2014)
	Coagulase-negative staphylococci (19.8%)	*E. coli* (12.3%)			
	S. pneumoniae (4.9%)	*H. influenzae* (4.9%)			
		K. pneumoniae (4.9%)			
7.	Coagulase-negative staphylococci (32.9%)	–	Ahwaz, Iran	Oct 2005–Mar 2006	Khosravi et al. (2007)
	S. aureus (12.9%)				
	S. pneumoniae (8.6%)				
8.	*S. pneumoniae* (20.76%)	*N. gonorrhoeae* (20.76%)	New Delhi, India	NA∗	Sherwal and Ak (2008)
	Coagulase-negative staphylococci (20.76%)	*Moraxella* spp. (19.13%)			
	S. aureus (19.13%)	*P. aeruginosa* (4.92%)			
	S. pyogens (0.55%)	*K. pneumoniae* (2.74%)			
		E. coli (1.1%)			
		Proteus spp. (0.33%)			

(continued)

Table 3.1 (continued)

S. no.	Bacteria		Study location	Study duration	References
	Gram-positive	Gram-negative			
9.	Coagulase-negative staphylococci (27.4%)	*K. pneumoniae* (14.5%)	Northwest Ethiopia	Sep 2009–Aug 2012	Muluye et al. (2014)
	S. aureus (21.0%)	*E. coli* (8.1%)			
	S. pyogens (14.5%)				
	S. pneumoniae (11.3%)				
10.	Coagulase-negative staphylococci (39.0%)	*K. pneumoniae* (6.2%)	Raichur, Karnataka, India	Nov 2010–Sep 2011	(Namitha et al. (2014)
	S. aureus (32.8%)	*E. coli* (4.7%)			
	S. pneumoniae (14.1%)	*P. aeruginosa* (3.0%)			
11.	*S. aureus* (21.0%)	*K. pneumoniae* (6.3%)	Hawassa, Ethiopia	Dec 2012–Apr 2013	Amsalu et al. (2015)
	Coagulase-negative staphylococci (18.2%)	*P. aeruginosa* (4.9%)			
	S. pneumoniae (14%)	*E. coli* (4.9%)			
	S. pyogens (4.2%)	*H. influenzae* (4.2%)			
		Proteus spp. (3.4%)			
		Moraxella spp. (2.8%)			
12.	Coagulase-negative staphylococci (25.0%)	*K. pneumoniae* (10.0%)	New York, USA	Ma 2001–Jan 2003	Haas et al. (2005)
	S. aureus (19.0%)	*E. coli* (8.0%)			
		P. aeruginosa (8.0%)			
13.	*S. aureus* (17.0%)	*P. aeruginosa* (16.0%)	Kelantan, Malaysia	2001–2010	Rahman et al. (2013)
	S. pyogens (3.1%)	*H. influenzae* (8.3%)			
		K. pneumoniae (5.5%)			
		E. coli (4.0%)			

(continued)

Table 3.1 (continued)

S. no.	Bacteria		Study location	Study duration	References
	Gram-positive	Gram-negative			
14.	*S. aureus* (22.1%)	*P. aeruginosa* (13.7%)	ARMOR study using Miami microbiology database, USA	2005–2015	Miller (2017)
	Coagulase-negative staphylococci (6.7%)	*H. influenzae* (2.3%)			
	S. pneumoniae (2.4%)				

Values in parenthesis are their prevalence
*NA** is data not available, *ARMOR:* antibiotic resistance monitoring in ocular microorganism

In India, a broad study conducted on 4417 ocular samples from an eye care hospital in the Tirunelveli district of Tamil Nadu, between January 2002 and December 2007, found that 58.8% samples showed bacterial growth, 10.3% showed fungal growth, 0.34% showed acanthamoebic growth, and 0.32% showed mixed microbial growth, while no microbial growth was recorded in 30.2% of total samples. Moreover, the most prevalent bacterial species isolated were *S. aureus* (26.69%) followed by *S. pneumoniae* (22.14%). Coagulase-negative staphylococci were the most commonly isolated bacteria in endophthalmitis (53.1%), while *S. aureus* was most predominant (51.22%) in eyelid infections. Similarly, *S. pneumoniae* dominated (64.19%) in corneal and lacrimal apparatus infections, *Corynebacterium* species (71%) in conjunctivitis and blepharitis, *Haemophilus* species (66.7%) in conjunctivitis and dacryocystitis, *P. aeruginosa* (66.5%) in dacryocystitis and keratitis, *Moraxella catarrhalis* (63.83%) in dacryocystitis, and *Moraxella lacunata* (54.17%) in blepharitis (Bharathi et al. 2010b).

Another study conducted on 756 ocular infection samples from South India found that 97.35% were infected with single bacterial species, while 2.65% showed the presence of two or more species of bacteria. The most common group of bacterial isolates were gram-positive cocci (70%), gram-negative bacilli (15.85%), and gram-positive bacilli (10%). The most commonly isolated bacterial species were *S. aureus* (25.13%), *S. pneumoniae* (21.78%), and coagulase-negative staphylococci (18.29%). *S. aureus* was most predominant in conjunctival and eyelid infections, whereas coagulase-negative staphylococci dominated the intraocular tissue infections (Bharathi et al. 2010a).

4 Biofilms in Eye Infection and Their Role in the Enhancement of Antimicrobial Resistance

Previously, it was thought that microbes grow only in the planktonic mode. Later, it was discovered that the majority live in complex structures called biofilms. Such organized structures consist of microbial communities protected by extracellular

polymeric substances (EPS) that may either be formed on solid or liquid interfaces (Ahmad et al. 2017). In the biofilm mode of growth, cells exhibit altered phenotypes when compared to their planktonic counterparts, especially when interacting with each other, by regulating their transcription (Donlan 2002; Hall-Stoodley et al. 2004). It is the natural tendency of microorganisms to attach to biotic or abiotic surfaces to form biofilms. In fact, the National Institute of Health has estimated that more than 80% of infections are established by biofilms, imposing an enormous burden on cost of human health (Schachter 2003). A majority of infections are caused by the formation of biofilms by pathogenic or opportunistic pathogens, such as *E. coli*, *P. aeruginosa*, *S. aureus*, *S. pyrogens*, and *C. albicans*, on mucosal surfaces and biomedical devices (Donlan 2001; Douglas 2002).

The initial step of biofilms formation is irreversible in nature and starts with the attachment of microbes to any surface (Dongari-Bagtzoglou 2008), and the nature of initial attachment may vary, depending on the surface, such as mineral surfaces, synthetic polymers, mammalian tissues, and indwelling medical devices (Nikolaev and Plakunov 2007). The attached microbes then produce extracellular substances, forming a mesh-like structure. Traditionally, research on microbes, especially concerning the development of therapeutic agents, have been conducted on planktonic cells, due to their ease of study and the lack of understanding of the biofilm mode of life. As a consequence, most of the chemotherapeutic agents that exhibit excellent efficacy against free-living cells do not perform well against biofilms. The major reason underlying the failure of antimicrobial drugs in combatting such infections is the increased tolerances of microbes in the biofilm state. Indeed, bacteria in biofilms can be up to several hundred times more tolerant to antibiotics or other antibacterial agents (Khan et al. 2014). These biofilms are three-dimensional structures of EPS that cause gradients in oxygen, pH, and nutrients, resulting in different microniches throughout (Davies 2003), and there is physiological heterogeneity among cells due to physiological adaptations to such microniches (Stewart and Franklin 2008). Microbial cells residing near the surface of biofilms remain more exposed to oxygen and nutrients, thereby exhibiting more metabolic activity. However, the cells present in deeper regions of biofilms are less active and sometimes dormant. This heterogeneity produces a range of responses to chemotherapeutic agents in which metabolically active cells present at the surface are quickly killed, while the dormant cells remain relatively unaffected (Davies 2003). Adding to this, antimicrobial molecules are less likely to penetrate within the biofilm due to lower rates of diffusion. Taken together, this makes populations of cells in biofilms more tolerant, ultimately reducing their antibiotic susceptibilities from 10- to 1000-fold in comparison to their planktonic counterparts.

The colonization of healthy conjunctiva, lids, and tears is mainly by gram-positive bacteria, among which coagulase-negative staphylococci is most prominent, followed by *P. acnes*, *Corynebacterium* spp., and *S. aureus* (Willcox 2013). There are considerable rates (2% to 46%) of anterior chamber contamination by gram-positive commensal microorganisms, *S. epidermidis* being the most prevalent, on the ocular surface due to uneventful cataract surgery (Mistlberger et al. 1997; Bausz et al. 2006). The incidences of anterior chamber

contamination are considerably more numerous than the incidences of postoperative intraocular infection. This indicates that the anterior chamber is efficient in clearing bacterial inoculum, significantly inhibiting the progression to endophthalmitis, possibly due to rapid turnover of aqueous humor (Durand 2013). Most of the organisms of eye microbiota are capable of adhering to intraocular lens and posterior lens, and may become well established when they reach the posterior chamber. The static nature of vitreous humor provides suitable conditions for establishment of an infection when compared to aqueous humor (Bispo et al. 2015). Intraocular lenses, made of polymethylmethacrylate, sometimes gets contaminated with conjunctival bacteria (usually *S. epidermidis*) during insertion and may introduce the organisms to the posterior chamber (Vafidis et al. 1984; Doyle et al. 1995). The tendency of commensal microbes to form biofilms on the surface of intraocular lens is an important mechanism in the pathogenesis of post-cataract endophthalmitis that hiders the resolution of infection. Moreover, biofilm microbes are less likely to render culture positivity for the samples of aqueous and vitreous region (Melo et al. 2011).

Post-cataract endophthalmitis is usually caused by gram-positive bacteria that come from ocular surface microbiota. Coagulase-negative staphylococci, mainly *S. epidermidis*, are the most recurrently encountered bacterial pathogens in acute endophthalmitis (Schimel et al. 2013), while *P. acnes* frequently causes delayed endophthalmitis, which is also a persistent form of infection (Shirodkar et al. 2012). Both of these bacterial pathogens (*S. epidermidis* and *P. acnes*) are capable of adhering to, and forming biofilms on, intraocular lenses (Bispo et al. 2015). There is also evidence that these microbes can also form biofilms in the posterior capsular bag (Baillif et al. 2006; Adán et al. 2008). The commensal microbes of the ocular surface and the periocular tissues are the primary causative agents of postoperative endophthalmitis.

Polysaccharide intercellular adhesin proteins (encoded by the *icaADBC* locus), including accumulation-associated protein (Aap) and biofilm-associated homologue protein (Bhp), contribute to the formation of the biofilm matrix of staphylococci (Fey and Olson 2010). Many commensal isolates of coagulase-negative staphylococci, including *S. epidermidis*, isolated from healthy conjunctiva, have been reported to carry genes responsible for biofilm formation and maturation, suggesting that biofilm formation is a fundamental part of their lifestyle (Makki et al. 2011). Therefore, the *ica* locus and other related genes play a crucial role in staphylococcal biofilm formation. A study from Japan reported the prevalence of *ica*A-positive strains of *S. epidermidis* as 60% and 69.4% in isolates from the healthy conjunctiva of volunteers and patients undergoing cataract surgery, respectively, and that the majority of isolates gave positive results for slime production on Congo red agar (Suzuki et al. 2005). Likewise, a study from Mexico reported that 17% of commensal conjunctival isolates of *S. epidermidis* were able to form biofilms under in vitro conditions, and 26.7% were found positive for icaA and/or icaD genes (Juarez-Verdayes et al. 2013). From samples collected from staff and students of an institution in India, the occurrence of *ica* genes was recorded at 36% for coagulase-negative staphylococci (except *S. epidermidis*) (Makki et al. 2011).

Previously, many studies have shown the biofilm-forming ability of *S. epidermidis* on intraocular lenses, both under basic in vitro conditions (Okajima et al. 2006), and in models resembling the intraocular environment (Baillif et al. 2008). The strength of biofilm formation not only depends on the material used to make intraocular lens, but also on the genomic content of the *S. epidermidis* strain tested (Bispo et al. 2015). Strains which lack the *ica* locus form weaker biofilms on the surfaces of various intraocular lenses (Shimizu et al. 2006). In a comparative study, *S. epidermidis* ATCC 12228 (*ica* negative) and ATCC 35984 (*ica* positive) formed the strongest biofilms on acrylic lenses, followed by polymethylmethacrylate and 2-methacryloyloxyethyl phosphorylcholine surfaces (Okajima et al. 2006). Of note, surface modification of the acrylic intraocular lens with 2-methacryloyloxyethyl phosphorylcholine resulted in reduced biofilm formation, which may due to increased hydrophilicity (Hirota et al. 2005). Similar results were obtained for 2-methacryloyloxyethyl phosphorylcholine-modified silicone intraocular lenses (Huang et al. 2007). Foldable intraocular lens made of silicone supported stronger biofilm formation by *S. epidermidis* (ATCC 35984) when compared to polymethylmethacrylate intraocular lenses; however, the same effect was not observed for *S. epidermidis* ATCC 12228 (García-Sáenz et al. 2000).

In a model experiment with flow conditions similar to the anterior chamber, using a bioreactor, *S. epidermidis* formed biofilms on different intraocular lens materials. In this study, silicone was most accommodating to biofilm formation, followed by hydrophobic acrylic and polymethylmethacrylate (Baillif et al. 2008).

5 Emergence and Current Status of Drug Resistance in Ocular Pathogens

Parallel increases in antibiotic resistance among ocular gram-negative and gram-positive pathogens have been recorded, with increased resistance in other systemic pathogenic microorganisms of non-ocular origin (Sharma 2011b). Excessive use of antibiotics during systemic infections, along with unjust application of topical antibiotics to the eye, has contributed largely to the development of drug resistance (Fintelmann 2011).

The rise in antibiotic resistance of *S. aureus*, a leading causative organism in a majority of ophthalmic infections, makes treatment challenging (Sadaka et al. 2017). The first instance of antibiotic resistance among *S. aureus* was reported in the 1940s, soon after the introduction of penicillin (Uhlén et al. 1984), and reports of hospital-acquired penicillin resistance in *S. aureus* became more frequent in the following decades (Ashley and Brindle 1960). Similarly, almost immediately after the introduction of Methicillin in 1960, MRSA strains were reported (Jevons et al. 1963). Consequently, MRSA became one of the leading causes of hospital-acquired infections around the globe. The prevalence of methicillin resistance among staphylococci is continuously increasing, and MRSA infections are responsible for about half of the total *S. aureus* infections in hospital settings in the United States and some European countries (Sadaka et al. 2017; Walter et al. 2017). MRSA strains

evolved through the acquisition of a large DNA element, staphylococcal cassette chromosome mec (SCCmec), at specific sites in the *S. aureus* chromosomes (Enright et al. 2002; Zhang et al. 2009), which encodes modifications to the penicillin binding proteins, PBP-2a or PBP2b, which are specific proteins involved in the structural synthesis of bacterial cell walls, conferring resistance to both methicillin and other related beta-lactam antibiotics (Chambers 1997; Blair et al. 2015). Moreover, the development of *S. aureus* resistance against fluoroquinolones, ranging from first generation to advanced iterations, is also on the rise (Asbell and Sanfilippo 2017; Deguchi et al. 2018). MRSA has been identified as the causative agents of a majority of ocular infections, including endophthalmitis (Major et al. 2010), which are associated with bleb (Pierre and Tang 2010), endogenous endophthalmitis (Ho et al. 2011), trauma (Major et al. 2010), and other common infections (Fukuda et al. 2002), among which more than 50 percent of strains showed resistance against fourth-generation fluoroquinolones commonly used in the management of eye infections (Major et al. 2010).

The emergence of MDR in staphylococcal ocular pathogens has increased over the past decades (Asbell et al. 2008b; Haas et al. 2011; Chang et al. 2015; Asbell and Sanfilippo 2017; Miller 2017). Data collected from laboratories across the United States showed a nationwide rise in the prevalence of MRSA-associated ocular infections, from 29.5% to 41.6%, during the span of 5 years (from 2000 to 2005) (Asbell et al. 2008b). Furthermore, the ocular Tracking Resistance in the United States Today (TRUST) data, the Antibiotic Resistance Monitoring in Ocular MicRoorganism (ARMOR), the Surveillance Network for ocular *S. aureus* isolates, and surveillance studies showed that the prevalence of MDR for MSSA isolates is significantly lower when compared with MRSA isolates (Asbell et al. 2008a, b; Haas et al. 2011; Chang et al. 2015). Sadaka et al. (2017) reviewed the prevalence of MDR in *S. aureus* associated with endophthalmitis and found that MRSA represents the most significant ocular pathogen associated with *S. aureus* endophthalmitis cases (Sadaka et al. 2017). Emergence of reduced susceptibility to vancomycin and the rise in resistance to fluoroquinolones were also recorded. Chang et al. (2015) demonstrated that MRSA represented 30.7% (122 out of 398) of the total *S. aureus* keratitis isolates collected over a period of 20 years (January 1993 to December 2012) (Chang et al. 2015). When compare to MSSA, MRSA showed significantly more resistance to the majority of antibiotics tested, with the exception of vancomycin and polymyxin B. Al-Dhaheri et al. (2016) detected a rise in resistance frequency of keratitis *S. aureus* isolates against oxacillin, from 14.8% in 2011 to 27.8% in 2014 (Al-Dhaheri et al. 2016). Recently, ARMOR, a nationwide surveillance program, showed geographic variations in the prevalence of MDR in ocular pathogens over a period of 7 years (from 2009 to 2016) in the United States. It was observed that azithromycin resistance among *S. aureus* was the highest (49.4–67.8%) across all regions, whereas fluoroquinolone resistance was found to be 26.1%. Strains of *S. aureus* isolated from the southern region showed high resistance, particularly to methicillin (Asbell et al. 2018). However, a small decrease in methicillin resistance over the period of the study (2009–2016) was observed, which may indicate improved antibiotic management in recent practices (Asbell and Sanfilippo 2017).

Fluoroquinolones, generally used for topical application during ocular pathologies, include ciprofloxacin, ofloxacin, and norfloxacin (Hwang 2004), and more recently levofloxacin, gatiflooxacin, moxifloxacin, and besifloxacin (Cheng 2007). Resistance against fluoroquinolones commonly results from point mutations in the genes encoding DNA topoisomerase IV (Sanfilippo et al. 2011) and has emerged against iterations from the first to fourth generations (Marangon et al. 2004). In fact, studies have shown that there has been increase in overall resistance to fourth-generation fluoroquinolones among staphylococcal strains from ocular infections (Miller 2006, 2017; Major et al. 2010; Chang et al. 2015). In a study, 42 staphylococcal isolates originating from keratitis cases were evaluated for MDR. The authors reported that, out of the total number of isolates, 76.2% were resistant to the fourth-generation fluoroquinolones, moxifloxacin, while 30.9% and 40.4% of isolates showed resistance against ofloxacin and levofloxacin. Additionally, it was also detected that the majority of isolates (85.72%) were strong biofilm formers (Kaistha et al. 2011). An increase in resistance to fourth-generation (moxifloxacin and gatifloxacin) drugs was also detected for MRSA and MSSA strains isolated over the period of 20 years (1993–2012). However, the resistance of *S. aureus* keratitis strains was higher for second-generation fluoroquinolones than for fourth-generation representatives (Chang et al. 2015). A 2-year study concerning the evaluation of current susceptibility patterns of ocular pathogens from various countries in Europe demonstrated alarming resistances of MRSA to fluoroquinolones, particularly to second-generation fluoroquinolones, such as ciprofloxacin. The susceptibility rates for MRSA varies greatly for different fluoroquinolones, probably due to their structural differences. However, no systemic resistance breakpoint has been established for besifloxacin, as it has been developed exclusively for ocular infections (Sanfilippo et al. 2016). However, in contrast to earlier reports, Miller (2017) documented no significant difference in the susceptibilities of MSSA versus MRSA for moxifloxacin and ciprofloxacin over a period of 5 years. However, the author confirmed the continued rise in resistance among both MSSA and MRSA staphylococcal ocular pathogens to fluoroquinolones. Susceptibilities are also influenced by the availability and frequency of the use of the antibiotics. Because of this, prevalence rate are as high as 70% in regions such India, Brazil, and United States, where the availability and/or use of fluoroquinolones are high for both MSSA and MRSA. On the contrary, in regions where application is limited or minimal, such as in the Middle East, Africa, and Australia, resistance rates are comparatively low (Miller 2017).

Apart from *S. aureus*, many other drug-resistant ocular bacterial pathogens have been reported in different parts of the world. Before 2003, the prevalence of gentamicin- and gatifloxacin-resistant coagulase-negative staphylococci were ~19% and 2%, respectively (Morrissey et al. 2004; Kaliamurthy et al. 2005). However, according to a report published in 2006, gatifloxacin-resistant coagulase-negative staphylococci increased to 11% in normal ocular cases and 53% endophthalmitis cases (Harper et al. 2007). Importantly, all methicillin-resistant *S. epidermidis* isolates were resistant to gatifloxacin and moxifloxacin (fourth-generation fluoroquinolones), while remaining sensitive to besifloxacin, the latest fluoroquinolone (Haas et al. 2009). It is expected that bacteria have a lesser chance of developing resistance

against besifloxacin, a fluoroquinolone developed mainly for ophthalmic use, as it is not used for systemic applications. Since there are not CLSI-established breakpoints for besifloxacin resistance, its potential resistance can only be assessed by comparing MICs (Sharma 2011b). In vitro results have shown that besifloxacin exhibited lower MICs in comparison to azithromycin and fluoroquinolones against bacterial isolates associated with conjunctivitis (McDonald and Blondeau 2010).

The choice of antibiotics for the management of ocular infections caused by *Pseudomonas* is a challenging task to ophthalmologists, owing to the recent increase in antibiotic-resistant isolates. In 1986, a study found that most *P. aeruginosa* strains isolated from contact lens-associated corneal ulcers were resistant to cephalothin, ampicillin, neomycin, and tetracycline (Mayo et al. 1986). Because of this, ciprofloxacin replaced aminoglycosides as the drug of choice against keratitis caused by *P. aeruginosa*. Ciprofloxacin exhibits broad-spectrum antimicrobial activity and hence is a good choice for the treatment of bacterial keratitis. However, due to its overuse, there has been a considerable increase in ciprofloxacin resistance among *P. aeruginosa* isolates (from <1% in 1994, to 4% in 1998, and finally to 29% in 2003) (Mayo et al. 1986; Chaudhry et al. 1999; Alexandrakis 2000). It is now common that *P. aeruginosa* isolated from keratitis and endophthalmitis patients exhibit MDR (Chew et al. 2010; Pathengay et al. 2010).

Other species of bacteria are also involved in ocular infections and exhibit various degrees of antibiotic resistance. For example, *Nocardia* and non-tuberculous mycobacterial species, although rare in eye infections, are an important cause of scleritis and keratitis. Amikacin is used in the treatment of such infections, and to date, most ocular isolates are susceptible to it (Sridhar et al. 2001; Reddy et al. 2010). Recently, *Corynebacterium* species have been recognized as ocular pathogens. Most of species of *Corynebacterium* are part of the normal eye and skin flora; however, *C. diphtheriae* has been reported to cause infection (Sharma 2011b), and *C. macginleyi* has been documented for its involvement in keratitis and conjunctivitis (Funke et al. 1998; Joussen 2000). Previously, almost all *C. macginleyi* isolates were sensitive to numerous antibiotics, including fluoroquinolones; however, a study in 2008 found that 11 out of 16 isolates of *C. macginleyi* from patients with conjunctivitis or going for surgery were resistant to certain fluoroquinolones, such as norfloxacin, ciprofloxacin, and levofloxacin (Eguchi et al. 2008). Lastly, antibiotic resistance among *Enterobacteriaceae* isolates associated with ocular infections is rare. In 2004, it was reported that only 3.4% of isolates were resistant to gatifloxacin, 5.1% to ciprofloxacin, and 8.5% to gentamicin (Morrissey et al. 2004).

6 Conclusion and Future Directions

There are variations in the distribution and proportion of bacterial isolates in different ocular infections, and both gram-negative and gram-positive bacteria can be responsible, though gram-positive species are the major contributors. From above literature survey, it has become clear that emergence of MDR in bacteria causing eye infection is similar to other diseased conditions. Multidrug resistance among

staphylococci, in particular, has increased in prevalence over the last few decades. To mitigate the further emergence of MDR among ocular bacterial pathogens, physicians should consider the judicious prescription of antibiotics and comply with etiologic approaches for the management of such infections.

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Antibiotic Resistance in *Campylobacter jejuni*: Mechanism, Status, and Public Health Significance

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Abstract

Emergence of antibiotic resistance is a never-ending process in the bacteria due its vast capacity to resist and acquire various resistance mechanisms against antibacterial drugs. *Campylobacter* is a well-known pathogenic bacteria to human and animals and survive in different environment including foods. Species of campylobacters is responsible of gastritis and diarrheal and other diseases. Common resistance mechanisms present in Gram-negative bacteria include modification in the target site of antibiotic, inability of the antibiotic to reach its target by expressing major outer membrane proteins (MOMPs), efflux action of the antibiotic through CmeABC pumps, and inactivation or modification of the antibiotic. The plasmid along with chromosomal encoded genes are responsible for resistance. Mutation and acquisition of resistance genes are the common genetic mechanism found in *Campylobacter* spp.; considering the widespread occurrence of drug-resistant campylobacters in the environment, specific strategies to control the emergence and spread are needed. In this chapter, we have reviewed the recent literature on the mechanism of resistance and current status of prevalence of *Campylobacter jejuni* in the environment and its significance in human health.

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1 Introduction

Campylobacters are spiral-shaped Gram-negative foodborne pathogenic bacteria which come in the family of *Campylobacteriaceae*. On the basis of morphological characteristics such as curved, spiral, or rod-shaped, the genus *Campylobacter* consists of 25 different species; two are provisional species and while eight are subspecies (Kaakouch et al. 2015). The natural habitat of *Campylobacter* species is believed to be the intestinal tracts of wild birds, poultry, pets (cats and dogs), cattle, and pigs (Abulreesh et al. 2017). The major primary cause of human campylobacteriosis is the intake of poultry-contaminated products. However, contaminated water, raw milk, and the handling of wild birds are also important sources of human infections (Abulreesh et al. 2006). Campylobacters are major cause of foodborne infections; it accounts for around 550 million foodborne illnesses annually, with 33 million cases of mortality worldwide, where children under the age of 5 years old are frequently susceptible, with estimated 220 million cases per year (Azrad et al. 2018). *C. jejuni* is the most common cause of human's gastroenteritis all over the world, whereas *C. coli* leads to total 1–25% of diarrheal infections associated with *Campylobacter* (Havelaar et al. 2015; Kaakouch et al. 2015; Natsos et al. 2016). Besides the clinical symptoms of gastroenteritis, *C. jejuni* can be responsible for further health complications, such as extraintestinal infections ranging from bacteremia to meningitis; furthermore, *C. jejuni* is a major risk factor for postinfectious complications like reactive arthritis, bowel syndrome, and Guillain-Barre syndrome (GBS), a neurological disorder causing immobilized muscles (Fitzgerald 2015; Kaakouch et al. 2015; Otigbu et al. 2018). Dissemination of *Campylobacter jejuni* to human populations occurs most commonly via the consuming foods of animal origin like incompletely cooked poultry meat or contaminated poultry products and unpasteurized milk. The ingestion of contaminated drinking water is also considered as an important route in transmission of the organism, while the handling of domestic pets, poultry, and wild birds is an established source of the infection (Abulreesh et al. 2006, 2017; Pitkanen 2013; Natsos et al. 2016). Although many of the infections of *Campylobacter* are mild, self-limiting, and generally resolved within a few days to weeks without follow-up of antibiotics, prolonged or complicated infections sometimes may occur, especially in young, elderly, and immune-compromised persons (Fitzgerald 2015; Kaakouch et al. 2015; Otigbu et al. 2018).

According to the World Health Organization (WHO), "antibiotic resistance" is the mechanism adopted by bacteria that makes them unresponsive toward antibacterial drugs (WHO 2018). A simple use of antibiotics creates the antimicrobial

resistance in microbes; however, the irrational consumption of antibiotics in humans including animals has developed the alarming situations and provided the opportunity for rising of superbugs also (WHO 2018). The antibiotic resistance is always developed by the bacterial cells using either natural (intrinsic) mechanisms such as modification of bacterial cell enzymes, restriction for the penetration of antibiotic in bacterial cell, and changes in the drug target site or acquired mechanisms such as antibiotic target modifications, reduction or barring cell permeability for penetration of drug, decreased influx of antibiotics by expressing efflux pumps, drug-inactivating enzyme production, and metabolism of an alternate pathway that bypass the mode of action of drug and biofilm development (Munita and Arias 2016).

One of the controlling measures of campylobacteriosis is fluid therapy which is most effective, whereas treatment with antibacterial agents is required in immunocompromised patients with severe symptoms. The most commonly used antimicrobial agents which is effective against *Campylobacter* infections are the class of macrolides (e.g., erythromycin) and fluoroquinolones antibiotics (e.g., ciprofloxacin). Tetracyclines have been regarded as an alternative drug of choice in treating the complications of campylobacteriosis; however, their use is limited (Wieczorek and Osek 2013). In recent years, the apocalypse of antimicrobial resistance and its spread has become a serious dilemma to public health in both developing and developed countries. *Campylobacter jejuni* with resistance to agents belonging to a wide number of antimicrobial classes like fluoroquinolones, macrolides, beta-lactams, tetracyclines, and aminoglycosides have been detected in clinical and environmental samples worldwide (Zhu et al. 2006; Luangtongkum et al. 2009; Mozina et al. 2011; Kaakouch et al. 2015; Geissler et al. 2017; Silva et al. 2018; Sierra-Arguello et al. 2018).

There are a number of contributing factors that have been recognized that are responsible for emergence and spread of antibiotic resistance in bacteria. These factors include indiscriminate use of antibiotics in routine clinical practice, particularly the unnecessary consumption of antibiotics during the course of viral infections, prescription of broad-spectrum antibiotics, and the overuse of antimicrobial agents in agricultural practices (Zhu et al. 2006; Luangtongkum et al. 2009; Reddy and Zishiri 2017). Antimicrobial resistance can spread easily through countries via travelling from one continent to another; migration of a large number of population to other countries due to increase in globalization, either for work or tourism, is believed to be common cause of the spread of antibiotic-resistant bacteria globally (Memish et al. 2003; von Wintersdorff et al. 2014; Anjum et al. 2016). Campylobacters have an intense alarming restriction modification system allowing bacteria to prevent the entry of foreign genetic material inside the cell. There is a built-in transformable system in *C. jejuni*, and the acquisition of various resistance genes from other related or unrelated bacteria has been described (Iovine 2013; Wieczorek and Osek 2013). Because of all these reasons, the study of the resistance mechanisms in *Campylobacter jejuni* is important for both human and veterinary medicine.

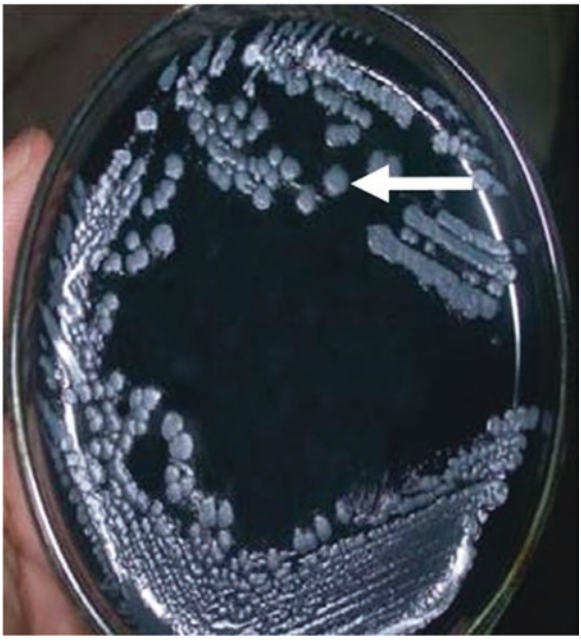

Fig. 1 *Campylobacter jeuni* colonies on modified charcoal cefoperazone deoxycholate agar (mCCDA), blood-free agar

The genetic elements that are involved in the resistance mechanisms may be either plasmid borne or chromosomal, representing a combination of indigenous and/or acquired genes. In *Campylobacter jejuni*, the mechanisms of antibiotic resistance involve (i) antibiotic target modification or alteration of its expression (i.e., mutations in DNA gyrase), (ii) accessibility of antibiotic to reach its target site (i.e., major outer membrane protein or MOMP expression, (iii) antibiotic extrusion through efflux pumps (i.e., such as CmeABC efflux pumps), and (iv) inactivation or modification of the antibiotic (i.e., β-lactamase production) (Iovine 2013; Wieczorek and Osek 2013; Allos et al. 2015). The purpose of the present review is to highlight the resistance mechanism acquired by *Campylobacter jejuni* against different antibiotics generally used for the treatment of Campylobacteriosis (Figs. 1 and 2).

2 Antibiotic Resistance Mechanism in *Campylobacter jejuni*

2.1 Resistance to Quinolones

The fluorinated 4-quinolones are a group of wide variety of antibiotics that are found effective against various infectious diseases. Few examples of the group include ciprofloxacin, moxifloxacin, and gatifloxacin. These groups of compounds possess carboxylic acid group at 3rd position of primary ring structure, whereas some newly

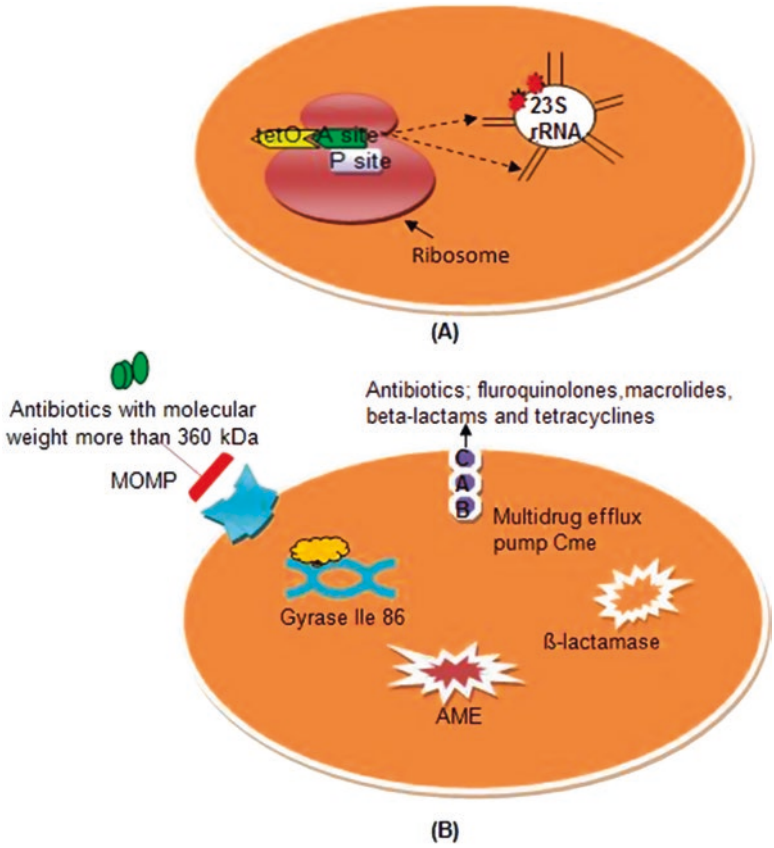

Fig. 2 Mode of antibiotic resistance in *Campylobacter jejuni*. (**a**) The ribosome is the site for the two main mechanisms of antibiotic resistance. Tetracycline is restricted at the A site through the binding of the tetO protein but for continued protein synthesis it still allows the access of tamino-acyl trNA. Point mutation in the domain v region of 23S rRNA (as shown in black) at position 2075 mainly and less frequently at position 2074 (indicated by red stars) decreases macrolides binding affinity and causing resistance toward these antibiotics. (**b**) The entry of most antibiotics having molecular weight larger than 360 kDa or negatively charged is restricted by the two major outer membrane proteins (MOMP). The most common mode of fluoroquinolone resistance is substitution of Thr-86-ile in DNA gyrase that confers high level of resistance toward such antibiotic. The resistance against fluoroquinolones, β-lactams, macrolides, and tetracyclines is contributed by CmeABC; multidrug efflux pump to aminoglycoside-modifying enzymes (AME) such as amino-glycoside phosphotransferase are the major mechanism of resistance of aminoglycoside, while β-lactam resistance is contributed by β-lactamases of the penicillinase type including Ambler class D OXA-61

identified fluoroquinolones antibiotics also have fluorine substituent at 6 position and a piperazine group at position 7 (Pomeri 2011).

The first documentation on quinolone-resistant *Campylobacter* was described during the late 1980s and suggested that such resistance was derived from the

acquisition of fluoroquinolone-resistant strains from animal sources (Iovine 2013). Furthermore, numerous research studies have highlighted that resistances emerged with injudicious usage of antimicrobial drugs particularly fluoroquinolones, in the form of growth supplements in food animals or as therapeutics in poultry flocks (Kovac et al. 2015; Collado et al. 2018; Khan et al. 2018; Wieczorek et al. 2018). It was also found that several clones of *Campylobacter* were selected by the fluoroquinolone drugs suggesting that the resistance did not arise from the spread of single resistant clone (Kovac et al. 2015; Collado et al. 2018).

Fluoroquinolones inhibits synthesis stages of *C. jejuni* DNA that causes cell death. These antibiotics target two important enzymes of bacterial replication machinery, topoisomerase IV, and DNA gyrase which participate mutually in transcription of bacterial DNA, replication repairing of DNA, and its recombination. The products of topoisomerase genes and gyrase are topoisomerase IV and DNA gyrase, respectively, whereas ParC and ParE and GyrA along with GyrB subunits pairs of gyrase and topoisomearse IV, respectively. It is found that the predominant resistance determinants of fluoroquinolone are chromosomally encoded and *Campylobacter jejuni* cells are devoid of plasmid-borne quinolone-resistant determinants like qepA and qnr, aac(6′)-Ib-cr have not distributed. Although the genes that encode topoisomerase IV (parC/parE) enzyme also participate in quinolone resistance in other Gram-negative bacteria, such genes are not present in *Campylobacter jejuni* (Ge et al. 2013; Iovine 2013; Wieczorek and Osek 2013; Gouvea et al. 2015; Tang et al. 2017a, b).

In Campylobacters including *C. jejuni*, the fluoroquinolone resistance is due to point mutations, which occurs through amino acids substitution in the resistance-determining region (QRDR) of quinolone in DNA gyrase A (GyrA) (Iovine 2013; Wieczorek and Osek 2013; Tang et al. 2017a, b). The quinolone-resistance-determining regions (QRDR) are localized inside DNA-binding domain that are making surface core of DNA gyrases (Ge et al. 2013). In *Campylobacter* species, several different single GyrA mutations such as Thr86Ile, Thr86Lys, Thr86Val, Asp90Asn, Asp90Tyr, and Thr86Ala have been recognized which are associated with fluoroquinolone resistance (Iovine 2013; Wieczorek and Osek 2013; Tang et al. 2017a, b). The most commonly observed mutation that leads to Thr86Ile substitution in the DNA gyrase causing high-level resistance to this group of antibiotics is the C257T. The mutations A70T, T86K, and D90N are some other but less commonly reported and do not show significant importance in quinolone resistance as high as found in Thr86Ile mutations (Iovine 2013; Wieczorek and Osek 2013; Tang et al. 2017a, b). A high resistance to ciprofloxacin has been observed due to point mutation Thr86Ile whereas Thr86Ala linked with resistance to nalidixic acid and lower ciprofloxacin resistance (Wieczorek and Osek 2013; Iovine 2013). Furthermore, double point mutations in the gene gyrA together with Asp90Asn or Asp85Tyr or Pro104Ser have also been previously reported (Iovine 2013; Wieczorek and Osek 2013). *C. coli* and *C. jejuni* are devoid of secondary target for the fluoroquinolones antibiotics facilitating the rise of

Table 1 Mode of antibiotic resistance in *Campylobacter jejuni*

Class of drug	Antibiotic	Resistance mechanism
β-lactam	Penicillin, oxacillin, ampicillin	1. Inactivation of the antibiotic by β-*lactamase* enzyme (*penicillinase*, OXA-61)
		2. Decreased membrane permeability of most anionic and Molecular weight higher than 360 kDa antibiotics due to MOMP
		3. Efflux action through CmeABC and possibly others
Fluoroquinolone	Ciprofloxacin, nalidixic acid	1. Modification of the target of enzyme DNA *gyrase* (Thr-86-ile; also Asp-90-Asn, Ala-70-Thr)
		2. Efflux action of CmeAB
Macrolides	Azithromycin, erythromycin	1. Mutations in 23S ribosomal RNA (rRNA)
		2. Contribution of mutations in ribosomal proteins L4/L22 is probably minor
		3. Efflux action through CmeABC and possibly others
		4. Decrease in membrane permeability due to MOMP
Aminoglycosides	Gentamycin	1. Modification of the antibiotic by aminoglycoside-modifying enzymes (AphA, Aade, Sat)
		2. Contribution of efflux action is not clear
Tetracyclines	Tetracycline	1. Modification of the target ribosomal A site by TetO binding
		2. Efflux action through CmeABC and possibly others
		3. Contribution of decreased membrane permeability due to MOMP is not clear

fluoroquinolone-resistant phenotype (Luangtongkum et al. 2009; Iovine 2013; Frasao et al. 2015; Tang et al. 2017a, b) (Table 1).

The decrease in permeability of outer membrane and an extrusion of drugs are two other mechanisms that have been found responsible for quinolone resistance in *Campylobacter jejuni*. The CmeABC multidrug efflux pump has been recognized as the major efflux mechanism that works in coordination with GyrA mutations and leads to antibiotic resistance toward the several antibiotics including fluoroquinolones and macrolides. CmeABC efflux pump is encoded by an operon of cmeC, cmeB, and cmeA genes (Ge et al. 2013; Tang et al. 2017a, b). These three genes encode an outer membrane protein channel, an inner membrane drug transporter, and periplasmic fusion protein, respectively (Iovine 2013; Wieczorek and Osek 2013). In addition, emergence of quinolone resistance during therapeutic treatment has also been well identified. It is predicted that 10% of *Campylobacter* enteritis patients treated with a fluoroquinolone harbor quinolone-resistant strains. This resistance development to fluoroquinolone has reported within 24 h of treatment with this group of antibiotics. However, prolonged treatment with these drugs, particularly in the immunocompromised patients, is also one of the risk factors observed associated with resistance. On the other hand, a naturally existing resistance toward the fluoroquinolone (ciprofloxacin) was also observed in the populations of bacteria found in environmental and food samples. The variation in DNA gyrase (target site of fluoroquinolones) was observed

to greater extent after DNA sequence analysis. It was also reported that there is same type of alterations in the target site sequence as found in isolated strains from clinical sources without applying any selective pressure (Alfredson and Korolik 2007; Weiczorek and Osek 2013; Reddy and Zishiri 2017).

2.2 Resistance to Macrolides

A majority of macrolide antibiotics are produced from Streptomyces and related genera. Erythromycin was the first macrolide antimicrobial isolated from natural product of Saccharopolyspora erythraea (Gaisser et al. 2000). Now the macrolides have become a drug of choice that has been regarded as safe and effective antimicrobial agents against most of Gram-positive as well as Gram-negative microorganisms involving *Campylobacter jejuni* and other members of this genus (Arsic et al. 2017). Macrolide antibiotics are large molecules with molecular weight larger than 700 kilobase (kb). The mechanism of action of these antibiotics is to bind reversibly to the 50S subunit of bacterial ribosomes on the P site and thus inhibiting protein synthesis. The associated members of the macrolides group include erythromycin, clarithromycin, azithromycin, telithromycin (a type of ketolide), tilmicosin, and tylosin. Erythromycin is considered as treatment of choice for campylobacteriosis. The latter two members (Tylosin and tilmicosin) are approved for the use as veterinary medicine (Hao et al. 2016).

The principal mechanisms of resistance to macrolides in *Campylobacter jejuni* include modification of target site and alteration in membrane permeability and efflux pump (Iovine 2013). The high-level macrolide resistance is facilitated by synergistical act of target modification and efflux pump mechanism by the organism. The high-level macrolide resistance has conferred due to point mutations within domain V of the 23S rRNA gene, in the peptidyl-encoding region at the position of 2074 and 2075 (corresponds to the position of 2058 and 2059 in *E. coli* numbering). The substitution at 2075 position has been observed more commonly (Bollinger and Kathariou 2017). Usually, *C. jejuni* comprises of mutated three copies of 23 s rRNA genes present in macrolide-resistant organisms. However, few strains of *C. jejuni* with decreased MICs to macrolides have been reported to carry only two copies of mutated genes and suggested macrolide dosage effect on genes responsible. *C. jejuni* strains having single point mutations in 23S rRNA genes have not been observed. On the other side, mutations (usually insertions) in the L22 ribosomal proteins (insertional mutation at position of 86 or 98) and L4 (G74D) have found responsible to macrolide resistance. However, these mutations are not recognized as major mode in case of tetracycline (Arsic 2012; Weiczorek and Osek 2013).

It is also observed that the greatest hurdle for the generation of macrolide resistance in strains of *Campylobacter jejuni* have been found with increased frequency compared to that of fluoroquinolone resistance. It is observed that tylosin resistance was developed after the administration of this antibiotic as growth promotion dose for several weeks in poultry. It is also found in a competition experiments that macrolide resistance imparts a fitness cost over fluoroquinolone-resistant *Campylobacter*.

These two important factors along with decreased spontaneous mutation frequency foremost to macrolide resistance (approximate 10^{-10}/cell/generation) making them drug of choice for treating complications of Campylobacteriosis. In multidrug efflux pump, a mechanism was adopted by *C. jejuni* against the action of macrolides antibiotics where CmeABC contributes an important role. It performs in a synergistic way with 23S rRNA mutations and exhibits elevated level of resistance toward this group of antibiotics. In macrolide-resistant mutant strains that lack mutations in 23S rRNA gene, antisense-mediated gene silencing of cmeA or disruption of cmeB gene causing deactivation of the CmeABC efflux pumps that leads to reversion into a macrolide-sensitive phenotype. It is also found that CmeG is assumed to act as efflux transporter, also contributing resistance to macrolide that has been examined in insertional mutagenesis experiments in erythromycin resistance vs. the wild-type parent of CmeG. It was revealed from the experiment that CmeG can cause an eight-fold reduction in erythromycin resistance. In *C. jejuni*, another mechanism for macrolide resistance that includes alterations in permeability of outer membrane mediated through expression of major outer membrane porin (MOMP) which is the product of chromosomal porA gene. These porins makes transmembrane pores in outer membrane facilitating the passive diffusion of many antibiotics including hydrophilic molecules in Gram-negative bacteria. The various properties of these pores such as size and ionic charge determine the selectivity for various molecules passing through these pores. In *C. jejuni*, these MOMP forms cation-selective pores which are smaller in size as found in the *E. coli*. Thus, it checks the influx of many antibiotics having molecular weight more than 360 kDa like the case of antibiotics macrolides having molecular weight more than 700 kDa, since macrolides have been found to show significant therapeutic efficacy against the *C. jejuni* strains. Therefore, such drugs must have the potential to cross the barriers of outer as well as cytoplasmic membranes. For this facilitation, these porins make an aqueous environment for the movement of hydrophilic molecules whereas hydrophobic macrolides are relatively considered to gain entry inside the cytoplasm through a "hydrophobic pathway in Gram-negative bacteria" (Iovine 2013).

2.3 Resistance to Tetracyclines

The tetracyclines resistance in *Campylobacter jejuni* is contributed by the (*O*) gene, which is widely distributed in the bacterium (Dasti et al. 2007). These tetracyclines bind to magnesium cations (Mg^{2+}) and pass through the outer membrane porin channels followed by periplasmic space where they dissociate from magnesium and passively travel into the cytoplasm to bind with 30S ribosomal subunit. The primary antimicrobial action of tetracycline is carried out through directed steric hindrance via binding to 30S ribosomal subunit on the A site. Thus, it hinders the easy movement of charged amino acyl transfer RNA (tRNA) and consequently inhibits elongation of peptide. The (*O*) gene encodes ribosomal protection proteins (RPPs), and it is localized on 45–58 kb self-transmissible plasmid. The (*O*) gene has been shown

to provide increased expression of tetracycline resistance (512 mg/L). The studies demonstrate that these RPPs have been found to recognize an open bacterial ribosomal A site and then bind to it. This induces a conformational change and release bounded tetracycline molecule. Tetracycline antibiotics are the subject of ribosomal protection protein (RPP)-mediated resistance that includes Tet(M) and Tet(O). The typical tetracycline binds to ribosome and inhibits elongation phase of protein synthesis by preventing lodgings of the aminoacyl-tRNA (aa-tRNA) on A site of ribosomal. The introduction of new amino acids is, therefore, prevented in the growing polypeptide chain. The presence of an insertion element IS607 showing similarity to IS607 present on the genome of *Helicobacter pylori* has been reported to found on (*O*)-carrying plasmids. Moreover, it is possible that dissemination and acquisition of (*O*) is mediated through mobile genetic elements other than transmissible plasmids. The G-C ratio, hybridization analysis, sequence homology, and codon usage demonstrate that the *Campylobacter* (*O*) gene was probably inherited through horizontal gene transfer from either *Enterococcus* spp., *Streptococcus*, or *Streptomyces*. The (*O*) genes that are showing 75–76% sequence homology with *Streptococcus pneumoniae* tet (M) genes with 40% G-C content (Alfredson and Korolik 2007; Iovine 2013).

2.4 Aminoglycoside Resistance

Aminoglycosides are also inhibitors of protein synthesis in various Gram-positive and Gram-negative bacteria. They are amino-modified sugars with a molecular weight that ranges from 445 to 600 kDa positively charged and hydrophilic in nature. The most commonly used members of aminoglycosides group include neomycin, amikacin, tobramycin, gentamicin, streptomycin, and kanamycin. Firstly, the aminoglycosides bind to the negatively charged membranes of bacteria followed by reversible attachment to the 30S subunit of the ribosome. This second phase of interaction is rapid but reversible compared with initial slow and weak electrostatic interaction. The aminoglycosides are transferred across the bacterial cytoplasmic membranes is with the involvement of electron transport system, ATP and oxygen. The antimicrobial activity of aminoglycosides is contributed by two major modes: (i) interfering with the nascent polypeptide peptide chain translocation from the A to the P site ribosome that leads premature termination of protein synthesis, and (ii) interfering with proofreading activity, leads to incorporation of mismatched amino acids, thus making the protein dysfunctional. In *C. jejuni*, one of the major mechanisms of resistance to aminoglycoside is through the expression of aminoglycoside modifying enzymes that are usually plasmid mediated (Iovine 2013; Garneau-Tsodikova and Labby 2016).

The first incidence of aminoglycoside resistance was detected in *C. coli* which was mediated by aphA-3 gene product, i.e., a 3′-aminoglycoside phosphotransferase enzyme that had been previously known to confer resistance of kanamycin in *Staphylococcus* and *Streptococcus*. This aphA-3 gene is frequent cause of

aminoglycoside resistance in *Campylobacters* including strains of *C. jejuni*. In some of the strains, aphA-3 is found to be localized downstream of IS607, an insertion sequence that shows similarity with IS607 present in *H. pylori*. On the other hand, in some of the strains, aphA-3 is found to be present with genes that encode streptothricin, encoded by sat gene, an acetyl transferase and streptomycin resistance (encoded by aadE, a 6′-adenylyl transferase). The existence of a similar type of resistance cluster present in strain of *Enterococcus* spp., strongly suggests that there is significant contribution of horizontal transfer mechanisms in *Campylobacter* spp. Other strains of *Campylobacter* harbor mosaic plasmids that carry various aminoglycoside resistance determinants along with transposon or insertion sequences of Gram-negative (i.e., *Salmonella*, *E. coli*, and *H. pylori*) and Gram-positive bacteria (i.e., *Enterococcus*) including tetO. The acquired inheritance of resistance plasmids by sensitive *C. jejuni* strains makes a huge repertoire of multidrug-resistant phenotype in the environment that leads a clinical challenge in human as well as veterinary system. Other common genes that provide resistance to kanamycin include aphA-1 and aphA-7. Both types of genes were found on the plasmids of *C. jejuni*. The aphA-7 composed of same G-C content as the chromosomal DNA of *C. jejuni* advocating that such genes intrinsic in *Campylobacter* while aphA-1 and aphA-3 are thought to be acquired by means of horizontal gene transfer. There are few reports available on ribosomal protein S12 mutations (encoded by gene rpsL) in *C. coli* that imparts resistance to streptomycin. However, such type of similar mutations has not been identified in *C. jejuni* strains. Additionally, the significance of efflux pump transporters in resistance of aminoglycoside has not been clear. In a study, it was revealed that putative efflux pump inhibitors phenylarginine-β-naphthylamide and 1-(1-naphthylmethyl)-piperazine did not cause any reduction in the kanamycin minimum inhibitory concentration (MIC) of five *C. jejuni* strains indicating that they are less important compared to plasmid-borne modifying enzyme for aminoglycoside resistance (Alfredson and Korolik 2007; Iovine 2013).

2.5 β-Lactam Resistance

The antibiotics belonging to β-lactam group have variety of chemical compounds which comprises of a β-lactam ring required for their antimicrobial action. The most frequently used members of this group include penicillins, cephalosporins, monobactams, and carbapenems. Each member of this family can be characterized on the basis of presence of various side chains that provides specialized properties such as increased tolerance to stomach acid, pharmacokinetics, and hydrolysis by β-lactamase enzyme. These antibiotics act by binding to bacterial cell wall, inactivating the expression of bacterial peptidoglycan transpeptidase (also referred to as penicillin-binding proteins) that acts as a catalyst in a final cross-linking step of cell wall synthesis. Therefore, resulting effect weakens the cell wall with alteration in its structural integrity that leads to osmotic lysis and consequently cell death. In *Campylobacter jejuni*, the three most common means of β-lactam resistance mechanisms that have been

frequently observed include (i) enzymatic destruction of antibiotics through chromosomally encoded β-lactamase, (ii) alterations in outer membrane porins that leads reduced uptake of drug, and (iii) efflux-mediated resistance. In *Campylobacter jejuni,* the increased expression of β-lactamase (penicillinase-type) corresponds to ampicillin, amoxicillin, and ticarcillin resistance, and such high level of resistance can be overcome by using inhibitors of β-lactamase, i.e., sulbactam, clavulanic acid, and tazobactam. A few years back, OXA-61, a class D β-lactamase, has been found in *Campylobacter jejuni* and other related species that shows resemblance to other type of OXA genes present in *Fusobacterium*, *Pseudomonas*, and *Acinetobacter* and mediating resistance toward penicillin, ampicillin, oxacillin, piperacillin, carbenicillin, and amoxicillin-clavulanate. From the previous studies, it has been postulated that OXA-61 have increased prevalence in human populations including veterinary animals. Consequently, two genes that encodes metallo-β-lactamase type of enzyme have been identified. However, their increased expression leads to β-lactamase resistance is a matter of new findings yet. The data on the ubiquity of resistance to β-lactam is not generally accessible from various antibiotic resistance monitoring authorities such as NARMS, due to no or least use of β-lactams against *Campylobacter jejuni*. In *C. jejuni*, the cation-selective MOMP are responsible for extrusion of most β-lactams which are anionic in nature similar to the macrolides or having molecular weight more than 360. The small molecular size of ampicillin (MW 333 kDa), imipenem (MW 299 kDa), and cefpirome (MW 347 kDa) and presence of partial positive charge are consistent with easy passage through MOMP and susceptibility of such antibiotics in the absence of another mechanism of resistance such as production of β-lactamase. The amoxicillin antibiotic having MW 365 kDa appears to impede the well-organized passage via MOMP. However, its partial positive charge is responsible for the influx through MOMP. Thus, non-MOMP-dependent mechanism may bring out alternatively its entry. Therefore, the efflux pump CmeABC may also facilitate β-lactam resistance. In *C. jejuni* strain 81-176 and another strain cmeB insertional mutagenesis have resulted in approximately 32-fold increase in ampicillin susceptibility. The mutants of cmeB show four times more susceptibility toward ampicillin related to the parent strain found in a study by using NCTC strain 11,168. The overexpression of cmeB may result rise in ampicillin resistance by fourfolds approximately. Similarly, it is also observed in human outbreak of *C jejuni* strain 11,168 and 81-176 that putative efflux pump CmeDEF have been inactivated by means of insertion mutagenesis of cmeF that can only lead to an increase in cefotaxime and ampicillin resistance by twofolds. However, in *C. jejuni* strain 11,168, inactivation of the CmeG efflux pump did not alter resistance of cefotaxime. The conclusion is therefore that putative CmeABC is most powerful efflux pump transporter for β-lactam class of antibiotics (Alfredson and Korolik 2007; Iovine 2013; King et al. 2017; Palzkill 2018).

3 Status of Resistance in *Campylobacter jejuni* to Various Antibiotics

Fluoroquinolone resistance in *Campylobacter jejuni* was first reported in the late 1980s, four decades ago, and since then, it has been increasing regularly in most countries all over the world. The resistance to fluoroquinolone among *Campylobacter jejuni* isolates of animals or food of animal origin and human has been observed. In Asian countries such as India, 80% isolates have been reported to be fluoroquinolone resistant, whereas 77% have been found in Thailand. In China also higher resistance rates of 95.8–99.0% toward the ciprofloxacin antibiotic has been reported or *Campylobacter* isolates isolated from swine. The quite similar resistance incidences of 91% and 85.4% have been observed also in the South Africa and United Arab Emirates, respectively. The emergence of fluoroquinolone resistance in Spain was evaluated between 1993 and 2003 in Europe. The statistically significant increase of 46.7% for nalidixic acid and 52.2% for ciprofloxacin was observed. Similarly, in the United Kingdom, an increase in fluoroquinolone resistance from poultry-isolated strains of *Campylobacter* was also observed, whereas in Poland, 47.9% and 90.2% of these resistant isolates were found ciprofloxacin resistant from 1994–1996 to 2005–2008, respectively. The proportion of ciprofloxacin-resistant human *Campylobacter* isolates in Germany was observed in 41–46%, whereas 42% of *C. jejuni* strains isolated from chicken were ciprofloxacin resistant in 2001. In the mid-1990s, the sarafloxacin and enrofloxacin were permitted in poultry flocks as growth promoters, contributing resistance to fluoroquinolone in the United States, and resistance among *Campylobacter* isolates from humans was expanded from 1.3% to 10.2% between 1992 and 1998. Several studies, on the other hand, show a lack or even low number of *Campylobacter* isolates resistance to fluoroquinolones. In Grenada 9.4% of the strains of *campylobacters* were found resistant only, whereas in Norway, Finland, no strain was found resistant. In another study from Denmark, it was found that resistance rates to tetracycline, nalidixic acid, and ciprofloxacin in travel-associated infections were significantly much more pronounced as compared to acquired infections domestically. From 2006 to 2007, the occurrence of these types of resistance was raised. In Australia, quinolone resistance among *Campylobacter* strains was found low. Furthermore, this resistance is allocated to the rare consumption of antibiotics for the diarrhea treatment and also utilization of fluoroquinolones in food-producing animals (Weiczorek and Osek 2013). In a study conducted by Vaishnavi and her colleagues at Institute Ethical Committee between May 2009 and January 2013 in India, the study was aimed to evaluate the burden of campylobacter infections in northern region of India. The pediatric and adult patients complained with diarrhea were screened in this study. A total of 1145 patients were screened for the isolation of *Campylobacter* species, and in 2.6% samples, the *Campylobacter* species were identified (Mukherjee et al. 2013). After analyzing antimicrobial sensitivity test, it was found that 23.3% isolates of *Campylobacter* species were found ciprofloxacin resistant (Vaishnavi et al. 2015).

Generally, the macrolides are considered as optimal treatment of choice infections associated to *Campylobacter.* Macrolides resistance in human *Campylobacter* isolates, however, has become a serious public health concern in several countries. The resistance of macrolides among *Campylobacter* strains has been found for a longer time at a stable and low level. However, the resistance to the erythromycin and other macrolides in *Campylobacter jejuni* is increasing gradually. As mentioned above, resistance to fluoroquinolone is globally distributed; the macrolides have become therapeutic drug of choice in campylobacteriosis, which also leads to the development of resistance. *Campylobacter* isolates recovered from poultry in China showed 8.9% resistance to erythromycin, and 26.7% and 13.9%, azithromycin and clindamycin, respectively, among *C. jejuni* strains. In Poland, the percentage of *Campylobacter* strains is an intermediate resistance toward erythromycin which has been increased significantly. These poultry isolates of *Campylobacter* were obtained between 1994 and 1996, whereas an increased resistance of 49.3% and 88.9% was observed between 2005 and 2008. In addition to this, *Campylobacter* isolated from human clinical samples have reported to express a decreased level of erythromycin resistance still found in several countries. Recently in northern India a study was conducted by our group where strains of *Campylobacter jejuni* were isolated from poultry meat and its related products. These isolates were assessed for the antimicrobial resistance against eight classes of antibiotics including macrolides by disc diffusion method. The study revealed drug resistance (97.0%) among *Campylobacter jejuni* isolates. Higher resistance (81.1% and 59.4%) was observed toward the cephalosporin (cephalothin) and tetracycline, whereas fluoroquinolone (ciprofloxacin), quinolone (nalidixic acid), macrolide (azithromycin, erythromycin), and aminoglycoside (gentamycin) showed a lower resistance from 6.9% to 8.9% (Weiczorek and Osek 2013; Khan et al. 2018).

The tetracyclines antibiotics were discovered in the late 1940s that showed activity against Gram-positive and Gram-negative bacteria. In the past, because of their heavy usage for both veterinary medications and in humans, their use in present days is somewhat limited (Iovine 2013). Fallon et al. (2003) studied antimicrobial-resistant pattern of 78 *C. jejuni* strains to 8 different classes of antibiotics by disc diffusion assay. The higher rates of resistance were recorded to tetracycline (20.5%). Ge et al. (2002) determined antimicrobial susceptibilities of three seventy-eight *Campylobacter* isolates and found that resistance to tetracycline antibiotics was highest (82%) related to the resistance of doxycycline (77%). In a study, a large-scale survey was conducted in seven European countries during the period from 2004 to 2007 for evaluating the antimicrobial resistance pattern of *C. jejuni*. The average tetracycline resistance varied between 23% and 33% (EFSA 2010). In India, a study was conducted by Khan and his colleague in 2012 for examining the antimicrobial status of *C. jejuni*. They examined thirty isolates of *C. jejuni* against nine antibiotics where azithromycin and nalidixic acid found most effective antibiotics against majority of examined isolates (Khan 2012). The higher resistance was observed against tetracycline (60%). The close similar resistance level was also observed few years back in India (Nigatu 2007).

Campylobacter jejuni causes systematic infection in humans that can be treated with aminoglycosides. Gentamycin (an aminoglycoside) is considered as drug of choice for such treatments. Generally, gentamycin resistance is lower and stable. However, in the

last decade, an increase in gentamycin resistance has marked. A significant resistance (12.2%) was observed in human isolates in the United States in 2011, while a slightly higher resistance (18.1%) was observed in retail isolates (Yao et al. 2017). In Japan, the antibacterial profile was assessed for *Campylobacter jejuni* and *Campylobacter coli* isolates in beef cattle and pigs from 25 farms. A higher resistance (66.6%) was observed in *C. jejuni* isolates against oxytetracycline. None of the isolate was found resistant toward the gentamycin in the survey (Haruna et al. 2012). Similar observation against gentamycin was observed also in Jordan where no resistance was noticed among *Campylobacter jejuni* isolated from layer farms. Highest resistance (100%) toward both ciprofloxacin and tetracycline was observed (Al Natour et al. 2016).

β-lactam class of antibiotics are generally not recommended for treating *Campylobacter* infections; however, due to regular increase in resistance toward the common antibacterial treatment choices, these β-lactams are considered as alternative chemotherapeutic agents. In Australia, the resistance mechanisms of *Campylobacter jejuni* poultry and pig isolates toward the different antimicrobials including ampicillin were assessed. The study showed extensive resistance (33.3–60.2%). The antibiotic resistance gene bla were found among 82.6–92.7% of these ampicillin-resistant isolates (Obeng et al. 2012). Sierra-Arguello and his colleagues observed a high resistance (65%) toward the ampicillin among *Campylobacter* species including *Campylobacter jejuni* isolated from Brazilian broiler slaughterhouses (Sierra-Arguello et al. 2015). A total of 98 *Campylobacter* isolates from vegetable farms and retail farms were assessed for antimicrobial susceptibility in Malaysia. The study suggested that approximate half of the isolates (51.0%) had multiple antibiotic resistance including toward ampicillin. However, study also suggested that the contamination in vegetables was due to cross-contamination and *Campylobacter jejuni* in small-scale vegetable production was less exposed toward the random use of antibiotics (Huat Tang et al. 2016).

4 Conclusion: Problems and Future Concerns

Antibiotic-resistant *Campylobacter jejuni* has been considered as a serious public health problem worldwide due to increased prevalence regularly. This is leading to the significant compromise in chemotherapeutic effectiveness of current antimicrobials used against the human campylobacteriosis treatment. The antimicrobial resistance trends are showing an accessible association between the usage of antibiotics in veterinary industry and human *Campylobacter*-resistant isolates. The dissemination and spread of antibiotic resistance among pathogenic bacteria is mediated through the acquisition of resistance genes by means horizontal gene transfer mechanisms. It is also marked that resistance determinants in *Campylobacter jejuni* can also be from other Gram-positive bacteria in addition to its own *Compylobacter* genus. These genes may incorporate into plasmids or chromosome through integrons or insertion sequences on transposons. It is possible that the spread of these resistance determinants both within and outside of *Campylobacter* genus. In the past few years, it is also observed that *Campylobacter jejuni* may also develop biofilms on various abiotic surfaces that may help in survival in the environment of the bacterium. Therefore, it is possessing

resistance towards the various key antibiotics used against the *Campylobacter jejuni* (Bae et al. 2014; Szcepanska et al. 2017). The resistance incidences to the several key antibiotics playing crucial role in the treatment of *Campylobacter* infections has raising regularly and several resistance patterns to the diverse array of antibiotics are markedly emerging in all over the world. Resistance to fluoroquinolones resistance in *Campylobacter* in many countries has limited their effectiveness as a treatment of choice for *campylobacter* infections. Although the fluoroquinolones are still prevailing drug in several countries such as Australia, for treating campylobacteriosis, resistance toward antibiotic in *Campylobacter* remains a public health challenge. An upgraded research initiatives are therefore essential to understand the underlying factors responsible for persistence and the transmission of *Campylobacter* resistance to fluoroquinolones in various hosts and environmental settings. It is also going to be a matter of interest to identify that how the strength of *Campylobacter* influences resistance to fluoroquinolones. In addition, new fluoroquinolones that show efficacy against *Campylobacter* strains resistant to ciprofloxacin and employing the new schemes of treatment avoiding resistant mutant selection of fluoroquinolones should also be evaluated (Tang et al. 2017a, b).

Similarly, *Campylobacters* erythromycin resistance is raising at the top regularly in some countries. However, erythromycin (macrolide) incidence of resistant *Campylobacter* in human is still somewhat low and stable. The drug is also considered as the choice of medication for the treating the *Campylobacter jejuni* infection currently. More studies are required to get deep insights that how under the selective pressure of macrolide *Campylobacter jejuni* resistance emerges. Gentamycin also found effective antibiotic against *Campylobacters*. The resistance of *Campylobacter jejuni* to specific antibiotics or multiple antibiotics could be due to dissemination of resistance patterns in the environment increasing by the misuse of antibiotics among the general population. Veterinary prescription of antimicrobials is also contributing the problem of resistance (ElHadidy et al. 2018). An estimated 50% in tonnage of total antimicrobial production in North America and Europe is used in poultry and food-producing animals. Such worldwide consumption of antimicrobials for the control of disease and growth promoters in animals has been identified as paramount cause of resistance emergence in bacteria that can disseminate from animals, often through food products that leads to infections in human. The prevalence of foodborne-resistant *Campylobacter jejuni* has increased due to increase in number of foodborne infections and the corresponding use of antimicrobials also. Global concern over the use and misuse of antimicrobials and subsequent emergence of antibiotic-resistant microbe has increased during the last decade. In developing countries, constant and irrational consumption of antibiotics in both human medicine and veterinary coupled with current understanding of transfer of antibiotic resistance between different bacteria making it important for monitoring the susceptibilities of pathogenic bacteria toward an array of antibiotics (Okeke and Edelman 2001; Bisht et al. 2009). The environmental eco-social status overcrowding, close association between human and animals, and poor hygienic conditions in the area of study might have significantly contributed role in dissemination of *Campylobacter jejuni* in developing countries like India. The transmission of the bacteria in food chain and indiscriminate use of antibiotics might

have resulted in increased emergence of resistance to common antibiotics used in veterinary and medical sciences (Vila and Pal 2010). Since multiple type of antimicrobials clinical treatment of campylobacteriosis are redundant, new generation of antibiotics and novel treatment schemes must be evaluated to prevent the fluoroquinolone-resistant mutant selection. It is expected the advanced molecular approaches, like proteomics and genomics, could provide deeper insights into the mechanistic view of molecular machinery involved in antimicrobial resistance development in *Campylobacter*. Some important steps can be followed to limit the dissemination of antibiotic resistance such as:

(i) Use antibiotics only when prescribed by a certified health practitioner and follow the complete prescription.
(ii) Never use leftover antibiotics and do not share them with the others.
(iii) Prevent infections by regular washing of hands and keep important vaccination up to date.
(iv) The antibiotics used for treating infectious diseases in animals should be given under supervision of veterinary practitioner.
(v) Vaccinate the animals to reduce the need for antibiotics and develop the other alternatives also.
(vi) Introduce good practices at all stages of food production and processing from both plant and animal sources.
(vii) Implement the international standards or guidelines proposed by WHO, FAO, or any national organization for using antibiotics.

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Mechanisms of Biofilm Development, Antibiotic Resistance and Tolerance and Their Role in Persistent Infections

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Abstract

Bacteria frequently form biofilms in response to stress factors that include exposure of planktonic cells to subinhibitory concentrations of antibiotics. When these attach to a surface, they switch to the biofilm mode of growth and undergo a phenotypic shift in behaviour. During this process, a large suite of genes are differentially regulated to develop a biofilm, which protect them from killing by antibiotics. This leads to the persistence of biofilm infections and the mechanisms used to protect bacteria in biofilms distinct from those that are responsible for conventional antibiotic resistance as well as tolerance. This tolerance to antibiotics is contributed to by multiple factors such as poor antibiotic penetration, nutrient limitation adaptive stress responses, slowed metabolism and the formation of persister cells. The present chapter deals with the introduction to biofilm and their mechanism to achieve antibiotic resistance as well as tolerance properties including their role in persistent infection with some advancement in biofilm research.

Keywords

Biofilm · Tolerance · Persistence · Antibiotic resistance · Challenge in chemotherapy

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1 Introduction to Biofilm

A biofilm is community of microorganisms where cells are stuck to each other and often also to a surface. These adherent cells become embedded within a slimy extracellular matrix that is composed of extracellular polysaccharides also called extracellular polymeric substances (EPS) (Chakraborty et al. 2018). Cells associated with a biofilm produce a polymeric conglomeration of EPS, DNA and proteins. They have been metaphorically described as "cities for microbes" as they have three-dimensional structure and represent a community lifestyle for microorganisms.

Biofilms may get developed on any surface including living as well as non-living surfaces. They can be prevalent in natural, industrial, hospital settings and public sectors. The new microbial cells in a biofilm are not as physiologically same as planktonic cells of the same organism. Unlike biofilms, planktonic cells are single cells that may float or swim in a liquid medium (O'Toole and Kolter 1998).

Microbes are governed by many different factors to form biofilms (O'Toole and Kolter 1998) which may include cellular recognition of specific as well as nonspecific attachment sites on a surface or by the exposure of planktonic cells to subinhibitory concentrations of antibiotics (Hoffman et al. 2005). Cells undergo a phenotypic shift in behaviour in which large suites of genes are differentially regulated when they switch to the biofilm mode of growth (An and Parsek 2007).

A biofilm may be considered a hydrogel that is a complex polymer containing many times its dry weight in water. Biofilms are entire biological systems where bacteria organize themselves into a coordinated functional community. These biofilms can attach to a surface such as a tooth, rock or virtually any surface and may include a single species or a diverse group of microorganisms. A biofilm usually begins to form when a free-swimming bacterium attaches to a surface, multiples and begins to produce EPS.

1.1 IUPAC Definition of Biofilm

"*Aggregate* of microorganisms in which all cells that are embedded within a self-produced matrix of extracellular polymeric substances (EPSs) adhered to each other and/or to a surface." EPS is a polymeric conglomeration which is composed of extracellular *biopolymers* in various structural forms and also referred to as slime (Vert et al. 2012).

1.2 Formation of Biofilms

The formation of a biofilm begins when free-floating microorganisms attach to a surface and/or adhere to each other (O'Toole and Kolter 1998). This process starts with the first colonist bacteria, which adheres to the surface initially through weak, reversible adhesion via van der Waals forces (Danhorn and Fuqua 2007). If the

colonists are not separated from the surface, then they can anchor themselves permanently using pili which are cell adhesion structures.

Bacteria with high hydrophobicity may have reduced repulsion between the bacterium and the extracellular matrix (Danhorn and Fuqua 2007), *reducing biofilm formation. While a* few species of bacteria are not able to attach to a surface of their own due to their limited motility, they may be able to anchor themselves onto the matrix or to bacteria colonists. In general, non-motile bacteria cannot recognize surfaces or aggregate together as easily as motile bacteria (Danhorn and Fuqua 2007).

During surface colonization, bacterial cells are capable of communicating using quorum-sensing (QS) signals such as N-acyl homoserine lactone (AHL). Quorum sensing (QS) enables transfer of information connected with biocidal agents' resistance and the mechanisms of their activation. Thus, biofilm-forming bacteria are the cause of many chronic, recurrent and persistent infections (Karthik et al. 2018). Once colonization has taken place, a combination of cell division and recruitment leads to further biofilm maturation. Matrices cover the entire bacterial biofilms with polysaccharides and some other component which may also contain material including components of blood as erythrocytes and fibrin and soil particles. The final stage of biofilm formation is also known as dispersion where the biofilm is completely established and planktonic cells may be released either actively or passively.

Steps Involved

Following are the five major stages of biofilm development (Monroe 2007):

1. Attachment
2. Microcolony formation
3. Three-dimensional structure formation and maturation
4. Detachment

1.2.1 Attachment

Bacteria make a reversible connection with the surface and/or already adhered other microbe to the surface to form biofilms and a solid–liquid interface develops at that place. This interface can provide an ideal environment for microorganism to attach and grow (e.g. blood, water) (Costerone et al. 1999). Rough, hydrophilic and coated surfaces will provide better environment for biofilm formation. Nutrient concentrations, flow velocity and temperature play an important role in increased attachment; apart from these factors, occurrence of locomotor structures on cell surfaces is also important and may possibly provide an advantage in biofilm formation when there are mixed community and these structures include flagella, pili, fimbriae, proteins or polysaccharides (Donlan and Costerton 2002).

1.2.2 Microcolony Formation

As bacteria get adhered to the physical surface or some biological tissue with a stable binding, formation of microcolony takes place. This microcolony is developed from multiplication of bacteria in the biofilm which starts as a result of

chemical signals. The production of exopolysaccharide is activated at molecular level production of exopolysaccharide is activated when intensity of the signal cross certain threshold; therefore the bacterial cell divisions take place within the embedded exopolysaccharide matrix, which finally result in microcolony formation by this way using such chemical signal (Mckenney 1998).

1.2.3 Three-Dimensional Structure Formation and Maturation

The stage of microcolony formation to develop biofilms is followed by expression of certain biofilm-related genes, and these gene products are required for the formation of EPS as the main structural material of biofilm. It has been reported that bacterial attachment by itself can trigger formation of extracellular matrix which is later followed by water-filled channels formation for transport of nutrients within the biofilm. Researchers have proposed that these water channels are like circulatory systems, distributing different nutrients to microcolonies and also removing waste materials from the communities in the microcolonies of the biofilm (Parsek and Singh 2003).

1.2.4 Detachment

Once the biofilm formation is completely done, the researchers have often noticed that bacteria present in microcolony now leave the biofilms itself on regular basis, and by doing this, the bacteria can undergo rapid multiplication followed by dispersal. A natural pattern of programmed detachment occurs for the detachment of planktonic bacterial cells from the biofilm. Due to some mechanical stress, it has been reported that sometimes the bacteria get detached from the colony into the surrounding while in most cases some bacteria stop EPS production and they get detached into environment. There are two ways for dispersing of biofilm cells; it may occur by either detachment of new formed cells from growing cells or dispersion of biofilm aggregates due to flowing effects or due to quorum sensing (Baselga et al. 1994). In biofilm, cells are removed from surface due to an enzyme action that causes alginate digestion. Phenotypic characters of organisms are apparently affected by the mode of biofilm dispersion. The dispersed cells have the ability to retain certain properties of biofilm like antibiotic insensitivity. The cells after getting dispersed form biofilm due to growth return quickly to their normal planktonic phenotype.

2 Properties of Biofilms

Biofilms are present as solid substrates submerged in or exposed to an associated solution. Once microorganisms get enough nutrients, a biofilm can quickly grow to be megascopic (visible to the naked eye). These biofilms consist of living organisms like bacteria, archaea, protozoa, fungi and algae; each group performs specialized metabolic functions. The social organization (cooperation/competition) within a biofilm depends on the various species present (Nadell et al. 2009).

3 Extracellular Matrix

The extracellular matrix consists of exopolysaccharides (EPS), proteins and nucleic acids (Branda et al. 2006). A large proportion of this matrix consisting EPS is more or less strongly hydrated; however, cellulose is one example of hydrophobic EPS which is produced by a range of microorganisms. The cells are encased by the matrix and present within the matrix and also facilitate communication among them through gene exchange as well as biochemical signals. Along with the cells, the EPS matrix also traps extracellular enzymes to keep them in close proximity to the cells; therefore, this matrix shows an external digestion system, and it allows for stable synergistic micro-consortia composed of different species (Wingender and Flemming 2010). Some biofilms contain water channels to facilitate nutrients distribution and signalling of biomolecules (Stoodley et al. 1994). This EPS containing extracellular matrix is strong enough; under certain conditions, biofilms can become fossilized (stromatolites).

Bacteria living in biofilms display different phenotypes from free-floating microorganisms of identical species, because the dense and guarded atmosphere of the biofilm permits them to associate and move in different ways (Vlamakis et al. 2008). One major advantage biofilms provide is an increased tolerance to detergents and antibiotics. This is due to many factors, including the dense extracellular matrix and outer layer of cells that shield the inside of the community. In some cases, antibiotic tolerance is increased a thousandfold (Stewart and Costerton 2001). Horizontal gene transfer is commonly expedited inside biofilms (Chimileski et al. 2014), which can increase the sharing of antibiotic resistance mechanisms (Molin and Tolker-Nielsen 2003). Extracellular DNA is also a major structural element of biofilms (Jakubovics et al. 2013) *and is therefore subject to* enzymatic degradation, which can weaken the biofilm structure and unharness microbial cells.

However, it has been observed that biofilms are not always less susceptible to antibiotics like the biofilm form of *P. aeruginosa* has no greater resistance to antimicrobials than do stationary-phase planktonic cells, although when the biofilm is compared to logarithmic-phase planktonic cells, the biofilm does have greater resistance to antimicrobials. This resistance to antibiotics in both stationary-phase cells and biofilms may be due to the presence of persister cells (Spoering and Lewis 2001).

4 Diversity of Biofilm Based on Taxonomy

Many different types of bacteria form biofilms. Some examples of biofilm-forming gram-negative species are *Pseudomonas aeruginosa or Escherichia coli*, while biofilm-forming gram-positive bacteria are *Listeria monocytogenes*, *Staphylococcus* spp., *Bacillus* spp and lactic acid bacteria, including *Lactobacillus plantarum* and *Lactococcus lactis*. Some bacteria form biofilms in aquatic environments like *Cyanobacteria* (Danhorn and Fuqua 2007).

Biofilms are formed by bacteria that colonize plants, e.g. *P. putida*, *P. seudomonas* and other related pseudomonas which are common plant-associated bacteria found on leaves, roots and in the soil, and the majority of their natural isolates form biofilms. Several nitrogen-fixing symbionts of legumes such as *Rhizobium leguminosarum* and *Sinorhizobium meliloti* form biofilms on legume roots and other inert surfaces (Joubert et al. 2006).

Along with bacteria, biofilms are also generated by archaea and by a range of eukaryotic organisms, including fungi, e.g. *Cryptococcus laurentii* (Joubert et al. 2006), and microalgae. Among microalgae, one of the main progenitors of biofilms are diatoms, which colonize both fresh and marine environments worldwide (Carl et al. 2014; Aslam et al. 2012).

5 Mechanism of Antibiotic Resistance

Bacterial biofilms are known for their tolerance to antibiotics and their reactive molecules which are produced by the host immune systems than planktonic bacteria. It has been estimated that the antibiotics resistance in biofilm cells can be up to 10,000 times more than the antibiotics resistance in planktonic cells (Costerton et al. 1995; Nickel et al. 1985). The antibiotic resistance was observed when Costerton and co-workers treated *P. aeruginosa* biofilm and planktonic cells with tobramycin, and then they found the planktonic cells could not survive greater than 50 μg/ml tobramycin whereas the biofilm cells could tolerate 1 mg/ml tobramycin (Nickel et al. 1985). Later, Abee and co-workers reported that with two different disinfectants, benzalkonium chloride and the oxidizing agent sodium hypochlorite, the effective inhibitory concentrations on *S. aureus* biofilms are 50 and 600 times higher than planktonic cells, respectively (Luppens et al. 2002). The reason(s) for increased antibiotic resistance by bacterial biofilms is/are not yet fully understood, but it is highly probable that multiple factors work together to protect biofilm cells from antibiotic treatment. Some of the possible mechanisms for antibiotic resistance exhibited by bacterial biofilms are discussed below.

5.1 EPS Matrix Protection

EPS matrix plays an important role in antibiotic resistance by limiting antimicrobial agents' penetration into the biofilm. Charged polysaccharides and eDNA can trap several kinds of antibiotics. The penetration property of antibiotics has been measured by the concentration at the base of the biofilm by Suci and co-workers. Results showed that ciprofloxacin concentration in *P. aeruginosa* biofilm was dramatically reduced, but not completely blocked (Suci et al. 1994). Steward and co-workers investigated the penetration limitation of ampicillin and ciprofloxacin on *K. pneumoniae*. Ciprofloxacin has a

much better penetration capability than ampicillin. As a result, biofilm cells could tolerate concentrated ampicillin, but their resistance to ciprofloxacin is poor (Anderl et al. 2000).

5.2 Horizontal Gene Transfer

Some bacteria can acquire antibiotic resistance via random mutations on genes. Others also harbour antibiotic-resistant genes on plasmids. Plasmids can be easily passed on to other cells by horizontal gene transfer. In biofilms, the frequencies of horizontal plasmid transfer are much higher than between planktonic cells. Studies on *S. aureus* biofilms showed that biofilms promote the spread of plasmid-borne antibiotic resistance genes by conjugation/mobilization (Savage et al. 2013).

5.3 Reduced Growth Rate

There is limited availability of oxygen and nutrients inside biofilms, so biofilm cells, especially those in the deep layers, have a slow metabolic rate, as well as growth rate and division rate. These features make biofilm bacteria insensitive to antibiotic drugs that target dividing cells. For example, the targets of β-lactams are dividing cells, so when they are used on *E. coli* biofilms, their bacteriolytic activity is diminished (Ashby et al. 1994).

5.4 Persister Cells

In biofilms, there is a small subpopulation of cells called persister cells (Lewis 2007; Keren et al. 2004). Their growth rate is zero or extremely slow. Most of the antibiotics that are currently used in the clinic, which target processes that are relevant for cell growth or division, are not effective against persister cells. Therefore, these cells act as disease reservoirs that could reactivate into infectious particles once the antibiotic stress has been removed.

5.5 Efflux Pumps

Efflux pumps allow bacteria cells to pump intracellular toxins out, including antibiotic drugs. Efflux pumps are also expressed in planktonic cells, but some efflux pump genes are upregulated in biofilm, indicating that they contribute to antibiotic resistance. Zhang and co-workers identified a novel *P. aeruginosa* efflux pump gene PA1874–1877, and the expression level of PA1874–1877 gene in biofilm is much higher than in planktonic cells (Zhang and Mah 2008). Efflux pump encoded by this gene increases the resistance to tobramycin, gentamicin and ciprofloxacin.

5.6 EPS Matrix Protection

EPS matrix provides physical protection to the aggregated biofilm cells. Lei and co-workers showed that exopolysaccharide alginate in *P. aeruginosa* biofilms kept biofilm bacteria from human leukocyte killing (Leid et al. 2005).

6 Mechanism of Antibiotic Tolerance

The antibiotic tolerance mechanisms in biofilms include failure of antibiotics to penetrate biofilms (Jolivet-Gougeon and Bonnaure-Mallet 2014), slow growth rate, altered metabolism, persister cells, oxygen gradients and extracellular biofilm matrix (Römling and Balsalobre 2012).

6.1 Failure of Antibiotics to Penetrate Biofilm

Antimicrobials may be prevented from penetrating the biofilm by its matrix acting as a barrier. Although prevention of penetration is no longer believed to be a significant factor, antibiotics can be prevented from penetrating if they bind to components of the biofilm matrix or to bacterial membranes (Walters et al. 2003; Chiang et al. 2013). Positively charged antibiotics such as aminoglycosides and polypeptides that bind to negatively charged biofilm matrix polymers are delayed in their penetration through biofilm. Besides, it is possible for biofilm to find retention places on medical or dental devices, where it is protected from antibiotics. It should also be recognized that the high density of bacteria in biofilms increases the selection of resistant bacteria under pressure from antibiotics by enhancing horizontal gene transfer and the frequency of mutation (Lazăr and Chifiriuc 2010).

6.2 Nutrient-Deficient Environment

Multiple microcolonies in the biofilm create a metabolically heterogeneous bacterial population (Hall-Stoodley et al. 2004; Lenz et al. 2008). In such environments, local diffusion gradients are developed, causing anoxic and acidic zones in the interior of the biofilm (de Beer et al. 1997). Zones that are nutrient-deficient can produce stationary phase-like dormant cells, which may be responsible for the general resistance of the biofilm to antibiotics (Walters et al. 2003; Fux et al. 2004). It is thought that limited penetration of nutrients rather than restricted access for antibiotics contribute to the general tolerance seen in biofilms towards antibiotics (Hall-Stoodley and Stoodley 2009).

6.3 Altered Metabolism

Within the biofilm population, cells with diverse genotypes and phenotypes coexist. This implies that distinct metabolic pathways are expressed based on the local environmental conditions in the biofilm. The metabolic activity of bacteria is high in the outer part of the biofilm, while it is low in the inner part (Werner et al. 2004; Pamp et al. 2008). The difference in physiologic activity is caused by limited oxygen and nutrient penetration due to consumption by bacteria present (Hall-Stoodley and Stoodley 2009). When bacteria grow in a biofilm, they may experience antibiotic tolerance through nutrient deprivation, which causes slow bacterial growth or starvation (Xu et al. 2000). Many antibiotics are directed against processes occurring in growing bacteria, such as replication, transcription, translation and cell wall synthesis. Therefore, increased antimicrobial tolerance will occur in biofilm bacteria with low metabolic activity located in the inner part of biofilms (Ciofu et al. 2014). As an example, the starvation of most amino acids, especially leucine, cysteine and lysine, or of glucose was found to induce biofilm tolerance to ofloxacin (Hall-Stoodley and Stoodley 2009).

6.4 Oxygen Gradients

The oxygen tension in the depth of biofilm is low. In *P. aeruginosa*, hypoxia increased antibiotic resistance through altering the composition of multidrug efflux pumps (Schaible et al. 2012). Microenvironmental hypoxia contributes significant development of antibiotic resistance in *P. aeruginosa* infecting cystic fibrosis patients. It has been found that in *P. aeruginosa* biofilms grown in vitro for 48 h with different antibiotics, oxygen limitation accounted for 70 % (depending on the antibiotic) or more of all the antibiotic tolerance (Borriello et al. 2004). It was found that oxygen penetrated about 50 μm into biofilm with an average thickness of 210 μm. 48-h colony biofilms were physiologically heterogeneous and most of the cells occupied an oxygen-limited stationary phase. The anaerobic environment within biofilms will most likely affect aminoglycoside antibiotic activity due to the downregulation of energy metabolism genes (Kindrachuk et al. 2011) and by triggering changes in gene expression (Taylor et al. 2014).

6.5 Presence of Persister Cells

Till date, there are no antibiotics that can kill all the pathogenic microorganisms; some organisms are always left unaffected after antibiotic treatment so-called persisters. This is a small subpopulation of bacteria that has entered a slow-growing or starving state and that is highly tolerant to be killed by antibiotics (Lewis 2012;

Hu and Coates 2012). The reduced metabolic rates of these cells make them less sensitive to antibiotics compared to active, exponential growth-phase bacteria. Because antibiotics must work on growing cells to destroy them, the hibernating cells can outlast the antibiotic and then repopulate the infectious site. This occurs especially in sites where immune components are limited, such as in biofilm. Thus, persisters are considered to be significant contributors to the persistence of biofilm infections.

7 Role of Biofilm in Persistent Infections

Nowadays, persistent infections have become a global challenge for human beings, claiming millions of lives every year and demanding huge medical and social resources. The host suffering tries to eliminate a pathogen while the pathogen tries to survive in the host; therefore, the development of persistent infections has been exemplified as a game of "Cat & Mouse". The simplest survival strategy employed by bacteria pathogens is to form a biofilm which is an amorphous and dynamic structure, and this biofilm is not only resistant to antibiotics but also resistant to host immune clearance. The biofilms provide an important reservoir of cells that can repopulate colonized sites and can lead to bacterial infections caused by different bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*) in cystic fibrosis pneumonia (Singh et al. 2000), *Escherichia coli* (*E. coli*) in urinary tract infections (Anderson et al. 2004) and *Mycobacterium tuberculosis* (*M. tuberculosis*) in human tuberculosis (Ojha et al. 2008). Biofilms are also responsible for persistent *Streptococcus mutans* (*S. mutans*) infections on tooth surfaces and the most nosocomial infections are persistent biofilm infections (Costerone et al. 1999; Costerton et al. 2003). It is estimated that, in developed countries, over 60% of treated infectious conditions are caused by biofilm formation. As a correlation between biofilm formation and bacterial persistence has been established (Balaban et al. 2004), the possibility of using drugs targeting biofilm formation in combination with the current antibiotics is emerging as a potential therapeutic approach for this type of bacterial persistent infection. Several anti-biofilm and/or biofilm control strategies, such as anti-adhesion, quorum-sensing disruption and selective targeted antimicrobial peptides, have been recently developed.

8 Advancements in Biofilm Research

Mostly it has been observed that the biofilm formation of infectious significance is found on "implant devices". The 2nd most common reason for ventilator-associated pneumonia (VAP) and catheter-associated urinary tract infections (CAUTI) was found due to *P. aeruginosa* which forms biofilms on endocardial tubes and catheters in CAUTI and VAP patients (Muhsin et al. 2018). Whereas other recent advancement in biofilm research has been applied to control energy crises as this approach is using microbial fuel cells (MFCs). These MFCs produce electricity, and they are

utilizing chemical energy found in organic and some inorganic compounds. Electrogenic microbes play a role in this process by accepting or donating electrons to an external object (electrode), while some non-electrogenic microbes are also involved as part of a synergistic electrogenic biofilm.

Another biofilm-related problem is caused by *Asaia* species, which form biofilms on plants used for the production of soft drinks, and may thereby contaminate the soft drinks even in the presence of a preservative. Additionally, biofilm resistance against antibiotics has reached an alarming state. Antibiotic therapy is not effective once the biofilm is matured. The Chinese medical herb i.e. *Herba patreniae* which degrades the mature biofilm of *P. aeruginosa* and its exopolysaccharide. A biofilm-growing mucoid strain can play a role in the exacerbation of cystic fibrosis. There are several antimicrobial agents used to treat this biofilm-forming strain of *P. aeruginosa*. For example, Ciprofloxacin has been shown to kill the bacteria found on the surface of the biofilm, whereas Colistin was shown to kill the ones found in the depth of the biofilm. There may also be many other possibilities that can be applied in the journey of treatment of biofilm-related infections some examples can be like inhibiting quorum sensing through breaking of matrix by alginate *lyase* or F-actin. For bacterial biofilm formation, quorum-sensing activity is very important, as revealed by genetic analysis of biofilms. There are many identified molecules produced by eukaryotes and prokaryotes to quench the quorum sensing, i.e. quorum quenching (Muhsin et al. 2018; Paramasivam et al. 2017).

9 Conclusions

Biofim formation is a two-stage biological process which is controlled by surface adhesions and cell-to-cell communication pathways. The aggregated bacterial cells which are protected and/or coated by extracellular matrix are insensitive to both nutritional stimulation as well as hostile attacks. In the human body, biofilms may trigger persistent infections with chronic inflammation. There is no single mechanism for antibiotic-induced biofilm tolerance or resistance, although a global response to various forms of stress may be a significant denominator. Probably, multiple mechanisms of tolerance and resistance act together, causing an increased overall level of resistance and tolerance. In this context, newly recognized genes for biofilm-detected antibiotic tolerance and resistance seem to be particularly important fellow players. Most studies on biofilm-induced resistance have been undertaken with *P. aeruginosa*. Such studies, although fundamental for our dawning knowledge of biofilm-specific antibiotic tolerance and resistance as such, may have limitations. The results from *P. aeruginosa* cannot uncritically be extrapolated to other forms of biofilm-induced chronic diseases which, often, are affected not by biofilms of single but of multiple species, where cells are subjected to a plethora of signals. However, *P. aeruginosa* can serve as a valuable model system for delineating biofilm-specific antibiotic tolerance and resistance mechanisms, and the results achieved with this bacterium should initiate studies on multispecies biofilm models in vitro and in vivo. A major setback in the treatment of

biofilm-related infections is the ineffectiveness of existing antibiotics due to the protective layers built by cells in the biofilm. There is therefore limited antibiotic penetration, so the community of sedentary cells persists even in the presence of antibiotics effective against their motile counterparts. The dispersion of matured biofilms may primarily require the disruption of the EPS matrix. Components of the EPS matrix such as alginate, Pel and Psl in *P. aeruginosa* biofilms could, therefore, be vital targets in the disruption of biofilm structure. With cells exposed, the drug compound may then exhibit bactericidal activity or act by dispersing the cells. On the other hand, the formation of new biofilms could be inhibited by preventing initial processes like attachment of cells to surfaces. This would be crucial in the development of medical and dental implants as they are easily colonized by biofilm-forming pathogenic bacteria. Nevertheless, the gap between observations made from an in vitro model and an in vivo model continues to be problematic. Choice of animal model, depending on how closely related the model is to humans, would be a critical parameter in the successful development of any anti-biofilm drug. Biofilm formation on indwelling medical devices greatly affects surgical and instrumental procedures and public health as well. It also has implications in non-device-related human-health complications. There is a need for an in-depth research to optimize measures for its prevention. Good hygienic conditions and practices are very necessary to avoid biofilm formation. With the passage of time, and with the advent of new technologies, progress has been made to remove and control biofilm-associated infections. However, new anti-biofilm strategies are necessary to handle biofilm-associated chronic infections.

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Developing In Vivo Infection Models with MDR Pathogens for Evaluating Compound Efficacy

Andrea Marra

Abstract

A key challenge in drug discovery is predicting the outcome between in vitro activity and clinical efficacy, but in antibiotic discovery, animal models do a credible job of bridging that gap. The ability to utilize the same epidemic, resistant strains and model dosing regimens to those of humans as well as the ability to target specific tissue sites is a powerful tool for designing clinical trials.

Keywords

Animal infection models · Multidrug-resistant pathogens · Antibiotic discovery

1 Introduction

In drug discovery, a key challenge is bridging the gap between in vitro activity and clinical efficacy; in antibiotic discovery, it is generally believed that animal models do a credible job of predicting clinical outcomes, given that these models can utilize the same strains responsible for disease, can determine the driver of pharmacokinetic/pharmacodynamic efficacy, and the magnitude required for a desired effect. Animals models can be designed for a specific infection site; they can be utilized to isolate resistant mutants, and they can be established using humanized dosing regimens. As such, animal models of infection can be a highly valuable and predictive bridge between in vitro drug discovery and early clinical evaluation.

A particular challenge not necessarily unique to antibacterial drugs is that of resistance; so, although it is the case that antibiotics are the only drug class that can cure their disease, they are in fact on a deadline from their very first use due to microbes' innate ability to develop resistance against them. Bacterial resistance to

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antibiotics is rapidly emerging as a major worldwide health threat, with the ESKAPE pathogens (*Enterococci*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Escherichia coli*) and multidrug-resistant (MDR) *Enterobacteriaceae* leading the pack in fending off the few treatment options that remain available to clinicians. In the United States alone, over 2 million illnesses and 23,000 deaths each year occur due to infections by resistant bacteria (Centers for Disease Control and Prevention 2013). *K. pneumonaie* carbapenemase (KPC) is rapidly spreading among *Enterobacteriaceae*, *P. aeruginosa*, and *A. baumannii* and has become endemic in many hospitals and long-term health-care facilities around the world (Endimiani et al. 2011). These are significant as they are resistant not only to most β-lactams and β-lactam-β-lactamase inhibitor combinations but to other antibiotic classes as well, including fluoroquinolone and aminoglycosides. In addition, the increase in MDR *Enterobacteriaceae* that harbor transmissible elements with diverse resistance determinants is troubling; particularly concerning are carbapenem-resistant *Enterobacteriaceae* (CRE) (Potter et al. 2016) and extended-spectrum β-lactamases (ESBL), the latter of which have been shown to carry along with them genes conferring resistance against up to eight antibiotic classes (Woodford et al. 2009; Coque et al. 2008). The $bla_{\text{CTX-M-15}}$ ESBL is a major cause of resistance to β-lactam antibiotics among *Enterobacteriaceae*, and *E. coli* isolates with this ESBL are major causes of community-acquired UTIs, with an associated 60% mortality rate. These isolates are also becoming more resistant to carbapenems, which can arise due to porin mutations and the acquisition of carbapenemases.

These organisms can cause a broad range of serious infections, including those of the respiratory and urinary tracts, intra-abdominal infections, and bacteremia. Data from the National Healthcare Safety Network indicates that up to 20% of all hospital-acquired infections were MDR; these have been steadily increasing over the past decade and it is clear that MDR pathogens are associated with higher morbidity and mortality and increased medical costs compared with susceptible organisms. The lack of available agents to treat these dangerous infections has resulted in the use of colistin and/or combination therapy involving an aminoglycoside antibiotic; this approach is often problematic due to efficacy, pharmacokinetics, and safety issues (Thaden et al. 2017).

2 Benefits and Utility of Animal Infection Models for Antibacterial Discovery

It is difficult to overstate the benefits of in vivo models to antibacterial discovery and the myriad ways they can contribute to our understanding of the host-pathogen relationships and responses. Antibiotics are the only curative drug class, and infection models are particularly relevant as they closely mimic the human disease. As outlined in Fig. 1, the main advantage of these models relies on the ability to utilize the causative agents of human infection, and from there a great deal of information

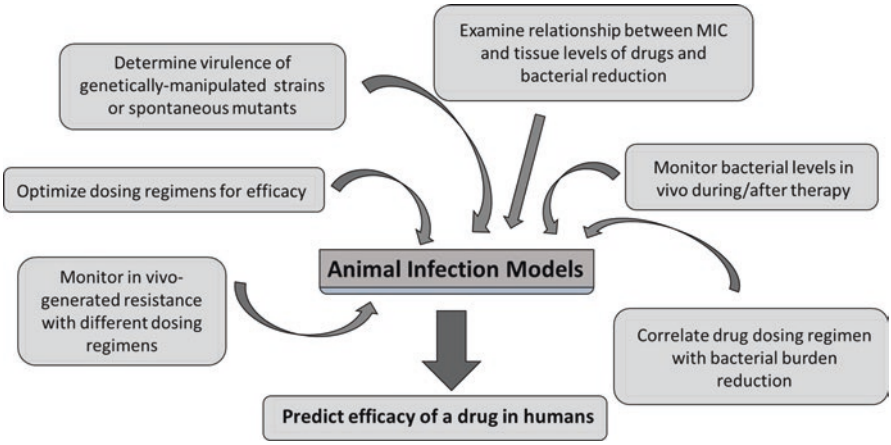

Fig. 1 Utility of animal infection models

may be gained, including the relationship between MIC and tissue levels of drugs and bacterial reduction; the correlation of dosing regimen with bacterial reduction; the development of resistant mutants with different dosing regimens; the kinetics of bacterial reduction after dosing; and how different mutations affect virulence. All of this information can aid in predicting how a drug will behave in a patient.

Prior to any studies involving animals, as much data as possible should be generated in vitro to help set doses and gain a better understanding of how an antibiotic will behave in vivo. Studies such as time-kills and hollow-fiber models using several strains encompassing a broad range of MIC values for the antibiotic of interest will be beneficial in that regard and should also help to prioritize compounds for evaluation in animal models.

3 The Challenge of MDR Pathogens

Access to a collection of recent clinical isolates is a valuable resource for evaluation of novel compounds in vitro as well as in vivo. Rodents are not always ideal hosts for human-derived pathogens, so when developing a new animal model, it is sometime necessary to provide the bacteria some assistance to help them colonize or to avoid the immune system. One way to do this is to passage a clinical isolate several times through an animal host via intraperitoneal, intranasal, or intravenous route of infection and then harvesting organisms 2–24 h later from the peritoneal cavity, lungs, or blood, respectively (Druilhe et al. 2002). It can also be advantageous to grow organisms under conditions that aim to mimic the host environment, such as medium that is reduced in iron, or in the presence of carbon dioxide, or in blood or urine (Marra et al. 2002). These activities often can potentiate bacteria to rodent infection by acclimating them via gene induction.

4 Adjuvants and Immunosuppression

The use of adjuvants in rodent models has also been proven enormously beneficial for establishing infections with human pathogens; these typically serve to "mask" the bacteria from the immune system (mucin, yeast) or prevent or slow clearance of the bacteria by the host (agar/agarose beads) (Table 1). These methods are model-specific but not usually organism-specific; other strategies include the use of iron-containing compounds or glucose diuresis to enhance kidney colonization in mice by bacteria. The former is used to cause microscopic damage to the kidney tubules so as to allow colonization whereas the latter appears to facilitate bacterial adherence to kidney epithelial cells via integrins and subsequent internalization and colonization.

Another approach is to assess the extent of infections in different mouse strains or by treating mice with immunosuppressive agents. The choice of mouse strain can be a crucial one for the establishment of murine models of infection, as it has long been known that certain strains are more susceptible or resistant to infection by different pathogens. For Gram-negative bacterial pathogens, several genetic loci have been identified that limit infection in some manner (Hagberg et al. 1984; Hormaeche 1979). Pilot studies are used to determine the minimum lethal dose (MLD; typically using 10-fold serial dilutions of inocula) and timing of endpoints for survivorship or tissue harvest and CFU enumeration. Generally, mouse strains for which organisms had the highest LD_{50}s also had the most prolonged infections when monitoring time to death (Hormaeche 1979).

Most commonly, immunosuppressive agents such as cyclophosphamide are often used in murine studies to deplete the number of neutrophils in the circulation, and infections are initiated at the timepoint when neutrophil numbers are near their lowest (Zak and Sande 1999). Such treatment allows a temporary advantage to the bacteria, enabling them to establish infection in the absence of

Table 1 Adjuvants and treatments to enhance rodent infection models

Adjuvant/treatment	Model	Reference
Immunosuppression (cyclophosphamide)	Multiple	Zak and Sande (1999)
Brewer's yeast	Intraperitoneal (acute systemic)	Sykes et al. (1977)
Hog gastric mucin	Intraperitoneal (acute systemic) pneumonia	Fothergill et al. (1937), Olotzki (1948), Tang et al. (2012)
Iron sorbitol citrate	Kidney abscess	Comber (1976), Comber et al. (1977)
Glucose diuresis	Kidney abscess	Keane and Freedman (1967)
Agar/agarose beads	Lung infection	Sawai et al. (1997), van Heeckeren and Schluchter (2002)
Microcarrier beads	Subcutaneous abscess	Ford et al. (1989)
Carrageenan	Kidney abscess	Alder et al. (2003)

an assault from the immune system. The literature variously reports different cyclophosphamide doses and schedules (Nakano et al. 1994; Zhi et al. 1988; Cryz et al. 1983); a regimen of two intraperitoneal injections of 150 and 100 mg/kg at four and one day prior to challenge is most commonly used and produces severe neutropenia by Day 4 (day of study start) that lasts approximately three days.

5 Antibiotic Susceptibility and Influence of Bacterial Strain Selection

Acinetobacter baumannii represents a clinical challenge in that it is an opportunistic Gram-negative pathogen, recent isolates of which have been identified as being resistant to all antibiotics but colistin and tigecycline (so-called extensively or extremely drug-resistant or XDR). This organism has the ability to readily acquire genetic material, namely, antibiotic resistance determinants, and also cause serious and varied nosocomial infections, notably causing >10% of all US hospital-acquired infections (HAIs); most troubling is the associated >50% mortality rate in patients with sepsis and pneumonia caused by *A. baumannii*. It has become a particular problem in intensive care units (ICUs), long-term health-care facilities, and military hospitals. With the recent emergence of colistin-resistance in this pathogen, new antibiotics are urgently needed. This organism has been notoriously difficult to manipulate in the laboratory, with most isolates rapidly cleared in rodent models and genetics still at the earliest stages. A 2014 paper by Jacobs et al. (2014) illustrates the value of a collection of recent clinical isolates that, coupled with systematic analysis via pulsed-field gel electrophoresis, a screening silkworm infection model and a mouse lung infection model could lead to the successful identification of a mouse-virulent, genetically manipulatable strains. The strategy selected one isolate from the diverse set of 33 *A. baumannii* isolates this strain, AB5075 is an MDR *A. baumannii* strain isolated from an osteomyelitis infection. Along with its ability to cause infection in the silkworm infection model and the mouse pulmonary infection model, this strain has also been shown to be virulent in a rat osteomyelitis model and a mouse wound infection model (Thompson et al. 2013). The ability to establish relevant in vivo infection models with the pathogen of great interest is an invaluable tool for the drug discovery scientist and has been a missing piece for this organism.

6 Reporting Data

One key point worth discussing is that of data reporting. Many investigators report in vivo results as total CFU recovered at the end of the study, showing treated and untreated groups side by side. This can be misleading, for example, in cases where the untreated control group has actually *declined* during the time period of the study, rather than remaining steady or increasing (preferred). As shown in Fig. 2, if data

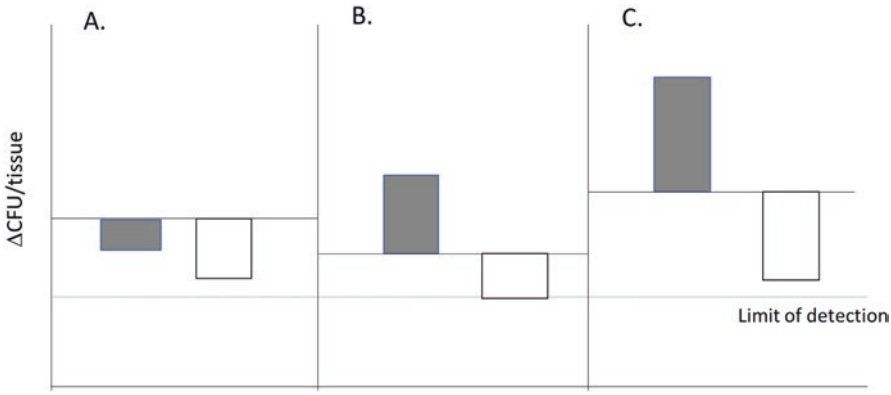

Fig. 2 Reporting in vivo results as ΔCFU from start of therapy as described in the text. Shaded bars, untreated control groups; unshaded bars, treated groups. Horizontal lines in each panel indicate the starting bacterial burden; the dotted line indicates the limit of detection

are reported as ΔCFU from the *start* of therapy, it rapidly becomes clear how robust the infection was, i.e., what the increase in bacterial burden in the untreated control group was relative to the treated groups. Panel A in Fig. 2 shows that the untreated control group is declining in bacterial burden over the course of therapy, so that the difference from the treated group is difficult to parse. Figure 2 also indicates an important point relative to the initiation of therapy: in Panel B, treatment was started when the bacterial burden was too low relative to the starting inoculum such that the maximal effect for the treated group cannot be observed due to limit of detection. Panel C shows a model that is optimally established, where the untreated control group is showing robust growth at the time of tissue harvest and the treated group can demonstrate maximal effect.

7 Specific Animal Infection Models by Site

7.1 Sepsis (Peritonitis) Models

One of the more straightforward murine infection models to establish is the sepsis or peritonitis model. In this model, mice (n = 5–10) are injected via the intraperitoneal route, taking care not to inject the intestines, with a number of bacteria sufficient to cause death in the untreated control group within 24–48 h. Investigators may preempt lethality by closely monitoring animals and noting clinical signs of lethargy, labored breathing, hunched posture, squinting, rough coat, and inability to access for water; once the latter is reached, it is unethical not to euthanize the animal. A more objective decision may be made by measuring the body surface temperature, if done properly this can be followed and guide euthanization decisions when a temperature of <28 °C is reached.

In the sepsis/peritonitis model, mice are dosed 30–60 min postinfection and the regimen can be either one or two doses given four to five hours apart; with rare

exceptions, efficacy tracks with MIC. One interesting paradox using this model is discussed in Endimiani et al. (2011), regarding the *K. pneumoniae* clinical isolates VA361 and VA406. Although both strains are *K. pneumoniae* carbapenemase (KPC)-producing strains that are non-susceptible to all β-lactams and clinically relevant β-lactam/β-lactamase inhibitor combinations, most of these agents had higher MIC values against VA-406 than against VA-361. Despite this difference in their in vitro activity, when these isolates were used to initiate a lethal septicemia in mice and treated with ceftazidime or ceftazidime-NXL-104, the ED_{50} for ceftazidime against VA-361 was higher than that against VA-406, as was that for ceftazidime-NXL-104. The speculation by the authors is that the two isolates possess virulence factors that affect the outcome of the infections; of note, a similar phenomenon occurred when ceftazidime and ceftazidime-NXL-104 were dosed against these two isolates in a thigh abscess model.

7.2 Thigh Abscess Models (Also Known as Skin and Skin Structure Models)

The murine thigh abscess model is the go-to model for pharmacokinetic-pharmacodynamic studies, as it offers a number of benefits to the investigator for evaluating compounds for efficacy: the infection entails an isolated tissue site that is not heavily vascularized, so in the neutropenic state the burden is on the antibiotic to eradicate the infection; the infection typically does not go systemic or cause lethality in the animal and depending on the infecting organism can be maintained for a week or more; the ability to infect both thighs in an animal gives duplicate samples.

This infection is usually initiated with an intramuscular injection to the caudal thigh muscles following the cyclophosphamide regimen noted in the "Adjuvants and Immunosuppression" section above. A PK-/PD-type study would last 24 h, in which case dosing is fractionated within this period. More routine study designs (say, for screening compounds) could run for 24 h or longer; a typical dosing regimen here would be 2 and 12 h or 2 and 9 h (to better fit into a work day) postinfection; animals are euthanized and tissues harvested at 26 h postinfection (24 h post first dose). For other studies, such as development of resistance in vivo, this model may be extended for up to a week, with sub-efficacious dosing employed.

An example of how some antibiotics can respond differently to *K. pneumoniae* KPC and non-KPC isolates with similar MIC values is detailed in a report by Hagihara et al. (2013). As discussed above, KPC-producing *K. pneumoniae* are resistant to carbapenems as well as other antibiotic classes. The interesting observation that was studied in Hagihara involved five KPC-producing and nonproducing isolates of *K. pneumoniae.* Against these five isolates, the carbapenems doripenem (similar MIC values 8 to >32 μg/mL regardless of KPC status) and ertapenem (MIC values of >32 or >64 μg/mL against all) were evaluated in a neutropenic mouse thigh infection study; doripenem and ertapenem were administered to mice in using a humanized dosing regimen. In this study there were two isolates against which doripenem had MIC values of 8 μg/mL, one a KPC producer and one a non-producer,

and three isolates against which doripenem had MIC values of 32 μg/mL (non-KPC producer) and 24 and >32 μg/mL (both KPC producers). Interestingly, treatment with doripenem resulted in substantial efficacy against the non-KPC-producing strains, >1.5 $\log_{10}$ CFU bacterial burden reductions, whereas against the KPC-producing isolates with similar MIC values the reductions in bacterial burdens were 0.5 $\log_{10}$ CFU or less. Ertapenem treatment did not demonstrate efficacy in this study. This study and that discussed in the sepsis section with the KPC-producing isolates VA-361 and VA-406 underscore the challenges presented by these organisms and the importance of animal models to help shed light on these questions.

7.3 Kidney/Urinary Tract Infections

There are two types of kidney abscess infections that can be established in mice: ascending and descending. In ascending models (Hagberg et al. 1983), bacteria are inoculated directly into the urinary bladder via a catheter and the organisms then migrate up into the kidneys. In the descending or hematogenous pyelonephritis model (Alder et al. 2003), the infection is initiated with a tail vein injection; *S. aureus* in particular will leave the bloodstream and colonize the kidneys very efficiently in this model. Kidney infections in mice can be challenging via the hematogenous route with Gram-negative bacteria due to the large numbers of organisms required to establish infection – levels that typically cause death from LPS overdose or bacteremia before kidney colonization. For these organisms or Gram-positive pathogens that do not colonize kidneys as well as *S. aureus* (such as *Enterococci*), iron-containing compounds such as iron sorbitol citrate (intramuscular injections given 18, 42, 66, and 90 h after infection) or carrageenan (0.2 mL of 0.2% solution injected iv 7 days before infection) may be administered (see Table 1) to increase kidneys' susceptibilities to bacterial infection. Another strategy that has been shown to enhance kidney infection with Gram-negative pathogens in mice is glucose diuresis, whereby the animals' drinking water is replaced with water containing 5% glucose, and their food is limited to 1 gram of food per day per mouse for 10 days. It is likely that the mechanism for this enhancement is due to the increased urination caused by glucose diuresis (Freedman 1966), and it has been shown that the attachment of certain Gram-negative organisms to bladder epithelial cells actually increases with shear stress (Thomas et al. 2002).

One benefit of this model that mirrors that of the human disease is the ability to harvest bladder and kidney samples to determine efficacy at both sites. In Soubirou et al. (2015), isogenic strains of a UTI-causing *E. coli* strain, one carrying a $bla_{\text{CTX-M-15}}$-containing plasmid, were used to initiate bladder and kidney infection in mice via the ascending route. Temocillin and imipenem were used to treat UTIs in mice caused by the two *E. coli* strains; the two antibiotics had MIC values of 4/8 and 0.5/0.5 μg/mL against the parent strain and the plasmid-containing strain, respectively. As temocillin has in vitro activity against this ESBL, it was of interest to determine how it behaved in this model when dosed every two hours using a humanized dosing regimen. The results indicated that temocillin was as effective as

imipenem in reducing bacterial counts of both strains in kidneys and no resistant organisms were selected; efficacy was observed when temocillin was dosed every four hours as well. This result indicates a possible non-carbapenem treatment for CTX-M-producing *E. coli* infections of the urinary tract.

7.4 Wound Infection Models

It has long been known that a large majority of human infections have a biofilm component (Akers et al. 2014; Percival et al. 2015; Zhao et al. 2013). These bacterial communities are made up of large numbers of densely packed organisms in a sessile, even multispecies community, contained within a matrix of polysaccharides, protein, and DNA. The nature of the biofilm affords protection for the pathogen from the host immune defenses as well as antibiotics; bacteria in biofilms are also thought to be in a slower metabolic state than planktonic bacteria which contributes to their antibiotic tolerance. Since biofilms can form on any surface, they can be found in chronic wounds and as a result cause delayed healing due to a prolonged inflammatory phase (Zhao et al. 2013). This seems to be especially true of biofilms in chronic wounds formed by pathogenic bacteria (Percival et al. 2015).

Wound infections have been modeled in rodent systems using various infecting organisms and different time frames. A simple wound infection can be initiated with full-thickness incision of about an inch in length along the dorsal region of the animal after shaving; this region is then infected with a bacterial inoculum and either stapled or sutured closed. A chronic wound model has been developed in mice with *A. baumannii*, that can last for up to 21 days if left untreated (Thompson et al. 2013). Not only for the reasons discussed above, this opportunistic pathogen has become a focus of antibacterial research, as infections caused by *A. baumannii* are a major cause of morbidity and mortality in injured military service members. This model relies on the mice being in a neutropenic state prior to infection, and on the day of infection, the animals are anesthetized and the dorsal area shaved, and a skin biopsy punch (6.0 mm) is used to create the wounds. The exposed areas are infected with bacteria; a dressing is placed over the wound and secured with adhesive. In the model reported by Thompson et al. (2013), antibiotic therapy is initiated 4 h postinfection and continued for 6 days; on Day 7 the dressing is removed and the wounds are monitored through Day 25. At the end of the study, animals are euthanized and the area surrounding the wound is excised, homogenized and plated for bacterial enumeration. In this model, wounds that were not infected healed within 13 days, whereas those that had been infected with *A. baumannii* AB5075 were still unclosed out to Day 21, underscoring the observation that the presence of bacterial biofilms in wounds substantially slows the healing process. Thompson et al. (2013) characterize this model in detail with photochronologies, scanning electron micrographs and longitudinal sections of wounds at different stages showing definite biofilm formations, as well as bacterial burdens after different antibiotic treatments. The simplicity of this model makes it likely that it can be applied to other pathogens; in addition, this model in particular is amenable to a bioluminescent readout so that

bacterial reduction can be monitored without the need for sacrificing animals. Most importantly, this model allows for the study of a challenging pathogen in a chronic infection model, the surface nature of this model allows for easy visualization of the healing process, and the infection can be maintained for up to three weeks.

7.5 Murine Lung Infections

Unfortunately for investigators, mouse lungs are efficient at clearing inhaled bacteria due to the phagocytic action of alveolar macrophages as part of the innate immune response to infection. An early study (Green and Kass 1963) showed that more than 90% of inhaled *S. aureus* CFU or 80% of inhaled *Proteus mirabilis* CFU are cleared within 4 h. What typically happens upon inhalation of bacteria is clearance due to mucociliary activity after attaching to mucins at the at the airway surface. Other microbicidal agents act to tamp down the infection, and epithelial cells signal immune cells to mount a defense (Williams et al. 2010). Although this makes establishing lung infection models in mice more challenging there are a few strategies that may be employed to aid in bacterial colonization.

The simplest infection route for mouse pneumonia model is via intranasal infection, whereby mice are anesthetized and allowed to inhale a 40–50 μL droplet of bacterial inoculum into the lungs; this method does not achieve bacterial access to deep regions of the lungs so some organism can be cleared via this technique. Intratracheal inoculation offers deeper lung penetration but is more labor-intensive and time-consuming for the investigator, and there are still some organisms that have difficulty establishing infections via this route. Adjuvants have been used to suspend the bacterial inoculum and slow bacterial clearance from the lungs.

Tang et al. (2012) describes a mouse pneumonia model of carbapenem-resistant *A. baumannii* that relies on intratracheal inoculation of a reasonable number of organisms (2.5×10^7 cfu) in a suspension of 10% porcine mucin. The authors indicate that acute pneumonia develops within 4 h in this model and that >90% of untreated control animals will succumb to the infection by 48 h postinfection. This model may also be used to quantify bacterial burdens in lungs at 72 h postinfection. The strain they use in this report is a clinical isolate, and so this method of infection could offer a strategy for testing novel antibiotics against an organism that has been a notorious challenge for animal modelers.

Another challenging organism for investigators is *P. aeruginosa*. This organism causes a range of opportunistic infections, and pulmonary infections are of particular interest due to the difficulties inherent in achieving activity against this organism and in obtaining adequate antibiotic levels in lungs to fight it. Agar or agarose beads have been used successfully for this purpose for different organisms. For decades researchers have found it useful to deliver *P. aeruginosa*-laden agarose beads intratracheally, as this allows penetration deep into the lungs and chronic infection for this organism (van Heeckeren and Schluchter 2002). Interestingly, acute pneumonia can be induced in mice injected intravenously with *S. aureus* enmeshed in agar

beads (Sawai et al. 1997); when other organisms were used to infect in this way (*S. epidermidis*, *Streptococcus pyogenes*, *P. aeruginosa*, or *K. pneumoniae*), only the *S. aureus* strains were recovered in mouse lungs at high levels (10^5–10^8 cfu) 7 days postinfection.

8 The Challenge of Metallo-β-Lactamases: A Real-World Example

Metallo-β-lactamases, such as NDM-1 (for New Delhi metallo-β-lactamase), VIM, and IMP, are broad-spectrum β-lactamase enzymes active against all β-lactams except aztreonam, which have been found in *E. coli* and *K. pneumoniae*, as well as other *Enterobacteriaceae* spp. NDM-1-producing isolates have been isolated from UTIs, lung infections, bacteremias, peritonitis, ABSSSIs, and device-associated infections. Most troubling is the association of NDM-1 with resistance to other antibiotic classes, such that NDM-1-containing isolates are multidrug-resistant, and in some cases resistant to all clinically available antibiotics; typically, the only options are colistin or tigecycline (Nordman et al. 2011).

Newer treatments for infections caused by NDM-1-producing *Enterobacteriaceae* are the focus of intense research efforts, in particular, optimizing drug combinations for these pathogens. Aztreonam is a β-lactam drug that is resistant to the activity of metallo-β-lactamases, though it is susceptible to serine β-lactamases, KPCs, and ESBLs; combining aztreonam with avibactam, a novel β-lactamase inhibitor with activity against ESBLs and serine β-lactamases, among other β-lactamases (but not metallo-β-lactamases), allows for a potential treatment option for these challenging organisms. By using in vitro tools and subsequent in vivo validation in a mouse thigh infection model much information may be gained regarding PK/PD driver and magnitude as rationale to moving to the clinic with a given regimen.

In Singh et al. (2015), multiple NDM-1-producing *K. pneumoniae* and *E. coli* isolates (n = 3) were studied in an in vitro hollow fiber infection model. Each isolate was resistant to aztreonam (MIC of 16 to >256 μg/mL); the addition of 4 μg/mL avibactam brought the MIC values to 0.125 to 8 μg/mL. In the hollow fiber system, the goal was to follow bacterial levels over time relative to drug levels, which simulated human drug exposures and half-lives of both aztreonam and avibactam. At specific time points samples were collected for bacterial enumeration and drug levels. Different hollow fiber study designs allowed for the identification of the PK/PD driver for each drug by fractionating the dose of the drug of interest and dosing the other drug at a fixed dose level. The results of the hollow fiber studies with different aztreonam regimens indicated that with once-daily dosing, bacterial rebound occurred, whereas no regrowth was observed when dosing was every 6 or 12 h. This study also pointed to the PK/PD driver (%*f*T>MIC) and the magnitude for a 1-$\log_{10}$ kill for the two isolates used (50 and 55% *f*T>MIC). This system was then used to confirm the PK/PD efficacy driver for avibactam and to define the optimum dose of avibactam in the combination. All of these results were then used in a mouse thigh infection model, and they showed that when their predicted PK/PD parameters were achieved, a maximal effect was observed.

Other strategies to help gain confidence in dosing regimens/combinations for in vivo studies include MIC combinations (determination of whether the combination has a lower MIC than the individual components), checkerboard studies (i.e., if the addition of an agent indicates synergy or even additivity then the combination may be worth testing in vivo) and especially time-kill studies with the combination showing enhanced or more rapid killing in vitro. Marshall et al. (2017) present a good demonstration of such an approach toward formulating a dosing combination for β-lactamase-resistant *Enterobacteriaceae* containing metallo-β-lactamases that was then used to treat and cure a treatment-recalcitrant *Enterobacter cloaceae* infection. Starting with a set of 21 isolates that were resistant to ceftazidime (21/21), aztreonam (19/21) and ceftazidime-avibactam (caz-avi; 21/21), they explored whether the addition of aztreonam to caz-avi would be a successful combination. They showed by disk diffusion and agar MIC that the addition of aztreonam at different concentrations could remarkably improve the MIC values against these isolates; five isolates required higher aztreonam concentrations (32 and 64 μg/mL) to be in the susceptible range. An in vitro time-kill kinetics study demonstrated the bactericidal effect of this combination against one of these five isolates at 8 μg/mL aztreonam with different sublethal levels of caz-avi; at 2 h, $\Delta 4$ $\log_{10}$ CFU decrease was seen for all drug concentrations relative to the growth controls with no rebound at 24 h. When this regimen was dosed in a neutropenic mouse thigh infection model, the addition of aztreonam significantly improved efficacy compared to either aztreonam or caz-avi, which needed to be dosed at 4-fold higher levels for similar efficacy.

9 Concluding Remarks

For antibacterial drug discovery, animal models provide the potential to address a host of questions in advance of the transition to human studies in order to reduce risk and improve efficacy. Although the in vitro – in vivo correlation is often quite good, there can be some disconnects, as described here; animal models can help to detect these issues early and mitigate them before they get to the clinic by developing dosing strategies or drug combinations. The benefits of being able to utilize recent clinical isolates make these models particularly relevant for studying novel antibiotics.

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Impediments to Discovery of New Antimicrobials with New Modes of Action

Paul S. Hoffman

Abstract

During the golden age of antibiotic discovery (1945–1970), little thought was given to the possibility that someday we would run out of them. It is shocking to admit that the last class of antibiotics was discovered over 30 years ago. What happened? The easy answer is that discovering new antibiotics is really hard, developing them is even harder, and once you get them to the clinic, there is little economic value for your efforts. This chapter seeks to explain some of the impediments to discovery of new antibiotics that include (1) the number of potential broad-spectrum "common" drug targets is small; (2) new pharmacophores are prone to early failure due to cytotoxicity, drug metabolism, or poor pharmacokinetics; (3) the general reticence to embrace and apply new technologies; (4) societal issues associated with their use and costs; and (5) the general lack of grant funding to support early discovery efforts. Despite these strong head winds, several concepts and approaches are discussed along with examples of what is working.

Keywords

Antibiotic pipeline · Antimicrobial · Amixicile · Antimicrobial challenges

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1 Introduction

Infectious diseases are one of the leading causes of death worldwide, yet the miracle antibiotics developed to combat them are being lost to the rapid emergence of drug resistance. Over the course of time and with a paucity of new ones, we find ourselves losing the war with the now drug-resistant microbes. Modern medicine is heavily dependent on antibiotics that enable many procedures including abdominal surgeries, bone marrow and organ transplants, and knee and hip replacements and manage immunocompromised patients and cancer patients (Cole 2014). While one might presume that new antibiotics would be discovered at a sufficient pace to replace those rendered obsolete by resistance, this has not happened. Worse, the antibiotics that have recently entered the clinic are not new, just derivatives of existing classes discovered over 30 years ago (Payne et al. 2007; Silver 2011; Devasahayam et al. 2010). Between 1995 and 2005, the pharmaceutical industry poured billions into the discovery effort as did national granting agencies and private foundations, and today there is little to show for these massive efforts as no new antibiotic class has made it to the clinic. The big questions partly addressed in this chapter are "why did these efforts fail, did we learn anything useful, and how can we be more successful"? With the emergence of "superbugs," the carbapenem-resistant enteric bacteria (CRE), *Acinetobacter baumannii*, and the methicillin-resistant and vancomycin-tolerant *Staphylococcus aureus* (MRSA), the urgency for new antimicrobials has reached the crisis point (Boucher et al. 2009). We are now losing the war, compelling the President's Council of Advisors on Science and Technology to convene a panel of experts to provide recommendations (Report to the President on antimicrobial resistance, Https://www.whitehouse.gov/sites/default/files/files/microsites/ostp/PCAST/pcast_carb_report_sept2014.pdf)). Additional reports by the PEW Charitable Trusts (A Scientific Roadmap for Antibiotic Discovery, http://www.pewtrusts.org/en/research-and-analysis/reports/2016/05/a-scientific-roadmap-for-antibiotic-discovery), and the Committee on Antimicrobial Resistance (CARB-X, http://www.carb-x.org/) to further define the problem and provide some solutions (Chaudhary 2016). These studies generally point to bottlenecks in early discovery, the urgent need for antibiotic stewardship, and the need for creating new chemical matter from which new antimicrobials might be derived. Even if new leads are discovered, getting them into the clinic still faces many challenges that include lead optimization, crossing the valley of death (translational gap between basic science and clinical development), regulatory challenges, and, finally, reimbursement (recouping costs of development which can be nearly a billion dollars) (So et al. 2011). This chapter will emphasize impediments to early discovery and the unique challenges for antibiotics and provide a few examples to illustrate strategies or approaches that appear to be paying off.

2 Headwinds of Antibiotic Development

Moving an antibiotic from early discovery to the clinic requires an investment of at minimum 500 million dollars and a timeline from lead to the clinic of 10 to 20 years (Gwynn et al. 2010; So et al. 2011; Fernandes and Martens 2017). These metrics perhaps explain why nearly all of the antibiotics that have entered the clinic in the past 10 years are derivatives of existing scaffolds of limited spectrum or utility (see Table 1). Historically, modifying existing antibiotics has provided more powerful analogues over many years, but the law of diminishing returns has been reached, and inevitable drug resistance has now caught up. Many of the antibiotics listed in Table 1 are second-generation derivatives of narrow-spectrum drugs that must be administered by IV. Drugs like dalbavancin, oritavancin, telavancin, and fidaxomicin are directed to Gram-positive bacteria (Van Bambeke 2015), but drugs to treat Gram-negative infections, including the superbugs, have proven more difficult to develop, mostly due to penetrability of the Gram-negative envelope (Page and Bush 2014; Jackson et al. 2018). The last new class of antibiotics developed for Gram-negative bacteria was the quinolones over 50 years ago.

While numerous companies manufacture and sell antibiotics, few companies currently are engaged in the creation of new ones. This is because the more traditional pharmaceutical commercial model has failed for antibiotics, despite antibiotic sales in the ~25 billion per year range (Payne et al. 2015). Despite the rapid emergence of antibiotic resistance, sales of generic antibiotics continue to account for between 75 and 85% of this market, and they are cheap. Basically, the health consumer is simply not willing to pay for new antibiotics that can cost thousands of dollars for a cure. Thus, the earnings potential for any new antibiotic is restricted by cost and by the likelihood that they will be held back and only used to treat infections that have failed standard therapies. Overcoming these headwinds will require fundamental changes in the way we develop and pay for these new antimicrobial therapeutics.

Table 1 Antibiotics recently entering the clinic. Antibiotics listed have recently entered the clinic. The vast majority of them are IV drugs used to treat infections such as MRSA. The combination drugs combine a cephalosporin or carbepenem with a β-lactamase inhibitor. These latter drugs are effective in treating most multidrug resistant Gram negative superbugs. PBP = Penicillin binding protein, β-lact = β-lactamase inhibitor

Antibiotic	Drug class	Target	Spectrum
Oritavancin	Lipoglycopeptide	Peptidoglycan	Gram positives
Tedizolid	Linezolid	Protein	Gram positives
Dalbavancin	Lipoglycopeptide	Peptidoglycan	Gram positives
Telavancin	Lipoglycopeptide	Peptidoglycan	Gram positives
Fidaxomicin	Tiacumicins	RNA	Gram positives
Ceftaroline-fosamil	Cephalosporin	PBP	Gram positives
Avibactam-ceftolozane	β-Lact inhibitor	PBP	Gram negatives
Avibactam-ceftazidime	β-Lact inhibitor	PBP	Gram negatives
Vaborbactam-meropenem	β-Lact inhibitor	PBP	Gram negatives

2.1 Why Is Antibiotic Development So Expensive?

The costs associated with developing a genuine broad-spectrum antibiotic are prohibitive partly because the FDA requires proof through clinical trials for all indications. Companies have to provide susceptibility data on a large number of strains, including drug-resistant ones (Gram positive and negative) and to demonstrate that the drug reaches therapeutic levels in all organs and tissues where the infections are likely to be found. For many of the new antibiotics listed in Table 1, a narrow indication is given, even when it is highly likely that the drug would be therapeutic for another pathogen. Gaining another indication is often not worth the cost. The frustration for physicians is that due to liability concerns, even if it is known that an antibiotic might be effective for an "off-label" indication, it would not be prescribed. This is not a good situation for patient or clinician. Historically, off-label use was a commonly employed strategy to gain new indications. It should be obvious that these restrictions on off-label use were introduced by the FDA by necessity to protect patients from false or unsubstantiated claims, and this is especially important in treating life-threatening infections where death is an endpoint. The FDA and European counterparts are working on solutions to this problem such as limited phase II or III trials in some of the other indications to mitigate expenses.

Early discovery is also an expensive and slow process, often requiring 8 to 10 years for lead optimization. Getting a lead through preclinical development and into a phase I trial can also cost millions of dollars and does not necessarily mean the drug will make it to the clinic. Most leads fail early due to toxicity or related safety issues, as unlike other medicines, antibiotics are given in high concentrations in order to achieve therapeutic blood levels. Historically, only one in five drugs entering phase I clinical trials will obtain FDA approval. As of December 2017, there were nearly 50 antibiotics in various stages of clinical development (http://www.pewtrusts.org/en/research-and-analysis/analysis/2014/03/12/glossary-for-the-antibiotic-pipeline). Most are in phase I. The list is updated from time to time because many are withdrawn for failure to meet primary or secondary endpoints often in comparison with similar approved drugs. In some cases, companies choose to terminate further development based on unfavorable market parameters.

Preclinical development also includes considerable work in animals prior to phase I study, as it is emphasized over and over that it is better to terminate a lead early than to have it reach the clinic and then be withdrawn. In some cases, toxicities and serious side effects do not manifest during clinical trials and only appear when patient numbers are sufficiently scaled up as exampled with the ketolide family and the quinolone trovafloxacin (Iannini 2002; Mitsugi et al. 2016). Finally, even very good antibiotic combinations like Avycaz (ceftazidime/avibactam), meropenem/vaborbactam, and others, designed to treat carbapenem-resistant enteric bacteria (CRE) including carbapenem-resistant *Klebsiella pneumoniae* (KPC), are market challenged by being reserved as last resort drugs, not to mention their expense relative to other options.

Avycaz earned less than 100 million in sales in its first 2 years (Fernandes and Martens 2017). This compares rather unfavorably with drugs launched in other

therapeutic areas (Januvia, Lyrica, etc.) that earned nearly 1.5 billion over the same period. Considering that nearly 25% of all visits to physicians is for infections (even more with pediatric visits) and the number of prescriptions written (mostly for cheap generic drugs), using costing metric based on Januvia as an example, would yield revenues of >30 billion in sales. Even treatment for hepatitis C runs around $70 thousand dollars for the drugs alone. In the absence of financial incentives, as outlined by CARB-X or governments picking up the tab, antibiotic development will remain far behind the more profitable therapeutic areas. Remarkably, John Rex, President of CARB-X, stated that for the US cost of maintaining the international space station, we could provide antibiotics for free to the US population for the year. Ultimately, it will come down to societal decisions on how tax revenues should be spent.

2.2 Investing in Early Discovery Initiatives

Both CARB-X and the European counterpart DRIVE-AB (Driving reinvestment in research and development for antibiotics and advocating their responsible use) have established priority pathogens lists and mechanisms for supporting development of new antimicrobials. To achieve this vision, DRIVE-AB used a research-based approach with significant stakeholder input to build policy recommendations to incentivize antibiotic research and development (R&D). CARB-X over the past two years has awarded millions of dollars to support early discovery projects. These include lead optimization and preclinical or preIND (Investigational New Drug) development to support regulatory approval for clinical trials. These arrangements usually involve partnership arrangements to support phase I studies, with an opportunity to apply for phase II funds which can be in the 50-million-dollar range. At the writing of this chapter, CARB-X funding is focusing on *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and drug-resistant *Salmonella*, *Shigella*, and *Neisseria gonorrhoeae*. Importantly for this round of funding, emphasis is placed on developing new antibiotic scaffolds and presumably includes new drug targets. It is easy to keep modifying existing antibiotics, but as the next sections will attest, it is especially difficult to find new antibiotic scaffolds and drug targets.

3 Finding New Drug Targets

On paper, this seems like a relatively easy exercise. After all, we have a massive database of sequenced genomes and annotations for all pathogenic bacteria to draw from. Using systems biology approaches, these genes and functional products can for the most part be assembled into common metabolic pathways, both catabolic and biosynthetic (Francisco et al. 2017). These approaches can be predictive of bottlenecks in pathways that might be targeted in drug discovery strategies (Chalker et al. 2001). For many selected pathogens, whole genome essentiality testing data also exists, useful in creating short lists of developable drug targets. The Venn

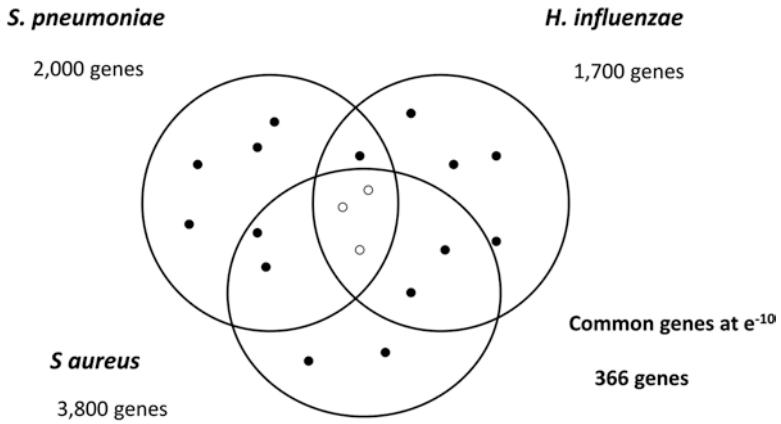

Fig. 1 Comparative genomic approach to finding common drug targets. This example is of three upper respiratory tract pathogens and using S.pneumoniae as the query genome, a BLASTP protein comparative analysis was performed. The Venn diagram shows the areas of overlap which reveals 366 common genes

diagram depicted in Fig. 1 shows how comparative genomics of a target group of pathogens, upper respiratory pathogens in the example, can generate a short list of common genes. By eliminating genes of unknown function, those encoding membrane proteins and proteins with human counterparts and non-essential genes, one can ultimately create a short list of ~100 potential candidates. Many of the genes on this short list encode ribosomal proteins, penicillin-binding proteins, and the topoisomerases, yes, the major known targets of most of the antibiotics currently in the clinic. Almost 95% of the 50 antimicrobials currently in clinical development target the known targets associated with protein synthesis, topoisomerases, and cell wall biosynthesis.

Having all of this information is not necessarily helpful or straightforward as essential target genes in one pathogen species may not be essential in another, and in some species, functional redundancies can overcome putative essentiality (Payne et al. 2007; Ibberson et al. 2017). Pathway redundancy is generally a function of genome size and must be taken into account for data generated from parsing through small genome-derived targets. Then there is the underground metabolism or moonlighting in the microbial world, enzymes with bifunctional capabilities that may utilize structurally related substrates and the like that enable microbes to basically fill in gaps in metabolic pathways or provide compensatory functions (D'Ari and Casadesus 1998). While there are still many genes of unknown function (25 to 30% of genomes), essentiality testing of them has proven that the grass is not necessarily greener in this pasture (Payne et al. 2007). Given all of this knowledge, why then are granting agencies still supporting clever screens to find new drug targets? One reason most pharmaceutical companies left the antibiotic discovery field around the turn of the century was that having used the tools of comparative genomics, they already knew that the number of new drug targets would be very small, perhaps

fewer than 100 (Gwynn et al. 2010). Due to the costs of target-based drug discovery initiatives and the abysmal failures of high-throughput screening of compound libraries to find leads (often one million dollars per high-throughput screen), these expensive approaches were abandoned (Payne et al. 2007).

As discussed by Payne et al. (2007), the postmortem from some 70 high-throughput screens (HTS) of selected targets just did not pay off as only 7% produced leads and none of these panned out. The GlaxoSmithKline (GSK) experience was typical of the industry. The problem was not with the chosen targets, which remain excellent choices, but with the notion that chemical libraries would contain antibiotic-like compounds [Authors note: Dr. Hoffman was an assistant director in the antiinfectives division at SKB/GSK during this period]. There are several key points that can be derived from this approach. First, these libraries of some half a million compounds or more represented a collection of all chemical matter created by the company regardless of therapeutic area. This also included libraries purchased from private or commercial companies and those acquired by mergers and acquisitions. Second, the libraries contained redundancies, duplications, and a good percentage of toxic matter such as detergents and ionophores. Thirdly, when compound libraries were analyzed based on compound mass cLogD/P (solubility measures) and potential penetrability of bacterial cells, few compounds possessed antibiotic-like properties. Finally, the notion that libraries optimized for antibiotic-like matter might fare better has also proven to be false (Lewis 2017). It has been estimated that over ten million compounds have been screened for antibiotic activity with no success. The very expensive and complete failure of HTS, be it target- or whole cell-based to find new antibiotics, has driven most of the pharmaceutical players out of the business.

What we can learn from these failures is that the list of protein targets from HTS initiatives might be analyzed differently, for example, by applying structural biology and rational drug design strategies (Durrant and Amaro 2015; Payne et al. 2015). In this regard, the NIH (NIGMS) has sponsored the crystallization of many microbial proteins, a potential starting point for picking targets amenable to rational drug design. These databases are accessible (Protein Data Bank, www.rcsb.org/; NCBI, ncbi.nlm.nih.gov; ExPASy proteomics portal, www.expasy.org/) and can be cross-referenced to information on enzyme function, essentiality, and perhaps more importantly to existing inhibitors. Even if existing inhibitors themselves are not developable as antibiotics, they can serve as tool compounds or templates for design of more antibiotic-like matter. It is important to emphasize that such strategies have been successfully applied to development of antivirals and HIV medicines. With few exceptions, the antibiotic discovery field has been slow to embrace these new technologies.

3.1 Repurposing Drug Targets

There seems to be an unwritten rule that once a target and an antibiotic inhibitor have been identified, it is more fashionable (innovative) to discover the next target. As

mentioned earlier, the vast majority of antibiotics used today hit only three classes of targets: ribosomes (protein synthesis), penicillin-binding proteins (cell wall biosynthesis), and DNA topoisomerase-gyrase (DNA replication) (Lewis 2017). Ribosomes are macromolecular machines composed of proteins and RNA and as a complex machine; there are many inhibitory opportunities that account for the diversity of existing small-molecule inhibitors. Similarly, penicillin binds to multiple highly conserved penicillin-binding proteins, and chemistries around the β-lactam ring have created a wide range of β-lactam classes (Bush and Bradford 2016). For the topoisomerases (DNA gyrase and topoisomerase IV), the target of fluoroquinolones, having two drug targets requires mutations to both in order to develop full drug resistance (Silver 2011). Thus, the fluoroquinolones are one of the most frequently prescribed of all classes of antibiotics. While quinolone resistance requires two steps, first-generation quinolones can provide the first step because of unequal inhibition of both targets. The driver for quinolone resistance in the clinical setting is often the quinolone merry-go-round (ciprofloxacin ➔ levofloxacin ➔ moxifloxacin) that can select for resistance to even the most advanced derivatives. One way to break the incremental cycle of next-generation analogue inhibitors is to find new chemical scaffolds that inhibit these good targets by different modes of action.

Given the great success of the fluoroquinolone antibiotics against the bacterial topoisomerase-DNA gyrases, there has been a push to create novel non-fluoroquinolone inhibitors (Mitton-Fry et al. 2017; Flamm et al. 2017). One of these that has advanced in clinical development is gepotidacin developed by GSK that attacks the same target but differently. Fluoroquinolones stabilize double-strand breaks in DNA, while gepotidacin stabilizes the pre-cleavage type II topoisomerase enzyme-DNA complex prior to DNA cleavage, generating single-strand breaks. The cartoon analogy is like wedging a stick in the mouth of the monster to prevent it from chomping down on the hero. Importantly, mutations associated with resistance to fluoroquinolones show no cross-resistance with gepotidacin. However, studies show that the major resistance to gepotidacin is associated with increased expression of efflux pumps. Conceptually, repurposing tried and true drug targets with new chemistries, as exampled herein, might be one way out of our current dilemma. This can be further aided by designing inhibitors that interact with invariant structurally constrained regions of the protein essential to functional activity.

4 New and Underdeveloped Scaffolds

The PEW study and others have emphasized the urgency for creating new chemical matter from which new antibiotics might be obtained (Wright et al. 2014). Creating new chemistries should not be limited to just finding new antibiotics, as all therapeutic areas are similarly in need of new drugs. It is also remarkable that from antifungals to statins, common core molecules such as azoles, imidazoles, indoles, and heterocycles can be identified as core elements. One must keep in mind that with new chemical matter come new opportunities in unexpected therapeutic areas. As an example, nitazoxanide (NTZ), an FDA-approved antiparasitic drug,

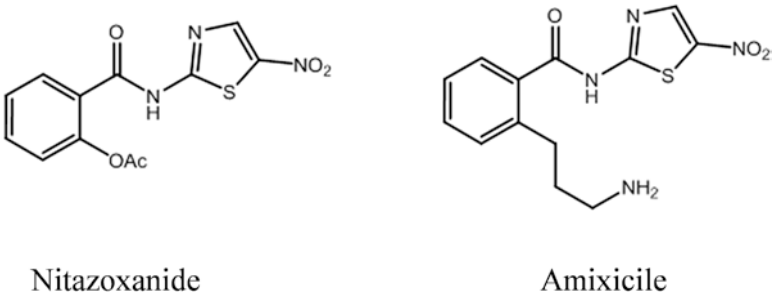

Fig. 2 Chemical structures

represents an underdeveloped scaffold considered promiscuous because in addition to its antiparasitic and antibacterial activities, it also possesses antiviral and anticancer activities (see Fig. 2) (Shakya et al. 2017; Qu et al. 2018). NTZ is currently in phase III clinical trials as a remedy for seasonal influenza and exhibits antiviral properties against a range of other viruses (Rossignol 2014; Tilmanis et al. 2017). There is also a rich literature on anticancer properties of NTZ that includes targeting colon cancer (CACO-2 cells) (Senkowski et al. 2015; Qu et al. 2018). One of the challenges obviously is distinguishing between non-specific phenomena such as generalized protein binding (nuisance compounds) and target specific activity that can be further developed via medicinal chemistry (structure-activity relationships) to drive lead optimization.

By interrogating the NTZ scaffold, we determined that the 2-amino-5-nitrothiazole moiety was responsible for the biological activity against the parasitic drug target pyruvate:ferredoxin oxidoreductase (PFOR) (Ballard et al. 2011). We created a library of >300 structural analogues representing new chemical matter with a wide range of antibacterial activity (Ballard et al. 2011). We discovered that chemistry can improve target specificity as exampled by amixicile (see Fig. 2), a potent PFOR inhibitor that no longer possess antiviral and anticancer activities (Warren et al. 2012). As seen in Fig. 3, we found that some analogues exhibited antibacterial activity against MRSA and *Bacillus anthracis*. By replacing the benzene ring of NTZ with thiophenes or furans, we created inhibitors of the Gram-negative bacterial chaperone/usher pathway of pili biosynthesis with apparent binding to BamA of the beta-barrel assembly machine, a novel drug target (Shamir et al. 2010; Chahales et al. 2016). Replacing the amino-nitrothiazole with a dinitrothiophene on the amixicile scaffold created a potent bactericidal therapeutic (in vitro) for *Mycobacterium tuberculosis*.

One group of analogues shows antiviral activity against Ebola, Zika, and hepatitis C viruses. Another group of analogues appears to be more potent than NTZ as anticancer agents and is in early clinical development. NTZ and analogues bind to peptidyl arginine deiminase (PAD2), thereby activating β-catenin citrullination (targeted for destruction) resulting in downregulation of Wnt signaling in cancer cells (Qu et al. 2018). It is interesting to note an overlap in spectrum between anticancer and antiviral activities, suggesting the possibility of common required host

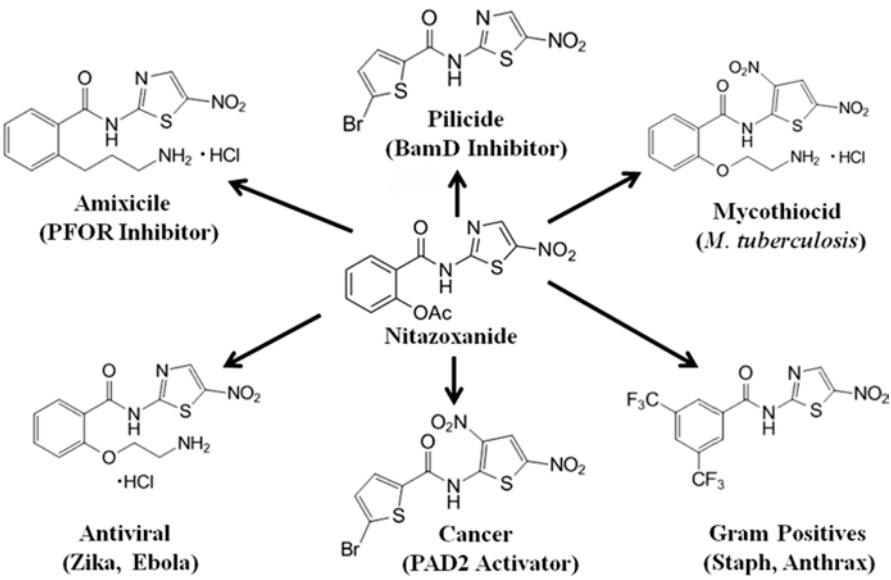

Fig. 3 Structure activity relationships and biological activities of nitazoxanite analogues

functions. While little is known regarding additional drug targets or their specificity, they do provide examples of how tinkering around with biologically promiscuous underdeveloped scaffolds can direct them to new therapeutic areas, a starting point for creating new medicines. The notion that promiscuous chemistries can be developed into selective medicines should not come as a big surprise given that life on earth is the product of millions of years of evolution. Thus, "Mother Nature is both lazy and does not bake from scratch." Thus, it remains a challenge to find antibiotics of sufficient selectivity that avoid off-target or unintended toxicities.

5 Central Metabolism and Drug Targets

It seems ironic in retrospect that the first target for antimicrobials was dehydropteroate synthase of the folic acid biosynthetic pathway, which led to the sulfa drugs that in combination with trimethoprim (co-trimoxazole) are still in use today (Wright et al. 2014). Trimethoprim inhibits dihydrofolate reductase. With the launch of penicillin and the discovery of streptomycin by Waksman in the 1940s, the "Waksman platform" of screening soil and other environmental samples for natural product antibiotics displaced medicinal chemistry and led to the golden age of antibiotic discovery (Lewis 2017). There was nothing wrong with the medicinal chemistry approach to produce antibiotics, as it managed to yield the antihistamines in the 1950s and later the myriad of medicines in use today.

In searching for new drug targets, one might consider targets associated with biosynthesis of other vitamins or highly conserved enzymes with unique catalytic

centers, cofactors (vitamins), or other attributes that may not have counterparts in humans or in mitochondria. One such unique target is the primitive enzyme pyruvate:ferredoxin oxidoreductase (PFOR) and related alpha-ketoacid oxidoreductases that are highly conserved through evolution and found in obligate anaerobic bacteria, anaerobic intestinal and vaginal parasites and in the *Epsilonproteobacteria* that includes *Helicobacter pylori* and *Campylobacter jejuni* (Horner et al. 1999; Hoffman et al. 2007). Importantly, these enzymes are not found in humans or mitochondria. With the exception of the *Epsilonproteobacteria*, PFORs utilize low-redox carrier ferredoxin as electron acceptor and are capable of chemically reducing synthetic prodrugs like metronidazole to DNA damaging toxic products (Sisson et al. 2000). Resistance to metronidazole is relatively rare in the anaerobes. The *Epsilonproteobacteria* utilize higher redox flavodoxin as electron carrier which does not reduce metronidazole (Hoffman et al. 2007).

A new class of PFOR inhibitor was recently discovered that attacks the catalytic center of the enzyme by competing with its substrate pyruvate (Hoffman et al. 2007; Kennedy et al. 2016). Both nitazoxanide (NTZ) and an optimized lead amixicile outcompete pyruvate for binding to the vitamin B_1 cofactor (thiamine pyrophosphate or TPP) of the enzyme (see Fig. 4). In the redox cycle of PFOR, activated TPP becomes protonated, and both NTZ and amixicile abstract the proton, thus inactivating the catalytic cycle of the enzyme. Proton abstraction reactions are not uncommon in the enzyme world, but in this case, the drug is interacting directly with the vitamin and not with the protein. The caveat for this mode of action is that mutations altering vitamin function are considered to be functionally lethal. In the nearly

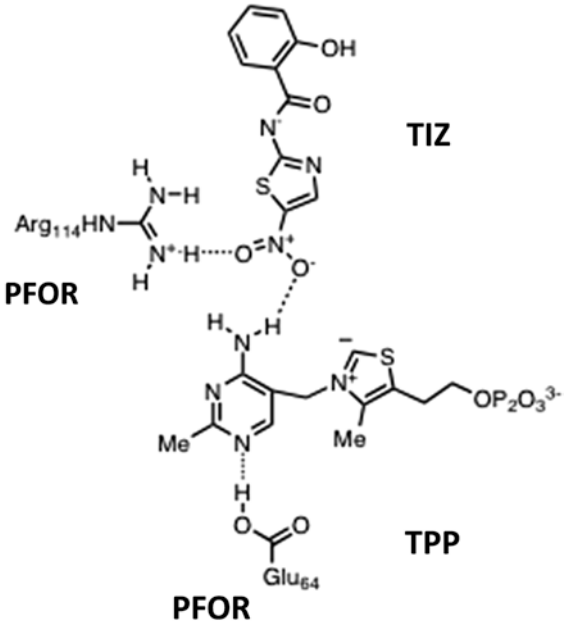

Fig. 4 Mechanism of action of nitazoxanide based analogues that target the amino-pyrimidine of the thiamine pyrophosphate cofactor of PFOR (Kennedy et al. 2016). TIZ = tizoxanide the deacetylated phenol of NTZ. Arg_{114} and Glu_{64} are essential to function

20 years that NTZ has been in the clinic, there has yet to be a report of drug resistance. We might learn from this example that novel catalytic mechanisms, perhaps involving vitamins or other cofactors, might similarly be amenable to inhibitor development. Amixicile is in clinical development and has a broad spectrum of action against Gram-positive and Gram-negative anaerobic bacteria, including those associated with periodontal disease, colitis caused by *Clostridium difficile*, and gastritis caused by *Helicobacter pylori* (Warren et al. 2012; Hoffman et al. 2014; Hutcherson et al. 2017).

6 New Medicinal Chemistry Rules

Toward the end of the GSK HTS failed exercise, and during the merger of SmithKline Beecham with Glaxo Wellcome, we often joked that by combining the chemical compound libraries from each company, a library of a million compounds would be created. The assumption that a bigger library would improve odds of finding a hit was mitigated by the likelihood that once redundancies and duplications were taken into account, the benefits would likely be incremental. This notion is supported by the reality that chemists build compounds from relatively standard groups of existing precursors such as found in ChemDraw and related software. The lack of chemical diversity and a need for new chemistries is a major conclusion from the Pew study and discussed by Wright et al. (2014), Payne et al. (2007), and Lewis (2013, 2017).

At the turn of this century, new antibiotic chemistry was guided by Lipinski's rule of 5 ($\leq$5 hydrogen donors; $\leq$10 hydrogen bond acceptors; molecular weight under 500 Da; and ClogP $\leq$5) (Lipinski et al. 2001). These rules tended to predict whether a molecule would be absorbed following oral administration, but did little to aid improving penetration or accumulation within bacterial cells, particularly for the Gram negatives. Obviously, Lipinski never met an antibiotic as vancomycin, daptomycin, erythromycin, and fidaxomicin, to mention a few, do not conform to these rules. A second shortcoming of existing chemistries is that tens of millions of compounds have already been screened to the point of exhaustion. The majority of compounds in existing libraries exhibit lipophilic properties that are not suitable for antibiotic development (Wright et al. 2014; Lewis 2017). It is important to point out that we are in pretty good shape when it comes to treating infections caused by Gram-positive pathogens. For the Gram negatives, it is a different story. What is obviously needed is a new set of rules for predicting which compounds might be absorbed by Gram-negative bacteria.

Accumulation of molecules into Gram-negative bacterial cells requires passage through two biological membranes: a lipopolysaccharide-coated outer membrane (OM) and a cytoplasmic inner membrane (IM). Passage of molecules through the OM requires that they be small enough to pass through the water-filled porin protein channels (generally $\leq$500 Da). This is why vancomycin, fidaxomicin, and daptomycin (large molecule antibiotics) show no potency against Gram-negative bacteria. A second challenge to penetration and accumulation of antibiotics in Gram-negative

bacteria is they express many classes of efflux pumps that expel compounds from within. So, getting drugs into and keeping them there is a big challenge facing medicinal chemists (Richter et al. 2017).

Hergenrother's group took a fresh look at why chemical matter found in existing libraries is not suitable for development of antibiotics to treat Gram-negative bacteria (Richter et al. 2017; Richter and Hergenrother 2018). Using a diverse group of 180 compounds in a model system using *Escherichia coli*, they found major differences over retrospective studies and came up with a new list of five predictions. They found that compounds containing an amine that were amphiphilic and rigid and had low globularity tended to accumulate in Gram-negative bacteria. Charge was also important as compounds with a positive or negative charge were more likely to accumulate than compounds with a neutral charge. Cheminformatics could benefit from knowing that addition of a primary amine, for example, to a low accumulating lead, might improve its penetrability and now the MIC (minimal inhibitory concentration). Similarly, distance between hydrophobic and hydrophilic regions of a compound also tends to favor accumulation. An example from our drug discovery effort is amixicile, where replacement of an acetyloxy group on the benzene ring with an aliphatic primary amine improved solubility and bioavailability of an otherwise poor pharmacophore (see Fig. 2). It is highly likely that an in silico screening of compound libraries using Hergenrother's rules could produce subsets of more attractive library material to support development of Gram-negative therapeutics (recently discussed by Richter and Hergenrother 2018). Importantly, new efforts employing machine learning and rational drug design can also benefit from this knowledge (Durrant and Amaro 2015). Finally, repurposing or optimizing existing antibiotics to favor penetration of Gram-negative bacteria is also possible.

7 Targeting Gram-Negative Bacterial Systems

While Gram-negative bacteria share a considerable number of drug targets with Gram-positive counterparts, existing antibiotics like linezolid and vancomycin are not effective against them. One strategy for developing antibiotics selective for Gram-negative bacteria is to target unique and perhaps common attributes of Gram-negative bacterial pathogens such as extracytoplasmic macromolecular assembly systems. These would include type III and type IV secretion systems, LPS biosynthesis components, beta-barrel assembly machine (BAM), lipoprotein assembly system, and the disulfide bond (DsbA) system. Mitigating attractiveness of these targets is that their natural substrates are often hydrophobic molecules which may favor more lipophilic inhibitors. Other attractive targets already in the discovery effort include the chaperone/usher pili and type III secretion systems where inhibitors are thought to function more as antivirulence weapons (Chahales et al. 2016; Berube et al. 2017). It has been hypothesized that defeating virulence functions, which are not likely to be essential for viability, will defang them and enable the host immune system or other antibiotics to eliminate the infection (Dickey et al. 2017).

8 Natural Products Versus Synthetic Antibacterials

Historically, the Waksman platform was the driver for the "Golden Age" of antibiotic discovery, but by the 1990s, it had essentially been played out. The shift to screening of synthetic chemistry libraries has proven unproductive (Payne et al. 2007). So, where do you find new antibiotics? There has been a recent resurgence in natural product screening based on development of new approaches to grow and screen uncultivatable microbes for antibiotics (Lewis 2013, 2017; Ling et al. 2015). This strategy yielded teixobactin which is active against Gram-positive bacteria and is in clinical development. This is a good example where innovation overcomes an existing impediment and opens an avenue with great promise. In general, the downside for natural products is preexisting resistance mechanisms that have also evolved over millions and spread rapidly once the new antibiotic has entered the clinic. One only has to look at the rapid spread of colistin, the drug of last resort for treating carbapenem-resistant superbugs (Sun et al. 2018). Another issue with natural product antibiotics is that they are optimized for their particular niche and generally will require considerable lead optimization (chemistry) to improve attributes associated with pharmacokinetics, toxicology, and drug metabolism for use in humans.

In contrast, synthetic antibiotics, particularly those against a new target for which there is no natural product inhibitor, might avoid the evolution of natural resistance penalty. However, many synthetic antibiotics are prone to mutation-based drug resistance as exampled by the leucyl-tRNA synthetase inhibitors (O'Dwyer et al. 2015). In this example, mutations occurred in the proofreading arm of the enzyme and not in the catalytic center, allowing partial function. In contrast, the topoisomerase-gyrase inhibitor gepotidacin, which appears to bind to a conserved region of the enzyme, appears to be less prone to mutation-based drug resistance. Certainly, this appears to be similar for amixicile and nitazoxanide in their mode of action against TPP of PFOR. In summary, there is no "right" approach to creating sorely needed antibiotics, and given our current plight, learning from the past and not making the same mistakes will likely enable us to prevail.

9 Early Discovery Challenges

Perhaps the greatest impediment to developing new antibiotics is the scientific community itself. In this respect, the one question that arises over and over again: "if no new antibiotic has been discovered in 30 to 50 years, then where are the experts to guide us into the future"? Having half a century of expertise on what not to do is not particularly encouraging. Our peer review infrastructure is populated with experts on what not to do. Thus, any new idea or strategy is often thwarted by the age-old argument "we already tried that." For young early-career scientists interested in developing new antibiotics, peer review is a significant impediment and begs the question of who can judge the science? It is not surprising that talented young scientists in tenure-track positions would avoid this area. Moreover, academic tracks

previously viewed as important to antibiotic development (medicinal chemistry and microbial physiology) have contracted over the past 30 years.

These are important issues in addressing who will make the next-generation antibiotics. While CARB-X and DRIVE-AB provide funding for those with advanced leads, early discovery, a period of 8 to 10 years, is completely dependent in the United States on National Institutes of Health grants and in particular from the National Institutes of Allergy and Infectious Diseases, where pay lines are 6% or less (Wright et al. 2014). This means that 94% of scientists are excluded from the game, which has been going on for nearly 20 years. The entire scientific research enterprise in the United States is under acute funding stress – another crisis. It follows that well-intentioned calls for proposals by NIAID for antibiotic development are essentially poached (and rightfully so) by the 94 percenters who are excellent scientists desperate for funding. Thus, the chance that any government initiative will result in new antibiotics has odds worse than the HTS outcome. The success rate for NIH funding resulting in new antibiotics compared to all other medicines is less than 1% (Galkina et al. 2018). There are no easy solutions to the funding crisis.

10 Concluding Remarks

The PEW study rightfully pointed out that until new chemical matter is created, the prospects for new antimicrobials will remain an unmet medical challenge. Similarly, the number of viable new drug targets is small and unlikely to lead to new medicines. The attractiveness of repurposing known antibiotic drug targets as exampled by gepotidacin shows promise, especially as rational drug design and structural biology become better integrated into discovery plans. New innovative strategies for searching for natural product antibiotics in the environment as outlined by Kim Lewis (2017) are likely to provide new scaffolds for future drug development. The antibiotic discovery field needs to attract scientists from a wide area of disciplines to work toward a common goal. This also begs a new funding structure to support high-risk high return ventures.

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Part II

New Antibiotic Drug Discovery Approaches and Progress

Endophytes: A Hidden Treasure of Novel Antimicrobial Metabolites

Palak Arora, Tanveer Ahmad, Sadaqat Farooq, and Syed Riyaz-Ul-Hassan

Abstract

An endophyte is a microorganism which colonizes the healthy tissues of the host plant without causing any symptoms of disease. The relationship between the endophyte and the host ranges from latent phytopathogenesis to mutualistic symbiosis. Endophytes obtain nutrition and protection from plants and, in return, help their hosts to adapt to different ecological stress conditions by producing certain functional metabolites. Consequently, endophytes are usually metabolically more active than their non-endophytic counterparts. By virtue of their functions in nature, endophytes produce multitude of natural products, particularly those having potential antimicrobial activities. As all the plants analysed for endophytism have been found to possess such organisms, endophytes represent a comparatively unexplored as well as a huge reservoir of bioactive metabolites. In this chapter, an effort is made to present an overview of the potential of endophytic microorganisms as a source for antimicrobial agents.

Keywords

Endophytes · Antibiotics · Volatile organic compounds (VOCs) · Fungi · Secondary metabolites · Natural products

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1 Introduction

The discovery of new antimicrobial agents is imperative for the treatment of infections caused by drug-resistant pathogens. Microorganisms are a well-known reservoir of bioactive natural products having huge potential in the field of pharmaceutical, industrial and agricultural applications (Demain 1999; Keller et al. 2005). From the foremost antibiotics, such as penicillin and streptomycin, to other life-saving drug molecules, like rapamycin and cyclosporin, microorganisms have contributed numerous molecules to natural product repositories that have the potential to treat human diseases. Natural products derived from microbial sources have been an important source of novel drugs (Clardy and Walsh 2004; Khosla 1997; Sieber and Marahiel 2005). Further, most of the anticancer and antimicrobial drugs currently available in the market are either natural products or their derivatives (reviewed by McAlpine et al. 2005). In comparison to other natural sources, such as plants, microorganisms are both highly diverse and poorly explored. Reports based on estimation of microbial population unfolded that only about 1% of bacteria and 5% of fungi have been identified and characterized, whereas the rest remain unexplored (Heywood 1995; Staley et al. 1997). The contribution of microorganisms to the pharmaceutical industry is further limited by the potentiality of orphan biosynthetic pathways that do not express themselves under optimum conditions (Bok et al. 2006; Hertweck 2009). However, the vast array of techniques pertaining to the growth and manipulation of microorganisms, such as media engineering, co-culture, chemical induction, epigenetic modulation and metabolite re-modelling, coupled with fermentation technology for scale-up, make them suitable for the production of useful natural products, both known and novel (Bok et al. 2006; Knappe et al. 2008; Bergmann et al. 2007; Schroeckh et al. 2009; Riyaz-Ul-Hassan et al. 2012). Hence, microbiologists explore unique niches including extreme environments, such as ocean beds, geothermal vents and cold desserts, in search of novel strains with promising bioactive potential (Staley et al. 1997).

In the recent past, it has been observed that much of the wealth of microbial biodiversity with complex biochemistry and secondary metabolite production resides in plant tissues (Strobel 2006). Interest in such microorganisms, termed as endophytes, increased immensely with the discovery of the billion-dollar anticancer drug, Paclitaxel, which was discovered in an endophytic fungus isolated from *Taxus longifolia* (Stierle et al. 1993). Since this ground-breaking discovery, numerous bioactive molecules have been isolated from endophytic fungi (Strobel 2006; Wang et al. 2011a; Deshmukh et al. 2015). Endophytes share a symbiotic association with the plant host, growing in the interstitial spaces of tissues without causing any adverse effects on the host. The interaction between the partners may vary from a mutualistic association to a balanced antagonism (Strobel and Daisy 2003). Due to their asymptomatic nature, endophytic microorganisms remained a hidden reserve until their potential was realized in the recent years.

As much of the previous research focused on exploring the host-plant metabolites in the endophytic partner (Stierle et al. 1993; Puri et al. 2006; Kusari et al. 2009), the theory of horizontal transfer of the gene clusters, coding for the

secondary metabolites of the host, and other interactions between the plant and its endophytes received much impetus (Strobel and Daisy 2003). Interestingly, later studies suggest that the endophytes possess biosynthetic pathways independent of the plant host (Staniek et al. 2009). However, it could also be logical that the microorganisms produce similar metabolites to those of their endophytic partners, given that they would have more chances of thriving in plant tissues if they were resistant to the present metabolites, thus favouring the findings that many endophytes produce the metabolites of their hosts.

Endophytic microorganisms may influence the ability of the plants to function in the specific environmental conditions. They may also impact the structure of the plant communities by playing crucial roles in colonization, coexistence, competition and dynamics of soil nutrients (Clay and Holah 1999). In other cases, herbaceous plants and grasses are associations with dominant endophytes that produce toxic alkaloids, thus providing protection against herbivores (Braun et al. 2003). Endophytes in woody plants are known to play specific defence roles to prevent them from pathogens (Strobel 2003). Overall, the biology and biochemistry of endophytic microorganisms is a novel emerging field with multitude of ecological outcomes.

Endophytes are metabolically more active than their free-living counterparts, and thus, they have the potential to produce exceedingly high numbers of secondary metabolites, which are often bioactive and of low molecular weight, and are produced as families of related compounds, with production often correlated with a specific stage of morphological differentiation (Keller et al. 2005). Several reasons are attributed to the increased metabolic activity of endophytes. Firstly, the organism needs to evolve in order to survive in the tissues of the plant, thus activating the production of molecules that help in the evasion of host defence mechanisms. Secondly, there exists a balanced antagonism between the endophyte and its host, resulting in the production of several phytotoxins by the microbial symbiont (Strobel and Daisy 2003; Strobel 2006). Recently, it has also been proposed that the chemical constituents of the host plant may bring about permanent epigenetic changes in the endophyte, thus turning on some of its otherwise ‘silent’ biosynthetic pathways (Riyaz-Ul-Hassan et al. 2012).

Endophytes have been known to produce volatile organic compounds (VOCs) with specific or nonspecific antimicrobial activities (Mitchell et al. 2010; Meshram et al. 2013) and may be involved in nature to build microenvironments that kill or inhibit pathogenic microorganisms (Riyaz-Ul-Hassan et al. 2012; Strobel et al. 2011). VOCs are important in the functioning of both atmospheric and soil ecosystems and have potential applications in biotechnological fields, viz. agriculture, industry and medicine. Surprisingly, no two microorganisms, even those that are morphologically and genetically identical, produce the same array of VOCs under similar growth conditions (Kudalkar et al. 2012).

The whole genome sequencing of microorganisms ushered a new area in the field of natural product research and drug discovery. The available knowledge about genetics and enzymology of natural products synthesized from microorganisms have expedited the identification and analysis of gene clusters involved in

biosynthesis of natural products in sequenced microbial genomes (Fischbach and Walsh 2006). Genome analysis of one of the first sequenced microbes, *Streptomyces coelicolor*, revealed that there are many more gene clusters encoding biosynthetic pathways than there are known natural products of the organism (Bentley et al. 2002). Similar observations have now been reported for several diverse, sequenced microorganisms, such as *Aspergillus* (Bok et al. 2006), *Streptomyces avermitilis* (Ikeda et al. 2003), *Saccharopolyspora erythraea* (Oliynyk et al. 2007), *Pseudomonas fluorescens* (Paulsen et al. 2005) and *Salinispora tropica* (Udwary et al. 2007). These studies revealed that many novel natural compounds are still unidentified and thus unexplored from natural sources and indicated that the withdrawal of big pharmaceutical companies from natural product drug discovery was premature. Over the past several years, genome mining for new natural products and biosynthetic pathways has become a rapidly advancing field (Corre and Challis 2007; Challis 2008). These findings strongly support the one-strain-many-compounds (OSMAC) approach, according to which varying growth conditions can positively influence the metabolite profile of microorganisms. Therefore, a multitude of potentially useful natural products still awaits discovery (Peric-Concha and Long 2003).

In this chapter, we review the potential of antimicrobial compounds obtained from endophytic microorganisms with potential and touch-up on the board techniques employed in the field of endophytic biology.

2 Why Endophytes?

In the battle against the increase of drug-resistant pathogens, there is an urgent need for novel alternatives to currently used antibiotics. Exploration of the unique niches of biodiversity leads to the discovery of new natural products, and the perusal of literature suggests that the microorganisms residing within the plant are an enormous untapped source of potential bioactive molecules (Menpara and Chanda 2013).

For a better understanding of why endophytes have been playing a key role in antimicrobial research, it is necessary to review their role in nature. Endophytes colonize internal plant tissues without causing any symptoms of disease. They are diverse at the species level, phylogenetically abundant, ecologically primed, evolutionarily strong and are an unexplored group of taxonomic, genetic and functional diversity. Endophytes are ubiquitous and have been found in every studied plant. Microbes enter tissues of the plant through the roots or wounds or rather by creating wounds through the production of enzymes like cellulases. It is still unknown why plant's defence mechanisms are ineffective against colonization by endophytes or why plants and endophytes coexist. Regardless, there exists a symbiotic (mutually beneficial) relationship between a plant and its endophytes; the endophytes are benefitted by their access to plant nutrients, and the plant is benefitted by protections provided by the endophytes against pathogens, the promotion of plant growth and increased tolerances to biotic and abiotic stressors. Recently, it was shown that plant microsymbionts produce a variety of secondary metabolites that not only play a major role in providing defences to the host but which also aid in specific

interactions and communication with the plant (Brader et al. 2014). Due to the constant process of microbial strain development by passage through various stages of plant growth and development, as well as their acquired ecological functions, endophytes have evolved into proficient producers of bioactive secondary metabolites (Strobel et al. 2004; Porras-Alfaro and Bayman 2011; Nalli et al. 2015).

Endophytes, in particular, assist their hosts in evading pathogens by producing antimicrobial secondary metabolites. The potential antimicrobial activity of these strains may be due to their evolution over billions of years in diverse ecological niches and natural habitats (Strobel et al. 2004; Aly et al. 2011; Mousa and Raizada 2013).

Many studies have revealed a novel role of endophytes in the improvement of plant physiology, where some are known to interact directly or indirectly with mineral and nutrient uptake by the host plant (Singh et al. 2011a). In one study, it was revealed that endophytic fungi present in drought-tolerant species not only exert their action through the storage and secretion of sugars and alcohols but also through triggering minor changes in leaf physiology, which ultimately leads to reduced transpiration losses (Auge et al. 2008). It has also been seen that under heavy metal stress, endophytes protect the host plant by reducing metal accumulation and transport (Yamaji et al. 2016).

Some endophytic microorganisms are known to confer their own ecological functions, such as thermal tolerance, to the plants. They can also affect community structure and microbial interactions, which are the lead determinants of biodiversity in plants, and can interact with the systems of the host plant by influencing the availability of nutrients and by their ability to provide resistance to biotic and abiotic stress. Importantly, endophytes can be modified in such a way that their positive effects are exploited. For example, *Leifsonia xyli*, a xylem-inhabiting bacterial endophyte, has been genetically modified with a gene from *Bacillus thuringiensis*, thereby producing delta-endotoxin, which is active against insects in nature, especially Lepidoptera and Coleoptera (Mills et al. 2001). Endophytes can also be used as biological control agents (BCAs) and are advantageous over conventional BCAs due to their ability to be directly applied to the seeds, thereby avoiding the treatment of a large number of established plants (Ezra et al. 2009).

Various groups have been working on endophytes, and a significant amount of literature is available on the field. However, as stated before, the primary focus has been in isolating promising plant metabolites from the endophytes of the host, with considerable success (Puri et al. 2006; Kusari et al. 2009). Other works have focused on using endophytes as growth-promoting agents in various plants/crops (Tiwari et al. 2010; Singh et al. 2013; Wani et al. 2017). The endophytes studied have been mostly acquired from individual plants sporadically, but significant efforts have been made to bioprospect the endophytes from different locations (Raviraja 2005; Puri et al. 2006; Shweta et al. 2013; Qadri et al. 2013, 2014; Arora et al. 2016; Yu et al. 2010; Yedukondalu et al. 2017). Considering the enormity of the biodiversity, concerted efforts are needed to tap the endophytic microorganisms for bioprospection. It seems also logical to isolate and characterize sustainable microbial compounds from these endophytes and to use new biology for known endophyte-produced molecules in order to discover bioactivities that have so far not been elucidated.

3 Endophytes as a Source of Bioactive Antimicrobials

Bioactive molecules from endophytes have potential uses in medicine, agriculture, cosmetics and the food industry (Strobel and Daisy 2003; Shukla et al. 2014). Classes of bioactive metabolites obtained from endophytes include, but are not limited to, alkaloids, cytochalasins, polyketides, terpenoids, flavonoids, steroids, cyclohexanones, depsipeptides, lactones, lignans, peptides and quinines with antimicrobial, anticancer, antioxidant, insecticide and immunosuppressant potential (Fig. 1) (Guo et al. 2008; Kharwar et al. 2011; Mousa and Raizada 2013). Thus, numerous bioactive molecules of microbial origin have been characterized from endophytes, and many more await isolation (Mousa and Raizada 2013).

The extraction of secondary metabolites from the endophytic isolates is a crucial step (Fig. 2). It is affected by a number of factors, including solvent used and the methods employed for extraction. The evolution of the microorganism, which may have incorporated genetic information from its host plant, is known to directly influence the production of secondary bioactive metabolites that help them to adapt and carry out specific functions, such as protection the host from insects, pathogens and grazing animals (Gouda et al. 2016).

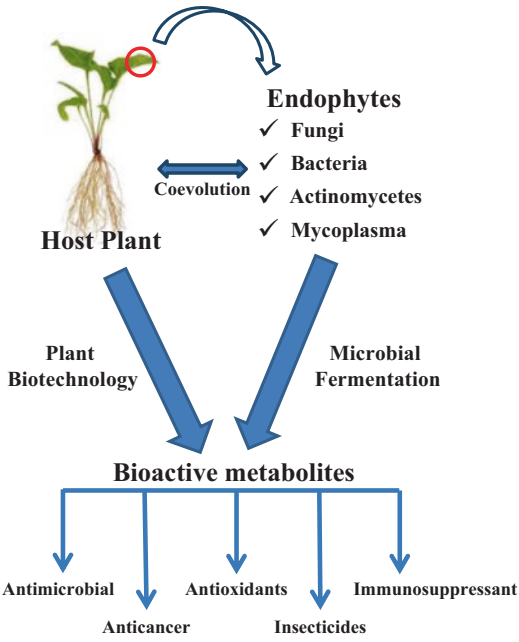


Fig. 1 Plant-endophyte symbiotic relationship along with their potential applications. Endophytes are associated with plants in various forms including fungi, bacteria, actinomycetes and mycoplasma. They are known to produce bioactives that serve as antimicrobial, anticancer, antioxidant, insecticide and immunosuppressant compounds

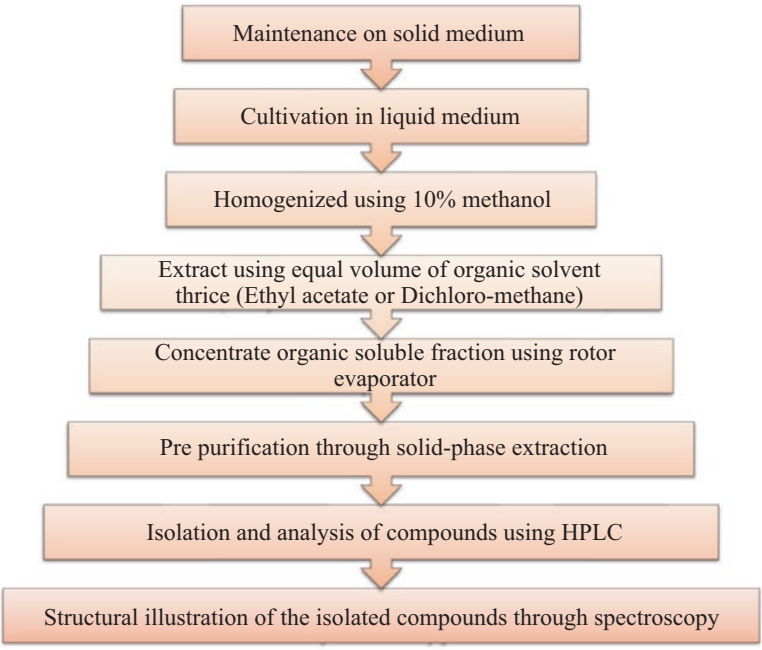


Fig. 2 Extraction of secondary metabolites from endophytes

A list of endophytic microorganisms, with their host plants and identified antimicrobial activities that have been discovered in recent years, is provided in Table 1, and the chemical structures of potential secondary metabolites isolated from endophytes are illustrated in Fig. 3. Some of the most promising agents are discussed below:

- **Leucinostatin A**, produced by the endophyte, *Acremonium* sp., which originated from *Taxus baccata*, has exhibited antimicrobial activity against *Pythium ultimum* with a 50% inhibitory concentration of less than 1 μmol (Strobel et al. 1997).
- **Ecomycins** belong to a novel family of lipopeptides containing uncommon amino acids, such as β-hydroxy aspartic acid and homoserine, which exhibit antimycotic potential. Ecomycin A, B and C are isolated from *Pseudomonas viridiflava*, a plant-associated bacterium having significant bioactivities against a broad spectrum of human and plant pathogens. Ecomycin B, in particular, exhibited the most potential, with an MIC of 40 mg/ml against *Cryptococcus neoformans* and 31 mg/ml against *Candida albicans* (Miller et al. 1998).
- **Cryptocandin**, a unique lipopeptide with significant antimycotic activity, was isolated from the endophytic fungus *Cryptosporiopsis quercina*. It was reported with the MIC value of 0.03–0.07 μg/ml against the fungal pathogens *Candida albicans*, *Trichophyton mentagrophytes* and *Trichophyton rubrum*. It was also found to be active against a number of fungal phytopathogens, including *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Strobel et al. 1999).

Table 1 A list of endophytic microorganisms, with their host plants and identified antimicrobial activities that have been discovered in recent years

Endophyte	Host Plant	Activity against pathogens	References
Colletotrichum sp.	*Artemisia annua*	*Rhizoctonia cereal*, *Phytophthora capsici* and *Helminthosporium sativum*	Lu et al. (2000)
Colletotrichum gloeosporioides	*Artemisia mongolica*	*Bacillus subtilis, Staphylococcus aureus* and *Sarcina lutea*	Zou et al. (2000)
Phomopsis longicolla	*Dicerandra frutescens*	*B. subtilis* and *S. aureus*	Wagenaar and Clardy (2001)
Paenibacillus polymyxa, *Bacillus* sp. and *Pseudomonas poae*	*Panax ginseng*	*Achlya klebsiana* and *Pythium spinosum*	Adhikari et al. (2001)
Streptomyces sp.	*Monstera* sp.	*Cryptococcus neoformans*	Ezra et al. (2004)
Nodulisporium sp.	*Juniperus cedre*	*B. megaterium*, *Chlorella Fusca*, *Microbotryum violaceum* and *Septoria tritici*	Dai et al. (2006)
Phomopsis cassia	*Cassia spectabilis*	*Cladosporium cladosporioides* and *C. sphaerospermum*	Silva et al. (2006)
B. amyloliquefaciens	*Scutellaria baicalensis Georgi*	*Streptococcus thermophilus*, *Saccharomyces cerevisiae*, *Botryodiplodia theobromae* and *Penicillium expansum*	Sun et al. (2006)
Botryosphaeria mamane	*Garcinia mangostana*	*S. aureus* and MRSA	Pongcharoen et al. (2007)
Phomopsis sp.	*Excoecaria agallocha*	*Candida albicans* and *Fusarium oxysporum*	Huang et al. (2008)
Ampelomyces sp.	*Urospermum picroides*	*S. aureus, S. epidermidis* and *Enterococcus faecalis*	Aly et al. (2008)
Phomopsis sp.	*Garcinia dulcis*	*Mycobacterium tuberculosis*	Rukachaisirikul et al. (2008)
Phoma sp.	*Saurauia scaberrinae*	*S. aureus*	Hoffman et al. (2008)
Penicillium sp.	*Acrostichum aureum*	*S. aureus* and *Candida albicans*	Cui et al. (2008)
Penicillium sp.	*Cerbera manghas*	*S. aureus*	Han et al. (2008)
Edenia gomezpompae	*Callicarpa acuminate*	*P. capsici, P. parasitica, F. oxysporum* and *Alternaria solani*	Macias Rubalcava et al. (2008)
Coniothyrium sp.	*Sideritis chamaedryfolia*	*Escherichia coli* and *B. megaterium*	Krohn et al. (2008a)
Dinemasporium strigosum	*Calystegia sepium*	*B. megaterium*	Krohn et al. (2008b)
Chaetomium globosum	*Viguiera robusta*	*S. aureus* and *E. coli*	Momesso et al. (2008)

(continued)

Table 1 (continued)

Endophyte	Host Plant	Activity against pathogens	References
Xylaria sp.	*Ginkgo biloba*	*S. aureus, E. coli, S. typhi, S. typhimurium, S. enteritidis, A. hydrophila, Yersinia* sp., *V. anguillarum, Shigella* sp. and *V. parahaemolyticus*	Liu et al. (2008)
Microdochium bolleyi	*Fagonia cretica*	*E. coli* and *B. megaterium*	Zhang et al. (2008)
Pestalotiopsis sp.	Lichen *Clavaroids* sp.	*S. aureus*	Ding et al. (2009)
B. subtilis	Wheat	*Gaeumannomyces graminis* var. *tritici* (Ggt)	Liu et al. (2009)
Nodulisporium sp.	*Erica arborea*	*B. megaterium*	Dai et al. (2009)
B. licheniformis, B. pumilus, Bacillus sp.	*Platycodon grandiflorum*	*P. capsici, F. oxysporum, Rhizoctonia solani* and *Pythium ultimum*	Islam et al. (2010)
Enterobacter sp., *B. subtilis*	*Raphanus sativus L*	*E. coli, Pseudomonas aeruginosa, Salmonella enterica, S. enteritidis, S. typhimurium, Shigella flexneri, Shigella sonnei, B. cereus, Listeria innocua, L. ivanovii, L. monocytogenes* and *S. aureus*	Seo et al. (2010)
Pichia guilliermondii	*Paris polyphylla*	*A. tumefaciens, E. coli, P. lachrymans, R. solanacearum, X. vesicatoria, B. subtilis, S. aureus* and *S. haemolyticus*	Zhao et al. (2010)
Paenibacillus sp.	*Manihot esculenta*	*R. solani*	Canova et al. (2010)
Burkholderia sp.	*Huperzia serrata*	*P. capsici, F. graminearum* and *Sclerotinia libertiana*	Wang et al. (2010)
Phoma sp.	*Salsola oppositifolia*	*B. subtilis* and *E. coli*	Loesgen et al. (2011)
Microdiplodia sp.	*Lycium intricatum*	*Legionella pneumophila*	Siddiqui et al. (2011)
Streptomyces sp.	*Kandelia candel*	Methicillin-Resistant *S. aureus* and Vancomycin-Resistant *Enterococcus faecalis*	Ding et al. (2011)
Penicillium chrysogenum	*Marine red alga Laurencia* sp.	MRSA, *P. fluorescens, P. aeruginosa* and *S. epidermidis*	Gao et al. (2011)
Fusarium oxysporum	*Cinnamomum kanehirae*	MRSA and *B. subtilis*	Wang et al. (2011b)
Phomopsis longicolla	*Bostrychia radicans*	*S. aureus* and *S. saprophyticus*	Erbert et al. (2012)
Diaporthe phaseolorum	*Laguncularia racemosa*	*S. aureus* and *S. typhi*	Sebastianes et al. (2012)

(continued)

Table 1 (continued)

Endophyte	Host Plant	Activity against pathogens	References
B. amyloliquefaciens	*Memecylon edule, Tinospora cordifolia*	*B. subtilis, E. coli, S. aureus, Pseudomonas aeruginosa* and *Candida albicans*	Bhoonobtong et al. (2012)
Dothideomycete sp.	*Tiliacora triandra*	*S. aureus* and MRSA	Senadeera et al. (2012)
Aspergillus sp.	*Bruguiera gymnorrhiza*	*S. aureus* and *B. subtilis*	Li et al. (2012)
Nigrospora sp.	*Pongamia pinnata*	MRSA, *E. coli, P. aeruginosa, P. fluorescens* and *S. epidermidis*	Shang et al. (2012)
Pestalotiopsis mangiferae	*Mangifera indica*	*B. subtilis*, *P. aeruginosa* and *K. pneumoniae*	Subban et al. (2013)
Coniothyrium sp.	*Salsola oppositifolia*	*E. coli* and *B. megaterium*	Sun et al. (2013b)
Microsphaeropsis arundinis	*Pinus* sp.	*S. aureus*	Luo et al. (2013)
Bacillus sp., *Pseudomonas* sp.	*Plectranthus tenuiflorus*	*S. aureus, E. coli, Klebsiella pneumoniae, Streptococcus agalactiae, Proteus mirabilis* and *Candida albicans*	El-Deeb et al. (2013)
B. amyloliquefaciens, B. methylotrophicus	*Panax notoginseng*	*F. oxysporum, Ralstonia* sp. and *Meloidogyne hapla*	Ma et al. (2013)
Lewia infectoria	*Besleria insolita*	*S. aureus*	Casella et al. (2013)
B. subtilis, Pseudomonas fluorescens	*Centella asiatica*	*Colletotrichum higginsianum*	Rakotoniriana et al. (2013)
B. subtilis, C. flaccumfaciens, Ps. Fluorescens, P. ananatis	*Panicum virgatum L.*	*Trichoderma virens* and *Rhizoctonia solani*	Gagne-Bourgue et al. (2013)
Cryptosporiopsis sp.	*Viburnum tinus*	*B. megaterium*	Saleem et al. (2013)
B. subtilis, B. licheniformis	*Codonopsis lanceolata*	*Phytophthora capsici*, *F. oxysporum* and *Rhizoctonia solani*	Kang et al. (2013)
Streptomyces sp.	*Polygonum cuspidatum*	*Aspergillus niger*, *Aspergillus fumigatus*, *Klebsiella pneumoniae, S. aureus* and *B. subtilis*	Sun et al. (2013a)
Aspergillus sp.	*Bauhinia guianensis*	*B. subtilis, E. coli, P. aeruginosa* and *S. aureus*	Pinheiro et al. (2013)
Phialophora mustea	*Crocus sativus*	*C. albicans*	Nalli et al. (2015)
Phoma sp.	*Glycyrrhiza glabra*	*S. aureus* and *S. pyogenes*	Arora et al. (2016)
Diaporthe terebinthifolii	*Glycyrrhiza glabra*	*Candida albicans*	Yedukondalu et al. (2017)

- The antifungal agent, **cryptocin**, was isolated from an endophytic strain of *Cryptosporiopsis quercina* from the inner bark of the stems of *Tripterygium wilfordii*. Cryptocin is a unique tetramic acid exhibiting antimycotic activity against *Pyricularia oryzae* with the MIC value of 0.39 μg/ml. It also possesses activity against a wide variety of plant-pathogenic, but not human-pathogenic, fungi (Li et al. 2000).
- The continual natural occurrence of cyclohexane epoxides and the exploration of their biological activities have gained interest within pharmacologists, biologists and chemists (Marco-Contelles et al. 2004). **Jesterone** and **hydroxy-jesterone** are potential cyclohexenone epoxides recovered from the fungal endophyte, *Pestalotiopsis*

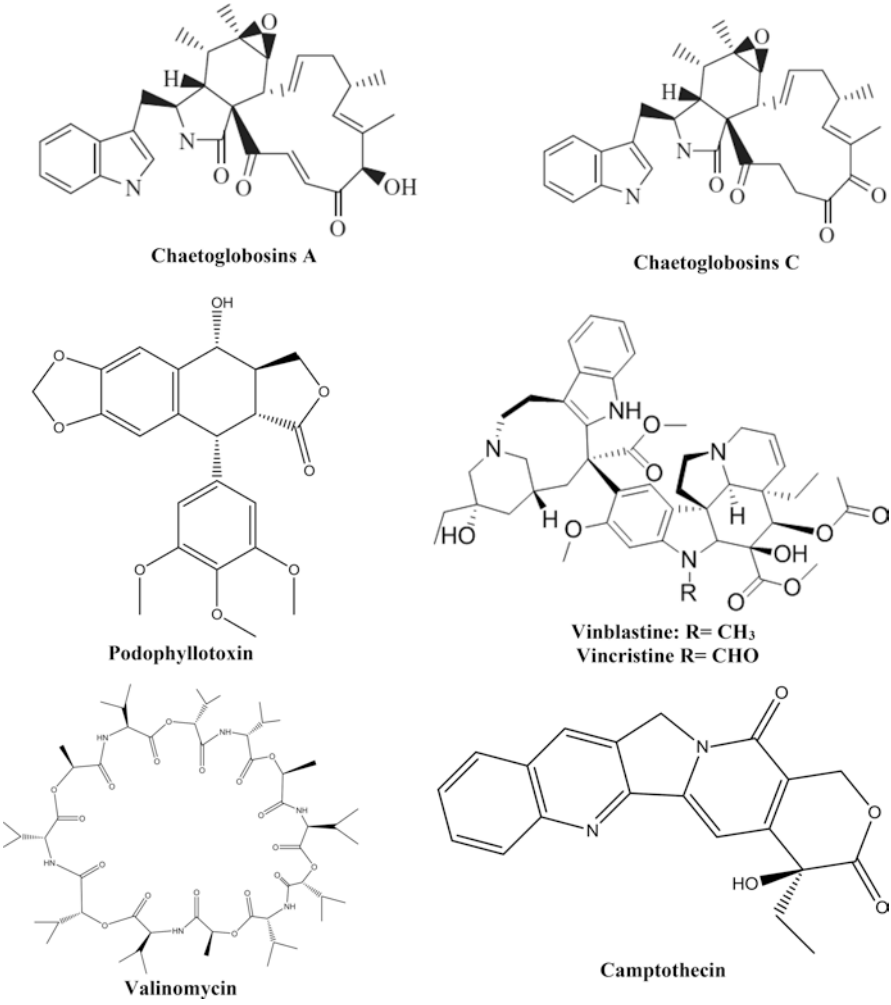
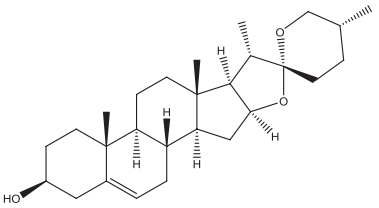
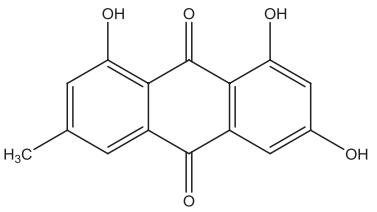


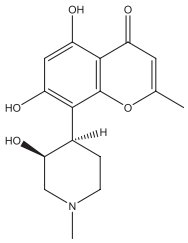
Fig. 3 Chemical structures of potential secondary metabolites isolated from endophytes



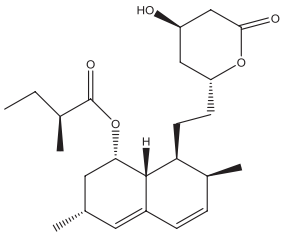
Diosgenin



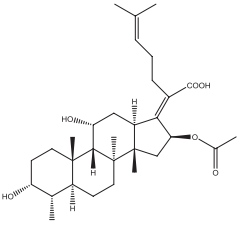
Emodin



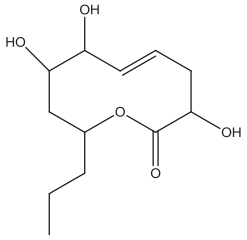
Rohitukine



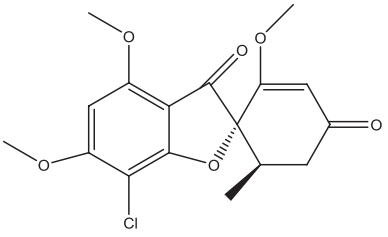
Lovastatin



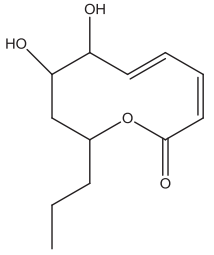
Fusidic acid



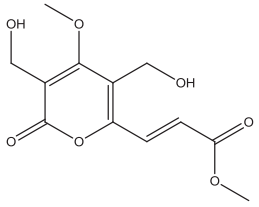
Phomolide-G



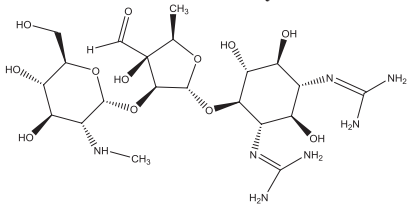
Griseofulvin



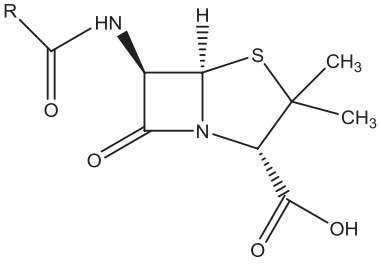
Xylarolide



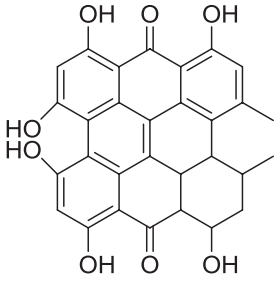
Rosellisin



Streptomycin



Pencillin R: variable group



Hypericin

Fig. 3 (continued)

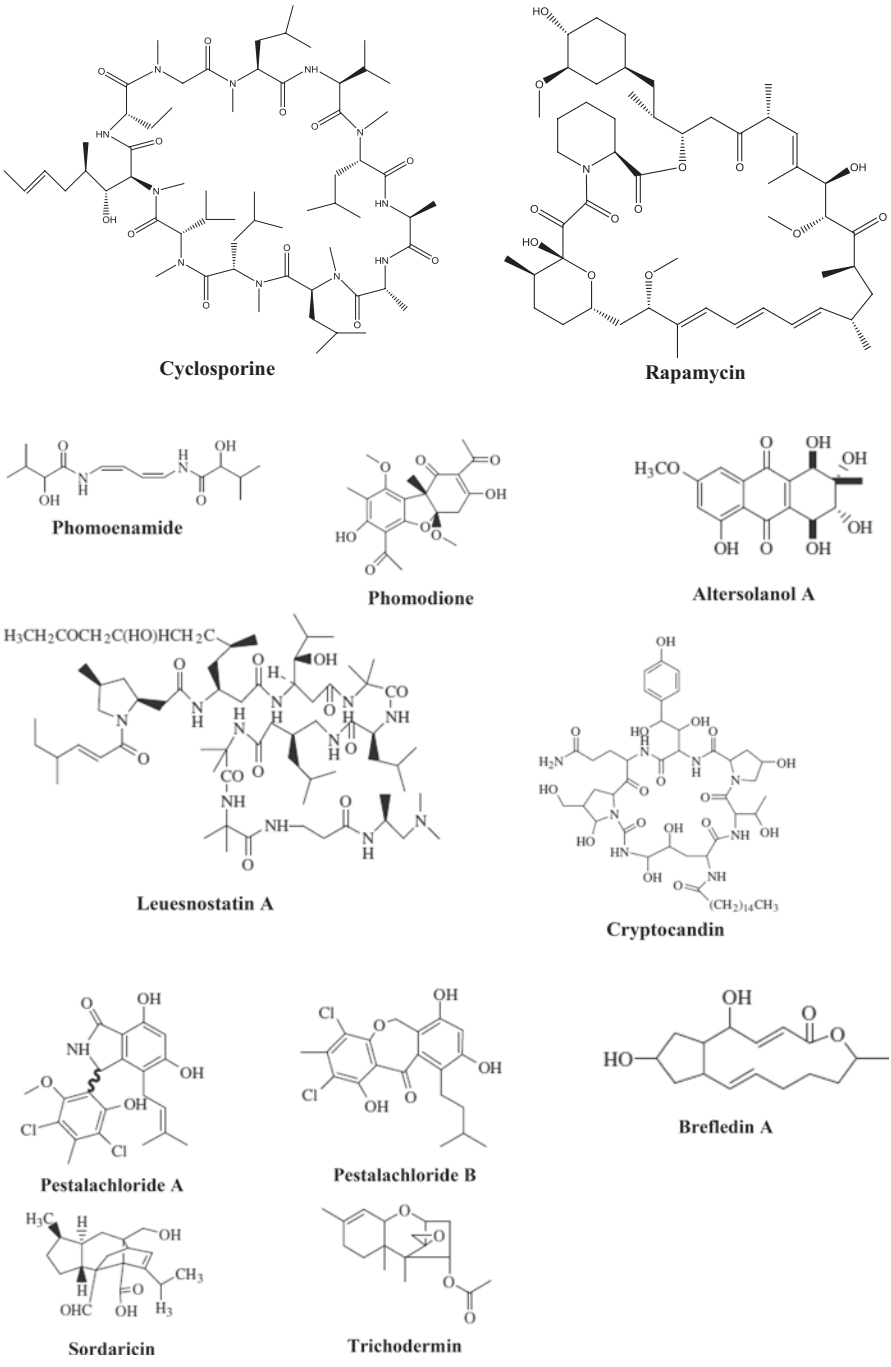


Fig. 3 (continued)

jester, with bioactive potential against *Pythium ultimum*. In particular, it showed selective antimycotic activity against the oomycetous fungi, which are some of the most phytopathogenic of all disease-causing fungi (Li and Strobel 2001).

- **Pestacin**, from *Pestalotiopsis microspore*, an endophytic fungus indigenous to Papua New Guinea, exhibits moderate antifungal and antioxidant activity (Harper et al. 2003).
- **Brefeldin A** is a fungal metabolite, exhibiting antitumor, antimitotic, antifungal and antiviral activities (Harri et al. 1963). Brefeldin A has been isolated from an endophytic culture of *Cladosporium* sp. associated with *Quercus variabilis*.
- **Echinocandins** are a group of lipopeptides with potential antifungal activities against *Candida albicans*, *Candida parapsilosis* and *Candida guilliermondii* (Grover 2010). Several echinocandins (A, B, C, D and H) have been isolated from endophytic *Cryptosporiopsis* sp. and *Pezicula* sp. (Noble et al. 1991). The antimicrobial compounds, ergosterol and 5a,8a-epidioxyergosterol, were isolated from the endophytic fungus *Nodulisporium* sp., which is associated with *Juniperus cedre* (Dai et al. 2006).
- **Munumbicins** were described as a novel group of antibiotics with a broad range of activity against many human pathogens and fungal phytopathogens. Four munumbicins (A, B, C and D) were isolated from *Streptomyces* sp., an endophyte of the medicinal plant, *Kennedia nigriscans*, also known as snake vine. In particular, munumbicin B exhibits the MIC value of 2.5 μg/ml against *S. aureus* (including MRSA), and munumbicin D showed activity against the malarial parasite *Plasmodium falciparum,* displaying the IC_{50} value of 4.5 ng/ml (Castillo et al. 2002). In another study, the structurally similar munumbicins E-4 and E-5 were isolated from *Streptomyces* sp. Both E-4 and E-5 showed potent activity against *Pythium ultimum* and were also active against *Plasmodium falciparum*, with IC_{50} values of 0.50 and 0.87 μg/m, respectively (Castillo et al. 2006).
- Five new octaketides, named the **cytosporones** (A, B, C, D and E), from the culture broth of two endophytic fungi, *Cytospora* sp. and *Diaporthe* sp., were isolated from the tissues of *Conocarpus erecta* and *Forsteronia spicata* plants, respectively. Cytosporones D and E displayed strong antibacterial activity, with an MIC for cytosporone D against representative strains of *E. faecalis*, *S. aureus* and *E. coli* and the fungus *C. albicans* of 8, 8, 64 and 4 μg/ml, respectively (Brady et al. 2000). In another report, cytosporone B and C were isolated from *Phomopsis* sp., an endophytic fungus of mangrove, and these compounds inhibited *C. albicans* and *F. oxysporum*, with MIC values ranging from 32 to 64 mg/ml (Huang et al. 2008).
- **Altersolanol A**, isolated from *Ampelomyces* sp., is an endophyte of the medicinal plant, *Urospermum picroides*, and has exhibited antimicrobial activity against the bacterial pathogens *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* at MICs of 12.5 and 12.5–25 mg/ml, respectively (Haraguchi et al. 1992; Aly et al. 2008).
- **Phomoenamide**, an antibacterial alkaloid, was isolated from the endophytic fungus *Phomopsis* sp., along with five more metabolites: phomonitroester, deacetyl phomoxanthone B, dicerandrol A, (1S,2S,4S)-p-menthane-1,2,4-triol and uridine. Phomoenamide exhibited moderate activity with the MIC value of 6.25 mg/ml against *Mycobacterium tuberculosis* (Rukachaisirikul et al. 2008).

- **Pestalachloride A and B** have been isolated from and endophytic strain of *Pestalotiopsis adusta* and showed significant antifungal activity against three phytopathogens, including *Fusarium culmorum*, *Gibberella zeae and Verticillium aibo-atrum* (Li et al. 2008).
- The **sordarin** family of compounds, characterized by a unique tetracyclic diterpene core, inhibits protein synthesis in fungi (Liang 2008). Sordaricin, produced by an endophytic fungal isolate *Xylaria* sp., recovered from the leaves of *Garcinia dulcis*, displayed moderate activity against a variety of fungal pathogens (Pongcharoen et al. 2008).
- **Chaetoglobosins**, well-known mycotoxins, have gained interest due to a large number of biological activities, viz. cytotoxic, antifungal, phytotoxic and nematicidal (Li et al. 2014). Till now, more than 40 chaetoglobosins have been reported from the cultures of some fungi, most belonging to the genus *Chaetomium*. Chaetoglobosins A, G, V, Vb and C were characterized from the culture of an endophytic strain *C. globosum* isolated from the leaves of *Ginkgo biloba* (Qin et al. 2009).
- The **non-nitrogenous methyl phenalenones** produced by a fungal endophyte, *Coniothyrium cereal*, possessed potential antimicrobial activity. Coniosclerodione, coniosclerodione, (–)-cereo lactone and (–)-scleroderolide showed strong antimicrobial activity against *S. aureus* SG 511. Z-coniosclerodinol, (S, S)-sclerodinol and coniolactone inhibited the growth of *Mycobacterium phlei* in agar diffusion assay. Also, trypethelone strongly inhibited the growth of *M. phlei*, *S. aureus* and *E. coli* (Elsebai et al. 2011a, b).
- **Azaphilones** or **azaphilonoids** are a structurally variable family of fungal polyketide metabolites exhibiting a wide range of significant biological functions, including antimicrobial, antiviral, cytotoxic, anticancer and anti-inflammatory activities (Gao et al. 2013). Pestafolide A, a novel antifungal azaphilone, has been isolated from the solid cultures of an endophytic isolate, *Pestalotiopsis foedan* (Ding et al. 2008). Four new azaphilone-derived molecules, Phialomustin A–D, were isolated and characterized from an endophytic fungus, *Phialophora mustea*, obtained from *Crocus sativus*. Compounds C and D displayed significant antifungal activities, with IC_{50} values of 14.3 and 73.6 μM against *Candida albicans* (Nalli et al. 2015).
- **Diketopiperazines** are the smallest cyclic peptides known for having important biological activities, such as antifungal, antibacterial, antitumor, antiviral, anti-hyperglycaemic and glycosidase inhibition. They also have the potential to disrupt bacterial biofilm formation (Carvalho and Abraham 2012). Five new sulphide diketopiperazine derivatives, penicibrocazines A–E, were isolated from the culture extract of *Penicillium brocae*, an endophytic strain recovered from the tissues of the marine mangrove plant *Avicennia marina.* Compound B showed antimicrobial activity against a few of the test pathogens, with the MIC values ranging from 0.25 to 64 μg/ml (Meng et al. 2015). Also, the chemical investigation of an endophytic strain, *Phoma* sp., associated with *Glycyrrhiza glabra*, led to the isolation of two thiodiketopiperazine derivatives. Both of these compounds inhibited the growth of several bacterial pathogens especially that of *Staphylococcus aureus* and *Streptococcus pyogenes*, with IC_{50} values of less than 10 μM. In addition, the compounds strongly inhibited biofilm formation by both of the pathogens (Arora et al. 2016).

- Two new fatty acid-derived metabolites, **diapolic acids A and B,** were isolated from the crude extract of *Diaporthe terebinthifolii*, with moderate antimicrobial potential against *Yersinia enterocolitica* (Yedukondalu et al. 2017).
- **Javanicin**, a highly functionalized naphthoquinone, was recovered from an endophytic fungus, *Chloridium* sp., isolated from the fresh tissues of neem. It exhibits strong antibacterial potential against *P. aeruginosa* and *P. fluorescens*, with thee MIC value of 2 μg/ml (Kharwar et al. 2009).

In the past years, natural and biological control agents against insects, pests and diseases affecting plants have attracted more attention as a way to reduce the use of insecticides and pesticides in agriculture biotechnology. In this regard, endophytes have gained much focus as a promising source of such agents. Earlier studies have reported that biological control of many plant diseases could be achieved by using antagonistic endophytes. Different bacterial species, namely, *Alcaligenes* spp., *Kluyvera* spp. (de Assis et al. 1998), *Pseudomonas fluorescens*, *P. alcaligenes*, *P. putida*, *Flavobacterium* spp., *Bacillus megaterium* (Reiter et al. 2002), *B. pumilus* (Benhamou et al. 1998), *Microbacterium* spp., *Clavibacter michiganensis*, *Curtobacterium* spp. and *B. subtilis* (Zinniel et al. 2002), and fungal species, namely, *Coniothyrium carteri*, *Fusarium larvarum*, *Truncatella spadicea* (Qadri et al. 2014), *Trichoderma harzianum*, *Porostereum* sp., *Alternaria* sp., *Alternaria alternata* and *Botrytis fabiopsis* (Wani et al. 2016) have been reported as endophytes that were inhibitory to plant pathogens. The fungi *Fusarium* and *Neotyphodium* have been found active against nematodes and *Triticum* spp., respectively (Pocasangre et al. 2000; Tunali et al. 2000).

Thus, a myriad of antimicrobial bioactivities have been recovered from endophytic species, and it is believed that these bioactivities can aid in solving the current threat of drug-resistant pathogens.

4 Antimicrobial Volatile Organic Compounds (VOCs)

VOCs are considered important chemicals produced by microorganisms in the environment that impact the kinetics of the ecosystem and vice versa (Wheatley 2002). Under optimum conditions, VOCs produced by microorganisms are consistent and reproducible. The discovery of the mycodiesel-producing organism *Ascocoryne* sp. (Strobel et al. 2008; Griffin et al. 2010), and further exploration of antimicrobial VOCs of *Muscodor* species (Strobel 2006a), led to the conclusion that fungal isolates produce diverse batteries of VOCs having potential applications in industrial as well as agriculture. In this chapter, the NIST database chemical terminology has been used for naming the VOC compounds.

In agriculture, the interest in fungal VOCs is for their potential as biological control (biocontrol) agents to combat fungal pests through the employment of a more environmentally sound pest management strategy, namely, by reducing fungicide use on crop plants (Morath et al. 2012). That is, since VOCs are naturally occurring, they have the potential to be used as possible alternatives to hazardous fungicides, pesticides and insecticides (Kanchiswamy et al. 2015).

Several potential VOC-producing endophytes with great industrial and agricultural potential have been reported in the last two decades. These compounds belong to different chemical classes, such as terpenoids and benzene derivatives, naphthalene derivatives, cycloalkanes, alcohols, organic acids, ketones and aldehydes, and often have antimicrobial potential, suggesting that these volatile substances may play an important role in nature to create microenvironments free of challenging microorganisms (Riyaz-Ul-Hassan et al. 2012; Strobel et al. 2011). Some of the most promising endophyte produced VOCs and are detailed in the following paragraphs.

More than 28 volatile organic compounds were isolated from the fungal endophyte *Muscodor albus*, associated with *Cinnamomum zeylanicum*, which are found to be potent antimicrobials, as they completely inhibited the majority of the test pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtilis* and some fungal pathogens, killing them within a period of 3 days. These VOCs are mixtures of gases belonging to five classes, made up of alcohols, organic acids, esters, ketones and lipids, among which the most effective were the esters, with 1-butanol-3-methyl-acetate having the highest activity (Strobel et al. 2001).

Another strain of *Muscodor*, namely *Muscodor crispans*, was also found to produce antimicrobial VOCs, namely, propanoic acid, 2-methyl-1-butanol, 3-methyl-1-butanol, 3-methyl-acetate, 2-methyl-2-methyl butyl ester and ethanol. The VOCs of the fungus were effective against a wide range of plant pathogens, including the fungi *Pythium ultimum*, *Phytophthora cinnamomi*, *Sclerotinia sclerotiorum* and *Mycosphaerella fijiensis* (the black sigatoka pathogen of bananas), and the serious bacterial pathogen of citrus plants, *Xanthomonas axonopodis pv. citri*. In addition, the VOCs of *M. crispans* killed several human pathogens, including *Yersinia pestis*, *Mycobacterium tuberculosis* and *Staphylococcus aureus*.

Artificial mixtures of fungal VOCs have also been constituted and evaluated for antimicrobial potential (Mitchell et al. 2010). A synthetic mixture of the VOCs from *M. crispans* demonstrated antimicrobial effects against a broad range of human and plant pathogens, including fungi, bacteria and oomycetes. *Pythium insidiosum* is an oomycete capable of causing a life-threatening disease in humans, called pythiosis. The synthetic mixture, at amounts as low as 2.5 µl, significantly reduced the growth of all *P. insidiosum* isolates by at least 80% (Krajaejun et al. 2012). VOCs produced by *M. yucatanensis* were also found effective against several fungi. Epigenetic modulation of this organism was found to induce the production of several new VOCs and other molecules (Qadri et al. 2017).

An endophytic fungus of *Persea indica*, identified as *Hypoxylon* sp., produced 1,8-cineole, 1-methyl-1,4-cyclohexadiene, the tentatively identified (+)-.alpha.-methylene-.alpha.-fenchocamphorone, and several other unidentified compounds. Six-day-old cultures of this endophyte displayed maximum antimicrobial activity against several pathogens. This was the first report of the production of 1,8-cineole by a fungal culture (Tomsheck et al. 2010). It was later found that epigenetic modulation of this endophytic fungus resulted in phenotypic changes, as well as modulation VOC profiles (Riyaz-Ul-Hassan et al. 2012). Similarly, the endophyte, *Hypoxylon* sp., produced a unique an array of bioactive VOCs, including 1,8-cineole. The

organism uniquely produced a series of ketones, including acetone; 2-pentanone; 3-hexanone, 4-methyl; 3-hexanone, 2,4- dimethyl; and 2-hexanone, 4-methyl, and 5-hepten, 2-one, and these account for about 25% of the total, *Hypoxylon*-produced VOCs. The VOCs of this isolate were selective active against a number of plant pathogens and induced the death of *Phytophthora palmivora*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, and a 100% inhibition of *Phytophthora cinnamomi*, with only slight to no inhibition of the other pathogens that were tested. From this work, it has becoming increasingly apparent that each isolate of this endophytic *Nodulisporium* spp., including *Daldina* sp. and *Hypoxylon* spp. teleomorphs, seems to produce their own unique set of VOCs (Riyaz-Ul-Hassan et al. 2013).

An endophytic *Phomopsis* sp. was found to produce a unique mixture of VOCs, including sabinene, which is a monoterpene with a peppery odour, only previously known from higher plants. Additional VOCs produced by this organism were 1-butanol, 3-methyl; benzene ethanol; and 1-propanol, 2-methyl and 2-propanone. The gases of *Phomopsis* sp. also possessed antifungal properties, with the IC_{50} values for an artificial gas mixture varying between 8 and 25.65 μl/ml (Singh et al. 2011b). The endophytic fungus *Phoma* sp., associated with the creosote bush, also produced a mixture of VOCs, including a series of sesquiterpenoids, some alcohols and several reduced naphthalene derivatives. The gases emitted by *Phoma* sp. possessed antifungal properties, and the compounds were markedly similar to that of a methanolic extract of the host plant (Strobel et al. 2011).

Thus, endophytic fungi are capable of producing unique arrays of VOCs with antimicrobial activities, having applications in several fields. These applications include alternate fuels, perfumery, biodegradation and decontamination of human and animal wastes, biofumigation, and post-harvest food processing, to name a few.

5 Exploration of New Endophytic Metabolites by Culture-Independent Methods

Molecular approaches have conservatively estimated that microbial diversity is highly unexplored, with only about 1% of bacteria and 5% of fungi characterized so far. Surprisingly, less than 1% of microorganisms can be cultivated by with current laboratory techniques (Amann et al. 1995). Thus, culture techniques inadvertently prejudice our perspective on microbial diversity, including that of both prokaryotic and eukaryotic phyla (Connon and Giovannoni 2002). In particular, endophytic fungi are well known for their potential to produce diverse and active secondary metabolites. The identification and characterization of microbial communities in the environments have been reformed by the use of molecular methods involving PCR amplification of rRNA and conserved protein genes, such as histones and beta tubulins (Vianna et al. 2009; Tejesvi and Prakash 2009). Traditional methods, such as restriction fragment length polymorphisms (RFLP), terminal restriction fragment length polymorphisms (T-RFLP), single-strand conformation polymorphism (SSCP) and quantitative PCR (qPCR) (Laguerre et al. 1994; Lee et al. 1996; Dunbar et al. 2000; Takai and Horikoshi 2000), have been in practice for more than two

decades, but they can only be applied for the identification of microorganisms and not for functional screening. Now, with the development of next-generation sequencing techniques, researchers have gained new methods, such as metagenomics and metaproteomics (Felczykowska et al. 2012; Jang et al. 2012), that have enabled the functional screening and identification of candidate gene-encoded proteins from endophytes for their use in agricultural, food and pharmaceutical industries. Recently, many bioactive compounds have been discovered by means of metagenomics, particularly antibacterials, such as indigo, turbomycins, violacein and nocardamine, all of which were isolated from soil samples (Banik and Brady 2010).

6 Conclusion

Endophytes constitute an enormously diverse microbial resource for bioprospecting, due to the fact that they are usually metabolically proficient. Their capability to produce vast spectrums of natural products is attributed to their diverse functions in nature. In fact, similar organisms isolated from different plants in the same region, or those isolated from the same plants in different regions, may produce different metabolites. These endophytes may play an important role in conferring pathogen resistance to their host by virtue of their metabolites, and therefore, they are of particular interest for the isolation of novel antimicrobial agents. The search for endophytes should be preferentially conducted in the areas of high biodiversity, as their diversity is directly linked to that of the plants. Preference for bioprospecting may be given to taxonomically novel microorganisms, as novel taxonomy may lead to the discovery of new natural products. Once isolated, endophyte potential may be explored through use of media manipulations, inducers, epigenetic modulators, fermentation technology, co-culture and other biotechnological approaches. Thus, given their potential to aid in the fight against antimicrobial resistance, a concerted effort is needed to explore the vast array of endophytes for the discovery of novel antimicrobial agents.

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Alternative Therapies to Antibiotics to Combat Drug-Resistant Bacterial Pathogens

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Abstract

The unabated emergence and spread of antimicrobial resistance (AMR) within both nosocomial and community environments is the driving force behind the urgent need to discover novel antimicrobial agents. However, owing to the challenges faced during conventional drug discovery programmes and the concomitant paucity of new drugs, it is prudent to focus on non-conventional approaches that could serve as alternatives to antibiotics. These approaches include all non-compound approaches that target pathogens other than antibiotics. Although these alternatives may or may not be absolute replacements of antibiotics, they can certainly be used in prophylaxis and in combination therapies with antibiotics to reduce the overuse and help prevent AMR. The advantage of this approach includes specific inhibition of pathogens without effecting the host's commensal beneficial microbiome. This is in direct contrast to antibiotic therapies which disturb the commensal bacteria, leading to increased risks of *Clostridium difficile*-associated diarrhoea, vaginal *Candida albicans* infections and the exacerbation of asthma and allergic diseases. Although a consistent efficacy is lacking, switching to alternatives will certainly reduce antibiotic abuse to a large extent and consequent resistance. Further development of these specific approaches is warranted to improve deliverability, potency and reliability. Thus, the investigation of novel non-antibiotic approaches for the prevention of, and protection against, infectious diseases should be stimulated, and such approaches must be high-priority research and development projects. The alternative approaches to antibiotics include immunomodulation, competitive exclusion of pathogenic bacteria via probiotics and their combination, natural and synthetic antimicrobial peptides, antibodies, bacteriophages and phage lysins. These alternative strategies are considered in this chapter.

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1 Introduction

The discovery of antibiotics has revolutionized the treatment of infectious diseases, but due to their extensive misuse and the resistance mechanisms inherent to bacteria, there is a widespread emergence of resistance against these antibacterial agents or AMR. This leads to the emergence of antibiotic-resistant strains against a new antibacterial as soon as it hits the market (Fig. 1). Poor financial incentives and resulting slow pace of newer antibacterial development exacerbates the current scenario. Owing to the challenges faced in the current drug discovery programme, there is a shift in interest towards non-conventional approaches. These include immunomodulators, antimicrobial peptides, probiotics, antibodies, bacteriophages and phage lysins. One common advantage with all of these is their specificity towards their target, a feature lacking in conventional antibiotic therapy. Antibiotic non-specificity causes disruption of the commensal gut microflora, resulting in the growth of some atypical organisms such as *Clostridium difficile*, a leading cause of diarrhoea in patients being administered antibiotics, and *Candida albicans*, which causes thrush or the enhancement of allergic diseases. Currently, these new approaches are being developed as adjuncts to conventional therapy or as prophylactic agents to reduce the overuse of the antibiotics. As most of these approaches are in clinical or preclinical stages, it is difficult to ascertain their efficacy, but with time they may emerge as potent alternatives to the conventional antibiotic approach. Currently, many alternative approaches are being studied and validated, but in this chapter, only those approaches for which validated data exists are discussed.

2 Immunomodulation

Immunomodulation is one of the promising approaches in the search of alternatives to antibiotics, which can be employed as a prophylactic and as an adjunctive therapy to enhance the effectiveness of antibiotics in the treatment of infections. Successful antimicrobial therapy depends on an appropriate immune response, which is achieved by coordinated efforts of innate and adaptive immunity, both of which target pathogenic organisms. Infectious organisms can subdue the normal immune responses of host with the help of their virulence factors and other mechanisms to propagate and cause disease. At times, abnormal immune system activity such as hyper host inflammatory responses is also partly responsible for pathogenesis of these organisms (Clark 2007). Thus, the potential to modulate immune responses by either enhancement or suppression, depending upon the need, is a potent therapeutic approach to work against infections and immune disorders (Ulevitch 2004; Hamill et al. 2008).

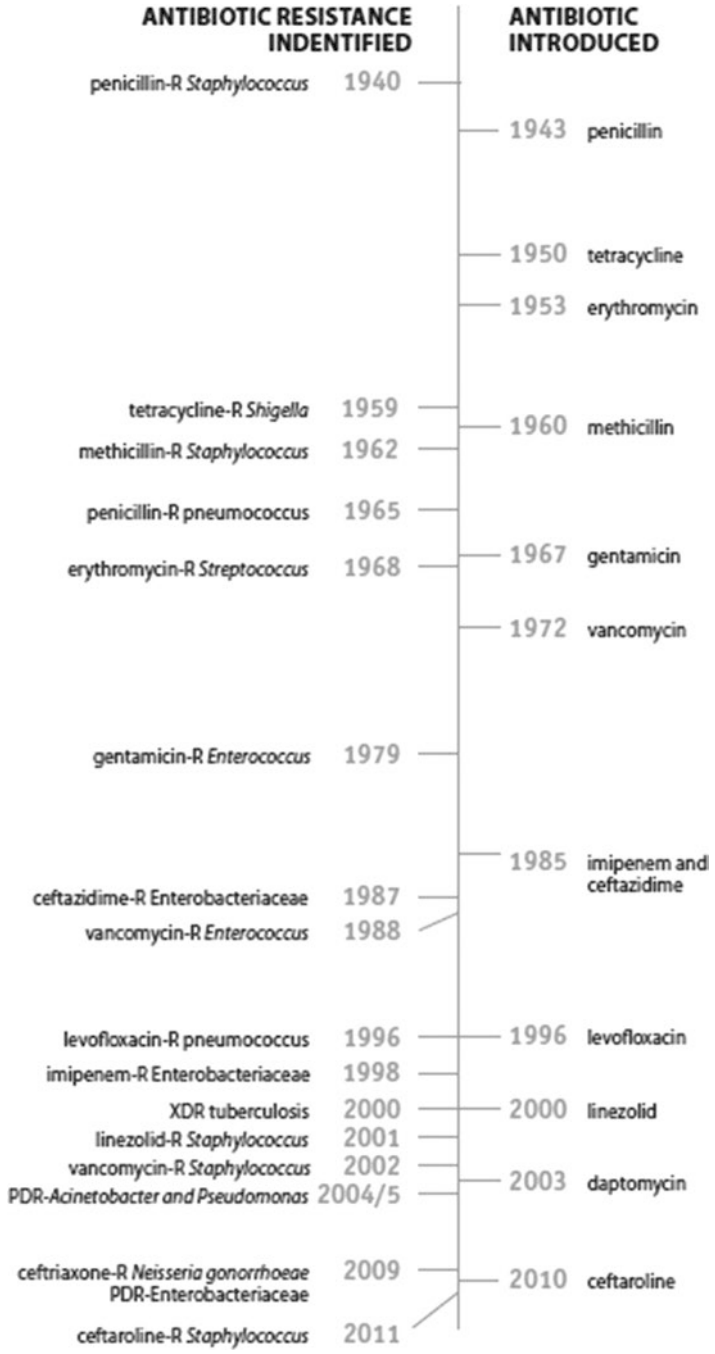

Fig. 1 Antibiotic discovery and identification of resistant bacterial strains

Currently, recombinant forms of natural immunomodulators produced by host defence system are being used for the prevention and treatment of infections in conjunction with antibiotics (Hancock and Sahl 2006). As anti-infectives, they work mainly by enhancing host innate immune responses, which may otherwise be lacking due to an acquired or congenital defect in the immune system function. While the stimulation of immune responses is necessary for the effective therapy, the aim should be to enhance protective innate antimicrobial immunity while keeping a check on inflammation-induced tissue injury (Karin et al. 2006). This is achieved by controlled stimulation of innate immunity without an increase in systemic pro-inflammatory responses (Hancock et al. 2012).

The primary advantage of these therapies is avoidance of AMR generation since the host's immune response is being exploited for deriving therapeutic benefit and not targeting the pathogen. That is exactly how vaccines have been able to evade resistance in pathogens over decades (Scherer and McLean 2002). Additionally, due to the non-specific nature of innate immunity, its stimulation can provide a broad-spectrum protection against various bacterial infections. Thus, immunomodulation can be used as a prophylactic measure since the protection can be provided beforehand by activating the immune response without the need to know the actual causal agent. However, stimulation of general action of the innate immune system can sometimes be detrimental and have adverse effects on increasing the severity of the disease (Antonelli et al. 2010). Also, there is inadequate target validation for bacterial infection, a high risk for side effects, variable responses and polymorphisms in patient populations and responses specific to bacterial species and strain. Hence, a proper and detailed understanding of the mechanisms of the innate immune system will aid in furthering immune stimulation therapies into translational research. The range of immunomodulators that have been able to significantly stimulate host innate antimicrobial immunity in different ways are described further (Hancock et al. 2012).

1. Agonists and antagonists of major classes of pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs).
2. Innate defence regulator peptides (IDRs).
3. Bacterial signalling molecules such as cyclic nucleotides and *N*-acyl homoserine lactones (AHLs) used in quorum sensing.

2.1 TLRs and NLRs Agonists as Immunostimulants

Toll-like receptors (TLRs) are pattern recognition receptors having a key role in innate immune system and responsible for recognizing a range of ligands that are mostly signature bacterial molecules such as cell envelop components, nucleic acids, flagella, etc. The TLRs include TLR1–TLR10 in humans, and each receptor is specific for their ligands (Trinchieri and Sher 2007). Upon sensing bacterial signature molecules, TLRs trigger signal transduction pathways such as the mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) pathways, resulting in the activation of innate immune cells. This further promotes the release of pro-inflammatory cytokines

following the induction of antimicrobial effector functions. Thus, if the TLR-driven innate immune pathways have to be modulated as desired, molecules that are analogous to the natural microbial ligands are used. Agonists of the innate immune pathway have a similar effect on the production of immune response promoting protective responses, potentially enhancing inflammation. On the contrary, antagonists suppress immune pathways and potentially harmful inflammation and in some cases might also repress protective mechanisms. Hence, in disease conditions where prompt immune response is required, agonists are suited, while where increased inflammatory response is detrimental, as in the cases of sepsis, antagonists must be used (Hancock et al. 2012). As an example, the lipid analogon **eritoran** (from Eisai Pharmaceuticals) acts as a TLR4 antagonist in cases of severe sepsis. Last reported in 2017, the results of a phase II and III clinical trial showed no better effect in reducing mortality in patients with severe sepsis than already present interventions (Tidswell et al. 2010; Opal et al. 2013). Thus, although there is evidence that TLR modulatory therapies can be used as preventive measures against microbial infections or in adjunction with antibiotics or vaccines, there has been no widespread usage since they have been widely implicated in inflammatory disorders and infectious diseases associated with inappropriate hyperactivation of immune responses.

Unlike TLRs, which are transmembrane receptors, NLRs are a family of cytoplasmic innate immune receptors that sense microbial cell wall fragments to induce inflammation and direct antimicrobial activities (Werts et al. 2011). Within the NLR family, three receptors NOD1 (nucleotide-binding oligomerization domain-containing (1), NOD2 (nucleotide-binding oligomerization domain-containing (2) and NLRP3 (NOD-, LRR- and pyrin domain-containing (3) are chiefly responsible for detecting peptidoglycan fragments generating immune responses via triggering of NF-κB and MAPK signalling (Sorbara and Philpott 2011). NOD1 senses diaminopimelate (DAP)-containing muropeptides in Gram-negative bacteria, and NOD2 recognizes muramyl dipeptide (MDP) found in all bacteria, while NLRP3 detects both microbial and endogenous ligands (Hancock et al. 2012). NLR agonists have been used to enhance both innate and adaptive immune responses. For example, alum is a NLRP3 agonist which is the most highly used adjuvant for vaccines, but its mode of action, as well as whether its activity in vivo requires NLRP3 activation, is still under debate (Spreafico et al. 2010). Testing of the potential for using NOD1 and NOD2 agonists, as human vaccine adjuvants, is currently in early stages. Thus, agonist and antagonist molecules of PRRs look promising as an adjuvant approach with antibiotics.

3 Innate Defence Regulator Peptides (IDRs)

IDRs are synthetic compounds based on sequences of host defence peptides (HDPs), with both anti-infective and immunomodulatory activities. Host defence peptides are the peptides produced by innate immune cells, including leukocytes and epithelial cells, in all multicellular organisms (Zasloff 2002). The amino acid composition of HDPs is biased towards cationic and hydrophobic residues. HDPs have important roles in the innate defence mechanisms, especially at mucosal linings and epithelial

surfaces. Natural HDPs have both immunomodulatory and antimicrobial activities where the antimicrobial activity is usually mediated or is a result of its stimulation/modulation of the host's immunity (Hancock et al. 2012). The combination of immunostimulatory effects, such as chemokine induction and regulatory functions such as the suppression of pro-inflammatory cellular responses to LPS, make HDPs highly desirable since they have a mechanism of action which avoids strong detrimental systemic pro-inflammatory responses (Hancock and Sahl 2006). Natural immunomodulatory HDPs have been exploited as general templates for the synthesis of synthetic IDR peptides. One such synthetic IDR peptide IDR1 was designed to have no direct antimicrobial activity but worked entirely through immunomodulatory activity in many animal models of antibiotic-resistant bacterial infections (Scott et al. 2007). Another recent example is IDR-1018, which is a small synthetic peptide derived by modification of bovine neutrophil HDP bactenecin. Although initially developed as an immune modulator with an ability to selectively enhance chemokine production while balancing the pro-inflammatory response, it is also reported to have anti-infective, anti-inflammatory, wound-healing and broad-spectrum antibiofilm activities. This shows its strong potential as an adjunctive therapy against antibiotic-resistant infections (Mansour et al. 2015).

Recent clinical trials have tested the efficacy of all kinds of peptide-based therapeutics, including the solely immunomodulatory host defence peptides, peptides with direct antimicrobial activities and their synthetic derivatives. While numerous such peptides have made it to the patents and research articles, very few have shown promising results in clinical trials with majority being tested for topical applications. This is mainly due to the reported less bioavailability, low metabolic stability or potential immunogenicity of this class of antibacterials. However, with a better understanding of the pleiotropic nature of natural peptides and how to design their admissible derivatives, fruitful results can be obtained (Kosikowska and Lesner 2016).

4 Bacterial Signalling Molecules

4.1 Cyclic Nucleotides and AHLs Stimulate Immune Responses

Two of the important second messenger molecules involved in signal transduction in a broad range of bacteria are cyclic di-GMP (c-di-GMP) and c-di-AMP. These molecules aid in bacterial intercellular communication. Mammalian cells encounter these molecules and respond to them by generating an immune response (Karaolis et al. 2005). Further research is required to discover the potential of these microbial molecules as immunomodulators and vaccine adjuvants. As with the other immunomodulators of microbial origin, the aim is to find the right balance between stimulation and regulation of immune responses.

Similar to cyclic nucleotides, acylated homoserine lactones (AHLs) are small molecules which are used as second messengers to communicate among bacterial organisms within a population in quorum sensing (Camilli and Bassler 2006). AHLs play roles in virulence and biofilm formation, for example, in *P. aeruginosa* (Smith

et al. 2002). AHLs can be exploited in two ways. First, their immunomodulatory property to generate innate immune response is similar to cyclic nucleotides, although AHL's receptors in host are yet unknown. Secondly, AHLs have a role in quorum sensing, so blocking bacterial communication through AHLs is another potential strategy for the treatment of infectious diseases (Mattman and Blackwell 2010). To achieve this, small-molecule inhibitors will act as antagonists of AHLs in bacteria. For example, blocking quorum sensing either by immunization (Miyairi et al. 2006) or by the use of small-molecule inhibitors (Wu et al. 2004) has shown to reduce mortality in a mouse model of *P. aeruginosa* lung infection. This proposes that quorum-sensing inhibitors could be an option for the treatment of patients with cystic fibrosis and *P. aeruginosa* infection. Garlic is also proposed as a quorum-sensing inhibitor in *P. aeruginosa* lung infections (Smyth et al. 2010). But again, side effects related to AHLs are still a cause of concern and need further research.

Finally, another exciting area which has emerged is the use of molecules to boost innate defences by stimulating host innate immune cells to produce natural peptides (HDPs). For example, repurposing of phenylbutyrate and vitamin D to enhance expression of innate antimicrobial cathelicidin peptide LL-37 seems feasible (Martineau et al. 2011; Liu et al. 2006; Raqib et al. 2006).

5 Antimicrobial Peptides (AMPs)

In 2010, Hancock et al. suggested that the term AMPs should be used when direct antimicrobial activity is observed, while the term 'host defence peptides' (HDPs) should be used when the concerned anti-infective activity is either the cause or result of modulation of the host immune response (Mayer et al. 2010). Other than these, peptides that show antibiofilm activity against bacteria that cause difficult-to-treat biofilm-associated diseases are also being developed (Pletzer and Hancock 2016). Thus, peptides with antimicrobial activity can broadly be classified into antimicrobial peptides with direct killing activity, antibiofilm peptides and host defence peptides (already discussed above under IDRs Sect. 2).

5.1 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are based on natural molecules that are found in a wide variety of organisms. These are generally 15–50-amino acid-long peptides that are mostly positively charged. They are ribosomally synthesized and post-translationally modified unlike natural product-peptide-based antibiotics, such as vancomycin, which are synthesized in part by peptide synthetases (Fox 2013).

The discovery of AMPs and their use as therapeutic agents has been increasing rapidly in recent years. Apart from deriving AMPs from natural sources, researchers are also putting their efforts into developing their synthetic analogues with enhanced antimicrobial activity with less cytotoxic effects (Fjell et al. 2011). The mechanisms of action of AMPs includes interaction with cell membranes, leading to membrane

disruption by interfering with physiological events such as cell wall biosynthesis or division and then translocation across the membrane to reach their intracellular targets (Brogden 2005). They exhibit broad-spectrum antimicrobial activity against a wide variety of Gram-positive and Gram-negative bacteria, fungi, eukaryotic parasites and enveloped viruses. Apart from the broad antimicrobial activity, the advantages of cationic AMPs include rapid onset of -cidal activity, potential low levels of induced resistance and potent anti-inflammatory activities (Zasloff 2002).

Although these advantages offer a promising therapeutic option, issues such as systemic and local toxicity; reduced activity based on salt, serum and pH sensitivity; susceptibility to proteolysis; pharmacokinetic (PK) and pharmacodynamic (PD) issues; sensitization and allergy after repeated application; natural resistance; confounding biological functions (e.g. angiogenesis); and high manufacturing and screening costs have prevented a therapeutic breakthrough for systemic treatments. Studies are needed that establish why they have largely not been used systemically and steps to overcome these problems such as new formulations (Koczulla and Bals 2003; Bradshaw 2003; Yeaman and Yount 2003).

Many AMPs have been discovered till now and reached the phase III clinical trial stage with some initial successes like polymyxin (Falagas and Kasiakou 2006; Zavascki et al. 2007; Landman et al. 2008). Some of the AMPs that are currently in various stages of clinical trials include POL7080128 (Roche), which is currently in phase II clinical trial for treatment of *P. aeruginosa* infections; NVB302129 (Novacta Biosystems) for *C. difficile* infections in phase II trials; AP-13864, AP-13964 and AP-11464 (Adenium Biotech) in pre-phase I trial for *S. aureus*, urinary tract and *C. difficile* infections; and human lactoferrin 1-11 (hLF1-11) which is a 'lactoferrin' derivative being developed for the treatment of bacterial and fungal infections in haematopoietic stem cell transplantation (HSCT) recipients (Adenium Biotech pipeline, Polyphor POL7080, Novacta Biosystems NVB302, Velden et al. 2009). Thus, if the deficiencies posed by AMPs are overcome by further research on improving their toxicity and administration issues, the gap between the list of AMPs claimed as potent drug candidates in the patents or related scientific articles and the real outcomes of the clinical trials can be filled.

5.2 Antibiofilm Peptides

Peptides that specifically inhibit biofilm formation have been identified and are in preclinical development. Bacterial biofilms are structured aggregates of bacteria embedded in a matrix comprised of polysaccharides, proteins, lipids and extracellular DNA. Biofilms strongly attach to both living (in chronic infections such as endocarditis, osteomyelitis, lung colonization in cystic fibrosis, etc.) and non-living surfaces (catheters, stents, mechanical heart valves, contact lenses, etc.) to form multicellular communities. Biofilms are a bacterial growth adaptation to environmental stress that enables them to resist stress, including the host immune system and antibiotics, making them difficult to treat and eradicate (Sutherland 2001) (De la Fuente-Núñez et al. 2013). Currently, there are no approved drugs

which specifically target bacterial biofilms that cause two thirds of all clinical infections and demonstrate a 10- to 1000-fold increase in adaptive resistance to antibiotics (Pletzer and Hancock 2016).

In recent years, a distinct set of cationic amphipathic antimicrobial/host defence peptides have been discovered that have unique structure-activity relationships differing from the antimicrobial peptides acting against planktonic bacteria. Thus, these can act even against the bacteria resistant to antimicrobial peptides. These antibiofilm peptides are known to inhibit biofilms formed by a broad range of resistant Gram-negative and Gram-positive bacteria at concentrations less than needed to kill planktonic bacteria (Pletzer and Hancock 2016). The antibiofilm peptides such as cathelicidin peptide LL-37 and IDR 1018, also known for their immunomodulatory activities, act at biofilms produced by *Pseudomonas aeruginosa* and other representative pathogenic bacteria at concentrations far less than their required MIC for planktonic growth. LL-37 exhibits maximum 80% inhibition of *Pseudomonas aeruginosa* biofilms at a concentration equal to a quarter of its MIC (64 μg/ml). The peptide is deduced to be affecting the two major quorum-sensing systems of *P. aeruginosa*, namely the Las and the Rhl systems which may be responsible for hindrance in biofilm formation and also damage the preformed biofilms (Overhage et al. 2008). The antibiofilm peptides 1018, DJK-5 and DJK-6 act by inhibiting small signalling nucleotides, (p)ppGpp, also called alarmones, which are a part of the stringent response by biofilm-forming bacteria. The bacteria synthesize two small signalling nucleotides, guanosine 59-diphosphate 39-diphosphate (ppGpp) and guanosine 59-triphosphate 39-diphosphate (pppGpp), collectively denoted (p)ppGpp for biofilm development. IDR 1018 exhibits potent broad-spectrum antibiofilm activity (MIC_{100} 2–20 μg/ml) against a diverse range of bacteria including *Pseudomonas aeruginosa*, *Escherichia coli 0157*, *Acinetobacter baumannii*, *Burkholderia cenocepacia*, *Salmonella enterica* serovar Typhimurium 14028S, *Klebsiella pneumoniae* and *Staphylococcus aureus* (MRSA) but weak antibacterial activity for planktonic cells (MIC_{100} >256–64 μg/mL). The peptide is able to inhibit biofilm formation, cause cell death in preformed biofilms and also disperse cells in maturing biofilms at 0.8 μg/ml in vitro (de la Fuente-Núñez et al. 2014). IDR 1018 also acts synergistically with standard antibiotics against bacterial biofilms (Reffuveille et al. 2014). These antibiofilm peptides have also shown activity in animal models of biofilm infections further pressing the need for their development (de la Fuente-Núñez et al. 2015).

6 Probiotics

Probiotics are living microorganisms that are beneficial to health and have shown antibacterial activity. Probiotics are most commonly called ‘good bacteria’ since they are intentionally fed to hosts for modulating their gut microbial community towards health, while prebiotics are comprised of molecular precursors that expand the existing gut microbiome and have been shown to have antimicrobial properties. Synbiotics are a combination of both probiotics and prebiotics (Schrezenmeir and de Vrese 2001).

Due to the lack of knowledge about the complete composition of gut microbiota, which is a consortium of more than 500 different bacterial species, the exact mechanism of how probiotics aid in improving health and fight infections is still under research (Round and Mazmanian 2009). The importance of this host mucosal microbiota in shaping the immune system and allaying the adverse effects of medical interventions such as antibiotic regimens is a major research area (Willing et al. 2011). Thus, designing specific immunomodulators that can help in maintaining, enhancing and restoring mucosal homeostasis is necessary. Probiotics comprise bacteria that are part of natural mucosal microbiota including *Lactobacillus* spp. and *Bifidobacterium* spp., *Streptococcus salivarius* and *Escherichia coli* str. *Nissle* 1917. Hence, the action of probiotics is similar to the interactions between the host and its natural microbiota. This includes interactions with mucosal epithelial cells and innate and acquired immune cell systems resulting in modulation of host immune responses (Lebeer et al. 2010).

Probiotics are also supposed to exhibit antibacterial activities by competitively excluding colonizing pathogenic bacteria, probably by bacteriocin-mediated action. Probiotics are involved in both pro- and anti-inflammatory effects resulting in enhanced immune defences and promoting the maintenance of mucosal homeostasis. It has also been reported that chemokine production and NF-κB signalling are inhibited by *S. salivarius* str. K12, but TLR signalling and secretion of innate immune mediators are activated in response to *E. coli* str. *Nissle* 1917 in epithelial cells (Schlee et al. 2007, 2008; Cosseau et al. 2008; Hafez et al. 2009). The administration of probiotics has shown promise in a range of clinical trials in patients with infectious and inflammatory diseases (Senok et al. 2009; Twetman and Stecksen-Blicks 2008), and the ease of synthesis, administration and lack of toxic effects in long-term treatments make it a favourable choice as an alternative option to conventional antibiotics.

7 Antibodies

The use of antibodies for the treatment of infectious diseases dates back to the early 1890s in the form of serum therapy by Emil von Behring and Shibasaburo Kitasato, but its use declined with the advent of the ‘antibiotic era’ (Behring and Kitasako 1890). However, now that many organisms are developing resistance to currently used antibiotics, the ease with which monoclonal antibodies can be made as a result of hybridoma technology has regenerated interest in antibodies as alternative therapies that replace or potentiate existing antibiotic therapy (Wright et al. 1992).

Currently, there are three FDA-approved mAbs and nine are in clinical trials. All three are used as an adjunct therapy to existing antibiotics as they do not have bactericidal activity. As of July 2018, there are nine mAbs in the clinical pipeline, of which five bind to bacterial cell surfaces and have shown bactericidal activity in preclinical studies (DSTA4637S, 514G3, MEDI3902, Aerumab, Aerucin) and the other four exert their effect via toxin neutralization (MEDI4839, ASN100, Salvecin, Shigamab).

Raxibacumab (Abthrax) is a human IgG1 monoclonal antibody against *Bacillus anthracis* protective antigen which was approved by FDA in December 2012 for the treatment of inhalational anthrax, in combination with appropriate antibacterial drugs and for prophylaxis of inhalational anthrax when alternative therapies are not available.

Binding of raxibacumab with protective antigen (PA) prevents the entry of lethal factor (LF) and oedema factor (EF) into cells and prevents progression of the disease. It was the first biological product approved by the FDA under the Animal Rule which is applied when it is not possible or ethical to conduct trials in humans and the drug is approved with efficacy measured only in animal models. Its efficacy in the treatment of inhalational anthrax was tested in murine models, rabbits and monkeys. The animals were aerosolized with *B. anthracis* spores. Treatment with raxibacumab (40 mg/kg body weight) was initiated when PA was detected in serum (28–42 h) or when temperatures rose above body temperature for 2 h. New Zealand white (NZW) rabbits showed 44% survival and cynomolgus macaques showed 64% survival as compared to placebo-controlled groups (0% survival). A combination of raxibacumab with levofloxacin showed increased survival, 82%, as compared to 65% in the drug-alone group. A single intravenous dose of 40 mg/kg has been recommended as observed in simulated animal experiments (Migone et al. 2009).

Obiltoxaximab (Anthim, ETI-204) is a chimeric IgG1 mAb with high affinity for PA. It was approved by the FDA in March 2016 for the treatment (in combination with appropriate antibacterial drugs) and prophylaxis of inhalational anthrax under the animal rule. Its treatment is recommended with a premedication of diphenhydramine, and close observation as hypersensitivity or anaphylaxis was observed in nearly 10% of individuals in a phase I clinical trial. Therapeutic efficacy was determined in animal models challenged with aerosolized *B. anthracis* spores. Treatment with obiltoxaximab was initiated after definitive signs of systemic anthrax were observed. NZW rabbits showed 62–93% survival and cynomolgus macaques showed 31–47% survival as compared to a placebo-controlled group with 0–6% survival. Combination of obiltoxaximab with levofloxacin, ciprofloxacin and doxycycline showed increased survival as compared to the drug-alone group. A single intravenous dose of 4–16 mg/kg has been recommended as observed in simulated animal experiments (Greig 2016).

Bezlotoxumab

One of the most common side effects of antibiotic therapy is the loss of normal gut flora which eventually may result in the germination of *Clostridium difficile* spores present in the gut. Upon germination, they secrete toxin A and B which bind to the intestinal epithelium, are endocytosed and lead the formation of pores in the epithelial lining. These toxins are glucosyltransferase, inactivate the Rho family of GTPase

and cause actin depolymerization leading to structural disintegration. All this increases the colonic wall permeability and a serious condition termed as '*Clostridium difficile*-associated diarrhoea'.

Bezlotoxumab (Zinplava) is a human IgG1 monoclonal antibody of 148 kDa, against *Clostridium difficile* toxin B. Bezlotoxumab has high affinity for the toxin and prevents its binding to host cells and concomitant damage to the intestinal epithelial. It was approved in October 2016 by FDA to 'reduce the recurrence of *Clostridium difficile* infection (CDI) in patients 18 years of age or older who are receiving antibiotics for CDI and are at high risk for recurrence'. X-ray crystallography studies have shown that the Fab fragment of bezlotoxumab binds to two epitopes towards the N-terminus of toxin B and this binding sterically hinders the carbohydrate-binding pocket of the toxin, thus preventing its attachment to the intestinal epithelium.

The efficacy of bezlotoxumab was tested in various animal models to evaluate its effectiveness as mono-therapy or combination therapy. A study conducted on murine models showed significantly lower intestinal epithelial cell damage, haemorrhage and necrosis in mice treated with the combined monoclonal antibodies (actoxumab-bezlotoxumab) compared to the untreated groups. In another study, conducted on hamsters to assess the mortality benefit, primary CDI was induced by the administration of clindamycin 24 h prior to orogastric administration of *C. difficile* spores. Antitoxin A antibody (50 mg/kg/day) and antitoxin B antibody (10–50 mg/kg/day) alone or as a combination were administered intraperitoneally in different groups 4 days prior to the administration of *C. difficile* spores. A mortality of 100% was observed in the untreated group due to development of severe CDI. The group which was administered a combination of monoclonal antibodies showed 45% reduction in mortality.

A similar study was conducted in a mouse model of CDI. Mice were treated with clindamycin to induce CDI and then treated with vancomycin or mAb, followed by inoculation with *C. difficile* spores. The four treatment groups were combination therapy (actoxumab-bezlotoxumab), combination of monoclonal antibodies and vancomycin, vancomycin alone and placebo. The groups were under observation for a period of 28 days. The group receiving the combination of vancomycin and monoclonal antibodies showed an 80% survival rate. The group treated with vancomycin alone had a 40% survival rate and all mice in the placebo group died by day 4 of the study. Intestinal microbial diversity analysis in mouse models elucidated that monoclonal antibodies helped in the restoration of the original gut microbiota, which was not observed in any treatment group.

In phase I clinical trial, it was safe and well tolerated up to a tested dose of 20 mg/kg. No serum-generated anti-bezlotoxumab antibodies were detected in immunogenicity studies in trials. The recommended dose after PK studies came out to be 10 mg/kg. In phase II and phase III trials, it was established as safe and effective monoclonal antibody for the treatment of *C. difficile*-associated diarrhoea (Markham 2016).

Suvratoxumab (MEDI4893) is a novel human monoclonal antibody against *Staphylococcus aureus* alpha toxin (AT) in clinical trial for the prevention of ventilator-associated pneumonia (VAP) caused by *Staphylococcus aureus*. Alpha toxin is a 33 kDa protein encoded by the *hla* gene on *S. aureus* chromosome, which,

in its heptameric form, traverses the cellular membrane as a complete beta barrel pore and causes cell death. Suvratoxumab binds to a novel epitope on the rim domain of AT which prevents its binding with its receptor (ADAM10) on the cell membrane. This binding of mAb has also been hypothesized to block oligomerization of AT monomers due to steric hindrance and thus AT neutralization. Suvratoxumab was generated by the introduction of a YTE mutation in the Fc region of a previously known anti-AT mAb LC10, which increased its half-life in humans but decreased its serum exposure in mice. Thus, LC10 was used in preclinical animal testing in spite of suvratoxumab.

In a *S. aureus*-induced pneumonia model, 7-week-old C57BL/6 J mice were passively immunized with LC10 (at 20, 12, 6, 3, 1.5 or 0.5 mg/kg) 24 h prior to intranasal infection with 10^8 CFU and observed for 7 days. LC10 prophylaxis resulted in a dose-dependent increase in survival relative to the control. In a similar experiment with *S. aureus* clinical isolates, LC10 was shown to increase survival significantly as compared to control. LC10 prophylaxis also reduced bacterial burden from lungs and kidneys and reduced bacterial dissemination in a murine pneumonia model. Intraperitoneal LC10 also showed synergy with vancomycin and linezolid. A phase I clinical trial was completed in the USA that evaluated the safety, tolerability and pharmacokinetics in healthy adults after intravenous administration of escalated doses of LC10.

Additionally, a phase II trial entitled 'randomized, double-blind, placebo-controlled, single-dose, dose-ranging Study of the Efficacy and Safety of MEDI4893, a human monoclonal antibody against *S. aureus* Alpha Toxin in mechanically ventilated adult subjects' is ongoing in multiple European cities (Yu et al. 2016).

Salvecin (AR-301/KBSA301) is another fully human monoclonal IGg1 antibody developed as an adjunctive therapy to standard of care (SoC) for ventilator-associated *S. aureus* pneumonia (VASP). It binds and neutralizes AT, thus prevents AT-mediated lysis of host cells. It was discovered by screening B-cell lymphocytes of a patient who had *S. aureus* infection. Its mode of action is independent of the antibiotic resistance found in the organism and is thus active against both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA). Salvecin met its primary endpoints in phase I/IIa studies, and it was shown that patients receiving Salvecin along with SoC antibiotics spent less time under mechanical ventilation as compared to patients who receive placebo and SoC antibiotics. Also, the blood bacterial burden was consistently lower in patients receiving Salvecin than in the control group patients (Ardis Pharmaceutics).

514G3 is a human mAb against staphylococcal protein A (SpA), a key virulent determinant expressed in all clinical isolates. SpA binds to the Fc domain of human IgG and allows *S. aureus* to escape from antibody-dependent phagocytic killing. 514G3 displaces SpA bound to IgG and makes its opsonophagocytosis possible. In phase II clinical trials, it provided enhanced protection as compared to control groups (Huynh et al. 2016).

MEDI-3902 has a dual mode of action. It targets the type III secretion system and Ps1exopolysaccharide of *P. aeruginosa*. The type III secretion system delivers toxins and effector molecules into host cells, and Ps1 exopolysaccharide has roles in attachment to host cells and biofilm formation. Intravenous administration (5–15 mg/kg) provided a 100% survival after a lethal challenge in bacteraemia and acute pneumonia models in mice, rabbits and pigs with *P. aeruginosa* infection (Warrener et al. 2014).

Aerumab (AR-101, panobacumab) is a human IgM mAb directed against the O-antigen of *P. aeruginosa* lipopeptide which accounts for 20% of VAP cases caused by *P. aeruginosa.* Aerumab binding to the antigen enhances bacterial clearance through phagocytosis or complement-mediated killing. Aerumab successfully cleared phase I/II clinical trials with a cure rate of 86% as compared to 64% in the placebo and antibiotic group (Secher et al. 2013).

Aerucin (AR-105) is a fully human IgG1 antibody currently under development which is targeted against *Pseudomonas aeruginosa* alginate. In preclinical studies, it has displayed good protection against lethal dose challenge of several *P. aeruginosa* strains in murine bacteraemia and acute pneumonia models. In phase I clinical trial, it was found safe and well tolerated up to a dose of 20 mg/kg. A global phase II trial in ventilator-associated pneumonia patients is expected to conclude in June 2019 (Aridis Pharma).

Shigamab is directed against the Shiga toxin (Stx) of *Escherichia coli* which is major cause of haemorrhagic colitis and which later manifests into haemolytic uremic syndrome (HUS). Shigamab is a combination of two mAbs Stx1 and Stx2 which are directed against Shiga toxin 1 and Shiga toxin 2, respectively, and are the key virulence factors in the pathogenesis of HUS. In a murine survival model, 20 mg/kg of Shigamab gave a survival of 90%. It showed no adverse effects in phase I clinical trial (Bitzan et al. 2009).

8 Bacteriophages

Bacteriophages are naturally occurring viruses that infect, replicate in and later kill their bacterial host. They are highly specific for the bacterial hosts they infect and don't affect the eukaryotic cells. In their amplification process, bacteriophages tend to kill their host and are unaffected by antibiotic resistance and are able to disrupt bacterial biofilms producing strong local therapeutic effects (Quan-Guo and Buckling 2012). Wild-type bacteriophages are considered an alternative to classical antibiotics. They are self-replicating and can replicate in their host bacterium and thus can be used in small doses. Their specificity to their host doesn't disturb the local microbiota. As bacteriophages can replicate within their host, there is a lower

chance of them becoming resistant to their host as both viruses and bacteria co-evolve with each other.

Along with this, there are certain disadvantages as bacteria possess a constantly evolving anti-phage systems like CRISPR-Cas, phage exclusion and restriction modification systems. Although the presence of such system puts a question mark to whole phage therapy, these mechanisms are not universally present, and phage can also develop countermeasures to these resistance mechanisms. Phage therapy differs from conventional antibiotics in that whole phages poorly diffuse as they are much bigger in size as compared to antibiotics, so they are to be dosed in lower number. As they replicate inside their host, their number further increases, so more phages than what is dosed will be present in the patient, and that too can change over time. What is sampled at the time may or may not be reflective of initial dose, resulting in complex pharmacodynamics and pharmacokinetics (Abedon et al. 2017).

AmpliPhi Biosciences (APHB) is the biopharmaceutical company focusing on the discovery, development and commercialization of bacteriophage-based therapies for the treatment of infections caused due to antibiotic-resistant bacteria. Three of its products, AB-PA01, AB- CD01 and AB-SA01, are in various phases of clinical development.

AB-PA01 (AmpliPhage-001) is a four-phage product being developed for the treatment of *Pseudomonas aeruginosa* lung infections in cystic fibrosis (CF) patients and also its MDR strains. AB-PA01 has demonstrated a broad range of in vitro activity against CF and non-CF *P. aeruginosa* clinical isolates. In murine lung infection model, it has demonstrated efficacy similar to meropenem (Pabary et al. 2015). As of April 2018, AB-PA01 has initially been tested in three patients under emergency conditions as intravenous and as inhaled bacteriophage therapy to treat life-threatening *Pseudomonas aeruginosa* infections not responding to antibiotic therapy. It was found to be safe and well tolerated with no treatment-related adverse effects.

AB-SA01 is a three-phage product developed to treat *Staphylococcus aureus* infections. In the preclinical studies, it has displayed excellent in vitro activity against clinical *Staphylococcus aureus* strains including methicillin-resistant and vancomycin-resistant isolates. In the neutropenic and immunocompetent mouse model of acute pneumonia, it displayed activity comparable to vancomycin. As of April 2018, under emergency conditions, it was used to treat life-threatening *S. aureus* infections in five patients not responding to antibiotic therapy. It was found safe and well tolerated with many cases of microbial eradication (AmpliPhi Biosciences Corporation).

AB-CD01 (Ampliphage-004) has been developed for the treatment of infections caused by *Clostridium difficile.* Initial in vitro characterization demonstrated efficacy against 86% of clinically relevant *C. difficile* ribotypes, including the hypervirulent strains RT014/020 and RT027. It has shown effective cure rates in murine infection models. A phase I clinical trial has begun enrolling patients (www.sec.gov/Archives/edgar/data/921114/000114420415001613/v398507_ex99-1.htm).

9 Lysins

Lysins are bacteriophage-encoded peptidoglycan hydrolases which rapidly degrade bacterial peptidoglycan resulting in cell lysis and death. Lysins have several advantages over antibiotics: they are specific for the pathogen and thus perform their function without disturbing the normal flora. They have a quick mode of action, there is less chance of developing resistance to lysins, and most importantly they have the ability to kill pathogens colonizing mucosal surfaces, an avenue which was previously unavailable (Fenton et al. 2010). Few examples in clinical trials are as follows:

SAL-200 is an anti-staphylococcal endolysin derived from bacteriophage SAL-1. It showed synergy with SoC antibiotics in vitro and in vivo in an animal model of bacteraemia. It has successfully cleared a phase I clinical trial with no adverse effects (NCT01855048).

Cpl-1 is a pneumococcal lysin which showed good activity against antibiotic-resistant *S. pneumoniae*. A 2000 mg intravenous dose of Cpl-1 in a mouse model 1 h after infection reduced pneumococcal titres from a median of 4.70 $\log_{10}$CFU/mL to undetectable levels (<2.00 Log_{10}CFU/mL) after 15 min. Compared to the lysin-treated mice, only a 20% survival rate was seen in untreated mice (Resch et al. 2011).

10 Conclusions and Future Directions

Antibiotics have become the mainstay of medical treatments. Unfortunately, their extensive misuse has contributed to the generation of AMR, which is significantly impacting healthcare systems worldwide. Thus, there is a concerted push towards finding alternate strategies to combat the rising menace of drug-resistant bacterial infections. These alternate methods demonstrate a lot of potential in combating bacterial infections; however, a number of issues need to be resolved before mass utilization can be practically achievable in the clinic. Interestingly, a number of these therapies could potentially be utilized as a prophylactic measure, rather than as a therapeutic.

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In Silico Molecular Modelling: Key Technologies in the Drug Discovery Process to Combat Multidrug Resistance

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Abstract

Drug discovery using advanced computational biology approaches is an emerging field in medical science and holds the promise towards identification of new drugs. The multidrug resistance in bacterial strains is a matter of serious concern specifically related to the pathogens associated with public health. Numerous strategies have been developed in the recent past to combat the MDR concerns. However, still due to upcoming new evolution mechanisms of bacterial strains, the issue has been addressed only to a limited extent. Pertaining to the limitations of molecular techniques, multiple in silico approaches are in trend with great advancements. This chapter is focused toward the description on several in silico techniques for drug discovery with an idea of target identification, namely, virtual screening, molecular docking, MD simulation, QSAR and pharmacophore modelling. In addition to multi-target identification, the structural genomics has also been illustrated which involves the three-dimensional structure predictions of proteins for better understanding to design drugs against MDR.

Keywords

Target identification · Virtual screening · Molecular docking · MD simulation · QSAR · Pharmacophore modelling · Multi-target identification · Structural genomics

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1 Introduction

Multidrug resistance or multiple drug resistance (MDR), manifested by specific microorganisms to multiple antimicrobial drugs, has emerged as a major threat to the public health worldwide. Almost all the pathogenic organisms including bacteria, viruses, fungi and parasites are capable of exhibiting multidrug resistance; however, the most frightening are MDR bacteria which resist multiple antibiotics important to public health. With the constant deployment and overuse of homo- or heterogeneous antibiotics, MDR has become the rule rather than exception among some species of bacteria. The resistant to antimicrobials has been developed in bacteria by many ways, namely, enzyme inactivation, decreased cell permeability, target protection, target overproduction, altered target site/enzyme, increased efflux due to overexpression of efflux pumps and aggregation of genes with each one coding for resistance to a certain agent on R-plasmid or transposons (Nikaido 2009).

Consequently, an expanding generality of pathogenic MDR bacteria is a matter of serious concern, which needs attention globally. These bacteria are generally associated with clinical infections originated from health centres and longstanding care provisions. Ordinarily, aged and seriously unwell people are highly influenced with the conditions gruesomely adverse. Specifically, low- and middle-income countries (LMICs) are influenced the most, as the exposure of antimicrobial resistance (AMR) is a prime medical distress in these countries. With the advancements in earnings, extravagant antimicrobials and an imperfect regulation of non-prescribed disposals of antibiotics, the cases of MDR advancements are constantly increasing specifically in LMICs (Lim et al. 2016). Moreover, the communication between the health workers and diseased personnel broadcasts MDR bacteria to new hosts. The new treatment strategies relying on novel targets, nanoparticles, combination therapy and so on are, however, able to regulate the resistance even when the upcoming new strains of MDR bacteria pose towards the concern. Altogether, the scenario today focuses towards the development of new therapeutic agents or strategies to effectively combat the resistance in bacterial pathogens.

2 Evolution of MDR Bacteria

Antibiotic-resistant bacteria are defined as those bacteria which cannot be regulated or eliminated by antibiotics. These resistant bacteria originate MDR on resistant plasmid by the gathering of genes for resistance against certain agent. These plasmids are defined as bacterial supplement chromosomal segments that convey genes communicating resistance to either single or multiple antibiotics. The potentiality of transmission through association between the different species of bacteria and consequential participation in the appearance and dispersion of MDR analogous with bacterial infections in humans of resistance plasmids is not as famous. The process of transferring the plasmids from a bacterial cell to the next one is often called conjugation, which is done by the association of cells that proceed through the transmission of a replica of plasmid DNA to a receiver from a donor (Clewell 2014).

Since 1930s, various antibiotics are used to treat bacterial infections, but due to the assembly of different MDR genes present on bacterial plasmids, these antibiotics are not sufficient to heal bacterial infections such as β-lactamase (bla), the less famous MDR gene which hydrolysis lactam ring CO-N bond of penicillin. Penicillin and cephalosporin are antibiotics hydrolyzed by MDR bacteria (Drawz and Bonomo 2010; Lynch III et al. 2013; McArthur et al. 2013; Okeke et al. 2005). The prime reason for the occurrence of MDR is expanding of bla enzymes (up to 250aa to 386aa) within plasmids (Bush and Jacoby 2010; Jacoby 2009; Palzkill 2013). Innumerable antibiotics such as ciprofloxacin, streptomycin, neomycin, azithromycin, polymyxin, chloramphenicol and tetracycline had been devised from mire sculpt *Penicillium notatum* since 1928 have resulted into the termination of infection to certain extent (Forsberg et al. 2012; King and Strynadka 2013). However, the upcoming issues in the recent past viz. complexity and diversity between *bla* and *aac* genes has lead to the development of MDR strains thereby arising the serious concern towards the development of novel therapeutic agents against the resistant bacterial strains.(Paulsen 2003).

Consequently before proceeding for any therapeutic treatment, there is an urgent need to understand the delicacy of the antibiotic-resistant ability of bacteria. Recently, numerous MDR methods have been developed, among which polymerase chain reaction (PCR) detection of actual MDR gene compound has been advised. After applying different methodologies, still there are various PCR that has to be performed by using cent of PCR primers for some hundreds of MDR genes, which is unfeasible and costly (Altschul et al. 1997; Bairoch and Apweiler 2000; Chakraborty 2016).

3 Current Strategies

The encouraging biotechnological approaches suggested various drug-resistant treatment strategies. As traditional methodologies fail to give healthier outcomes due to resistance augmentation and lengthy and noxious effects. These severe consequences lead to the advancement of novel and promising techniques (Ahmad and Mokaddas 2010; M.-Y. Chen et al. 2017; Shenoi et al. 2009).

Recently, various therapies, namely, genotype-based method, non-immune toleralizing approaches, phage therapy, use of quorum-sensing inhibitors, hemoperfusion devices, liposome-based cytotoxic inhibitors and monoclonal and polyclonal antibodies were found as better alternatives for non-antibiotic inhibitors of bacterial disease expansion (Opal 2016). Apart from it, optic microscopy, electron microscopy, serology and antigenic detection techniques are the used as conventional modes of action.

3.1 Quorum Sensing

Quorum sensing (QS) is a phenomenon by which the bacteria or autoinducers used to communicate and coordinate their signals, thereby increasing the functionality cell density via tissue invasion and leading to an amendment in gene expression. These also have a crucial role in regulation of bacterial pathogenicity via virulence, production of antibiotic, competence, conjugation and biofilm development. When the compound attains a threshold concentration, the signals, that is, quorum sensing, triggers their gene expression.

Many remarkable natural and chemical compounds have the ability to block the QS and showed better results in systemic infectivity. For instance, acyl homoserine lactones are synthesized by LuxI-type enzymes; LuxR-type regulators are responsible for expression of QS inhibitors. The inhibition of QS is called quorum quenching (QQ). QQ compounds inhibit the quorum-sensing receptors which enhance the selectivity and resistivity of MDR diseases (Miller and Bassler 2001).

3.2 Genotype-Based Methods

Owing to high prevalence of the MDR diseases worldwide and pacing towards its quick and fast assessment, various genotyping and molecular spotting methods have been implemented. According to this technique, molecular drug-resistant mutations in the MDR isolates from the different cases are scrutinized in respect of their ancestral history. This pedigree analysis will reveal several control strategies against MDR diseases.

3.3 Hemofiltration Devices

These devices are helpful in binding and removing the array of bloodstream pathogens. Extracorporeal membrane oxygenation (ECMO) and continuous renal replacement therapy (CRRT) are the commonly used filtration methods which maintains the fluid balance and electrolyte disturbances when used in combination (H. Chen et al. 2014; Sulakvelidze et al. 2001; Zewdie et al. 2018).

3.4 Phage Therapy

Viruses that infect bacteria are known as bacteriophages. These viruses attach to the bacterial cell wall and inject their genetic material. They have the capability to replicate multiple times within the host cell, and ultimately, the bacterial cell wall ruptures releasing viral particles out. Conventionally, this phage therapy relies on the use of naturally occurring phages to infect and lyse bacterial cell at the site of infection. Since bacteriophages do not infect human cells, they have been regaining their strengths in medical therapies to treat various bacterial diseases like MDR. These

may also be modified into bioengineered phages and purified phage lytic proteins vaccines further for better cure results (Keen and Adhya 2015). The phage therapy came into existence as an alternative or a better supplement to antibiotic treatments, although several lawful impediments are obstructing the usage of phage therapy in current remedial methods prompting some ethical issues (Sulakvelidze et al. 2001).

3.5 Liposome-Based Cytotoxic Inhibitors

Engineered artificial liposomes are designed to inhibit the toxins released by bacteria on lysis of several cell layers including innate immunity system. Sometimes this non-antibiotic mechanism defends the human cells from lysis so they can also act as carrier for drug delivery. Liposomes neutralize a range of toxins released by bacteria which is essential for the cure of antibiotic bacteria and the resistance caused by these bacteria (Henry et al. 2015). The use of these liposome-based therapies helps in enhancing pharmacokinetics and better bio-distribution of the drugs. It also aids in increasing the target selectivity and specificity with lesser toxicity level, thereby trouncing the resistivity of bacteria (Schiffelers et al. 2001).

3.6 Immune Therapies in MDR

The concept of immunotherapy is derived from the expansion of therapies like serum therapy to monoclonal and polyclonal antibodies in curing MDR bacteria (Abate and Hoft 2016). Immune therapies could investigate the infectious disease in latent phase, and have the ability to modulate the immune system, thereby averting a self-damaging inflammatory reaction.

3.6.1 Monoclonal Antibodies (mAbs)

mAbs are basically identical immunoglobulins which are generated from a single B-cell clone. They recognize unique epitopes or binding sites present on a single antigen. These mAbs adhere themselves to the infected cells so that the immune system finds them and block the signals of infectious cells. This process is known as antibody-dependent cell-mediated cytotoxicity (ADCC) (http://www.cancerresearchuk.org; https://www.pacificimmunology.com).

3.6.2 Polyclonal Antibodies (pAbs)

These antibodies are the set of antibodies of different B-cells acquainted with numerous epitopes on the same antigen. Recent finding suggests the multi-epitope peptide (MEP) vaccines which target many antigens and believed to be a better method of treatment against MDR. These are quick to produce and highly stable, best for finding specific proteins in multiple assay types (https://www.pacificimmunology.com).

3.7 Non-Immune-Toleralizing Approaches

These approaches can be explained by three strategies: tolerance, avoidance and resistance. Immunological *tolerance* refers to the condition when the body fails to generate an immunologic reaction, that is, it fails against an antigen. It reduces the chance of infection on host fitness and does not directly affect the pathogen burden. *Avoidance* refers to the behavioural mechanism shown by the host that is being changed according to the risks of infection detected by the host. *Resistance* refers to the host protection strategy from infection by detection, destruction and neutralization of the attacked pathogen (Medzhitov et al. 2012; Rasmussen et al. 2014).

4 Need for in Silico Perspective

The prompt extension of MDR bacteria in nature has been cautioned by the researchers and the advices related to the antibiotic usage in patients as well as in the development of animals and farmland should be minimized. Conventionally, examining of MDR bacteria depends on the infection-restraint individuals to develop the extensive index of lab outline and is in-depth exertion, practice based and incorrectly susceptible physically. The strengthening of computer advancement and data analysis, prompt operation and examination of a huge number of electronic data turn practicable making it highly suitable to resolve the issues. For the purpose of infection regulation supervision, some computer-affiliated automated systems have been developed (Kho et al. 2008; Pittet et al. 1996). The main hypothesis behind these systems is that an automated MDR bacteria supervision approach will ease and upgrade the refinement of infection regulation (Wu et al. 2009).

With the advancement in in silico approaches, bioinformatics has become a new era of handling the biological data with a good rate and storage capability leading towards better understanding. Thousands or more than thousands of MDR genes have been examined nowadays by colour dideoxy method of whole genome sequencing and facile study of a huge number of sequences by some in silico programs like BLAST (Altschul et al. 1997; Bairoch and Apweiler 2000). In addition, thousands of MDR genes have been perceived and sequenced. The main aim is as high as the expansion of coherent drugs and antimicrobial factors, expansion of new magnified bacterial strains to regulate pollution, expansion of more than better and simple deal vaccines, the expansion of protein biomarkers for different bacterial problems and for the better interpretation of host bacteria interaction to regulate bacterial infections. To achieve this target, the research work using bioinformatics can be classified into three categories, which are as follows:

I. The present exploratory wet-lab data's investigation.
II. Retrieval of additional information through the use of mathematical modelling.
III. A coherent method which assimilates exploring systems with mathematical modelling.

Bioinformatics research work currently has facilitated the automation of genome sequencing, coherent genomics and proteomics databases, genome differentiations to recognize the function of genome, extraction of metabolic pathways, gene expression investigation to retrieve control pathways, clustering analysis and data mining, etc. to acquire DNA–protein and protein–protein communications. All this has assisted in designing 3D structure of proteins and docking between proteins and bio-chemicals for enlightened drug design and comparative analysis between non-pathogenic and pathogenic strains (Ordonez et al. 2017). Success of bioinformatics approaches have escalated the traverse of biological finding by motorized study of a huge number of microbial genomes. Now researchers are on the edge to amplify all this knowledge to decipher the cellular mechanisms at standard level as the successfully developed bioinformatics methods have the ability to ease with

1. Causes of disease's investigation.
2. Drug designing rationally or vaccinations.
3. Ameliorate value potent factors for bioremediation by shearing out numb ends.

Thus, the execution of bioinformatics tools and methods to examine the expanded data originated in molecular biology, genomics, proteomics and transcriptomics is attaining strength (Bansal 2005). Furthermore, the aggregate information obtained is stored in database's form and literature to create molecular portray and to retrieve data allied epidemiology of pathogens has been become ascending too (Hogeweg 2011). Hence, the tradition of using bioinformatics tools and methods in pathogen recognition and entering data, recognizing markers for instant reading and therapy, sanctioning customized intercessions and forecasting patient consequences are vital (Carriço et al. 2013). The supervision of pathogen breakout in suppressing infectious diseases is very crucial nowadays under the scope of bioinformatics approaches (Saeb et al. 2017).

4.1 Target Identification

The process under which the identification of direct molecular target like protein or nucleic acid of a micro-molecule has been done is called *target identification*. Target identification is sighted as discovering the edge factor/target of a drug clinically. To identify, used methods may be depend on preposition of biochemistry, biophysics, genetics, chemical biology, bioinformatics or other disciplines.

Conventionally, bacterial drug targets are recognized through costly and time-taking genetic tests, biochemical tests and cellular assay. In silico approaches for target identification has transpired spawning an astounding attainment in the area of drug designing. Researchers are using different methodologies for target identification of bioinformatics including homology-based testing, Drug Bank database and structure-based drug ability study of PDB structures and suggested that generating more in silico techniques result in better understanding of metabolic network in

drug designing in a more comprehensive conception of bacterial infections and therefore present an improved counsel for drug discovery (Perumal et al. 2008).

The bioinformatic tools comprise a salient position in enterprising genomic, transcriptomics and proteomic data to attain prescience into the molecular medium that recognize prospective targets. Genomics and proteomics methods have generated a prototype turn in the drug designing means. Here, we discuss about the ongoing situation of skill for few of the bioinformatics techniques to recognize drug targets, counting recognizing and characterizing new components of affluent target groups and its functionality, projecting disease apposite genes, and assembling gene networks and protein interaction networks. Furthermore, we have focused on the investigation process of drug target by applying systems biology's approach and studied few data initiative to pinpoint drug targets. There are many bioinformatics tools and means which can be applied to pinpoint apparent drug targets, though affirmation of these targets is still a necessary procedure which necessitates an acknowledgement of the functionality of the gene or protein in the disease and is steadily based on laboratory work (Jiang and Zhou 2005). Accordingly, there are multiple bioinformatics approaches to identify the target for these bacterial infections as we have already mentioned above; here, we will pinpoint these emerging techniques under target identification (Fig. 1).

Implementing bioinformatics techniques from target identification to clinical trial phases through drug discovery pipeline assures time reduction and the cost of drugs. It offers various tools to screen the bundle of compounds by virtual screening (Lengauer and Rarey 1996) tending towards finding the best possible lead compound.

A range of applications like exploring best hit, scaffold hopping, fragment-based drug designing, pharmacophore mapping, structure activity relationship, similarity search and selective profiling are employed to pursue several tasks in computational biology. Docking studies reveals the interactions between the target protein and the respective ligands within the pocket sites or with a flexible docking mechanism (Kukol 2008).

This section of the chapter explains the importance of the computational techniques, which helps in combating the MDR diseases in a simpler way as compared

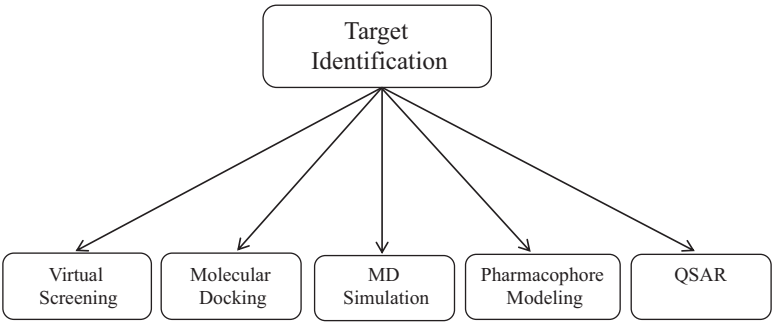

Fig. 1 A flowchart showing various in silico techniques of target identification

to the higher cost and time-taking experiments. This in silico approach needs to be explored as they augment vigorous route towards successful therapies against bacterial infections.

4.1.1 Virtual Screening/High Throughput Screening (HTS)

Computational-aided drug design (CADD) is an indispensable module in the drug discovery cycle (lead identification to clinical phase trials). CADD is enriched with several tools, out of which virtual screening is foreseen as one of the best components to refine hit lead compound from the large libraries. Pacing towards explicating several unique drug targets against bacterial diseases (MDR), CADD in sequence with the experimental methods, that is, in vitro/in vivo experiments, leads to the development of novel drug-resistant antibiotics. CADD is also assisting in creating structure–activity relationship (SAR) data for approaching towards a low time and cost-effective drug-designing procedure (Yu and MacKerell 2017).

High-throughput screening (HTS) is basically the filtration of the most potent compound at each step to the next progressive one from the library of a large and structurally diverse dataset via subsequent experimental assays. Although sometimes the compound itself became a hindrance due to the presence of heavy functional groups, causing steric barrier and thereby affecting the biological (IC50) activity of the compound, so these get eliminated as they create misleading data (Leach and Gillet 2007). In best hit to lead phase, HTS has always been a choice for the researchers but because of higher cost and time-taking process, the pharmacy companies moved towards computational approach, that is, virtual screening (VS). It is an in silico counterpart of the HTS. VS can be explained by the assaying stack of compounds and can analyse a vast amount of data in very less time-using iterative algorithm. The increased employment of virtual screening and the combinatorial chemistry assist the researchers to generate ideas of devising and scrutinizing vast compound libraries with higher conformational flexibility (Lavecchia and Di Giovanni 2013). After human genome project, numerous targets have been identified which are a boon for computational biology, thereby magnetizing faster and cheaper screening approaches in drug discovery pipeline (Hopkins and Groom 2002).

The purpose of VS can be used at various steps of analysis, namely, in screening out the best and novel possible hit compound by having drug likeliness and surpassing the pharmacokinetic properties (Cheng et al. 2012). This hit compound demonstrates the pharmacological activity of the target protein, which will be gradually optimized in the later stages of drug discovery pipeline.

On the basis of the structural and bioactivity data, Wilton et al. in 2003 proposed four major classes of VS:

- Structure-based virtual screening (SBVS).
- Ligand-based virtual screening (LBVS).
- Machine learning through neural network.
- Database searching for 3D pharmacophore mapping (2).

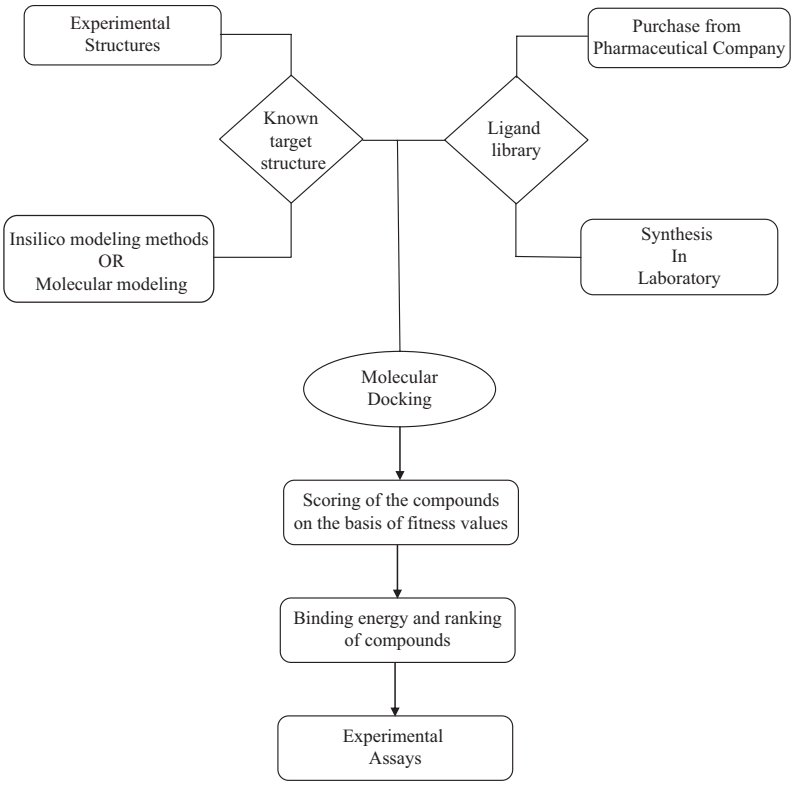

Fig. 2 Flowsheet of LBVS

SBVS is applicable when the three-dimensional (3D) structure of the protein is available and then virtual screening is done on the basis of the existing structures; on that basis, a novel compound can be identified mimicking biological target structure. It eases the process of identification of lead compound by quick, fast and cost-effective property (Leach and Gillet 2007; Solt et al. n.d.). SBVS can be further classified as ***molecular docking*** which is based on predicting a binding energy between the ligand and the target protein with the help of a predefined binding pocket site in the target protein. Docking also helps in revealing different pocket sites of the ligand due to conformational changes in the receptor (Shin et al. 2015). This method is also known as docking-based virtual screening (DBVS) (Lyne 2002). Most frequently used docking software are GOLD, FlexX, AutoDock, Glide, Dock, etc. The flowsheet of the DBVS can be understood through Fig. 2.

LBVS is applicable when the inhibitor is known, that is, the screening is done with the help of a number of ligands, their experimental values, orientation, etc. (Leach and Gillet 2007; Solt et al. n.d.). Disparately from SBVS, this approach relies on the similarity in arrangement of the chemical descriptors along with the bioactivity in

compounds. Nevertheless, LBVS is the more efficient and acceptable method of filtering the compounds (Solt et al. n.d.).

Several machine learning approaches, namely, neural network, SVM (support vector machine), etc., have also been implicated to enhance the analysis of the correlation and regression data. When VS is performed on the basis of the features as well as bioactivity, a novel pharmacophore model is generated with mapped features and quantitative structural data. Then a screening process known as 'database searching' is used.

Similarity search screening, like the study of pharmacological as well as pharmacokinetic properties (***ADMET*** – adsorption, distribution, metabolism, excretion and toxicity), helps in focusing on deriving the molecular interaction (ligand–protein interactions) of the several multidrug-resistant diseases (Solt et al. n.d.).

ADMET is equipped with some pharmacokinetics criteria as it reveals the drug property inside the body.

Drug-like rules like *Lipinski, PAINS, Veber, 3/75 Pfizer, LiliMedChem, Egan, Oral PhysChem, GSK 4/400, Ghose, Muegge* and *Jorgensen's* are some filters which refine compounds on the basis of their standard values.

Further, VS can also be availed by checking whether the compound is *PPI friendly* or not, or if it is an inducer or non-inducer of *phospholipidosis* (Fig. 3).

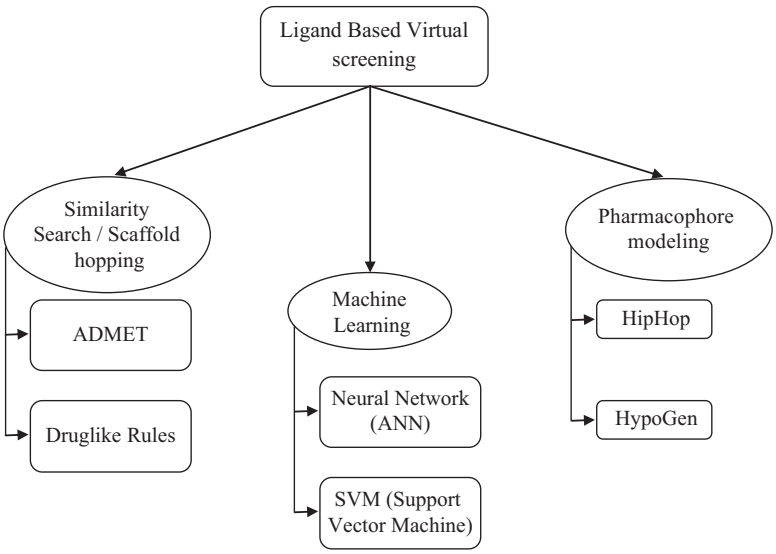

Fig. 3 Ligand-based virtual screening

4.1.2 Molecular Docking

In general, a molecular docking technique implies a method to predict a preferred orientation of one molecule bound to another molecule to form a stable complex (Arabnia and Tran 2015). The physiological interactions between molecules, which are biologically admissible as in case of nucleic acids, proteins, lipids and carbohydrates portray a key part in signal transduction phenomenon inside the cells. Additionally, the conformational stability of generated complex of the two interacting molecules majorly influences the efficacy of signals. Thereby, to project and predict the stability and varsity of transducing signals at the level of protein–substrate or protein–protein interactions, docking analysis studies has been found to be quite useful.

Virtual screening of millions of compounds in parallel against a specific receptor along with their structural and conformational orientation analysis have been the key utilities of molecular docking studies in the modern era of lead discovery (Rohs et al. 2005). Nowadays, molecular docking has turned into an important standard constituent of any drug-designing toolbox, with ease of accessibility and faster computing algorithmic abilities (Guedes et al. 2014). Increasing computational power and algorithmic performances has allowed to dock libraries of ligands to a single target in real time with enhanced accuracies (Agarwal et al. 2015; Seeliger and de Groot 2010).

At the procedural level, it requires a structural database to retrieve a receptor molecule in an appropriate structural file format and a chemical compound database to look for promising interacting lead molecules. A number of software, for example, AutoDock, Discovery studio, FlexX, GOLD and Hex, are available for molecular docking analysis, allowing a framework to ligands according to their propensity to interact with target protein or DNA (Gschwend et al. 1996). These docking softwares deploy a quest of algorithms like fragment-based methods, Monte Carlo algorithms, genetic algorithm, molecular dynamics, point complementarity methods and distance geometry methods as a part of their search and scoring functions to predict the best bound complex. Furthermore, molecular docking technique also facilitates rigid body docking method involving both target and ligand as rigid molecule, and flexible docking method in which both interacting molecules are treated as flexible (Alder and Wainwright 1959; Parrinello and Rahman 1981). Moreover, molecular docking studies can be importantly used for parallel screening of multiple newer leads against a newly established receptor/target in MDR phenomenon, significantly curtailing the time and cost factor involved in expensive wet lab studies (Fig. 4).

4.1.3 Molecular Dynamics Simulation

Molecular dynamics (MD) studies allows mimicking the behaviour of the molecules at the in silico platform as a function of time. Simulating Newton's second law of motion, F = m.a, MD simulations express the conformational stability of the receptor protein in complex with ligand molecule with the propagation of time. MD simulations has seen its vast applications in the field of molecular modelling studies

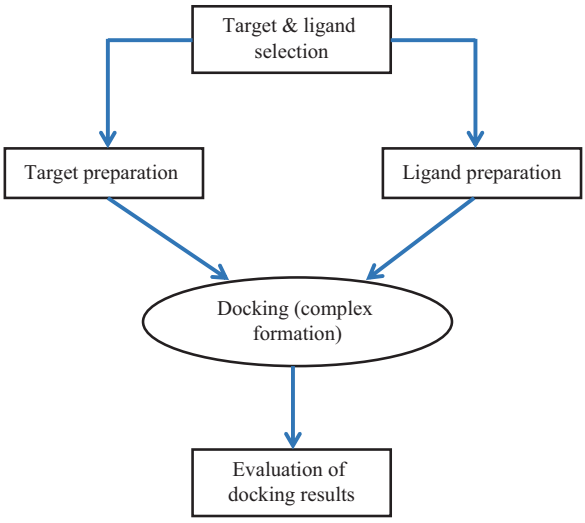

Fig. 4 Flowchart of steps involved in molecular docking

and drug designing in understanding the stability and energy parameters of the interacting lead molecules against a specific target.

Molecular dynamics techniques analyse the physical motion of atoms and molecules and emit a sight of the dynamic progression of the system for a stable phase of time. It yields a computerized informational data of molecular complexes, assimilating Newton's laws of motion governing their stability and energy parameters (Petrenko and Meller 2010, Phillips et al. 2005).

The quality of the results derived from a standard MD simulation largely depends upon the starting conformation of the systems. MD simulations yield different programs to analyse interaction of protein with protein, ligand and nucleic acid separately (Case et al. 2005).

MD simulation can be accomplished through multiple strategies like NAMD (Dror et al. 2012), GROMACS (Duan et al. 2003), AMBER (Foloppe and MacKerell Jr. 2000) and CHARMM. All these strategies have significantly upgraded their formulary worldliness and collateral interpretation and are capable of providing up to approximately 10 to 100 ns/day/workstation/cluster (Schuler et al. 2001). MD simulation of a protein involves different parameters, namely force fields (AMBER03 (Daggett 2006), CHARMM27 (Pronk et al. 2013), GROMOS96 (Case 2002) etc.), volume (NVT) and pressure (NPT). Force fields are used to produce informative data of potential energy of the atoms of a molecular assembly. Basically, MD simulation process is accomplished following the steps mentioned in the flowchart (Fig. 5).

MD simulation is accomplished via multiple steps like energy minimization, which involves prediction of the minimal energy globally based on the site of side chains atoms that show the configuration of specific dispositions of atoms in which the exact appealing force on each atom attains a maximum equilibration dynamics in which NVT (volume regulation) and NPT (pressure regulation) take place. MD

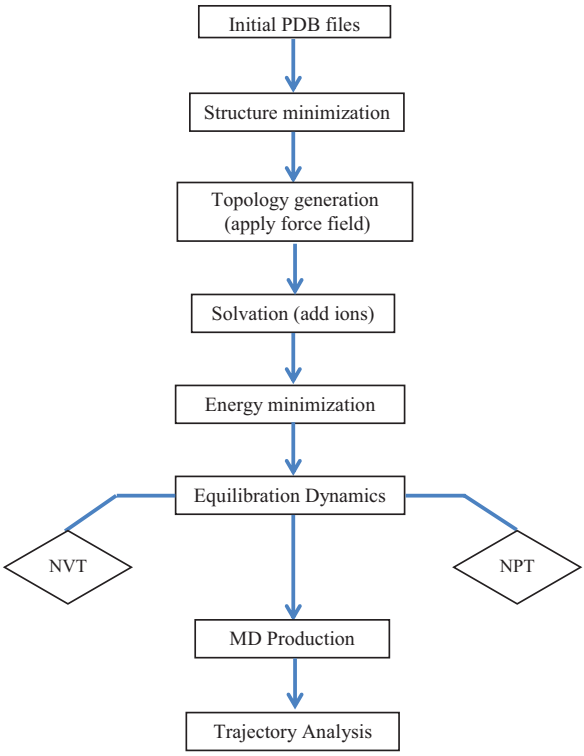

Fig. 5 Flowchart representing the steps involved in MD Simulation of a protein

production involves trajectories development, which are further run for specific analysis. Analysis of these trajectories involves the calculation of these following quantities:

1. Radius of gyration.
2. Root mean square deviation (RMSD).
3. Root mean square fluctuation (RMSF).
4. Interface-associated labels such as density of groups and electrostatic potential.

A normal MD simulation run generates gigabytes of data for better understanding and technical visualization (Patodia et al. 2014). A normal drug designing process involving interaction of a lead molecule with a specific target (novel target in case of MDR) can be precisely studied at *the* in silico level using MD simulation studies, emphasizing specifically on the its stability and energy factors. This significantly simulates the real-world wet lab experimental set up of interacting molecules on in silico platform.

4.1.4 Pharmacophore Modelling

In 1890, Paul Ehrlich proposed the concept of pharmacophore. Ehrlich concluded that there are some fundamental features present in the molecule which define its

biological activity (Khedkar et al. 2007). Later, Schueler implied a new concept of electronic features along with the steric parameters responsible for defining a biological activity of a molecule (Güner and Bowen 2014).

These steric and electronic features together define the interaction, that is, hydrogen bond formation, hydrophobic interaction, aromaticity and the presence of positive- and negative-charged ions between the bioactive molecules. Pharmacophore mapping makes it easier to understand these interactions.

Earlier pharmacophores were characterized by a three-point feature, that is, inter-feature distance of three atoms only. However, in case of availability of more functional groups, excluded volumes may be considered to illustrate the pharmacophoric features. 3D pharmacophore models comply the excluded volumes which imitate the conformations of the active site and avert the coordination with the low active and non-active molecules (Verma et al. 2010).

These days, four-point pharmacophore method delineates the ***4-point 3D pharmacophore model*** with a diverse set of bioactivity and higher molecular resemblance, which aids in describing the steric and chemical parameters of the receptors and ligand interaction (Mason et al. 1999). Pharmacophore mapping can be explained via generating the QSAR models with two main approaches:

- Structure-based pharmacophore modelling (SBPM).
- Ligand-based pharmacophore modelling (LBPM).

4.1.4.1 Structure-Based Pharmacophore Modelling (SBPM)

Structure-based pharmacophore modelling basically works on the principle of receptor–ligand docking algorithm. Docking algorithm relies on search and scoring-based parameters.

Search parameter predicts the orientation and conformation of the ligand inside the pocket of the receptor while scoring parameter predicts the best binding energy from a set of ligands bounded to the same protein (Leach and Gillet 2007). There are several tools working on the docking principle like *GOLD, FlexX, AutoDock* and *Glide* (Halgren et al. 2004; Kramer et al. 1999; Morris et al. 2009).

Structure-based pharmacophore models can be generated with an *apo* (free) form or with a *holo* (protein–ligand complex) form. The structure of the protein can be fetched from experimental (X-ray, NMR crystallographic) data, the *apo* form requires only the binding pocket of the receptor while the *holo* form requires interaction-generated file (potential interactions in the docked complex) to achieve a best featured pharmacophore model (Pirhadi et al. 2013). When the ligands are not known, then the steric and electronic spaces of the receptor molecule are analysed by an interaction of map-generating tool LUDI, HS-Pharm (knowledge-based method) and GRID package within the pocket site (Qing et al. 2014). Structure-based methods have their valuable significance in the identification of best hit tending towards lead optimization, virtual screening, scaffold hopping and multi-target drug design. They offer unique assets over other screening approaches like CoMFA based on ligand structures and features (Mannhold et al. 2006).

4.1.4.2 Ligand-Based Pharmacophore Modelling (LBPM)

Ligand-based approach has been utilized in the absence of 3D structure of protein, while it is totally based on the library of ligands of the target protein and their bioactivity data. They contribute in the lead refinement and optimization mounting towards generating a pharmacophore model embossed with the requisite features for binding to the target protein. This built model results in more novel molecular interactions and chemical function with the protein. Ligand-based pharmacophore modelling can be further elucidated by both quantitative and qualitative chemical descriptors. These are based on two types of algorithms.

(a) **HipHop.**

When the bioactivity or IC50 value of the compounds are not known, and the ligand dataset are mapped on the basis of the common, that is, chemical/functional groups and the steric features present in them, then the model building is done by a qualitative approach known as *HipHop*. The random selection of compounds with common features results in a novel pharmacophore model which is further iteratively mapped with the database screening to find out the best lead compound.

4.1.4.3 Workflow of HipHop

HipHop is based on the common features mapped from the training set excluding the bioactivity. The aligned or mapped model possesses a maximum of five common features out of eleven features, namely hydrogen bond acceptor (HBA), hydrogen bond acceptor lipid (HBAL), hydrogen bond donor (HBD), positively charged (PC), negatively charged (NC), positively ionizable (PI), negatively ionizable (NI), hydrophobic (HY), hydrophobic aliphatic (HYAL), hydrophobic aromatic (HYAr) and ring aromatic (RA) in the Catalyst tool of DS (discovery studio). The principle works on matching the conformers in 3D spaces and superimposing them with a definite certain tolerance. The results are analysed on the basis of best-fitted drug molecule, and then after database screening, a compound having maximum common features along with highest fit value is considered as lead compound which is further scrutinized by molecular docking and simulation studies.

(b) **HypoGen.**

HypoGen in Catalyst works on quantitative approach and uses almost the same protocol as in HipHop. Based on the training set of the compound library with the bioactivity and the common features, the protocol is divided into three consecutive phases:

- Constructive (most active).
- Subtractive (both highly active and least active).
- Optimization phase (least active).

4.1.4.4 Workflow of HypoGen

Working on the hit and trial hypothesis, by modifying the common features, excluding volume and providing a definite uncertainty value (either 3 or 2), the hypothesis

is generated and analysed on the basis of four main factors, namely, cost difference (total cost−null cost), configuration cost, root mean square deviation (RMSD) and correlation value. All these inferences are represented in bit values, and the program catalyst builds a pharmacophore model by applying Occam's razor principle. According to this principle, among all the generated hypotheses, the less bit cost value is the accepted value. RMSD is the difference between the experimental and the estimated binding affinity. It must lie within 1. The weight cost is derived by the difference in the actual and ideal weights of the features. It must be 2, as the more the weight, the more the conformational change in the compound. The estimated binding affinity depends on the fit value of the hypotheses (Mohansinh 2015). Following are the standard values for the result analysis of a generated model:

- Configuration cost must be below 17, as higher cost means more hypotheses leading to take more time to compute results.
- Cost difference = 40–60.
- RMSD equal to or less than 2.
- Correlation must be 1 or less than 1.
- Weight = 2, and configuration cost must be below 17.

Pharmacophore modelling methods have generally come up as boon in the field of drug discovery in specifically designing drugs in the cases where the receptor/protein structure is unavailable. In the quest of exploring and availing novel targets in MDR phenomenon, these pharmacophore modelling methods can promisingly be used to search or design novel analogues as prominent leads against them.

4.1.5 QSAR

QSAR (Quantitative structural Activity relationship) is most accepted approach of novel lead identification and optimization in the drug discovery pipeline (T. Wang et al. 2015). To minimize the outlay, delay in the finding the hit compound to clinical trial phase of the drug development process, Ligand based drug designing, fragment based methods, machine learning approaches etc. are been commonly implemented in the pharmaceutical research

Despite of presence of huge crystallographic data (X-ray and NMR) in the protein databanks (PDB, SCOP, CATH etc.) still function of more than a few number of proteins are uncertain (Divakar and Hariharan 2015; Todeschini et al. n.d.). Thus, the use of tools like QSAR, Quantitative structure property relationship (QSPR), Quantitative structure toxicity relationship (QSTR) for drug leads is becoming extensively significant in predicting the physicochemical, pharmacokinetic and toxicological criteria of these leads (Fang and Xiao 2016)

QSAR are the analytical and demographic models built by correlating the drug's chemical, structural, steric as well as the biological activity parameters. Various linear regression analyses are used to derive the QSAR equations (Pinner 2007; Roy et al. 2015). The generated models discover the novel and best possible compound which is further mapped with the database screening process.

QSPR defines the model generation with the help of modeling and correlating the physiochemical feature and the invivo-invitro (biological data); while when the model building is done with the help of the toxicological data, then it defines the QSTR (Deeb and Goodarzi 2012)

QSAR recognize a molecule on the basis of following basic parameters (Unger and Hansch 1975)

- Lipophilicity (*logP*)
- Steric (conformation of molecule)
- Polarizability (molar refractivity)
- Electronic (charge and electrostatic potential)

Lipophilicity of a molecule is defined as the distribution of a drug molecule in a lipid; fats, oil etc. and it can be calculated as the ratio of concentration of drug molecule in an octanol phase (non-aqueous) to the concentration of drug molecule in water (aqueous phase). It is represented as *log P* or the partition coefficient (Mohansinh 2015)

$$\log P = [\text{drug}]\,\text{in octanol}] / [\text{drug}]\,\text{in water}$$

**Standard value for log P lies between 1 and 5*

Hansch and his fellows forged the substituent/hydrophobic constant (π) for the first time and proposed the following equation defining the phase and relation between the number of hydrogen and lipophilicity of drug molecule.

$$\mathbf{p_x} = \log \mathbf{P_x} - \log \mathbf{P_H}$$

In organic phase, π value increases, the lipophilicity $_{\text{(drug molecule)}}$ also increases while lipophilicity $_{\text{(Hydrogen)}}$ decreases and vice-versa in aqueous phase.

Hansch showed the relationship between molecular structure and bioactivity by demonstrating a Hammett substitution parameter (σ) as

$$\log(\mathbf{1/C}) = \mathbf{k1logP} + \mathbf{k2}\sigma + \mathbf{k3}$$

where C = drug concentration needed to reach the standard value at a given time, log P = lipophilicity and σ = Hammett substitution parameter. This formalism expresses both sides of the equation in terms of free energy.

Steric Parameter is evaluated by the spatial arrangement of heavy or bulky functional groups affects in conformer change the non-bonded interactions between drug and receptor protein influencing reactivity of drug molecule

Polarizability is the property to form an immediate dipole or dynamism to the system because of the electronic cloud.

Molar Refractivity (MR) is the total volume covered by an atom or the functional group. It is calculated by refractive index, density and molecular weight of the drug molecule.

Electronic Parameter is calculated by Hammett constant (σ) and the dipole moment of the drug molecule. It occurs because of the inductive and resonance effects, that is, the value of sigma (σ) depends on the *meta* and *para* substituted groups while *ortho* is independent of this effect (Leach and Gillet 2007).

Using in silico approaches, QSAR builds virtual models which analyse the interaction affinity between the molecules, their pharmacophoric features, for example, aromaticity, hydrophobicity, polarizability and hydrogen bond donor/acceptor. Generated QSAR models have proven to filter out appropriate compound with the accepted activity and properties. This is further stepped into the optimization process via molecular docking and simulation studies. Currently, the tools have been extended to ADMET property check through various online and offline applications like PreADMET and DS (Kwang 2005; Lill 2007).

4.1.5.1 Classification of QSAR

QSAR has been classified into the following categories on the basis of their dimensionality (Jani 2015):

- 1-D QSAR
- 2-D QSAR
- 3-D QSAR
- 4-D QSAR
- 5-D QSAR
- 6-D QSAR.

4.1.5.2 Role in MDR

P-glycoprotein (P-gp) transporters have a noteworthy role in multidrug resistance (MDR). So, we need to target these transporters to overcome MDR phenomenon. QSAR approach definitely marks a progressive step in drug discovery of MDR diseases as it helps in finding out the best possible hit with a definite fit value after mapping against a database. MDR has also been seen on uprise due to overproduction of P-gp transporters, so designing P-gp inhibitors could be a major step in MDR treatment (Nobili et al. 2012; Tan et al. 2013).

5 Multitarget Therapy (MTD)

Bacterial drug–resistant diseases deregulate the function of various membrane proteins and cellular pathways, which escort to several epigenetic alterations in the expression of genes. Recently, to combat these issues, multitargeted or combination therapies are employed (Nikaido 2009).

These combinational treatments are more puissant and rarely susceptible to the drug-resistant diseases. MTD can easily affect more than one target simultaneously and have shown remarkable results in infectious diseases, cancer, diabetes, etc. (Worthington and Melander 2013).

The line of attack on which MTD hypothesis works is to inhibit the respective target without affecting the healthy or surrounding state in the bacterial pathway system. The different combinations of drug molecules can be described as (Bernal et al. 2013; Worthington and Melander 2013)

- Mixing antibiotic two or more antibiotics.
- Mixing antibiotic with non-antibiotic adjuvant molecule.

Antibiotic combination therapy can be basically categorized into 3 major classes (Zimmermann et al. 2007):

(i) The drug molecule inhibits two or more separate targets by inhabiting within the cellular pathway or at different tissues.
(ii) Action of one target affects the function of other targets by one drug molecule. This is common in MDRs by inhibiting efflux pumps (Nikaido 2009) along with the resistance-causing factor by multi-target mechanism.
(iii) Simultaneously inhibition action at different positions on the same target.

These combination therapy aids in exploring the interaction proteins between cellular pathways can also be an advantageous approach towards MDR treatment (Worthington and Melander 2013).

Combinations therapy also explores the systems biology data as they are used as a probe to track the pathway analysis of infectious disease. They form clusters on the basis of synergy scores of denoting the multitargeted ligand interaction (Zimmermann et al. 2007).

6 Structural Genomics

Structural genomics studies tend to decipher and report the three-dimensional structural data of all the proteins encoded by a full genome sequence of a specific organism. Though structural genomics endeavours to specify the structures of every protein compressed by the genome rather than pivoting on any specific protein, it is predominantly divergent to conventional structural prophecy and is meant to acknowledge the functionality of each and every protein.

Structural genomics further facilitates easy comprehension of protein functions considering the adjacent connections of protein structure with its function. The studies involve the full-fledged experimental methods of structure determination, for example, X-ray crystallography, NMR to methods involving structure prediction studies on the basis of sequence or structural homology like homology modelling and threading. At the molecular level, to analyse the protein function and communication between proteins, the specification of all protein structures within a cell or organism is a must. Overcoming this tedious task, structural genomics studies offer an alternate method of splicing and structural determination techniques in and after protein synthesis variations (Y. Wang et al. 2009).

The National Institute of Health (NIH) in the United States has initiated a miscellaneous attainment called protein structure initiative (PSI) with the collaboration of many industries and educationalists specifically to carry out structural genomics studies. Their prime objective constitutes the enhancement of protein structure consciousness by implying structural genomic techniques and to revamp the strategies of structure specification. Similarly, an open protein structure annotation network (TOPSAN) has been developed by the Bioinformatics Core of the Joint Center for Structural Genomics (JCSG), which has been actively engaged in annotating protein structures emerging from high-throughput structural genomics centres. Determination and prediction strategies of protein structures at the structural genomics centres is basically accomplished through either traditional de novo methods and/or prediction-based, modelling-based methods. Modelling-based methods involve the current bioinformatics-based protein structure prediction strategies like homology modelling, threading techniques followed by ab initio methods (Gupta et al. 2014, Gibson and Muse 2002).

A significant research related to MDR phenomenon in the field of structural genomics has been seen as a development of a TB Structural Genomics Consortium primarily constituted to determine the structures of each and every protein and thus novel potential drug targets in Mycobacterium tuberculosis. Currently, more than 700 structures of proteins encoded by M. tuberculosis have been deciphered at the consortium. The centre has enabled identification of a number of target proteins for structure determination involving iron-regulatory proteins, extracellular proteins involved in pathogenesis and proteins determined to have novel folds.

Conclusively, structural genomics approach to determine the three-dimensional structures of every protein of a particular organism has been seen as a highly successful initiative in current scientific era for being time-saving, feasible, cost-effective and with the ability to propose novel drug targets and folds, specifically in the cases involved in MDR phenomenon.

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Computational Approaches for Antibacterial Drug Discovery

Prachi Srivastava and Neha Srivastava

Abstract

With the emerging problem of antibacterial drug resistance and resurgence of disease, there is immediate need for the development of new and effective therapeutic interventions to combat pathogens. Traditional methods of drug discovery are very expensive and time consuming, and carry high error rates. Computational approaches, on the other hand, predict drug targets and therapeutic agents with fewer side effects (i.e., minimal disease resurgence) and reduce the time and cost for discovery. Thus, the computational approaches have become a crucial part of drug development, as they streamline processing and testing in a cost-effective manner. This chapter highlights the significance and progress of computational approaches in antibacterial drug discovery.

Keywords

Anti-bacterial drug resistance · Disease resurgence · Disease pathogen · Computational approaches · Drug discovery

1 Introduction

Bacterial diseases, such as tuberculosis, pneumonia, cholera, diphtheria, meningitis, tetanus, Lyme disease, gonorrhea, and syphilis, are a leading global health threat and represent a major cause of morbidity and mortality. The disease pathologies are

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largely caused by the production of toxins or by an aggressive immune response to bacterial antigens. Although improved awareness about sanitation, advancements in vaccination, and the discovery of antibiotics have greatly minimized the impact of these diseases, many hurdles yet remain. The emergence of drug-resistant bacterial strains and the subsequent resurgence of diseases such as tuberculosis have reestablished infection as a prominent global threat.

Despite the rising problem of drug resistance, no effective alternative therapeutics has emerged to protect against these deadly bacterial infections. According to the World Health Organization (WHO), approximately 700,000 people die every year due to drug-resistant infections worldwide (de Kraker et al. 2016), with those numbers estimated to reach ten million by 2050. Currently available approaches to drug discovery are very expensive and time consuming, and thus there is a great need to develop new and advanced perspectives to overcome this problem. Advancements in computational methods are currently playing a key role in scientific research, not only in the discovery of novel drug targets but in regard to many other fields, such as genomics, proteomics, metabolomics, transcriptomics, systems biology, and molecular phylogenetics. These methods allow scientists to link disease symptoms to particular mutations, epigenetic modifications, and various other genetic and environmental alterations, thereby identifying potential drug targets and novel therapeutics with fewer side effects and potential for drug resistance (i.e., minimizing long-term adverse effects on human health while improving cost- and time-effectiveness). Thus, the current chapter focuses on the significant role and real-world applicability of computational approaches with respect to antibacterial drug discovery.

2 Genomic Approaches to Drug Discovery

Genomics approaches are regularly used for the detailed study of an organism's genetic code and are applied in the fields of DNA sequencing and recombinant DNA technology, and in understating the assembly, annotation, and interpretation of the structure and function of a genome (Mishra and Srivastava 2017). The rise of the genomics era has played a significant role in vaccine and drug development from sequence-based approaches and has provided a novel path to the investigation of underlying disease mechanisms. Genomic study is well suited for the identification of potential drug targets and the design of novel therapeutics and vaccines, as well as the prediction of their side effects on human health, and represents a promising approach to combatting drug resistance and disease resurgence.

Identification of potential antibacterial drug targets based on genome sequences is a major challenge, as the number of genes with unknown biological function is still high. The emergence of bioinformatics has played a crucial role in the identification of homologs of known genes by comparative genomic analysis of new sequences with biochemically characterized sequences of proteins/enzymes. The complete genome sequencing of bacteria provides insight into new information about the disease, its pathogenesis, resurgence, and drug resistance. Thus, genomics

is used to predict potential antibacterial drug targets for disease. After the completion of the first whole genome bacterial sequence of *Haemophilus influenza* in 1995, computational approaches have been vital in providing significant information about pathogens, pathogenesis, and antibiotic resistance. To date, thousands of bacterial genomes have been sequenced and many more are presently ongoing. The genome sequence of a particular bacterial pathogen can contain thousands of genes, providing a massive collection of potential antigens and drug targets. Thus, genomics can be used to identify potential drug candidates faster and more accurately than conventional methods. Genomic approaches are not only used in target-based screening studies but also in whole genome expression profiles to study the cellular response to therapeutic manipulation of an antimicrobial drug target. Additionally, genomics can be used in screening large numbers of herbal compounds possessing antibacterial activity and can identify novel structural classes of antibiotics. Thus, the genomics era has had a significant influence on vaccine and therapeutic development.

2.1 Reverse Vaccinology

Reverse vaccinology is defined as a genome-based approach to vaccine development. It uses computation to design novel vaccines by taking information present in the genome without the need to grow specific microorganisms in the laboratory. The first vaccine developed by reverse vaccinology was the meningococcal B (MenB) vaccine for the prevention of Neisseria meningitides (R. Rappuoli et al. 2012). In this study, the complete genome of MenB was sequenced and computationally analyzed. The selected in silico vaccine candidate was then expressed in *Escherichia coli* to allow for in vitro testing. This work represented a significant contribution to the field of vaccine development. Since then, the approach has been successfully applied in the development of vaccines against various organisms, including *Bacillus anthracis*, *Streptococcus pneumonia*, *Staphylococcus aureus*, *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Edwardsiella tarda*, and *Mycobacterium tuberculosis* (Motin and Torres 2009). The approach is also being used in the development of protein-based vaccines against antibiotic-resistant *Staphylococcus aureus* and *Streptococcus pneumoniae*, and to interpret transcriptomic and proteomic data in order to generate a short list of candidate antigens that can be used for in vitro analysis, thereby reducing the cost and time of downstream processing (Del Tordello et al. 2017). Thus, reverse vaccinology represents a revolutionary new vaccine development strategy that greatly improves upon conventional methods.

2.2 Next-Generation Sequencing

Present approaches for the identification of human pathogen are not sufficient to provide complete information related to disease pathogenesis. Next-generation sequencing (NGS) identifies the complete genome of an organism in a single

sequence. The results interpreted from these data provide, in detail, the underlying mechanisms of disease virulence and resistance, provides information on disease outbreak, and is being used to monitor the current and historic emergence of drug resistance in bacteria and other microbes. NGS technologies are used in various medical microbiology laboratories for the identification and characterization of causal pathogens, rapid identification of bacteria using the 16S–23S rRNA region, and in taxonomic and metagenomic approaches to the study of infectious disease. It is specifically used in the study of evolution and dynamics of drug resistance in bacterial pathogens (ECDC 2016). Various NGS software are available for analysis, including CLC Genomic Workbench (Qiagen) (Powell 2018), SPAdes (Lapidus et al. 2014), and Velvet (Zerbino 2010) for genome assembly analysis, multi-locus sequencing typing (Belén et al. 2009) (MLST) approaches, and conserved core genome (Ghanem et al. 2018) (cgMLST), or whole genome (wgMLST), for investigating genetic relationships (Chen et al. 2017). Many others, such as SeqSphere (Ridom) (Kohl 2014) and BioNumerics (Applied Maths, Biomérieux) (Hunter et al. 2005), or online tools, such as EnteroBase and BIGSdb (Bacterial Isolate Genome Sequence Database) (Jolley and Maiden 2010), are also available.

3 Proteomic Approaches to Drug Discovery

Proteomics is a science dealing with the global analysis of cellular proteins (Osman et al. 2009). It is defined as a complete set of proteins that are produced by an organism under certain conditions (Wasinger et al. 1995). In recent years, proteomics has become a powerful tool for the analysis of complex biochemical mechanisms, and identification of new protein structure, function, and protein–protein interactions. In addition, investigating protein profiles in response to antibiotic sensitivity and drug resistance can significantly contribute to the development of therapeutics for disease recurrence. Furthermore, with the emergence of bioinformatics it has become easier to retrieve information about specific genomes and proteomes for in-depth analysis. In short, proteomics has a diverse range of applications, including drug target identification and validation, efficacy and toxicity testing, and the investigation of drug mechanisms and activities.

3.1 Molecular Modeling

Protein 3D structure is an important perspective for structure-based drug design, as protein structure is vital to ligand binding. With the rapid emergence of homology modeling, it has fast become the first choice for protein 3D structure prediction (Srivastava and Tiwari 2017). Homology modeling techniques for structure prediction are based on sequence similarity to a homologous structure. It is an easy, reliable, low-cost, and less-time-consuming method than conventional means. The 3D structures of proteins provide valuable information about the underlying mechanisms and functions of the molecules, which plays a significant role in drug

discovery. Presently, over 137,000 experimental protein structures are available in the Protein Data Bank (PDB) (19).

Homology modeling involves several steps, including template identification, multiple sequence alignment, and model building based on the 3D structure of a template, as well as model refinement, optimization, and validation. Various studies have been conducted that reveal the significance of homology modeling in drug discovery. In one such study, the 3D structure of the N315 stp1 protein produced by a clinical strain of *Staphylococcus aureus* was predicted using homology modeling through modeler software (Jain et al. 2014). The predicted structure was then validated through PROCHECK, ERRAT, VERIFY-3D, and ProSA tools. Further, the structure was refined through GROMACS software to obtain a more stable and refined configuration. Thus, the predicted 3D structure of *Staphylococcus aureus* N315 stp1 provided valuable information about structure–activity relationships and interactions with the protein. Another study found that drug resistance in *Mycobacterium tuberculosis* is due to a multidrug efflux mechanism of the Mycobacterium multidrug resistant (MMR) protein (Malkhed et al. 2013), which belongs to small multidrug-resistant family of proteins (SMR). Thus, considering the MMR protein as a novel target, an in silico tertiary structure of protein was designed and constructed in order to identify a novel drug molecule for drug resistance in *Mycobacterium tuberculosis*. Another study on a virulent strain of CGSP 14 *Streptococcus pneumoniae* (Karavadi et al. 2014a, b) revealed that the genome codes for 2206 proteins, among which the polysaccharide polymerase protein (B2ILP9) and capsular polysaccharide biosynthesis protein (B2ILP4) act as efficient drug targets and were modeled through homology in order to discover the role of the proteins in the disease pathway of pneumonia. Hence, homology modeling plays a significant role in providing information about protein structure and function in less time, and in a cost-effective manner than conventional means, representing a boon for drug design and development. Various tools for molecular modeling are available, such as Modeller, I-TASSER, LOMETS, SWISS-MODEL, and Gromacs (Frantisek et al. 2007; Zhou et al. 2015; Chou 2004).

3.2 Virtual Screening and Molecular Docking

Virtual screening is defined as the process of screening the molecules from a library of chemical compounds based on their scoring and binding affinity to the specific target (Chen 1977). It is performed for the screening of most potential drug candidates based on their chemical properties, the Lipinski rule of five (Lipinski et al. 2001), their ADMET (Cheng et al. 2013) properties (absorption, distribution, metabolism, excretion, and toxicity), their interaction with the specific target, and their binding affinity as determined by a molecular docking study.

These methods have been used to screen a host of potential therapeutics. For example, in a study involving abnormal prion protein (PrP^{Sc}), which is responsible for the pathogenesis of prion diseases, structure-based drug discovery approaches were used to filter out the most promising compound with anti-prion effects,

showing high binding score and spatial interaction with the target, and remarkably reducing prion disease pathogenesis (Ishibashi et al. 2016). In another study, proteins identified from highly virulent strains of *Streptococcus pneumonia* (HUNGARY19A-6, D39, TIGR4, G54, CGSP14, TCH8431-19A) were modeled and validated (Nastasa 2018). Structure-based virtual screening was then performed to identify novel drug molecules against the proteins, and a docking study was utilized to analyze the protein–ligand interactions and binding affinity. Peptide deformylase protein (PDF), which is essential to the pathogenesis of several bacterial diseases, is used as an attractive target to identify novel antibacterial drug molecules based on binding affinity with hydrazine derivatives using virtual screening and molecular docking study (Karavadi et al. 2014a, b). Bacterial topoisomerase (khursheed 2013), a key target in antibacterial and anticancer drug discovery, was used to discover potential bacterial topoisomerase I inhibitors and structural motif using in silico screening. Various online and offline tools and software, such as *Pharmer, Catalyst, PharmaGist, Blaster, Anchor Query, Ligandscout, Autodock, Swiss-Dock,* and *GOLD*, are available for study and analysis (Sandhaus et al. 2018; Peter 2010; Kujawski et al. 2012; Jones and Rowland 2013; Parrott et al. 2014; Dubey et al. 2011).

3.3 Molecular Dynamic Simulation

Molecular dynamic (MD) simulation plays a very significant role in the drug discovery process. Through MD simulation, one can track the rapid processes of biological systems that can occur in less than a millisecond. It is used to study the physical movement of all the macromolecules, proteins, nucleic acids, atoms, and carbohydrates of biological significance (Cumming et al. 2013). Calculating the free binding energy of ligand–protein and protein–protein interactions is an important feature of the simulations.

Structure-based virtual screening with MD simulation and free energy calculation was used to study the activity of anon–peptide compound against falcipain 1 and 2 (FP-1 and FP-2), *Plasmodium*-produced proteins that catalyze hemoglobin degradation, and their analogs (D W. Borhani 2012). A South African natural compound, 5PGA, and five further potential compounds were identified as having inhibitory activity against FP-1, FP-2, and their analogs. In another study utilizing MD simulation of two *E. coli*–produced Resistance-Nodulation-Division (RND) transporters, AcrB and AcrD, which play a major role in multidrug resistance, researchers connected various specificity patterns of the two transporters to their physicochemical and topographical properties based on calculation of multifunctional recognition sites on the molecular surface (Musyoka et al. 2016). Another molecular docking and MD simulation study was performed on meropenem and imipenem, two antibiotics having different binding affinity for the efflux pump in *P. aeruginosa* and AcrB structures, revealed a greater susceptibility of meropenem over imipenem to the binding site of AcrB, and in-depth analysis identified a key residue involved in the binding interaction (Ramaswamy et al. 2017). These

examples illustrate how MD simulation is a very beneficial tool for structure-based drug design. Various MD simulation software is available, such as *Amber, CHARMm, Gromacs, NAMD,* and *Schrodinger's Desmond* (Bajic et al. 2016; Salomon-Ferrer et al. 2013; Brooks 2009; Phillips et al. 2005).

3.4 Toxicity Prediction

Determining the safety and toxicity of chemical compounds represents a crucial step in the drug discovery process. In silico toxicology prediction is a computational method used to visualize, analyze, simulate, and predict the toxicity of a chemical (David E. Shaw 2006). The aim of toxicity testing is to identify the effects of harmful chemicals on both the patient and the environment. Various parameters are involved in toxicity testing, such as the rate, frequency and dosages of exposure, ADMET (absorption, distribution, metabolism, excretion/elimination, and toxicity) properties, as well as other biological and chemical properties. In silico toxicology testing using computational approaches minimizes the use of animals while also reducing the cost and time requirements. Along with this, it also improves the safety and assessment of the chemicals. Various tools and software are available for computational toxicity testing, including *OSIRIS Property Explorer, ALOGPS, ADMET Prediction, Molinspiration,* and *TOPKAT* (Parthasarathi and Dhawan 2018; Dearden 2003; Tetko and Bruneau 2004; Ekins et al. 2017; Nadeem et al. 2015).

4 Antibacterial/Antimicrobial Databases

Biological databases are the most important feature of bioinformatics. They contain vast libraries of biological information, including genes, proteins, metabolic pathways, microarray data, next-generation sequencing data, and much more. Many antibacterial/antimicrobial databases are freely available that provide valuable information about the sequences, structures, and signatures of genes and proteins. This information plays a vital role in the drug discovery process in terms of gene/protein and screening of novel synthetic/herbal therapeutic compounds. Selections of important and useful antibacterial/microbial databases are listed in Table 11.1.

5 Future Perspectives

The emergence of drug-resistant bacteria is a major threat facing the world today, and there is a great need to improve the methods by which we investigate antibacterial drug-resistance mechanisms and to discover new therapeutic interventions to combat the issue. Traditional approaches to antibacterial drug discovery are very time consuming and expensive, and thus, the emerging field of computational approaches allows researchers to combine biological and chemical parameters in order to streamline the drug discovery pipeline in a time- and cost-effective manner.

Table 11.1 List of antibacterial/antimicrobial databases

Name of Database	Description	Website Address
Antimicrobial peptide database (APD)	Database of antimicrobial and peptides	*http://aps.unmc.edu/AP/*
Antimicrobial drug database (AMDD)	Includes a detailed repository of antibacterial and antifungal compounds, along with their basic information and properties	*http://www.amddatabase.info*
Antimicrobial compounds database	Provides information about antimicrobial compounds	*http://www.dsf.unica.it/~gmalloci/abdb/*
Collection of antimicrobial peptides ($CAMP_{R3}$)	Database of antimicrobial peptide family-based studies, including information related to sequence, structure, and signatures of peptides	*http://www.camp3.bicnirrh.res.in/*
The antimicrobial index	Contains information on microorganisms and antimicrobial agents	*http://antibiotics.toku-e.com/*
Data repository of antimicrobial peptides (DRAMP)	Includes information regarding sequences, structures, antimicrobials, physiochemical data, patents, references, etc.	*http://dramp.cpu-bioinfor.org*
PhytAMP database	Contains information about taxonomic, microbiological, and physicochemical data on small cysteine-rich antimicrobial peptides (AMPs)	*http://phytamp.hammamilab.org/*
Antibiotic resistance genes database (ARDB)	Provides information on antibiotic resistance	*https://ardb.cbcb.umd.edu/*
MIC database	Contains 2D structures of synthesized compounds/antibiotics, IUPAC names, smiles, MIC values, etc.	*www.trimslabs.com/mic/index.htm*
Antibacterial biocide and metal resistance genes database (BacMet)	Includes antibacterial biocide and metal resistance genes	*http://bacmet.biomedicine.gu.se/*
A database linking antimicrobial peptides (LAMP)	Provides a useful resource and tools for AMP studies.	*http://biotechlab.fudan.edu.cn/database/lamp*
MilkAMP database	Provides information on antimicrobial dairy peptides, including microbiological and physicochemical data	*http://milkampdb.org/home.php*
Antimicrobial consumption database (ESAC-net)	Provides data on antimicrobial consumption based on community and the hospital sector	*https://ecdc.europa.eu/en/antimicrobial-consumption/*

(continued)

Table 11.1 (continued)

Name of Database	Description	Website Address
The comprehensive antibiotic resistance database (CARD)	Provides information about resistance genes, their products, and associated phenotypes	*https://card.mcmaster.ca/*
Antimicrobial combinations database	Provides information on antimicrobial combination therapies	*http://sing.ei.uvigo.es/antimicrobialCombination/*

Combining computational methods with wet laboratory experiments provides efficient ways to understand the entire mechanism of drug resistance, as well as pathogen virulence and progression. Computational approaches play a significant role in predicting the functions, properties, and activities of antimicrobial agents and their interactions with therapeutic targets throughout the drug discovery process. Bioinformatics provides advanced tools and software that allow microbiologists to analyze and interpret high throughput experimental data in a cost-effective manner, while the use of various databases, tools, and software by R&D laboratories makes practical application that much easier to realize. It is foreseen that the continued synergy of experimental data with computational approaches will lead to a new age of antibacterial drug discovery.

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Efflux Pump Inhibitors and Their Role in the Reversal of Drug Resistance

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Abstract

The worldwide emergence of resistant bacteria to multiple antimicrobial drugs is one of the greatest hurdles to chemotherapy. Multidrug resistance (MDR) is the capability of pathogenic bacteria to survive lethal doses of antimicrobial drugs. One of the underlying mechanisms of survival under stressful conditions is the extrusion of drugs through membrane-embedded efflux proteins. These ubiquitous resistance elements, which confer resistance or cross resistance to multiple drugs, are considered MDR efflux transporters. Consequently, efflux pump inhibitors (EPIs) from various natural and synthetic sources have been developed to increase the therapeutic armamentarium for combating bacterial resistance and restoring the antibiotic activity. Owing to less toxicity issues than chemical-based EPIs, plant-based EPIs are gaining much importance, but none are yet undergoing clinical trials. In this review, we will introduce the concept of efflux pumps and their diversity and then provide a comprehensive understanding of efflux pump inhibitors from both plant and chemical sources, their mode of action and the recent advances in their development.

Keywords

Multiple resistance · Efflux pumps · RND transporters · EPI

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1 Introduction

In the present century, antimicrobial resistance (AMR) has become a global threat to chemotherapy of infection control as stated by data obtained from global surveillance (WHO 2014). Evolution of resistance leads to the emergence and spread of a large number of resistant bacteria globally that expresses sophisticated phenotypic or genotypic variants towards a variety of antimicrobial drugs. Antibiotic resistance has become a topic of interest in various science and political summits and has been referred to as a *slow motion tsunami* (Cox 2015). Bacterial resistance towards antibiotics develops as a result of mutations and/or gene acquisitions through genetic exchange mechanisms. Broadly, the mechanism of resistance is classified into three categories: first, production of drug hydrolysing or modifying enzymes; second, mutation in the transporters of antibiotics that hinders their entrance; and the third is by the impaired accessibility of an antibiotic to its target by using energy-dependent efflux pumps for its exclusion. In bacteria, active efflux pumps play an important role in both intrinsic and acquired multiple drug resistance as opposed to other various biochemical and molecular resistance mechanisms. Energy-dependent efflux pumps are ubiquitous and important to the drug resistance of all organisms from prokaryotes to mammals. In the case of humans, they are relevant contributors of anticancer drug resistance, and in bacteria, they contribute resistance to antibiotics (Amaral et al. 2012; Nikaido 2009). Efflux pumps were firstly described in the 1970s as the mechanism of resistance to P-glycoprotein in humans (Gottessman and Ling 2006) and resistance towards tetracycline in *Escherichia coli* (Levy S., 1992).

Considerable progress has been made in understanding the various types of efflux pumps prevalent in bacteria that are responsible for different types of drug resistance (Chitsaz and Brown 2017). Subsequently, researchers have found methods to inhibit efflux pump functions by using efflux pump inhibitors. The specific mechanisms involved may vary accordingly (Sjuts et al. 2016). Both synthetic and natural product-derived efflux pump inhibitors have been developed, and their clinical and therapeutic potential have been reported. In this chapter, we will provide a summary of recent updates on the diversity of efflux pumps and variance of both synthetic and natural efflux pump inhibitors and their significance in chemotherapy.

Drug efflux pumps are protein complexes embedded in the membranes of MDR bacteria. Their overexpression causes extrusion of structurally unrelated drugs and contributes to reduced susceptibly, thereby decreasing the concentration inside the cell to sub-toxic levels. This class of proteins are encoded by genes that are located on chromosomes or plasmids (Piddock et al. 2006; Nikaido and Pages 2012; Blanco et al. 2016). A generalized scheme of the five major families of bacterial efflux pumps is represented in Table 1.

Table 1 The five major families of bacterial efflux pumps)

Family of efflux pump	Organisms	Substrate used for extrusion	Energy source	Structural properties
ABC superfamily	*Lactococcus lactis, Staphylococcus aureus, E. coli*	Multiple antibacterial drugs	Hydrolysis of ATP	Multiple transmembrane helices of varying amino acids in the ATP-binding cassette
SMR family	*Acinetobacter baumannii, Staphylococcus aureus*	Acriflavine Benzalkonium	Proton motive force (PMF)	4 helices and primary structure comprises of 100–120 amino acids
MFS family	*E. coli* and *Staphylococcus aureus*	Acriflavine Benzalkonium	Proton motive force (PMF)	12 or 14 transmembrane helices
MATE family	*Vibrio parahaemolyticus, E. coli* and *Staphylococcus aureus*	Fluoroquinolones Aminoglycosides Cationic drugs	Na^+	12 putative transmembrane helices spanning the membrane
RND-type family	*Pseudomonas aeruginosa* and *E. coli*	Multiple antibacterial drugs	Proton motive force (PMF)	Multi-subunit complex

Bremner (2007) and Kumar and Pooja Patial (2016)

2 Major Classes of Efflux Pumps

Efflux pump transporters in the prokaryotic kingdom are classified into five major superfamilies based on their composition, energy source, number of transmembrane spanning regions and substrate specificity: resistance-nodulation-division (RND) superfamily, the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the multi-drug toxic compound extrusion (MATE) superfamily and a small multidrug resistance (SMR) family (a member of a much larger metabolite or drug superfamily). These efflux pump transporters are found in both gram-positive and gram-negative bacteria except the RND efflux pumps that are predominantly distributed in gram-negative bacteria. The genes encoding this class of transporter proteins can be chromosomally encoded or plasmid encoded (Piddock 2006).

The RND superfamily have a tripartite composition that includes an inner membrane transporter, a periplasmic adapter protein and an outer membrane channel and are extensively associated with antibiotic resistance such as AcrB in *Escherichia coli* and MexB in *Pseudomonas aeruginosa* (Li and Nikiado 2009). With advancements in molecular biology and biochemistry, there has been a growing

identification and characterization of MDR pumps in numerous problematic species of bacteria, particularly the pathogens belonging to ESKAPE group [*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* species], evoking their predominant role in clinical settings (Davin-Regli et al. 2016). Among gram-positive bacteria, the MFS family of efflux transporters is widely distributed like NorA efflux pumps in *Staphylococcus aureus* and PmrA from *Streptococcus pneumonia* (Jang 2016).

2.1 ATP-Binding Cassette (ABC) Family MDR Efflux Pumps

ABC transporters that are involved in drug extrusion are found in fungi and animal cells. There are only a few examples reported of ABC transporters in gram-negative bacteria, although MSbA transporters that export the biosynthetic intermediate of lipopolysaccharides carry out erythromycin extrusion when they are overexpressed in *Lactococcus lactis*. ABC pumps are composed of four domains in which two are hydrophobic and membrane-embedded and are responsible for substrate recognition and translocation. The other two domains are hydrophilic nucleotide-binding domains (NBDs) and serve as a site for ATP hydrolysis, which then generates the energy for the translocation of drugs (Crow et al. 2017). The most commonly studied ABC drug exporter in gram negatives is MacB of *E. coli*, which becomes functional when it comes into contact with the periplasmic MacA adapter and TolC of the outer membrane channel. Overexpression of MacA–TolC transporters increases the MIC of macrolide antibiotics and results in the emergence of resistance (Lu and Zgurskaya 2013). MacB also possesses moderate ATPase activity. ATP hydrolysis is triggered by the continuous presence of MacA, which becomes fully functional when it comes together with dimeric MacB and TolC trimer unit of the pump (Wilkens 2015).

2.2 SMR Transporters

SMR transporters are small, proton motive force-dependent drug transporters that consist of four transmembrane segments (TMSs). These proteins generally function as homodimers, but their arrangement is either in parallel or antiparallel and is a matter of controversy (Lloris-Garcera et al. 2013). Overexpression of these proteins by their corresponding plasmids causes reduced susceptibility to aminoglycoside antibiotics. EmrE, one of the transporters of the SMR superfamily, generates hyperosmotolerant phenotypes in *E. coli* also harbouring AcrAB proteins. Most of the common substrates of EmrE pumps are mainly quaternary ammonium compounds like osmoprotectants of *E. coli* and betaine. The overexpression of such pumps increases the susceptibility of cells to alkalinity in the medium and hyperosmolarity conditions as well (Bay et al. 2008). In the case of *S. aureus,* the SMR proteins like QacC are mainly plasmid encoded, while in other bacteria, the genes for the related components are also present on chromosomes. QacC carries out the efflux of biocides such as ethidium bromide and also quaternary ammonium compounds (Furi et al. 2013; Wassenaar et al. 2015).

2.3 Multidrug and Toxin Extrusion (MATE) Family

MATE transporters were firstly identified in *Vibrio parah*a*emolyticus* as sodium or cationic antiporter, i.e. NorM with 12 TMS (Kuroda and Tsuchiya 2009). The MATE family of drug transporters act as a contributing factor for resistance and are in recent discovery. Most of the work has been done with these pumps, and well reports are obtained in gram-negative bacteria including the crystal structures of four proteins out of them (Lu and Zgurskaya 2013). Some of the significant work has been done to determine the function and structure of MATE transporters in gram-positive bacteria, such as *S. aureus* MATE pumps like MepA. Interestingly, it was reported that in methicillin-susceptible strains, the majority of the population overexpresses MepA, but the reason behind this remains uncertain (Schindler and Kaatz 2016). MATE proteins are dependent either on Na ions or the proton gradient and are regulated by the MepR regulatory protein. Dimers in single and dimeric form bind to MepR and MepA, respectively. MepR belongs to the MarR family of proteins and consists of winged helix-turn-helix that retards the expression of its own gene as well as mepA (Jang 2016). Additional MATE efflux pumps in gram positive have been described like PdrM of *S. pneumoniae*, FepA of *Listeria monocytogenes* and Cda of *C. difficile* (Hashimoto et al. 2013; Gurein et al. 2014). The substrate utilized by FepA is larger in number since they decrease susceptibility to several dyes, ciprofloxacin, norfloxacin and biocides. A regulatory protein that belongs to the TetR group regulates the expression of *fepA*, and mutation in FepR increases the overexpression of *fepA* and thus reduces the drug susceptibility.

2.4 Major Facilitator Superfamily (MFS) Transporters

There are 74 families of MFS transporters, grouped on the basis of their sequence homology. Most of them are embedded in an inner membrane in a free state, and these pumps transport the drugs from the cytosol only to the periplasm. Antimicrobial agents have the ability to cross the lipid bilayer through diffusion, and once they are pumped out, they are unable to come, but in this case, MFS pumped out drugs can easily come to the cytosol. Thus, this kind of transporters is not responsible for high level of resistance. It was found that in *P. aeruginosa,* these pumped out drugs are easily captured in the periplasm by MexAB–OprM RND pumps, which are constitutive expressed, synergistically enhancing the activity of singlet pumps in the development of resistance (Choudhury et al. 2015).

In case of *E. coli*, MFS forms a tripartite efflux system that includes an outer membrane protein channel, Tol-C and periplasmic adapter proteins. These efflux systems include EmrB and EmrY and carry out the extrusion of a variety of substrates and uncouplers (Nishino and Yamaguchi 2008). A central cavity which is surrounded by hydrophobic and hydrophilic side chains is found in most of the MFS pumps. It was also noted that the loops formed through connections between H4, H5, H10 and H11 are localized towards the cytosol and responsible for substrate recognition and binding (Yin et al. 2000). A dimer of EmrAB is formed when EmrA

comes into association with EmrB. Among the singlet MFS transporters, MdfA pumps lead to MDR when they are overexpressed. In combination with the SMR-type transporter EmrE, these exporters carry out efflux of cationic agents like quaternary ammonium compounds (Tanabe et al. 2009).

NorA is an MDR pump predominantly found in *S. aureus* which is chromosomally encoded and also belongs to the MFS family of proteins. In *Escherichia coli* and *S. aureus*, expression of the plasmid-encoded *norA* gene leads to the resistance of hydrophilic quinolones like norfloxacin (Yu et al. 2002). MgrA is a global regulatory protein that controls expression of not only NorA pumps but also other pump proteins like TetG, NorB and NorC. Additionally, another regulatory protein involved in NorA expression is GntR which is able to interact with the promoters of NorC, NorB and NorA. In addition to interacting with these promoters, this regulatory protein reduces the transcription of NorG, leading to a fourfold decrease in fluoroquinolone susceptibility (Redgrave et al. 2014; van der Putten et al. 2018). A deletion of GntR does not affect NorA, NorB or NorC but does cause a significant increase in the transcription of the ABC family of transporters that play an important role in the susceptibility of *S. aureus* towards β-lactams (Foster 2017; Ranaweera et al. 2015).

2.5 Resistance-Nodulation-Cell Division (RND) Transporters

RND-type efflux pumps are predominant in gram-negative pathogens. These tripartite protein complexes span throughout the membrane and enable bacteria to pump antibiotics out of the cell. These protein assemblies are composed of an inner membrane protein (IMP), a periplasmic membrane fusion protein (MFP) and an outer membrane protein**.** The IMP exhibits selectivity towards drugs and carries out catalysis of the drug/proton antiport (Venter et al. 2015). The most widely studied RND-type efflux pumps are AcrAB–TolC in *E. coli* and MexAB–OprM in *P. aeruginosa* (Blair et al. 2014). In recent years, the elucidation of crystal structures of different efflux pumps that are complexed with their substrate or inhibitors has greatly increased our understanding of their specificities towards multiple substrates. The AcrAB pump protein of *E. coli* was the first crystal structure of an efflux pump to be resolved at 3.5 Å resolution (Murakami et al. 2002). Later the same research group identified the interaction of crystal structure with the substrates doxorubicin and minocycline. The crystal structure is comprised of a periplasmic headpiece, which constitutes a large portion and a transmembrane domain. The upper part represents the TolC docking domain, and the central region of the headpiece makes up the pore domain (Murakami et al. 2006).

The best characterized efflux pump is MexAb–AcrB produced by the opportunistic pathogen *P. aeruginosa* and confers resistance towards a broad spectrum of antimicrobials. The crystal structure of *P. aeruginosa* efflux pump components that closely resembles with *E. coli* tripartite AcrAB-TolC has also been reported and characterized (Sennhauser et al. 2009). There is structural similarity in the transmembrane domains of all RND-type transporters. The MexB docking domain in *P. aeruginosa* is composed of two sub domains, one of which forms a loop that inserts

into the docking domain of an adjacent pump subunit. These findings clearly demonstrate that RND efflux pumps and outer membrane channels have low affinity and interact in transient ways (Yamaguchi et al. 2015; Daury et al. 2016).

3 Efflux Pump Inhibitors (EPIs)

Overexpression of multidrug efflux pumps is the principle cause of antibiotic resistance in pathogenic bacteria that have increased MIC towards variety of antibiotics. These efflux transporters have become major resistance determinants for the efficacy of both old and new antimicrobial drugs (Hernado-Amado et al. 2016). The structure of efflux transporters not only provides a deep insight into the mechanism of extrusion of drugs but also makes the discovery of efflux pump inhibitors possible (Lomovskaya and Bostian 2006). Broadly speaking, the agents that cause inhibition of efflux, either through disrupting the proton motive force, interacting with proteins or inhibiting the efflux of pump-associated genes, are called efflux pump inhibitors. Because of the increasing evidence of resistance emerging in pathogenic bacteria, alternative therapeutic strategies that counteract antibiotic efflux are needed. The strategies for inhibiting efflux pump include:

(i) Structural alterations in the chemical design of existing antibiotics so that their binding affinity to the efflux pump protein sites is reduced.
(ii) Interference with the energy source that is needed for the activity of efflux pumps.
(iii) Downregulating the expression of genes required for the expression of active efflux pumps in bacterial cell envelopes.
(iv) Interference with the functional subunit of efflux pump complexes.
(v) Competitive or non-competitive inhibition of antibiotics towards substrate-binding sites in efflux pumps.
(vi) Hindrance in the activity of membrane channels responsible for antibiotic extrusion by inserting designed molecular plugs.

EPIs act as therapeutic agents since they have the potential to restore the activity of conventional antibiotics. The combination of EPIs along with antibiotics is expected to reduce the intrinsic resistance of bacteria towards a varying range of antibiotics, decrease the frequency of emerging resistant mutant strains and facilitate the reversal of acquired resistance in strains that have multiple targeted mutations. Lomovskaya et al. (2001) provided the following criteria that a potent efflux pump inhibitor should satisfy:

(a) It must potentiate the antibiotic activity against the resistant strain that has developed as a result of the drug efflux pump.
(b) It must have the potential to enhance the accumulation and reduce the extrusion of a substrate that shows specificity towards the efflux pump.
(c) It should not have outer membrane permeabilizing ability.

(d) It should not decrease the MIC of antibiotics that are not the substrate of the efflux pump.
(e) The proton gradient across the inner membrane must be unaffected by the EPI.

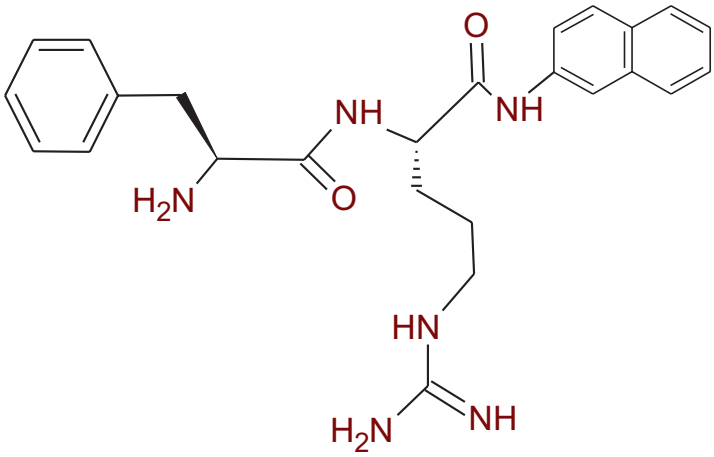
The use of a combination of EPI and antibacterial agents have the potential to enhance the activity of antibiotics against efflux pumps and lead to a reduction in mutant frequency. For example, in the case of the food-borne pathogen *Campylobacter jejuni,* the combination of PAβN with antimicrobials caused a 2000-fold reduction in the minimum inhibitory concentration of the substrate used against CmeABC efflux of *C. jejuni* and a 1000-fold decrease in the development of erythromycin-resistant mutants (McCrackin et al. 2016).

Several EPIs are used in combination with a photosensitizer dye in the presence of light and exhibit broad-spectrum antibacterial activity. The photoactivable dye generates reactive oxygen species and singlet oxygen, which kill microbial cells. Tegos et al. (2008) used a cationic phenodizidium dye, Toluidine Blue (TBO), under red light in combination with a variety of known EPIs that inhibit the NorA and MexAB–OprM efflux pumps of *S. aureus* and *P. aeruginosa*, respectively. Quinolone derivatives such as alkylaminoquinolone, alkoxyquinolone, chloroquinolone and pyrridoquinolone are also used as EPIs and exhibit structural similarity with the quinolone group of antibiotics (Pages and Amaral 2009). These derivatives inhibit antibacterial extrusion and increase antibiotic susceptibility in clinical strains of *Klebsiella pneumonia* and *Enterobacter aerogenes* (Cheveleir et al. 2004).

The efflux transporters between different gram-negative bacteria exhibit structural similarity so that the EPI used against pumps such as the AcrA–AcrB–TolC of *E. coli* can also be effectively used against other gram-negative pathogens. At the present time, two classes of EPIs have been extensively characterized such as pyridopyrimidines and peptidomimetics that exhibit broad-spectrum activity. A third class of EPIs have also been established known as quinolone derivatives (Sun et al. 2014; Aygul 2015). The chemical structure of some synthetic and natural efflux pump inhibitors are shown in Fig. 1.

4 Synthetic EPIs for Modulating the Reversal of MDR Phenotypes

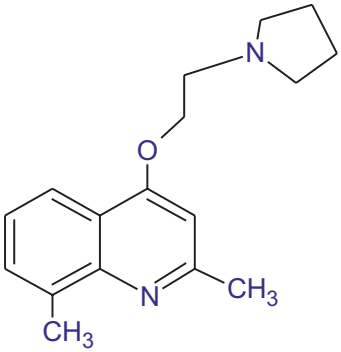
L-Phenyl alanine-L arginine β Napthylamide (PAβN) [MC-207,110] was the first compound developed as an EPI for gram-negative bacteria and is characterized as a dipeptide amide compound, MC-207,110. It inhibits all four efflux systems of *Pseudomonas aeruginosa,* MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY–OprM. Overexpression of the AcrAB–TolC efflux pumps of *E. coli*, *S. typhimurium* and *S. pneumoniae* is also reduced by combining PAβN with fluoroquinolones (Kourtesi et al. 2013). However, the use of PAβN is still limited because of issues associated with its bioavailability and toxicity (Bhardwaj and Mohanty 2012). A derivative of PAβN, MC-04,124, has been developed to target the overexpressed pumps of *Pseudomonas aeruginosa* with less toxicity and greater stability



PAβN

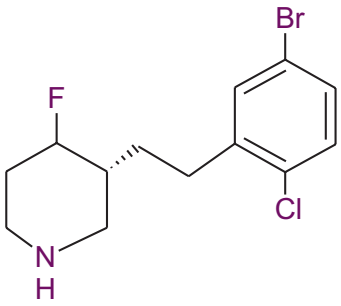
Quinolones

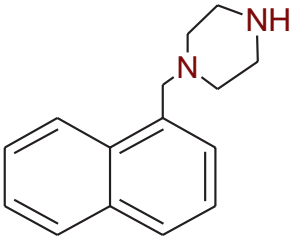
(2,8 di methyl-3-pyrrolidinethyloxyquinoline)



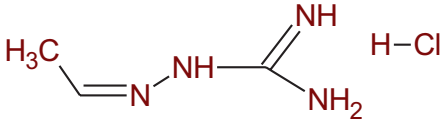
Arylpiperidines

3-fuolro-2-(2 chloro-5 -bromophenyl ethyl) pepridine



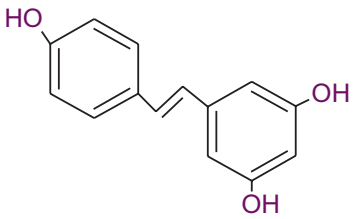


NMP

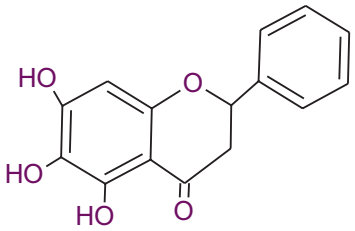


Aminoguanidine Hydrazone (AGHs)

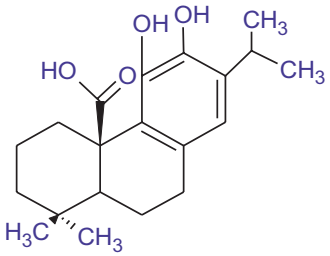
Fig. 1 Chemical structure of some synthetic and natural efflux pumps inhibitors is represented



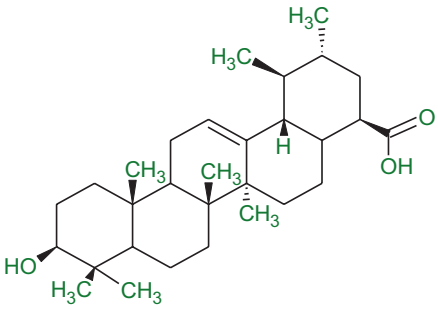
Resveratrol



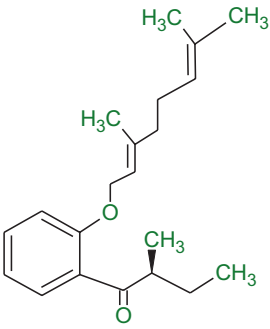
Baicalein



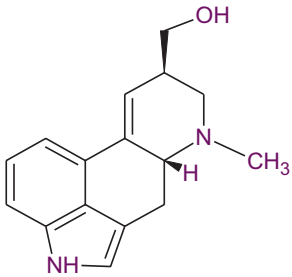
Carnosic acid



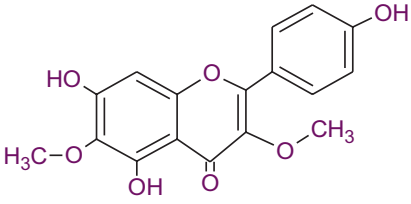
Ursolic Acid



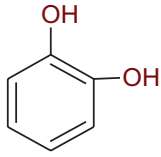
Olympcin A



Clavine Alkaloid



Sarothrin Flavanoid



Catechol

Fig. 1 (continued)

in biological fluids (Pages and Amaral 2009). Additionally, a number of pyranopyridine EPIs have been developed like MBX2319 that exhibits potent activity against the members of *Enterobacteriaceae*, but it has reduced activity in *P. aeruginosa* strains (Oppeman et al. 2014). The efficacy of this compound has not been tested in animal models and is the stages of lead optimization.

Quinoline compounds also show enhanced activity against the AcrAB–TolC efflux pumps of *Enterobacter aerogenes*. These compounds effectively increase the intracellular accumulation of chloramphenicol by inhibiting drug extrusion.

More recently, a molecular modelling and docking study conducted to determine promising EPI activities of aminoguanidine hydrazones found that they act as competitive inhibitors of the NorA efflux pump of *S. aureus* against norfloxacin. By binding with NorA, they restored the activity of norfloxacin and ultimately reduced resistance emergence (Dantas et al. 2018).

5 EPIs from Natural Sources

EPIs have been screened from both microbial and plant sources like in study two compounds, Ea-371α and EA-371δ, as effective efflux pump inhibitors through the microbial fermentation process have been identified (Lee et al. 2001). These two compounds have the potential to suppress the MexAB–OprM pumps of *P. aeruginosa.*

In developing countries, medicinal plants have had a long history to treat various health-related issues. Natural phytochemical compounds are routinely used as antibacterial agents, and their strength is their ability to synergize with antibacterial drugs, which can make antibiotics that are not used in infection control due to resistance, relevant again (Lee et al. 2011). These compounds cripple the resistance mechanisms of bacteria making, and due to the complexity of plant EPIs, bacteria may take decades to develop resistance to them (Wink 2012). Researchers have classified the phytocompounds that potentiate the activity of antibiotics and exhibit activity against multidrug-resistant pathogens as modifying, modulating or reversal agents (Fankam et al. 2017).

A well-known example of an EPI from a natural source is the plant alkaloid reserpine, obtained from the roots of the poisonous devil's pepper (*Rauwolfia vomitoria* Afz), which inhibits the *Bacillus subtilis* efflux pump Bmr (Ahmed et al. 1993). Phytochemistry has played a significant role in the search for EPIs against the NorA efflux pumps of *S. aureus*, and it has been reported that plant-derived EPIs containing varied chemical constituents, such as porphyrin phaeophorbide, acylated glycosides, flavones and isoflavones, have the ability to inhibit NorA pumps (Holler et al. 2012). Moreover, in case of gram-negative bacteria, it was found that a synergistic combination of cefixime and aqueous extracts of *Terminalia chebula* and gallactotannin, isolated from *Terminalia chebula,* enhances the antibiotic potential and competes with multidrug-resistant efflux pumps of *E. coli* as well (Bag and Chattopadhyay 2014).

Some plants produce a variety of antimicrobials along with compounds that inhibit the extrusion of antibiotics from the bacterial cell. This was first exemplified by the discovery of the antimicrobial compound berberine from berberis, a medicinal plant, which also synthesizes 5′-methoxyhydnocarpin (5′MHC), a *S. aureus* NorA efflux pump inhibitor, which reduces the MIC of berberine (Stermitz et al. 2000). Unfortunately, its use is limited because of toxicity issues. There are some EPIs of plant origin that have been patented, such as a tetrandrine-based EPI that reduces the resistance of *E. coli* to fluoroquinolones and also a natural compound called geraniol that is effective against *Enterobacter aerogenes* (Berti et al. 2010).

Maisuria et al. (2015) extracted maple syrup with methanol and demonstrated EPI activity against *P. aeruginosa* ATCC15692, *Proteus mirabilis* and *E. coli* ATCC 700928. The active compound catechol was isolated from maple syrup and found to inhibit ethidium bromide efflux but not to a larger extent than maple syrup extract itself. Lysergol which is a clavine alkaloid obtained from *Ipomoea muricata* (L) Jack (Convolvulaceae) exhibited efflux pump inhibitory potential against both sensitive and resistant strains of *E. coli.* It was reported that the active compound lysergol and its derivative 17-O-3″,4″,5″ trimethoxy benzyl lysergol have promising EPI activity compared to that of reserpine in an EtBr accumulation assay (one of the assays for the screening of efflux overexpressing strains), and it was shown that the YojI efflux pump of *E. coli* is the target of this compound (Maurya et al., 2013). In another case, the essential oil obtained from *Salvia fruticosa* Mill (Lamiaceae) by means of hydrodistillation is found to be active against the TetK pumps of tetracycline-resistant *S. epidermis*. It was reported that this oil caused a 63% reduction in the overexpression of Tet proteins (Chavanova et al. 2015). Furthermore, a known fatty acid, linoleic acid, was obtained from the herb *Portulaca oleracea* L (Portulacaceae) and found to inhibit EtBr efflux at a concentration of 64 mg/L compared to reserpine against MRSA strains. This unsaturated fatty acid restored the activity of erythromycin in overexpressing ABC efflux pumps of MRSA (Chan et al. 2015). The overexpression of the NorA efflux pump can be inhibited by Olympian A obtained from *Hypericum Oylmicum* L.cf. uniflorum. This patented acylphloroglucinol compound is active against *S. aureus* 1199B strains with decreased cytotoxicity at about 8.9 μM in cancer cell lines (Shiu et al. 2013). Previously, it was observed that carnosic acid from *Rosmarinus officinalis* can block NorA-induced efflux of EtBr in MDR *S. aureus* 1199B strains (Smith et al. 2007). In a study of triterpenes from plant sources, active compound such as karavilagenin C and balsaminagenin B from the medicinal plant *Momordica balsamina* L (Cucurbitaceae) was obtained and was checked for the EPI activity against MRSA COL_{oxa} (MRSA that are highly resistant to oxacillin) strains and *E. faecalis* ATCC 29212, and it was found that balsaminagenin B was most active at a concentration of 30 μM in *E. faecalis* cells (Ramalhete et al. 2016). Another triterpenes isolated from the same plant were Karavilagenin C and balsaminol F, found effective at a concentration of 3 μM in NorA pump overexpressed MRSA COL_{oxa} cells (Ramalhete et al. 2011a, b).

It can be emphasized that natural compounds are effective at inhibiting the SMR, MFS and ABC transporter superfamilies found in gram-positive bacteria; however, the transporters in gram negatives are highly complex because of the presence of an

outer membrane permeability barrier. Furthermore, there are certain compounds that inhibit human transporters. Thus, EPIs should be designed in such a way that can specifically target the efflux pumps of bacterial origin (Amaral et al. 2014). Recently, in a screening of a small molecule library containing 8000 molecules, a compound (IIR08027) was found to potently inhibit the proton-driven efflux pump AbeS of *Acinetobacter baumannii*. Although the molecule does not have growth inhibitory action, it restored the activity of ciprofloxacin against *Acinetobacter baumannii*-resistant strains (Bhattacharyya et al. 2017). In Table 2, we list some of the newly synthesized EPIs from natural sources. Previously identified EPIs are discussed elsewhere (Tegos et al. 2011; Abreu et al. 2012 and Kourtesi et al. 2013).

6 Mechanisms of Action of the Major Classes of EPIs

Bacterial efflux pumps are novel target for a variety of antimicrobial compounds for combating MDR resistance. EPIs are used as adjuvant to enhance the efficacy of antibiotics. Efflux pump inhibitors have been exploited from both natural and synthetic sources, including gram-positive and gram-negative bacteria. RND pumps in gram negatives play a predominant role in virulence and pathogenicity. EPIs that are targeted towards RND pumps are useful as adjunctive therapies in combination with antibiotics to reduce spread and emergence, decrease virulence and inhibit biofilm formation in *Klebsiella pneumoniae* and *E. coli* (Kvist et al. 2008; El-Banna et al. 2016). A variety of EPIs have been developed, but recent reports suggest that only three of the synthetic EPIs are optimized and in preclinical trials (Mahmood et al. 2016). Owing to the structural complexity of the RND-type efflux pumps, the mechanism of action of EPIs complicates their development and discovery. Generally, efflux pump inhibitors are designed to target pump components as depicted in Fig. 2.

6.1 1-(1-Naphthylmethyl)-Piperazine (NMP) and Analogs of Arylpiperazine

NMP was discovered as an EPI against *E. coli* by Bohnert and Kern (2005). This research group screened a library of N-heterocyclic compounds and found that phenylpiperazines act as effective potentiators of levofloxacin in AcrAB and AcrEF-resistant strains of *E. coli*.

Schuster et al. (2014) were the first research group to determine the mode of action of NMP-resistant mutants. They screened a library of NMR-resistant mutants that were able to grow on linezolid, and focusing their study on the binding and extrusion domains of the AcrB pumps in these mutants. Subsequently, they found a mutation in the amino acid residue, Phe610, near the substrate-binding pocket that plays an important role in the extrusion process. Mutated amino acid residues were also present across the G-loop that separates the distal and proximal binding sites (Eicher et al. 2012). A predicted study of MD simulations also suggested Nmp bound to these locations across the G-loop and allowed for the extrusion of linezolid

Table 2 A summary of clinically relevant MDR efflux pumps present in gram-positive and gram-negative bacteria

Protein family	Organisms	Antibiotics used/substrate	Efflux pump protein	References
ABC transporters	*S. aureus* *S. pneumonia* *E. faecium* *Salmonella typhimurium*	MC, SG EB, HO, FQ MC, SG	MsrA PatAB Sp2073–2075 MsrC MacAB	Nishino and Yamaguchi (2008), Boncoeur et al. (2012), and Hurlimann et al. (2016)
SMR transporters	*S. aureus* *P. aeruginosa* *E. coli*	EB, QA, CV, TPP EB, MV, AC AC, MU, EB, TPP	Smr/QacC Pasmr EmrE	Marchi et al. (2015) and Kenana et al. (2017)
MATE transporters	*L. monocytogenes* *S. pneumoniae* *S. aureus* *E. coli*	AC, TPP, FQ, CT, EB AC, DP, FQ, EM, CHL TG, QAC, EB, FQ AC, KM, nor, Cip	FepA PdrM MepA YdhE	Hashimoto et al. (2013), Guerinest et al. (2014), Tocci et al. (2013), and Radchenko et al. (2015)
MFS transporters	*L. monocytogenes* *S. aureus* *E. faecium* *S. pneumoniae* *E. coli*	AC, FQ, EB, BC AC, EB, NO, QAC, HO, FU EB, CT, FQ, TPP FQ, EB AC, TPP, CT, CH, CV EB, PT EB, EM, TPP, DP FQ, EB, AC TE CCMP, NA	Lde NorA NorB NorC QacA EfmA PmrA TetA EmrB	Lekshmi et al. (2017), Wendlandt et al. (2015), Kumar et al. (2013), and Reddy et al. (2012)

RND transporters	*P. aeruginosa*		MexAB–OprM MexCD–OprJ MexEF–OprN MexXY AcrAB AcrB AcrEF–TolC OqxAB EmrAB–TolC AdeABC AdeFGH AdeIJK AcrAB–TolC OqxAB AcrAB CmeABC Oqx AB AcrAB–TolC Cme ABC	Avrian et al. (2013), Krishnamoorthy et al. (2016), Singh et al. (2017), Goli et al. (2017), Zhang et al. (2018), Anes et al. (2015), Coyne et al. (2011), Hou et al. (2012), Beheshti et al. (2014), Perez et al. (2013), Perez et al. (2012), Su et al. (2017), Bialek-Davenet et al. (2014), Perez et al. (2013), Pérez et al. (2012), Su et al. (2017), and Yao et al. (2016)
	S. typhimurium *E. coli*	AG, CHL, TE, FQ, BL, NO AC, DOC, ERY, CIP CV, EB, AC, bile salts		
		CHL, FQ, TM MAC		
	Acinetobacter baumannii	AG, FQ, CE, MC, CHL		
	Klebsiella pneumoniae *Enterobacter cloacae* *Campylobacter jejuni*	FQ, CHL, SULF-TRI, TE MAC, FQ, TE, BL, NO TE, FQ, CHL, BL, RF, NO, CTX MC, CPH, FQ, TE, CL, BL CHL, COT, FQ, AG, BL, MAC MAC, AG, RF, CHL, FQ, CE, TE		

Abbreviations: *MC/MAC* macrolide, *SG* streptogramins, *EB* ethidium bromide, *HO* hoechst 33342, *CV* crystal violet, *CH* chlorhexidine, *TPP* tetraphenylphosphonium, *AG* aminoglycosides, *RF* rifampicin, *CHL* chloramphenicol, *TE* tetracycline, *COT* cotrimoxazole, *BC* benzalkonium chloride, *NO* novobiocin, *CTX* cefotaxime, *CE* cervimycin, *SULF-TRI* sulfamethoxazole, *TM* trimethoprim, *AC* acriflavine, *DOC* doxycycline, *CIP* ciprofloxacin, *ERT* erythromycin, *CCMP* carbonyl cyanide m-chlorophenyl hydrazine, *NX* nalidixic acid, *CV* crystal violet, *PT* pentamidine, *FU* fusidic acid, *DP* 4,6-diamino-2-phenylindole, *QA/QAC* quaternary ammonium compounds, AM amikacin, NOR norfloxacin, TG tigecycline, *BC* benzalkonium chloride, *KM* kanamycin, *FQ* fluoroquinolone, *CT* cetrimide, *BL* beta-lactams, *MU* mupirocin

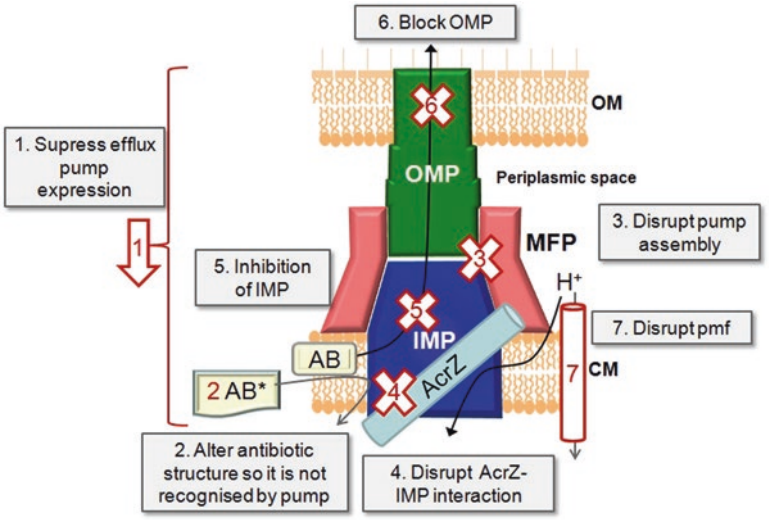


Fig. 2 Possible EPI targets of RND-type efflux pumps. (Source Venter et al. 2015)

in resistant mutants (Vargiu et al. 2014). This clearly demonstrates that NMP interferes with the G-loop and is responsible for substrate extrusion, inhibiting the action of AcrB. Similar to the mode of action of PAβN, NMP is able to cause conformational changes that lead to reduction in the width of the substrate-binding domain as predicted by MD simulations (Vargiu and Nikaido 2012; Bohnert et al. 2016) (Table 3).

6.2 Pyranopyridines (MBX2319)

MBX2319 is a novel inhibitor of the AcrAB efflux pumps of *E. coli,* reported by Opperman et al. (2014). The efflux proteins of other members of *Enterobacteriales* like *K. pneumoniae, Shigella flexneri* and *E. cloacae* are also inhibited by this compound. MBX2319 possesses no antibacterial activity (MIC≥100 μg/ml) but has profound effects on the MIC of antibiotics like beta-lactams, fluoroquinolones, chloramphenicol, linezolid and erythromycin, acting in the range of 3.1–12.5 μg/m as substrate for AcrB pumps. The underlying mechanism of this efflux transporter inhibition involves conformational changes in binding sites due to the functional rotation of AcrB. Once MBX2319 binds these sites, it hinders their rearrangement, and as a result, the translocation of proteins from the periplasm to the cytoplasm occurs. Additionally, a distal binding site, which is also the site for doxorubicin and minocycline, becomes shrunk when the compound binds to them (Nakashima et al. 2013). The results obtained through MD simulation closely resemble those from crystallographic methods and generate a molecular hypothesis that supports the mode of action of this EPI on the basis of biochemical and genetic experiments. The compound is effective against the RND pumps of gram negatives since it does not

Table 3 Examples of EPIs from natural sources against efflux pumps of MDR bacteria

Organisms affected by EPI	Plant sources	Bioactive compound of plant acts as an EPI	Pumps affected	Interaction of EPI with antibiotics	References
S. aureus	*Anadenanthera colubrina*	ND	ND	Aminoglycosides	Barreto et al. (2016)
P. aeruginosa	*Berberis vulgaris*	Palmatine and berberine	MexAB–OprM	Various antibiotics	Aghayan et al. (2017)
P. aeruginosa *E. coli* *Proteus mirabilis*	*Acer saccharum* Marshall	Catechol	ND	Cip	Maisuria et al. (2015)
Salmonella Typhimurium *Enterobacter cloacae*	Trimethoprim plants	Theobromine	AcrAB–TolC	Cip	Piddock et al. (2010)
B. subtilis *S. pneumoniae* *S. aureus*	*Rauwolfia Vomitoria*	Reserpine	Bmr NorA Tetk, N	Tet, Cip, Norf	Garvey and Piddock (2008) and Dhanarani et al. (2017)
Campylobacter jejuni *S. aureus*	*Alpania hainansis*	Alpha pinene Essential oil	CmeABC Cj1687	–	Kovac et al. (2015)
E. coli *P. aeruginosa*	*Digitalis lanate*	Lantocide C	MexAB AcrB	Carb Levx	Aparna et al. (2014)
E. coli	*Eucalyptus Tereticornis*	Ursolic acid	AcrA AcrB YojI TolC	–	Dwivedi et al. (2015)
S. aureus	*Portulaca oleracea L.*	Linoleic acid	MsrRA	Ery	Chan et al. (2015)
S. Epidermis	*Salvia fruticosa*	Essential oil	TetK	Tet	Chovanova et al. (2015)
Enterococcus faecium *S. aureus*	*Memeorandicabalsamina L.*	Balsaminagenin B Karavilagenin C	NorA	— —	Ramalhete et al. (2011a, b)
E. coli, S. aureus	*Thymus vulgaris*	Baiclain Baiclain	Tet K Tet K	Tet	Nguefack et al. (2009)
Arcobacter species	*Vitis vinifera*	Resveratrol	ND	–	Ferreira et al. (2014)

Abbreviations: *Tet* tetracycline, *Cip* ciprofloxacin, *Norf* norfloxacin, *carb* carbenicillin, *Ery* erythromycin, *Levx* levofloxacin, *ND* not discovered

possess any antibacterial activity nor has any additional targets like outer/inner membranes (Opperman et al. 2014; Nguyen et al. 2015).

6.3 D13–9001 and Pyridopyrimidinone Analogs

D13–9001 was discovered by Yoshida et al. (2007) and possesses in vivo activity and good solubility against MexAB–OprM overexpressing *P. aeruginosa*. The three-dimensional structure of this compound was elucidated by Nakashima et al. (2013). The hydrophobic tert-butyl thiazolyl amonocarboxyl pyridopyrimide moeity unit of D13–9001 comes into contact with a depression in the hydrophobic trap of the substrate-binding site. This narrow depression is lined with hydrophobic residues and branches off from the substrate translocation channels. The substrate-binding regions are surrounded by aceto amino-ethylene amino acetate moieties that are the sites for minocycline and doxorubicin. The crystal structures suggest that the binding sites are similar for both AcrB and MexB, as the compound strongly bound to the hydrophobic trap of the MexB binding domain and inhibited conformational changes required for pump activity (Nakashima et al. 2013). The silver lining of this study is the fact that these data lead to an understanding of the MOA of EPIs as well as provide information concerning the unknown binding sites of RND pumps.

7 Conclusion

Efflux-mediated antibiotic resistance can be abolished by screening or designing EPIs against multidrug-resistant organisms. EPIs have the promising potential to restore the activity of antibiotics by decreasing their MIC. Significant advancement has been made in understanding the physiology, mechanisms of action and regulation of MDR pumps. Efflux pump crystal structures bound to their substrate/specific inhibitors increase our knowledge of MDR pumps as well as aiding in the development of EPI. However, to date, there are still no efflux pump inhibitors that are used in treating human/animal infections caused by bacteria. However, EPIs have been developed from synthetic and natural sources and some of them are in preclinical trials. Meanwhile, numerous researchers have suggested that the secondary metabolites of plants display greater activity as EPIs against gram-positive than gram-negative bacteria. So, there is need to develop effective EPIs against gram negatives. A silver lining is the fact that in the near future, standardized methods and techniques for discovering EPIs from natural sources will likely be established. Future endeavours involve the search for EPIs that exhibit low cytotoxicity, improved solubility and a broad spectrum of activity against clinically significant different classes of efflux proteins.

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Medicinal Plants as a Reservoir of New Structures for Anti-infective Compounds

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Abstract

The continued emergence of antimicrobial resistance across a spectrum of infectious pathogens presents a clear and urgent threat to human health across the globe. This trend has been further complicated by a decline in the discovery of novel chemical classes for anti-infective development. Natural products – primarily microbial in origin – have historically served as a key resource for anti-infective drug discovery efforts. On the other hand, natural products from the plant kingdom have served as a source of traditional medicine for millennia, and yet they remain relatively unexplored. The aim of this chapter is to provide an overview of plant natural products and discuss their potential as a resource for ongoing and future drug discovery efforts to fill the anti-infective pipeline and combat antimicrobial-resistant infections.

Keywords

Antibiotic resistance · Pharmacognosy · Medicinal plants · Ethnobotany · Phytochemistry · Secondary metabolites

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1 Introduction

Antimicrobial resistance (AMR) has become a major point of concern in healthcare across the globe. In a recent report on AMR, it was estimated that it presently causes 700,000 fatalities annually and is projected to cause 10 million annually by the year 2050 (O'Neill 2016). The rate of discovery for new anti-infectives has slowed tremendously since the golden era of antibiotic discovery, and we have especially faced a drought in the discovery of new antibiotic classes since the 1980s (Silver 2011). Over the past two decades, all of the "new" drugs approved and brought to market were either initially discovered in the 1980s or prior or are "re-do" compounds – representing structural modifications of existing antibiotic core scaffolds. As a result, these antibiotics have also succumbed to common mechanisms of resistance – both intrinsic and acquired. Innovation in anti-infective drug discovery is recognized as one of the most important pieces in the strategy to addressing the burgeoning difficulties of infection control moving forward (van der Meer et al. 2014). The natural products contained in medicinal plants are very well-positioned to meet this need for innovation. This is due not only to the intrinsic chemical properties of plant natural products but also to their largely untapped diversity, the targeted approach of ethnobotany through which this diversity can be explored, and new technologies that further enable ethnobotanical drug discovery.

2 Traditional Medicine and the Ethnobotanical Approach to Drug Discovery

Of the estimated 390,900 species of plants on Earth, at least 28,187 species – or 7% – have been documented in the literature as having a medicinal use (Willis 2017). While there is no precise tally of how many of these species have been investigated in a comprehensive manner for their full pharmacological potential, based on knowledge of the current literature one can estimate that this number is in the low hundreds, if that. The prioritization of medicinal species for drug discovery efforts is a key component of the ethnobotanical approach to drug discovery (Cox and Balick 1994). The field of ethnobotany – or the study of how people relate to and use plants – has also been referred to as the science of survival (Prance 2007). This is because it is the study of how people use plants as a source of food, tools, shelter, musical instruments, toys, medicine, and more.

Wherever people have inhabited the planet, a form of indigenous medicine has thrived. In some cases, traditional systems of medicine – referred to as such in contrast to Western medicine – continue to serve as the primary modality of healthcare for people, and plants are a key ingredient to their pharmacopoeias. Indeed, a 2002 World Health Organization report estimated that in some parts of the world, up to 80% of the population uses traditional medicine to meet their medical needs (WHO 2002). These forms of medicine may be based in shamanism, with healers

trained via individual apprenticeships, while others ascribe to a more structured form of knowledge transmission through schools, such as in Ayurveda, Traditional Chinese Medicine, Tibetan medicine, and more. In all cases, knowledge of the natural world – particularly knowledge of plants – is key to the process of becoming a healer or traditional medicine practitioner.

2.1 Plant Secondary Metabolites

Why do plants play such a key role in traditional medicine? Plants manufacture a vast array of bioactive secondary metabolites – or natural products – for the purposes of enhancing their own chances of survival, and sometimes these metabolites are active against targets of interest to human medicine. Secondary metabolites are differentiated from primary metabolites in that they are not required for primary metabolic processes (e.g., photosynthesis and growth) but rather serve specialized roles as tools of communication with other organisms in the environment. For example, some secondary metabolites are responsible for plant colors, scents and flavors. Plants use other secondary metabolites to attract pollinators and seed dispersers; to protect themselves from microbial pathogens, predatory insects, and overzealous herbivores; and to compete with other species for resources essential to their survival such as access to light, water, and nutrients. The production of secondary metabolites is regulated by plants in response to environmental cues.

This chemical perspective can be applied to any practice of traditional medicine. A plant preparation that demonstrates therapeutic activity against a given indication contains at least one chemical at a high enough dose to pharmacologically perturb the disease state. Often, it is a combination of chemicals that work additively or synergistically to exert a pharmacological effect, whether that be inhibition of angiogenesis in a tumor or inhibition of an efflux pump in multidrug-resistant bacteria. This is the foundation of the ethnobotanical approach to drug discovery – the end goal of which is the identification and study of these chemicals that contribute to the therapeutic effects of a medicinal plant preparation.

2.2 The Ethnobotanical Approach to Drug Discovery

Ethnobotanical drug discovery begins with a targeted approach to selecting plants for study: traditional knowledge is consulted to identify plants with a history of use against an indication of interest (Cox and Balick 1994). This initial step confines the study to plants that have already been shown to carry the therapeutic potential of interest with relevance to the disease state being targeted within traditional systems of medicine. Thus, only plants with a very high likelihood of containing chemicals of interest are included in the study. There are a number of databases that can be consulted to identify medicinal plants of interest, including NAPRALERT, Dr. Duke's

Phytochemical and Ethnobotanical Database, and the Native American Ethnobotanical Database (Duke 1992–2016; Farnsworth 2018; Moerman 2018). In other cases, ethnobotanists working with a drug discovery team may also undertake primary research in collaboration with communities that have a recent history of medicinal plant use or currently use them. This approach of looking to nature for medically relevant chemistry is also known as bioprospecting. It often requires expertise across a number of disciplines, such as anthropology, botany, chemistry, ecology, linguistics, medicine, molecular biology, microbiology, and pharmacology. A multidisciplinary team-based strategy is thus often the most effective strategy.

2.2.1 Ethical Bioprospecting

Bioprospecting and the ethnobotanical approach to drug discovery requires implementation of rigorous standards of ethics in the project design and implementation. The International Society of Ethnobiology has designed guidelines for ethical research, and this has been adopted by a number of academic societies in this field, including the Society of Ethnobiology and Society for Economic Botany, with the expectation that their members adhere to the Code (ISE 2006). The Code focuses on 17 principles that embody the concept and implementation of traditional resource rights; these include principles of prior rights and responsibilities, self-determination, traditional guardianship, active participation, full disclosure, educated prior informed consent, confidentiality, respect, active protection, precaution, reciprocity, mutual benefit and equitable sharing, supporting indigenous research, the dynamic interactive cycle, remedial action, acknowledgment and due credit, and diligence. Similar to the Code of Ethics for these organizations, guidelines for bioprospecting research in genetically rich source countries have also been a topic of attention among the membership of the American Society of Pharmacognosy (Cragg et al. 1997).

Both the guidelines share common principles outlined in further detail in the United Nations Convention on Biological Diversity (CBD) and the Nagoya Protocol (UN 2011). The CBD is a multilateral treaty widely considered the key document regarding sustainable development. It was signed by many countries in the international community in 1992 and has three aims: (1) the conservation of biological diversity, (2) the sustainable use of its components, and (3) the fair and equitable sharing of benefits arising from genetic resources. The CBD asserted provider countries, their people, and representatives as stakeholders to be included in negotiations for plant-based drug discovery programs, providing a framework for the regulation and defining bioprospecting. The CBD treaty left many open questions as well, especially with respect to access and benefit sharing. To bring clarity to this issue, the Nagoya Protocol, a supplementary agreement to the CBD, was adopted in 2010. Importantly, it outlines mechanisms for equitable access and benefit sharing with genetic resource source countries (UN 2011). Legally binding, it serves to further clarify the issues of access and benefit sharing by setting out obligations for its contracting parties to take measures in relation to them. There are a number of case studies and useful resources for reference on this topic available on the CBD website (UN 2018).

2.2.2 Collection of Medicinal Plants

Following their identification either by literature or database searches, or through primary field research, the next step in the drug discovery process is to access the plant material. In the majority of cases these materials are not commercially available and must be wild-crafted, or found and harvested from wild populations. Such endeavors require expertise in botanical taxonomy and an understanding of which ecosystems the plants may be found in. Furthermore, the optimal collection time for plant identification – when it is in a reproductive state with fruits or flowers – must also be considered. The World Health Organization's Guidelines on Good Agricultural and Collection Practices (GACP) for Medicinal Plants covers a number of crucial protocols for collecting from wild populations, such as avoiding areas contaminated by environmental pollutants like road or agricultural field runoff and avoiding collection of CITES-protected species or species that are otherwise listed as threatened or endangered (WHO 2003).

Once the plants are identified in the wild, there are four types of samples that require collection: herbarium voucher specimens, DNA specimens, bulk specimens, and retention vouchers. Herbarium voucher specimens include vegetative and reproductive parts of the plant (e.g., a segment of a flowering tree branch or a whole small herb in fruit). Specimens are field pressed, or placed into a folded sheet of newspaper that has been given a collection number, and then squeezed flat in a plant press – a rectangular unit composed of two sets of wooden slats held together with thick straps. Upon return to the field research base station, the plants are more carefully arranged and subjected to drying under low heat. Multiple copies of vouchers for a single species are often collected in order to make deposits of the final pressed and labeled specimen in the scientist's home institution as well as at local institutions in the country or region where the collections took place.

DNA specimens are collected by taking leaf samples (roughly 1 square inch of leaf material) and storing them in labeled coin envelopes. All samples are numbered according to the same collection identification scheme for linking each of the samples (herbarium voucher, retention voucher, DNA, and bulk). The DNA samples can then be stored in sealed plastic bags with desiccant until they are taken to the lab for DNA extraction and characterization. This adds an additional level of evidence toward species identification and may be of use to other scientists engaged in various conservation efforts, such as in tracking the trade of medicinal plant products.

Bulk specimens are made of different plant tissues, often guided by traditional medical uses reported. For example, if the leaves of a plant are reported as being the key ingredients in a traditional medicine for a disease of interest, then the leaves should be the main focus of the bulk collection. Each plant tissue serves a different purpose for the plant as a whole organism, and as a result, each tissue also exhibits a different chemical profile than the others. In most cases, 40 g of dry material is plenty for an initial study on the chemical makeup and potential biological activity of each tissue. If the plant later becomes the subject of more in-depth studies aimed at the isolation of multiple individual compounds, then many kilograms of material may eventually be required, depending on the abundance, or percent yield, of the active compound(s) in the plant tissue.

In the field, bulk materials are typically collected and processed on the same day to avoid sample loss due to decay or mold. This may involve rapid field collection and later separation of plant tissues at the research base camp, where they are then chopped up into smaller segments and dried either in a desiccating cabinet at low heat or spread out in the shade in more arid countries, where they can dry without the aid of a heat source or dehumidifying machine. Once dried, samples may be packed up in vacuum-sealed bags with desiccant sachets for shipment back to the lab. Retention vouchers of this chopped up and then ground material are also collected upon return to the lab and stored in small plastic bags as a record of how the semiprocessed and ground material appears.

2.2.3 Extraction of Medicinal Plants

Bulk plant materials are processed according to a wide variety of techniques that have been developed for this purpose. Some examples include extraction in organic solvents either by maceration at room temperature or under pressure, with sonication, under heat, or under both heat and pressure. Other methods include extraction with water or by steam distillation for the extraction of essential oils. In some cases, the materials may be extracted in plant or animal-based fats. All methods have their own advantages and disadvantages with regard to extraction efficiency and the variety of compound classes that emerge from each technique. In any of these cases, the first level of extraction produces a "crude extract," which is composed of many different compounds – sometimes including hundreds to thousands of unique molecular entities. As such, any crude extract is in itself a chemical library, representing multiple core scaffolds and many derivatives of each scaffold.

In order to identify bioactive compounds from the crude extract, the framework of bioassay-guided fractionation is followed. Based on this strategy, chromatographic techniques are employed, such as partitioning, column chromatography, flash chromatography, and high-performance liquid chromatography (HPLC), in order to produce fractions of the crude extract for testing in the biological model of interest. The most bioactive fractions are selected for further fractionation, the fraction characterized, and the cycle of chromatographic separation and biological testing repeats until the most active fraction or single compound is identified (Fig. 1).

3 Characteristics of Plant Natural Products

With regard to anti-infective drug development, which until the 2010s focused heavily on classical growth-inhibitory mechanisms of action, microbial natural products as a resource were found to be extremely rewarding. Indeed, while 69% of all US FDA-approved antibacterials were natural products or derivatives thereof, as of 2016, 97% of these were contributions from microbes while a mere 3% came from plants (Patridge et al. 2016). During this time, however, plant natural products instead enjoyed exploration for various other indications, leading to several indispensable contributions to drug development. Some notable examples for cancer include vincristine and vinblastine from the Madagascar periwinkle (*Catharanthus roseus*), paclitaxel and derivatives thereof from yew (*Taxus*) species, and camptothecin and

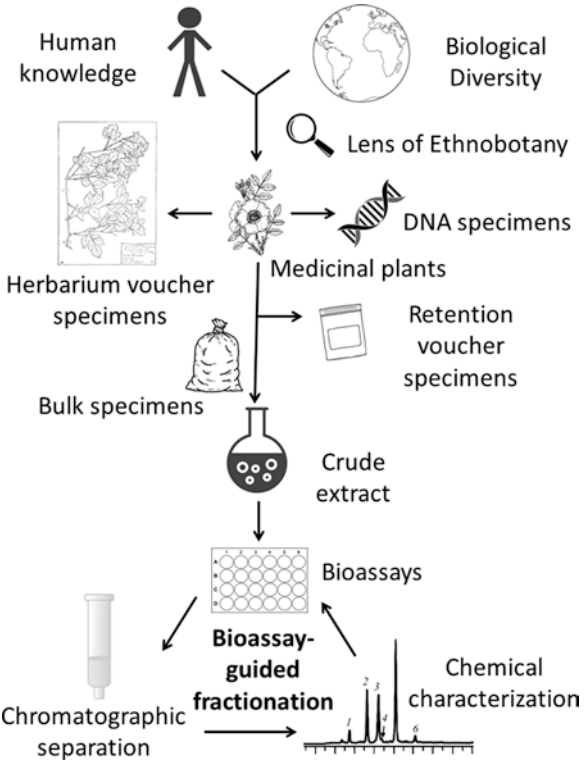

Fig. 1 Schematic representation of the ethnobotanical approach to drug discovery and bioassay-guided fractionation to identify bioactive molecules found in plants

derivatives thereof from *Camptotheca acuminata* (Cragg 1998; Moudi et al. 2013; Thomas et al. 2004). Also of note are the antimalarial artemisinin, isolated from sweet wormwood (*Artemisia annua*), and the Alzheimer's drug galanthamine, isolated from *Galanthus nivalis* (Heinrich 2010b; White et al. 2014).

It must be noted that the compounds contained in medicinal plants represent one of several chemical frontiers in which anti-infective drug discovery is being undertaken. Other very promising frontiers include marine natural products, microbial natural products, and recently developed complex synthetic small molecule libraries bearing the complexity of natural products (Gogineni et al. 2015; Rossiter et al. 2017). With that said, plant natural products remain largely underexplored (Kenny et al. 2015). Only about 15% of higher plant species have been phytochemically investigated, with only a tiny faction having been studied for anti-infective potential (Cragg and Newman 2013).

3.1 Innovation in Plant Natural Product Research

There are many attributes of plant natural products that make them highly desirable for drug discovery, particularly for anti-infectives. Among the chief chemical attributes is that they have a high tendency to occupy regions of the biologically relevant chemical space, which refers to all chemicals that are biologically active (Kellenberger

et al. 2011; Quinn et al. 2008; Wetzel et al. 2011). Indeed, plant natural products have already interacted with a number of proteins during their biosynthesis, and following this, they often function to interact with yet other proteins found in organisms in the ecosystem. Accordingly, their core scaffolds are privileged structures, occupying a region of the chemical space predestined for protein interaction (Maier 2015). Considering that plants have adapted the production of secondary metabolites to interact with their biological surroundings, this in fact makes sense.

Because plant natural products (and natural products in general) are metabolite-like and hence are substrates for biological transporters, they represent an exemption to Lipinski's rule of five, as stated by Lipinksi (Gedeck et al. 2010; Harvey et al. 2015; Koehn and Carter 2005; Lipinski et al. 1997). The observation that led to this exemption was that only four categories of orally active drugs fell outside the fold of Lipinksi's rules (antibiotics, antifungals, vitamins, and cardiac glycosides), and thus, these exemptions were summed up as "compound classes that are substrates for biological transporters" (Lipinski et al. 1997). Plant natural products, along with drug-likeness, also possess vast structural and chemical diversity in excess of many synthetic small molecule libraries (Harvey et al. 2015; Shen 2015). A large contributor to this diversity is the complexity of a large portion of plant natural products. This complexity in itself is yet another advantage, as it has been observed that infectious diseases remain one of the areas that often require chemically and structurally complex molecules (Morrison and Hergenrother 2014). Finally, screening of plant natural products, as well as natural products in general, has been found to be particularly relevant to therapeutic development against "non-druggable" targets (Keseru and Makara 2009).

In addition to providing innovation by way of unique chemistries, plant natural products also show promise with respect to novel mechanisms of action. This includes their potential for the development of both new classes of growth-inhibitory antibiotics and antivirulence drugs, and it is especially in these areas where plant natural products demonstrate great potential. The latter classes of anti-infective, antivirulence drugs have received the attention of research groups more recently, as the approach has been recurrently cited in the literature as a promising anti-infective method that could slow the development of AMR (Pieren and Tigges 2012; Wright 2016). In theory, and as supported preliminarily by a number of in vitro and in vivo studies of multiple microbial pathogens, antivirulence drugs would attenuate pathogenicity of the target microbe, relieving symptoms of infection and allowing for host immunity to clear the pathogen (Johnson and Abramovitch 2017; Salam and Quave 2018). In doing so, antivirulence drugs would exert less selective pressure for the development of resistance than growth-inhibitory drugs, as the latter clears non-resistant organisms, making way for resistant organisms to enrich the population. A few recent review articles demonstrate the strong promise of plant natural products in antivirulence drug discovery by covering the numerous such compounds

discovered to date (Dickey et al. 2017; Silva et al. 2016). Many antivirulence drug candidates are under investigation as both stand-alone treatments and adjuvants to classical antimicrobials. In the latter case, antivirulence drugs are predicted and have preliminarily been shown to potentiate antimicrobial efficacy.

3.2 Current Development of Anti-infectives

Epigallocatechin gallate, the most abundant catechin in tea, was found to inhibit virulence in *Streptococcus pneumoniae* in a nonbactericidal manner by preventing the oligomerization of pneumolysin and reducing the activity of sortase A, which helps anchor to the cell wall surface proteins that contribute to virulence (Song et al. 2017). Derivatives of hamamelitannin, isolated from American witch hazel (*Hamamelis virginiana*), are under investigation for enhancing vancomycin activity in biofilm-associated MRSA infections (Brackman et al. 2016; Vermote et al. 2017a, b). A derivative of 8-hydroxyquinoline, synthesized in roots of the diffuse knapweed (*Centaurea diffusa*), called INP1855 is being investigated for antivirulence activity against *Pseudomonas aeruginosa* (Anantharajah et al. 2016). INP1855 inhibits the injectisome and flagellar type III secretion systems and, very interestingly, was found in a synthetic small molecule library screen (Enquist et al. 2012).

We previously mentioned that one frontier besides plant natural products for anti-infective exploration is the frontier of complex synthetic small molecules that mimic the complexity of other natural products. To this extent, there are examples of such antivirulence small molecules that resemble plant natural product pharmacophores. For instance, Compound 22 is an isoquinolone mannoside, and virstatin is an isoquinoline, and they have been found to target the pili of *Escherichia coli* and *Acinetobacter baumannii*, respectively (Cushnie et al. 2014; Jarvis et al. 2016; Nait Chabane et al. 2014). As for growth-inhibitory plant natural products, up until now most of those that have been isolated from medicinal plants tend to exhibit weak potency and selectivity. An exception to this are a set of acylphloroglucinols from St. Johns Wort species (*Hypericum* spp.), which have demonstrated submicromolar MICs in clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) (Rahman et al. 2018).

On the way to isolating bioactive single compounds from plant extracts, bioassay-guided fractionation produces bioactive fractions. Our lab is currently studying fractions of two plant extracts for the isolation of compounds that inhibit the Agr system in *S. aureus*. The Agr system codes for the organism's quorum sensing system, which is its main mediator of virulence (Salam and Quave 2018). One enriched fraction of a European Chestnut (*Castanea sativa*) extract, as well as an enriched fraction of a Brazilian Peppertree (*Schinus terebinthifolia*) extract, demonstrated high bioactivity against *S. aureus* in vitro and no detectable resistance after drug passaging (Muhs et al. 2017; Quave et al. 2015). In a mouse skin infection model,

co-administration of either fraction with MRSA impaired pathogenesis without manifesting local or systemic toxicity. Of note, both plants were initially selected for study in primary screens of Agr inhibition due to their reported traditional medicinal uses for the treatment of topical infections.

4 Challenges in Studying Plant Natural Products

Medicinal plants are gaining renewed interest as sources of new therapeutics, although this has come about only after several decades of scientists' attention favoring combinatorial chemistry, synthetic small molecules libraries, and high-throughput screening (HTS) (Atanasov et al. 2015; Harvey et al. 2015; Li and Vederas 2009). The diminished attention to plant natural products had been a result of an embrace of the aforementioned resources as novel and promising, legal challenges, difficulty of chemical manipulation, challenges in the compatibility of plant natural products with HTS, and other inherent obstacles in ethnobotanical drug development.

4.1 Legal Challenges

Prior to the 1990s, there was little legal guidance as to plant access, sharing of benefits, and patenting with local governments, and many developing countries have been previously exploited. These issues created barriers to the access of genetic resources – including plant materials – in biodiversity-rich countries and was one major factor that discouraged pharmaceutical companies from plant natural product drug discovery (David et al. 2015; Kingston 2011). Furthermore, the synthetic compound libraries became more attractive because they did not include the many access and intellectual property issues that accompanied work on plant natural products (Butler 2004). Fortunately, many of these issues have been addressed by the CBD and Nagoya Protocol, which we have previously discussed.

4.2 Chemical Manipulation

Chemical synthesis approaches and derivatization of plant natural products were difficult challenges in lead optimization and resupply (Butler 2004). Total synthesis of a natural product or semisynthesis from another natural product of similar structure are extremely useful because they represent production options that may be more economical and efficient than isolation from plant material. As for derivatization, it is almost always performed on lead natural products in order to perform structure-activity relationship (SAR) studies and to develop analogs with enhanced pharmacological properties. And since 2014, it is a required step in patenting a natural product after new guidelines were issued by the United States Patent and Trademark Office that state that a patent claim must demonstrate a

"marked difference" from a known natural law, material, or phenomenon (Atanasov et al. 2015). In either case, the chemistry has often been difficult to implement due to the innate complexity of plant natural products, particularly in those with numerous oxygen-containing substituents and chiral centers. Whereas classical medicinal chemistry is dominated by C–N bond formation, natural product syntheses rely heavily on C–C bond forming reactions, often accompanied by the formation of hydroxyl groups which contribute to the increased number of asymmetric centers (Maier 2015). Other general characteristics contribute to the chemical complexity of natural products, such as partial or complete saturation of ring structures. Compared to plant natural products, synthetic small molecule libraries tend to include compounds of lesser complexity which are easier to synthesize or modify (Atanasov et al. 2015).

4.3 Compatibility with High-Throughput Screening

As mentioned above, a major obstacle for the inclusion of plant natural products in drug discovery programs had been the compatibility of plant natural products – and natural products in general – with HTS (Henrich and Beutler 2013; Koehn and Carter 2005). Often, HTS is performed using plant extracts or fractions thereof rather than single compounds as a component of a wide bioassay-guided fractionation approach. Investigating such a large number of botanical compositions via HTS presents many challenges such as sample preparation and assay design must complement each other, extracts and fractions must be carefully handled so as to minimize compound degradation and precipitation, and attention must be given to the possibility of assay interference and nonspecific effects. In all of these respects, plant natural products are more prone to failure than synthetic compounds. For example, plant extracts can contribute to the optical density of wells, they have a tendency to form precipitants, and they are likely to contain nuisance compounds such as tannins that nonspecifically bind to proteins and saponins which can interfere with cell-based assays via cell lysis (Barbehenn and Peter Constabel 2011; Hostettmann and Marston 1995).

Plant extracts also have a tendency to contain fluorescent or fluorescence-quenching compounds, interfering with the output of assays such as fluorescence gene reporter assays, often employed in HTS (Gul and Gribbon 2010; Henrich and Beutler 2013; Zou et al. 2002). Certain plant natural product classes such as chlorophylls, polyphenols, flavonoids, and fatty acids have also demonstrated a propensity to interfere with several HTS assays (Henrich and Beutler 2013). False-positive results can also be obtained if inorganic molecules such as heavy metals end up in the samples tested (Fernando et al. 2013; Hermann et al. 2013). This can occur, for example, in extracts if plants were collected from busy roadsides exposed to automobile exhaust, or it can occur in synthesized natural products if the synthesis utilized metals which then remained in the sample (Guan and Peart 2006; Zhai et al. 2016).

4.4 Deeper Challenges

As we will discuss in the next section, the vast majority of the challenges above have been overcome. There are several other challenges native to ethnobotanical drug discovery that present an intrinsic barrier to entry, a characteristic shared with all other drug discovery approaches. All tasks related to plant collection cannot be automated and must include the expertise of botanists for unambiguous identification, documentation, and herbarium preservation. Collecting sufficient amounts of plant material for isolation, especially if the plant was obtained abroad, can prove difficult when the need for more plant material increases substantially following lead identification, advanced preclinical testing, and especially with demonstration of clinical efficacy. Care must be taken to source plant material in a sustainable way in order to avoid situations similar to the classical "taxol supply crisis" (Cragg et al. 1993). Loss of biodiversity and loss of traditional ethnobotanical knowledge (TEK) also lead to accessibility issues where (1) isolation of a natural product of interest is no longer possible due to the endangered status of the plant of origin, and (2) this drug discovery approach loses its inherent advantage of being a targeted approach when the knowledge guiding this advantage ceases to exist. Unlike many other challenges in ethnobotanical drug discovery, these two appear to be growing worse over time, as climate change prevention efforts and ethnobotanical surveys have yet to gain sufficient momentum. It is because of these collection and resupply challenges, as well as the aforementioned accessibility issues, that microbial natural product drug discovery has often been preferred by pharmaceutical companies (Butler 2004).

The complexity of medicinal plant extracts also presents challenges. The chemical composition of plant material can vary, especially between different collection times, presenting challenges for chemical assessment. A bioactive agent may be present at such low concentrations that it is not detected. An assay may also fail to detect it if it is unstable in mixture or separated by fractionation from a synergist (Wagner and Ulrich-Merzenich 2009). If detected, often only small quantities of the bioactive agent are present, and with that, structurally related molecules are usually present as well and must be distinguished. Considerable time is often required to structurally characterize natural products to determine whether the molecule is already known. Due to these challenges and those described above, a prevailing sentiment in the field is that plant natural product drug discovery requires tremendous effort, hits are theoretically easy to miss, and the probability of duplication is high (Li and Vederas 2009). In the 2000s and into the 2010s, along with plant natural products, microbial and marine natural product research have seen a decline (Beutler 2009; David et al. 2015; Ortholand and Ganesan 2004). With many large- and medium-sized pharmaceutical companies having terminated their natural products programs, academic universities and start-up companies have been left to move forward the bulk of the research and development in this space.

5 Re-emergence of Plant Natural Products for Drug Discovery

Pharmaceutical companies had for the past three decades largely avoided the difficulties of natural product-based drug discovery, turning instead toward combinatorial and synthetic small molecule libraries for the discovery of new anti-infective drug leads via HTS (Beutler 2009; David et al. 2015). The results of these decades of drug discovery, however, did not meet expectations (Scannell et al. 2012). The hype for combinatorial chemistry and synthetic libraries was that drug leads would be delivered quickly and in vast amounts for any therapeutic area of interest, as compared to previous drug discovery methods (Butler 2004). In fact, as evidenced by a declining number of new drugs reaching the market, the hype was not realized (David et al. 2015; Kingston 2011; Scannell et al. 2012). Indeed, while 45 new drugs were approved by the US Food and Drug Administration (FDA) in 1990, less than half that amount, 21, were approved in 2010 (David et al. 2015; Kingston 2011). An important contributor to this low turnout is the narrow region of chemical space occupied by these libraries. At present, the decrease in drug approvals is in part revitalizing interest in natural product drug discovery, notwithstanding its complexity (Heinrich 2010a). A recent analysis of PubMed publication trends in this research area reflects a rapid increase in plant natural product research (Atanasov et al. 2015). This renewed interest coincides with specific major scientific and technological advances, which have addressed many of the challenges of ethnobotanical drug discovery: improved understanding of disease pathogenesis, improved natural product medicinal chemistry, increased compatibility of natural products with HTS, and advances in phytochemical analysis.

5.1 Advances in Medicinal Chemistry for Natural Products

Over the past 20 years, the field of synthetic chemistry has seen tremendous progress in the ability to synthesize and modify natural products (Szychowski et al. 2014). Modern synthetic methods have opened doors to transformations that had traditionally been difficult due to their selectivity and high yield. These synthetic methods can be divided into two categories based on what they aim to create: (1) large numbers of complex and diverse small molecules for building libraries or (2) small quantities of derivatives of a given natural product (Morrison and Hergenrother 2014). Successful strategies for the former include diversity-oriented synthesis (Cui et al. 2011; Schreiber 2000), diverted total synthesis (Szpilman and Carreira 2010), function-oriented synthesis (Wender et al. 2008), biology-oriented synthesis (Wetzel et al. 2011), complexity to diversity (Ciardiello et al. 2017), and biosynthesis-inspired synthesis (Baskar et al. 2011).

As for producing derivatives in a more controlled fashion, such methods can be divided into two further categories: the use of a single reactive group to alter structure or the use of multiple reactive groups (Maier 2015). Traditional methods that target a single reactive group generate multiple products in one reaction, making separation difficult and yields low (Appendino et al. 2001). In the mid-2000s, reagent control allowed for the selective synthesis of product (de la Torre et al. 2003, 2005), and in the 2010s, the process was systemized to yield more predictable product (Balthaser et al. 2011; Hutt et al. 2013; Ignatenko et al. 2013; Ignatenko and Tochtrop 2013). As for methods that use multiple reactive groups, recent work has formalized the process of multiple structural transformations to produce several highly complex derivatives (Huigens et al. 2013). Thanks to such synthetic methods, there are chemistries we can access now that may very well aid in the development of new therapeutics (Szychowski et al. 2014).

5.2 Improvements in HTS Compatibility with Natural Products

For HTS, particularly in campaigns that involve natural products, one must pay attention to many sample details, including maintenance of integrity, potential to interfere with assay readouts, and nonspecific and off-target effects. As discussed in the prior section, these were among the most challenging aspects of incorporating natural products into HTS campaigns. Now, most of these potential problems can be addressed by careful experimental design and management. Extracts have been and should continue to be screened in HTS due to the sheer biodiversity they contain, which is much less accessible in the form of isolated single compounds due to the difficulty of isolations at that scale (Henrich and Beutler 2013). The problems of low concentrations of bioactive compounds and high concentrations of nuisance compounds in extracts have been addressed by the trend of prefractionation (Harvey et al. 2015; Henrich and Beutler 2013). Prefractionation aims to split an extract into a small number of fractions of reduced complexity, concentrating nuisance compounds (such as highly hydrophilic or hydrophobic compounds) into some fractions, while other metabolites, more likely to be pharmacologically active, become enriched in others (Harvey et al. 2015). Additionally, these fractions are more amenable to chromatographic analysis than their parent extracts, facilitating bioassay-guided fractionation.

HTS assays can be categorized as either biochemical or cell-based. In both types, false activities can be identified by utilizing parallel, nonspecific, or off-target assays so as to examine effects such as the actual detection enzymes, reagents, and cell survival (Henrich and Beutler 2013). In biochemical assays, assay interference has been shown to be reduced by protocol modifications such as more stringent washes for certain assays, testing multiple doses, carefully selecting detection reagents, and addition of agents to reduce aggregation or nonspecific binding. In the last 15 years, such adjustments have led to the identification of modulators of a number of enzymatic targets via natural products HTS. Cell-based assays can be categorized as targeted (assays examining reporter strains, enzymatic activities, etc)

or phenotypic (assays examining growth, differentiation, etc). In these assays, there is a strong need for eliminating nonspecifically cytotoxic compounds. The presence of such compounds can be effectively assessed by running a cytotoxicity assay in parallel with the reporter assay (Ruocco et al. 2007; Woldemichael et al. 2006). Following cytotoxicity assays, sample libraries as well as cell lines can be cataloged with acquired data to record the cytotoxities exerted and experienced, respectively, aiding in identifying potentially problematic samples (Schulze et al. 2013). Additionally, secondary orthogonal screens, perhaps examining multiple doses, can help identify true active samples.

Indeed, the above advances make HTS of natural products more accessible than ever before. To boost access even further, the National Cancer Institute (NCI) has launched the NCI Program for Natural Product Discovery (NPNPD) in 2018 (Thornburg et al. 2018). Under this program, the NCI will create a prefractionated library using over 125,000 natural product extracts from the NCI's Natural Product Repository. This library will consist of over 1,000,000 fractions and will be made available free of charge in 384-well plates for screening by researchers against any disease. Importantly, NCI's Natural Product Repository is made up of extracts acquired through NCI Letters of Collection agreements with participating countries or their representatives, ensuring mechanisms for equitable access and benefit sharing in line with the spirit of the CBD and the Nagoya Protocol (UN 2011).

5.3 Emergence of Metabolomics and Ethnophytotechnology

Metabolomics and ethnophytotechnology represent two fields that stand to become strong enablers of ethnobotanical drug discovery thanks to recent technological advances. Metabolomics in its most general sense is the analysis of all metabolites in a biological sample. Advancements in chromatography and spectroscopy over the past decade have allowed for the acquisition of high-quality data, particularly when coupled in the form of with ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRMS) (Breton and Reynolds 2013; Rathahao-Paris et al. 2015; Seger et al. 2013). As for ethnophytotechnology, it refers to "the use of plant biotechnology to improve or enhance the inherent economic or culturally valuable traits of plants as described and influenced by ethnobotany" (de la Parra and Quave 2017). The field allows for the improved production, manipulation, and scientific understanding of natural products through advancements in technologies such as bioreactors and metabolic engineering.

In a metabolomics analysis of a plant extract, MS data provides a set of molecular features, which include molecular ions, adducts, and in-source fragments, among other data points (Allard et al. 2017). Molecular formulae can be deduced from an analysis of these data. MS fragmentation (MS/MS) data can then be used to link metabolites based on spectra or structure via an MS organization tool, and other information can be mapped to this organized data, including bioactivities, taxonomy, and gene sequences. This data set, now a molecular network, can be annotated against existing MS/MS databases, whether built experimentally or in silico, in order to

identify metabolites contained in the extract (Allard et al. 2016; Klein-Junior et al. 2017). Such a metabolomics analysis performed on fractions of a bioassay-guided fractionation campaign can inform researchers on a number of issues of interest, such as whether there are common pharmacophores among potentially bioactive compounds or whether certain compounds may act synergistically (Ngo et al. 2013). Such analyses can also be used in dereplication, the process by which already-known compounds in a natural product composition are identified to avoid duplication (Wolfender et al. 2015). Effectively, metabolomics transforms traditional bioassay-guided fractionation into a rapid identification process of valuable plant natural products.

While metabolomics facilitates the drug discovery process, ethnophytotechnology facilitates the resupply of plant natural products of interest. A major focus is their standardized and controllable production in vitro by way of bioreactors, where plant cell and tissue cultures reproducibility generate high yields (O'Connor 2015). To help increase yields further, TEK can be consulted to understand (1) timing and conditions when a medicinal plant is traditionally collected and (2) characteristics of populations of the medicinal plant that are traditionally favored for use (de la Parra and Quave 2017). Metabolomics can also be used to identify favorable timing and conditions by monitoring gene function and biochemical status of a plant (Harvey et al. 2015). By attempting different types of exogenous administration of hormonal elicitation or by genetically modifying cells to better meet optimal plant RNA or protein levels, appreciable improvements in yield can be achieved (Atanasov et al. 2015). Through metabolic engineering, enzymes can be altered or introduced to produce metabolite derivatives for SAR and lead optimization studies (Ochoa-Villarreal et al. 2016). *Agrobacterium*-mediated gene transfer and CRISPR technologies are being studied to this end (Chumakov et al. 2012; Loyola-Vargas and Avilez-Montalvo 2018). However, production of plant natural products need not only occur in plant cells and tissues; cultivable microbes including yeast have been successfully utilized (Galanie et al. 2015; Nakagawa et al. 2016). These technologies open up drug development opportunities for rare, endemic, or endangered medicinal plants, whose populations are too small for sustainable bulk collections (de la Parra and Quave 2017).

6 Conclusions

We have presented an overview of the current state of the ethnobotanical approach to drug discovery and the emerging opportunities for the discovery of new anti-infectives from medicinal plants. Although this pathway to discovery was once burdened by numerous hurdles in the access and examination of plant natural products, today scientists can benefit from the establishment of clear international platforms for ethical access to materials as well as from advancements in fields such as medicinal chemistry, high-throughput screening, metabolomics, and ethnophytotechnology. This progress is of great importance as it has further enabled the pharmacological exploration of the chemical space encompassed by medicinal plants. Indeed, the biological resource and potential for anti-infective drug development are there, and now, it is more accessible than ever before. We predict that with the spread of these technologies, plant natural products will prove an important source for anti-infective drug development.

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Essential Oils: Potential Application in Disease Management

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Abstract

Natural essential oils extracted from dietary plants and their constituents which include monoterpenes, sesquiterpenes, and phenolics play crucial role in different disease management. Several mechanisms such as antioxidant, enhancement of immune function, enzyme induction, and enhancing detoxification are responsible for different disease management. Essential oils are representing a promising source of active elements and an array of pharmacological properties, including antibacterial, antifungal, antiaging, etc. This study presents the overview of different action exerted by essential oils and discusses active constituents and their effect on disease control.

Keywords

Essential oils · Disease management · Ayurveda · Phytomolecules · Antibacterial

1 Introduction

Essential oils (EOs) are aromatic oily liquids extracted from plant material (flowers, buds, seeds, leaves, etc.) possessing antibacterial, antifungal, and antiviral properties which have been considered worldwide as an alternative source to treat

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infectious diseases and agents promoting food preservation (Belletti et al. 2008; Alviano and Alviano 2009; Safaei-Ghomi and Ahd 2010; Astani et al. 2010). EOs or their components have also been shown to exhibit antiviral (Bishop 1995), antitoxigenic (Juglal et al. 2002), and antiparasitic (Pandey et al. 2000) properties. The quantity of active components present in essentials oils differs among species and plant parts. These types of components have chemical origin from terpenes and their oxygenated derivatives, which are aromatic and phenolic compounds.

1.1 Use and Role of Essential Oils

The modern lifestyle could be stressful at some point of time which quickly becomes overwhelming. The wealth of natural products that we inherited from our ancestors in the form of plants and minerals might be utilized in medicines. Apart from medicinal use, natural extracts are predominantly used in essential oils and aromatherapy. Other important roles of essential oils include fragrance, bathing products, incense, perfumes, and cosmetics. Aromatherapy in the form of alternative medicine could be used, as well as further research to evaluate how plant extracts can help patients are still being carried out by the scientific community. Some essential oils like eucalyptus, peppermint, and tea tree oil are well known for their role in treating minor ailments. EOs have a broad-spectrum use such as in food, perfumes, and pharmaceuticals (Van Welie 1997; Van de Braak and Leijten 1999). The antibacterial properties of essential oils could be employed for manufacturing many products like dental root canal sealers (Manabe et al. 1987) and antiseptics (Cox et al. 2000). According to these reports, essential oils are rich sources of natural antioxidants and used in the treatment of many diseases.

1.2 Antibacterial Essential Oils

Essential oils consist of an odoriferous mixture of monoterpenes, sesquiterpenes, and aromatic compounds. Bacterial infections are caused by some agents such as *Staphylococcus aureus*, *Escherichia coli*, etc. leading cause of health hazard in the developing world. It is generally due to the partial admittance of the poorer economy population to integrated health care and treatments. To overcome this type of problem, there is a serious need to explore and develop natural and cheap agents which could be used as powerful substitutes to the current synthetic drugs. Essential oils are a very good alternative for this type of infections.

1.3 Antifungal Activity of Essential Oils

Synthetic drugs are of crucial concern due to the emergence of drug-resistant microbes, raising the reluctance toward the use of chemical-based products. In this context, natural products such as extracts or essential oils can play a fundamental role. EOs mainly consist of active molecules, well known for their medicinal properties such as anti-inflammatory, analgesic, and sedative (Masango 2005; Macwan et al. 2016).

Chemical treatments are most efficient, but strains develop resistance against it. EOs can be used as one of the most capable natural products for different types of infections (Kalemba and Kunicka 2003). Natural extracts obtained from different plants or herbs exhibited strong antifungal properties (Prakash et al. 2012; Lang and Buchbauer 2012; Ghalem 2016). Like other phytochemicals, EOs could mitigate microbial growth and development of biofilms through specific mechanisms (Hyldgaard et al. 2012).

1.4 Nematicidal Activity of Essential Oils

The parasitic nematode is one of the plant pest leading to an extensive monetary loss in the form of reduced crop yield and production. About (Masango 2005; Macwan et al. 2016) earlier, a report on eucalyptus essential oil demonstrated inhibition of root-knot nematode *Meloidogyne incognita* by *E. citriodora* and *E. hybrids* (Pandey et al. 2000). Ibrahim et al. (2006) reported that *Eucalyptus camaldulensis*-, *E. saligma*, *E. urophylla*-extracted essential oil caused mortality of *Meloidogyne exigua* and *M. incognita*.

1.5 Anticancer Activity of Essential Oils

Cancer is the world's second-largest cause of death. There is no active drug available in the market to treat most diseases like cancer. Natural products deal opportunities for drug discovery and play an essential role in cancer treatment. A large number of antitumor drugs are currently used worldwide from natural origin, isolated from medicinal plants, for the prevention of cancer. For pharmaceutical purposes, traditional medicinal herbs have been used, and dietary therapy is currently used in cancer treatment (Cai et al. 2004).

1.6 Antiaging Activity of Essential Oils

Aging is a natural sensation experienced by most species. Many of the research conducted over the past few decades have suggested that aging is not entirely an arbitrary deterioration of cells and tissues and is influenced by genetic pathways (Lithgow 2006; Kenyon 2010). Nowadays, many essential oils extracted from plants have increased responsiveness for various types of bioactivity, such as antibacterial, antiviral, and antifungal. The Juniper berry (*Juniperus communis* L.) essential oils (JBEOs) extracted from juniper berries have a lot of components, i.e., α-pinene, limonene β-caryophyllene, δ-cadinene, etc. reported as an in vivo antioxidant and antiaging agent (Pandey et al. 2018). *Trachyspermum ammi* L., traditionally known as carom seed, is used in India to cure severe human and animal disorders. This medicinal plant is used as a standard spice and is used as a stimulant and tonic, and its essential oil is also used to treat age-related disorders (Rathor et al. 2017).

1.7 Anti-Alzheimer's Activity of Essential Oils

Cholinergic dysfunction is primarily associated with diseases such as Alzheimer's and Parkinson's and neurodegenerative and psychiatric disorders. The extent of cholinergic affliction is maximum in Alzheimer's disease which is a progressive neurodegenerative disorder involving the death of cholinergic neurons. To date, only the use of acetylcholinesterase (Ache) inhibitors limits the therapeutic management of cholinergic dysfunction to provide symptomatic relief. Essential thyme oil increases neurotransmission through the modulation of levels of synaptic acetylcholine (Ach) and receptor activity of nicotinic acetylcholine (Sammi et al. 2017). Similarly, essential oils from *Hedychium gardnerianum* had shown acetylcholinesterase inhibition properties and radical scavenging properties (Arruda et al. 2012). The essential oil of *S. lavandulifolia* reported having acetylcholinesterase-inhibiting properties in striatum and hippocampus of the brain (Table 1).

1.8 Role of Essential Oils Against Human Pathogenic Bacteria

Some medicinal agents have been isolated from natural sources, along with an impressive number of new drugs. The presence of various life-sustaining components in plants has led scientists to investigate these plants for their potential uses in the treatment of certain infectious diseases. Natural medicine has long been accepted in many countries as an alternative to Western medicine practices. Infections caused by infectious microorganisms such as bacteria, fungi, viruses, or parasites are a vital pathological condition and one of the world's top ten causes of morbidity and mortality. Essential plant-isolated oils were studied for their antimicrobial activity against microorganisms, including many pathogens (Dorman and Deans 2000; Delaquis et al. 2002). The essential oils can be a powerful tool for reducing the development and spread of various microorganisms that are resistant to antimicrobials. Hence, the activity of various essential oils against the different pathogenic bacteria is listed in Table 2.

1.9 Multidrug-Resistant Bacteria

Antibiotics are used in large numbers for human treatment, as well as in animals and even fish in aquaculture, resulting in the selection of multiple drug-resistant pathogenic bacteria. Two mechanisms in bacteria can generate the resistance of many drugs. First, these bacteria can accumulate different genes for resistance to a single drug within a unique cell coding. Unusual gene accumulation occurs naturally on plasmids with resistance (R). Second, the increased expression of genes that code for multidrug efflux pumps, extruding a wide range of drugs, may also result in multiple drug resistance. The treatments are very complicated when infections are caused by multidrug-resistant bacteria; sometimes, few or no treatment options

Table 1 List of essential oils with different disease control properties

Plants	Essential oils	Different properties	References
Thymus vulgaris	Thyme oil	Antimicrobial, antioxidant cholinergic dysfunction	Sammi et al. (2017), Rota et al. (2008), and Zheng and Wang (2001)
Hedychium gardnerianum	Hedychium gardnerianum oil	Antithrombin, antibacterial, anti-acetylcholinesterase, and antioxidant activity	Medeiros et al. (2003) and Arruda et al. (2012)
Eucalyptus globulus	Eucalyptus oil	Antimicrobial, nematicidal, acaricidal	Fiori et al. (2000) and Pandey et al. (2000)
Lavandula angustifolia	Lavender oil	Antibacterial, antifungal, astringent, anti-inflammatory	Cavanagh and Wilkinson (2005) and Hammer et al. (1999)
Matricaria recutita L.	Chamomile oil	Antidiarrheal and antioxidant, neuroprotective, anti-allergic, anti-inflammatory, antimicrobial, anticancer	Ranpariya et al. (2011), Chandrashekhar et al. (2011), Bulgari et al. (2012), Silva et al. (2012), and Matić et al. (2013)
Melaleuca alternifolia	Tea tree oil	Antioxidant, anti-skin cancer, antibacterial, antiviral, antifungal, antiprotozoal, antitumor	Mantle et al. (2001), Greay et al. (2010), Mondello et al. (2006), and Carson et al. (2006)
Rosa damascena	Rose oil	Antidepressant, analgesic, hypnotic, antispasmodic, anti-inflammatory, anticonvulsant	Boskabady et al. (2006), Rakhshandah and Hosseini (2006), and Kheirabadi et al. (2008)
Juniperus communis L.	Juniper berry oil	Antioxidant, antiaging, antistress	Pandey et al. (2018) and Emami et al. (2007)
Anthemis Palestina	Anthemis oil	Antioxidant, antispasmodic, antihelicobacter, antimicrobial, antiproliferative	Bardaweel et al. (2014), Konstantopoulou et al. (1992), and Teixeira (2004)
Cinnamomum zeylanicum	Cinnamon oil	Antioxidant, anti-inflammatory, antidiabetic, antimicrobial, anticancer, neurological disorders	Mancini-Filho et al. (1998), Tung et al. (2010), Park et al. (2005), Jana et al. (2013), Marom et al. (2011), Hili et al. (1997), and Chou et al. (2013)
Trachyspermum ammi L.	Ajwain oil	Antiaging, antistress, antioxidants, antimicrobial, nematicidal, antihelminthic, antifilarial, antifungal, antibacterial, and antiviral agent	Rathor et al. (2017) and Chatterjee et al. (2013)

Table 2 In vitro and in vivo activities of essential oils against human bacterial pathogens

Name of essential oils	Bacterial pathogens	References
Sesame oil (*Sesamum indicum*)	*Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus cereus, Salmonella typhi, Shigella flexneri, Acinetobacter baumannii*	
Santhanathi oil (*Santalum album*)	*Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus cereus, Salmonella typhi, Shigella flexneri, Acinetobacter baumannii*	
Mustard oil (*Brassica nigra*)	*Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus cereus, Salmonella typhi, Shigella flexneri, Acinetobacter baumannii*	
Punga oil (*Pongamia pinnata)*	*Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus cereus, Salmonella typhi, Shigella flexneri, Acinetobacter baumannii*	
Tea tree oil *(Melaleuca alternifolia)*	*Alternaria* spp. *A. flavus*, *A. fumigates*, *A. niger*, *Blastoschizomyces capitatus*, *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Cladosporium* spp., *C. neoformans*, *Epidermophyton floccosum*, *Fusarium* spp., *Malassezia furfur*, *Microsporum canis*, *M. sympodialis*, *M. gypseum*, *Penicillium* spp.	Carson and Riley (1993) and Hammer et al. (2012)
Euphrasia rostkoviana	*C. albicans*	Novy et al. (2015)
Thuja sp. *(Thuja plicata*, *Thuja occidentalis)*	*P. aeruginosa*, *K. pneumoniae*, *S. aureus*, *E. coli*	Jirovetz et al. (2006)
Thymus zygis	*S. choleraesuis*, *S. typhimurium*, *E. coli*	Peñalver et al. (2005)
Salvia lavandulifolia	*P. vulgaris*, *P. aeruginosa*, *K. pneumonia*, *E. faecalis*	Jirovetz et al. (2006)

persist. In many cases, health-care workers have to use antibiotics that are more toxic to the patient. Many bacteria are multidrug-resistant, viz. *Staphylococcus aureus*, *Salmonella paratyphi*, *Shigella dysenteriae*, *Escherichia coli*, *Bacillus subtilis*, and *Candida albicans.*

1.10 Essential Oils Synergy with Antibiotics

Antibiotic therapy is one of the most critical treatments used in the fight against infectious diseases and since its introduction has greatly enhanced the health aspects of human life. In nature, the role of antibiotics remains unproven due to bacteria's responses by demonstrating different forms of resistance after new antibiotics for clinical use have been initiated. Antibiotic resistance can lead to failure of treatment, increased cost of treatment, as well as death rates and create even wider problem-controlled infection-controlled resistant bacteria from hospital to the community. Essential oils are produced from the secondary metabolism of aromatic plants, also known as volatile oils. The term is "essential" because it represents the

very essence of the plant and its essential part. Synergy occurs when combined therapy is used because the combined effect is greater than the sum of the individual outcomes. In past studies, when resistance to β-lactam antibiotics occurred, pharmaceutical scientists modified the β-lactam warhead periphery to obtain a more useful variant, and the penicillin and cephalosporin, second- and third-generation β-lactams, emerged. Occasionally, essential oils are synergistic enhancers because they are not capable of producing any significant inhibitory effects when used alone, but when used in combination with standard drugs, the combination impact surpasses their performance and improves antimicrobial activity (Gibbons et al. 2003). It has been found that synergistic action using essential oils reduces the minimum effective dose of antibiotics in infection treatment and reduces the adverse effects of antibiotics. Most importantly, a combination of antibiotics with essential oils targeting resistant bacteria may have a distinct mechanism of action, leading to new choices to overcome the microbial resistance attack. Because essential oils are multicomponent compared to many conventional antimicrobials that have only one target site, the exploitation of essential oils to prevent bacterial resistance is considered more promising. A new concept is the combination of traditional antimicrobial agents and essential oils (Table 3).

Table 3 The synergistic effect of essential oils and antibiotics in human pathogenic bacteria

Pair combinations	Pathogens	Methods	Interaction	References
Oregano/fluoroquinolones	*E. coli*	Broth microdilution	Synergistic	Si et al. (2008)
Oregano/doxycycline		Checkerboard assay		
Oregano/lincomycin				
Oregano/maquindox				
Pelargonium graveolens/norfloxacin	*S. aureus, B. cereus*	Agar dilution checkerboard assay	Synergistic	Rosato et al. (2007)
Lantana montevidensis/aminoglycosides	*E. coli*	Broth microdilution	Synergistic	
		Checkerboard assay		
Eugenol/vancomycin	*E. coli, E. aerogenes, P. vulgaris, P. aeruginosa, S. typhimurium*	Broth microdilution checkerboard assay	Synergistic	Hemaiswarya and Doble (2009)
Eugenol/β-lactams				
Rosmarinus officinalis/ciprofloxacin	*K. pneumoniae*	Broth microdilution	Synergistic	Van Vuuren et al. (2009)
		Checkerboard assay		
Eucalyptus/chlorhexidine digluconate	*Staphylococcus epidermidis*	Broth microdilution	Synergistic	Karpanen et al. (2008)
		Checkerboard assay		

(continued)

Table 3 (continued)

Pair combinations	Pathogens	Methods	Interaction	References
Zataria multiflora/vancomycin	*S. aureus* (MRSA and MSSA)	Broth microdilution	Synergistic	Mahboubi and Ghazian Bidgoli (2010)
		Checkerboard assay		
Aniba rosaeodora/gentamicin	*Bacillus cereus, Bacillus subtilis, S. aureus, E. coli, Acinetobacter baumannii, Serratia marcescens, Yersinia enterocolitica*	Broth microdilution	Synergistic	Rosato et al. (2007)
Pelargonium graveolens/gentamicin		Checkerboard assay		
Citrus limon/amikacin	*Acinetobacter* spp	Broth microdilution	Synergistic	Guerra et al. (2012)
Cinnamomum zeylanicum/amikacin		Checkerboard assay		
Coriander/chloramphenicol	*A. baumannii*	Broth microdilution	Synergistic	Duarte et al. (2012)
Coriander/ciprofloxacin		Checkerboard assay		
Coriander/gentamicin				
Coriander/tetracycline				

2 Conclusion

Essential oils play a vital role in traditional medicine for treating different types of infection worldwide for centuries and are important and alternative medicine for many diseases. Many studies focused on revealing the specific action mechanisms of essential oils and their active ingredients. In the current scenario, essential oils against bacterial, fungal, nematicidal, aging, Alzheimer's, etc. have been used effectively. This review emphasizes to make way for a new route of phytomolecules-based drugs for several disease management from the diverse pool of natural compounds.

3 Future Prospective

Essential oils, phytoconstituents, and herbal medicine are also necessary to manage the pathological conditions of several diseases. The active constituents from natural resources are presenting enormous scope for improved therapeutic application for the treatment of human disease. Therefore, it is time to decipher and identify our traditional knowledge regarding plant products and understand its recent advancements to fight against different disease management, to give it a worthy place.

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Exploration of Soil Resistome Through a Metagenomic Approach

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Abstract

Resistance toward antibiotics in microbes is considered to be one of the most significant challenges to modern medicine. The sum total of resistance genes against antibiotics in microorganism present in the soil is called the soil resistome and could serve as a source of resistance in microbes that can ultimately serve as a sink for drug discoveries. A deep knowledge of soil resistome and its multilateral interaction with advancement in drug development is essential for implementing suitable actions reducing spread of resistance in an efficient way. However, the soil resistome and its evolution are still in their infancy. The amalgamation of metagenomics with next-generation sequencing technology proved to be a robust methodological approach for exploring the soil microbiome, along with other related factors, especially the resistome. In this chapter, we have tried to incorporate the current knowledge on how the soil resistome is designed and discuss application of metagenomics to decipher hidden processes, particularly in respect to novel findings for medical diagnostics, controlling infections, and improving public health.

Keywords

Metagenomics · Antibiotic resistome · Rhizosphere · Microbial diversity

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1 Introduction

The soil is not only a natural reservoir of microbial diversity, but it also serves as a major source of the antibiotics used today in medical science. Most of the presently available antibiotics are synthesized as a bioactive compound of either bacterial or fungal origin. Microbial interaction within the soil presents a perfect model for understanding and developing novel drug discovery (Allen et al. 2015). Alternatively, the risk of emerging resistance in pathogenic strains has severely challenged this victory. Clinical environment is more prone to the occurrence of resistant pathogens due to introduction of new antibiotics (Lewis 2013). However, information related to the manifestation of "antibiotic resistance genes (ARGs)" in the soil are scarce. At present, culture-independent techniques—for example, metagenomics—have played an important role in revealing the diversity and existence of antibiotic resistance in soil. Deciphering the mechanisms related to antibiotic resistance may help in the identification of targets for the development of new drugs and effective antibiotics. Thus, gaining knowledge of ARGs will provide a platform to understand microbe-mediated diseases and their subsequent resistance. Earlier, Truman et al. (2014) illustrated underlying mechanisms of resistance for identification of novel antibiotics. They unravel the resistance mechanism against glycopeptide by *Streptomyces coelicolor* for the development of a novel screening procedure for antibiotic compounds. In the initial step of screening, the assay was performed to analyze cell wall stress using collection of bacterial extracts to analyze the inducers of sigE promoter, responsible for stress response in the cell wall. In the next step, mutant bioassay was performed to evaluate the extracts for activation of the sigE promoter, which in turn unveiled the presence of a glycopeptide antibiotic through the induction of resistance genes for glycopeptide. More recently, the culture-independent method has been used to identify a new antimicrobial by screening bacterial growth in diffusion chambers, in situ (Ling et al. 2015). This technique enabled the bacteria to access the required substrates from their natural environment. Using this approach, the author's revealed teixobactin which is a powerful antibiotic against clinically important Gram-positive bacteria. The authors suggested that although there was no resistance mechanism in *Mycobacterium tuberculosis* and *Staphylococcus aureus* strains against this antibiotic, they also recognized the role of horizontal gene transfer for acquiring resistant traits. For example, actinorhodin antibiotic cluster contains genes which encodes for export of proteins. Bacteria may develop resistance by acquiring mutations in the genes encoding antibiotic-related genes, e.g., DNA gyrase subunit A gene. However, pleiotropic effects occurring from these mutations may alter the metabolic pathways and consequently result in varying levels of resistance. Molecules having antibiotic activity at high concentration behave differently at low (but nontoxic) concentration, demonstrating main role in signaling and communication among bacteria, resulting in the induction of gene expression involved in virulence and further processes. An earlier study reported that some bacteria utilize antibiotics as an energy source increasing the possibilities of high antibiotic resistance in the natural environment

(Martinez 2008). Nevertheless, reports related to mechanisms of antibiotic resistance particularly in relation to soil are limited.

Soil contains particles of different size and composition, mineral, and organic compounds. Its heterogeneous structure creates a nexus of microenvironments allowing the existence of diverse microbial communities. Therefore, soil is considered to be a chief reservoir containing metabolic as well as genetic diversity and important source for identifying novel ARGs, increasing the area of knowledge about antibiotic resistance system. Over the decades, the metagenomics has revolutionized the area of soil microbiology by unearthing the hidden concepts. The alterations in emphasis from single isolate to diverse microbial communities, as well as from culturable to unculturable microorganisms, have reformed this field. Recently, Hug et al. (2016) established a new tree of life by aligning ribosomal protein sequences instead of 16S rRNA genes and was characterized to have an additional subdivision within the bacteria domain known as the candidate phyla radiation (CPR). Members included under the CPR group exhibit relatively small genomes with limited metabolic abilities, suggesting the existence of symbionts. It is interesting to observe that the lineages of CPR and other related groups without cultivable members contain maximum tree's diversity, signifying the role of culture-independent techniques for microbial diversity analysis. Certainly, these studies with the help of high-throughput next-generation sequencing (NGS) proved to unravel the soil ecological dynamics. Metagenomic technique involving both sequence-based and functional properties helped to demonstrate the functional and taxonomic diversity of microorganisms from natural environments. Sequence-based approach of metagenomic study comprises direct sequencing of total extracted DNA from an environmental sample. With the help of this approach, we can identify variation in the genes and diversity and abundance at taxonomic and functional levels along with microbial genome assembly. Advent of NGS technology has popularized the sequence-based metagenomics among scientific community and amplified the depth of sequencing by enabling full genome assembly. Moreover, combining different sequencing technologies resulting in short as well as long reads is considered to be more advantageous than using either technology alone (Sharon et al. 2015). Sharon et al. (2015) have successfully demonstrated the assembly of genomes having low abundance from complex terrestrial sediments. Short- and long-read sequencing has allowed researchers to demonstrate the abundance of microbial communities in a particular environment with numerous rare species. Alternatively, functional metagenomics is an important technique for identifying new genes of interest from diverse environmental samples. Typically, functional metagenomics encompasses creating metagenomic libraries; subsequently clones expressing the interested phenotype are being selected (e.g., antimicrobial or enzymatic activity, xenobiotic/antibiotic resistance, toxic compounds detoxifying, or environmental pollutant degrading). Since the phenotype-based screening provides important information about the gene function, therefore it could be employed for gene annotation. Recently, this approach was successfully used in the identification of amylolytic, cellulolytic, and lipolytic activities having potential industrial applications (Kim et al. 2015; Su et al. 2015; Alnoch et al. 2015). Functional

metagenomics is also useful for antimicrobial bioprospection (O'Mahony et al. 2015) and bioremediation of aromatic compounds present in soil system (Nagayama et al. 2015). Basically, ARG research involves functional metagenomics approach with the requirement of clone selection on agar plates added with antibiotics, followed by genetic content analysis. Soil microorganisms are important source of both antibiotic compounds and ARGs. Actually, more than 80% of all antibiotics being utilized in the clinic are derived from soil microorganisms, which are rich in antimicrobial resistance elements (Torres-Cortes et al. 2011). Therefore, exploring the resistome of soil will allow researchers to decipher the true diversity of resistance genes and will be helpful in identifying new resistance mechanisms, along with their ecological roles.

2 The Antibiotic Resistome

The antibiotic resistome is a framework to study resistance beyond the narrow point of view of the clinic, taking a wider outlook to incorporate resistance in all its forms, including within noninfectious environmental microbes (Perry and Wright 2014). The genetic and functional diversity in the resistome is vast and reflects the billions of years of evolution of microbes in close contact with toxic molecules of many origins (O'Brien and Wright 2011). These include inorganic metals and organic compounds made by microbes, plants, and animals (Li et al. 2016; Bérdy 2005). The molecules we term antibiotics are a subset of these toxic compounds (and here we must also include man-made compounds, as they are subject to the same evolutionary forces as natural substances).

In many cases, the molecular mechanisms that have evolved to evade nonspecific toxic molecules are identical and can be readily repurposed for antibiotic resistance. The sequencing of microbial genomes from diverse phyla and environments reveals that most (perhaps all) bacterial genomes harbor resistance elements, many in the form of intrinsic mechanisms (Cox and Wright 2013). These are the scars of the natural history of bacteria and the diversity of toxic molecules that they have encountered, including antibiotics.

Furthermore, many of these genomes display a variety of HGT signatures in the form of genes and pseudogenes encoding integrases, transposases, and gene sequences that confirm a long history of gene mobilization within and across microbial species and genera (Soucy et al. 2015). Such genomic resistance islands are found in many bacterial genera, frequently in the association of integrons, and can carry lots of resistance genes (Gillings 2014; Hamidian et al. 2015). The mycelial structure of many soil microbes may greatly facilitate HGT and the exchange of antibiotic resistance elements (Berthold et al. 2016). Mobile elements (e.g., plasmids and transposons) are found in many bacteria and offer facile routes of HGT that often do not respect species or genus boundaries. As a result, antibiotic resistance elements had diversified and moved across environments over the millennia. Analysis of the resistance gene makeup of environmental microbes in comparison

to naive antibiotic pathogens clearly shows that resistance elements are highly enriched in the former and rarer in the latter (Surette and Wright 2017). Waksman was among the first to observe the antagonistic interactions between environmental microbes comprising primarily bacteria and fungus (Waksman 1941). A more systematic study of the resistome of ~500 actinomycetes collected from a variety of soils revealed that these bacteria show resistance toward 7–8 antibiotics at 20 μg/mL (D'Costa et al. 2006).

In contrast, a survey of pre-antibiotic era *Salmonella* strains identified numerous plasmids but no resistance genes (Jones and Stanley 1992). The differences in antibiotic resistance before 1940 between pathogens and environmental microbes reflect the more challenging and diverse chemical ecology experienced by environmental bacteria in comparison with pathogens, many of which are commensal. Resistance genes present in bacterial genomes may not be expressed, and as a result resistance genotype may not correlate with phenotype as measured by standard minimal inhibitory concentration (MIC) in the laboratory (Perry et al. 2014). For this chapter, we consider antibiotic resistance to be driven by genotype, i.e., a bona fide resistance gene is one that confers protection from the antibiotic (increase in MIC) when expressed. We do not consider mechanisms of antibiotic tolerance such as persistence, biofilm formation, and stochastic gene amplification, though these do contribute significantly to the global challenge of antibiotic resistance.

3 Soil: Sink of Antibiotics Resistome

The soil has an enormous diversification in terms of biological, chemical, and physical properties. With regard to the microbial ecology, there is an existence of large number of niches comprising individual organisms and their group (Misra et al. 2017). The residents of soils include microbes (bacteria, fungi, archaea, cyanobacteria, protozoans, phages, and other viruses), plants, and larger animals, including nematodes, arthropods, worms, and burrowing mammals. Earlier, Waksman (1945) expounded on the complexity of soil microbe communities over 70 years ago. He summarized the interactions between microbes as associative (symbiosis, growth promotion, the liberation of nutrients from complex forms, consumption of oxygen), competitive (for nutrients or space), or antagonistic (production of growth-inhibitory substances). The latter can be passive, e.g., change in the pH of the local medium, exhaustion of nutrients, or active, in the form of excretion of toxic compounds, pigments, or lytic enzymes. Using culture-based approaches, an effort was made to analyze microbe-mediated production of selectively toxic metabolites (Waksman 1941). This realization not only launched the golden era of antibiotic discovery but also identified some of the first anticancer and antifungal agents as well as multiple other drug classes—from immune suppressants to cholesterol-lowering agents. Soils indeed offered a wealth of leads for new medicines, and mining of soil microbes for such compounds dominated efforts in the pharmaceutical field for decades. Bioactive compounds from soil microbe area are boon to drug

discovery but are rarely investigated for their intrinsic roles in producers or their impact on the ecology of the soil. Thousands of such compounds reported in the literature represent only a small part of the chemical space available to these organisms (Bérdy 2005). Sequencing of the genomes of soil microbes including bacteria of the genus *Streptomyces* and other filamentous actinomycetes reveals that they have the genetic capacity to produce a dizzying spectrum of compounds. Each actinomycete genome has on average 20 to >30 genetic programs encoding bioactive compounds, many of which are antibiotics (Katz and Baltz 2016). Similarly, filamentous fungi are important producers of secondary metabolites, with approximately 40–80 biosynthetic gene clusters per *Aspergillus* genome, for example (Inglis et al. 2013). The precise roles of most of these compounds in soil environments are rarely known with confidence (O'Brien and Wright 2011). Certainly, many do have antimicrobial activity, but whether this is their primary role is disputed (Davies 2006). The killing activity of antibiotics is concentration dependent, and sub-MIC concentrations can have pleiotropic effects on bacterial gene expression and metabolite production (Surette 2013). The fact that antibiotic quantities in soils are difficult to measure, and likely generally much lower than concentrations that can be secreted in the lab, fuels doubt that cell killing is their primary activity. Imaging mass spectrometry experiments demonstrate that production of secondary metabolites, including antibiotic, is highly dependent on growth conditions and proximity to other microbes (Traxler et al. 2013). Not surprisingly, what is evident from these studies is that there is a gradient where antibiotic concentrations are high close to the producing cells diminishing as compounds diffuse out into the medium. Furthermore, it is well known to researchers purifying antibiotics and other secondary metabolites that these are very frequently physically associated with producing cells. Microbes live on the micron scale, so although antibiotics may have multiple effects on adjacent cell metabolism and gene expression not directly related to cell death, as the proximity of cells increases, antibiotics very likely do have antibiotic activity. This hypothesis was supported by the studies demonstrating genetically diverse and extensive soil resistome evolved to attenuate antibiotic killing activity. The similarity of the molecular mechanisms of resistance observed in the clinic to those in soil bacteria, in particular, the self-protection mechanisms of antibiotic-producing bacteria, had been made over the past four decades (Marshall et al. 1998). D'Costa et al. (2006) have systematically explored the soil resistome revealing antibiotic resistance in spore-forming bacteria having ubiquitous and diverse nature. Resistance mechanisms include several metabolic processes in clinical isolates and also the strategies that are not recognized in pathogens. In another study that explored a broader spectrum of bacterial genera, 412 strains isolated from a variety of soils were tested for resistance against a panel of 24 drugs (Walsh and Duffy 2013). The majority (80%) were multidrug resistant, confirming the diversity and frequency of resistance in soils. Efflux mechanisms were the primary source of the multidrug phenotype, but drug inactivation was also prevalent, in particular for penicillin resistance. Common β-lactamases circulating in the clinic were not detected, pointing to the importance of the intrinsic β-lactamases.

4 Antibiotic Resistance: Possible Risks to the Environment

The environmental resistome is vast, ancient, and mobilizable. As such, it presents a risk to the development of resistance in pathogens and subsequent drug failure. Reports are available demonstrating circulation of resistance elements in pathogens belong to gene families that have their origins in the environment. Given the immense numbers of environmental bacteria and associated resistance genes accumulated over geologic time, it is a reasonable assumption that environmental bacterial genomes are the source reservoir of antibiotic resistance. That said, there are few concrete examples where the direct link between genes found in the clinic and those found in the environment is unimpeachable. The emergence of the *qnr* fluoroquinolone resistance genes, now widely circulating on mobile elements in pathogens, likely originated in environmental *Shewanella* species (Poirel et al. 2005).

Similarly, the Extended Spectrum Beta Lactamases (ESBL) of the Cefotaximases (CTX-M) family is thought to have emerged from bacteria of the genus *Kluyvera* (Rodriguez et al. 2004). Examination of the genomic context of the majority of resistance elements in environmental microbes reveals that these are embedded in genomes and rarely associated with mobilization elements (transposases, integrases, inverted repeat sequences). The history of antibiotics is that resistance, often via mobile genetic elements, inevitably follows use. The environment is a reservoir for these genes, but the acquisition by pathogens is not facile. Indeed, it is unlikely that antibiotics would have emerged as therapeutic successes in the first place. One is therefore left with the hypothesis that the use of antibiotics in bulk provides a strong selection for the stochastic capture of resistance genes by mobile genetic elements that can eventually be acquired by pathogens. This gene capture is unpredictable and can occur relatively quickly after antibiotic development, such as in the case of the serine β-lactamases, or only after decades of use, such as with vancomycin resistance. The frequency of these capture events correlates with the gene diversity and burden in the environment. For example, most surveys of resistance genes and phenotypes in nonpathogenic environmental bacteria identify β-lactamases and aminoglycoside-modifying enzymes as highly prevalent, and these were recorded as the first elements showing resistance present on mobile elements in sensitive pathogenic bacteria (Davies and Davies 2010). It is not unreasonable to hypothesize that antibiotics that show less abundant resistance gene diversity and frequency in the environment will similarly be slower to show resistance in the clinic. This criterion should be a valuable screen in antibiotic discovery to identify which scaffolds to focus on in drug development. Their cognition that the environment is a nearly boundless reservoir of antibiotic resistance has resulted in several studies that seek to estimate the risk of gene transfer to pathogens (Bengtsson-Palme and Larsson 2015; Martínez et al. 2015; Manaia 2016). This examination identifies so-called hot spots of gene transfer (manure, wastewater treatment plants), where mixing of pathogens, mobile elements, and resistance genes is more likely and therefore has greater potential to affect health (Gaze et al. 2013). The absence of contact between pathogens and environmental bacteria and the lack of enabling mobilization genes and sequences, for example, are mitigating issues that decrease the risk of gene

transfer into pathogens. Establishing more sound policies to avoid unnecessary risk of gene transfer, in particular for new antibiotics, should be a priority. Nevertheless, the history of antibiotics has demonstrated time and again that even low-frequency events will occur and antibiotic resistance is inevitable.

5 Metagenomics: A Tool to Explore Soil Resistome

Metagenomic technique has been used from the past few decades to identify resistance genes among the isolates from environmental samples (D'Costa et al. 2006; Wright 2007). The identified genes are responsible for producing compounds having antimicrobial activity and signaling molecules within bacterial community (Linares et al. 2006). Antibiotic resistome signifies collection of genes contributing to antibiotic resistance in microorganisms, having their origin either from nonpathogenic or pathogenic bacteria. This term comprises not only the genes involved in antibiotic production but also the precursor gene that could be evolved to behave as resistance element under selective pressure of the natural environment. Most of the resistance genes show ambiguity and sometime not naturally expressed in isolates from environment (Wright 2007). D'Costa et al. (2006) were the pioneers to establish this antibiotic resistome concept by screening antibiotics and constructing a library of *Streptomyces* sp. strains isolated from different environmental samples. In this way, the metagenomic approach has increased its breadth by being applied to different natural environment exploring genes and genetic elements responsible for resistance or resistance gene transfer commonly termed as "antibiotic extended resistome" (D'Costa et al. 2007). This approach has been proved beneficial for exploring diversity in different ecosystems, including nosocomial transmission, and human hosts. Moreover, it is useful for predicting future evolution of antibiotic resistance (Martínez et al. 2007).

There are several metagenomic-based reports demonstrating functional or sequence-based approach for characterization of ARGs present in natural environments (Table 1). The studies evaluated soil, water, sludge, and other samples from different environmental sources to assess genes responsible for antibiotic resistance comprising β-lactams, amphenicols, macrolides, fluoroquinolones, sulfonamides, aminoglycosides, polymyxins, lincosamides, phosphonic acids, tetracyclines, polypeptides, and trimethoprim. The only difference between functional and sequence-based metagenomic approach is that the latter can be used to identify known ARGs using in silico analysis by comparing from ARG databases, e.g., Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al. 2013) and Antibiotic Resistance Database (ARDB) (Liu and Pop 2008). In contrast, functional metagenomic approach can explore novel resistance mechanisms or could allocate new roles to previously identified proteins by classifying and separating possible open reading frames (ORFs). Perhaps, Amos et al. (2014) mark off quinolone resistance in a water sample along with assigning new characters to recombinase A (RecA) and regulatory protein X (RecX), previously known to be as ARGs. The authors established the role of RecX in modulating RecA which in turn is responsible for DNA damage repair caused due to ciprofloxacin-mediated inhibition of DNA

Table 1 List of different sources evaluated for antibiotic-resistant gene(s)/clone(s) analysis

S. No.	Sources	Phylum/genera of microbiome associated with different sources	Gene(s)/Clone(s)	References
1.	Maize	*Proteobacteria*, *Actinobacteria*	Antibiotic resistance gene for transporters, β-lactamases	Li et al. (2014)
2.	Paddy	*Actinobacteria*, *Proteobacteria*	Bac A, Sub E, Upp P, Amp C, Cmx A, Tet A, Tet G, Van A, Van H, Van R	Xiao et al. (2016)
3.	Arable soil	*Pseudomonas* sp., *Janthinobacterium* sp., *Psychrobacter pulmonis*	$bla_{CEP\text{-}0}$, $bla_{CEP\text{-}01}$, $bla_{CEP\text{-}02}$, $bla_{CEP\text{-}03}$, $bla_{CEP\text{-}04}$, $bla_{CEP\text{-}05}$, $bla_{CEP\text{-}06}$	Udikovic-Kolic et al. (2014)
4.	Apple	*Chitinophaga*, *Pseudomonas, Pedobacter*	AOAmox2, AOCarb3, AOCefta2, AOAmox1, AOCefta11	Donato et al. (2010)
5.	Arable soil	*Streptomyces* sp.	*van a*, *van b*, *van d*, *bla_a*, *bla_b*, *bla_c*	Nesme et al. (2014)
6.	Arable soil	*Actinobacteria*, *Verrucomicrobia*, *Acidobacteria*	Antibiotic resistance gene for mfs_ transporter, β-lactamase	Forsberg et al. (2014)
7.	Wood composts, activated sludge	*Proteobacteria*, *Gammaproteobacteria*, *Enterobacterales*, *Enterobacteriaceae*	NHMcSpc1 (LC306682), mgSpc1 (LC306679), mgSpc2 (LC306680)	Miyazaki and Kitahara (2018)
8.	Mariculture system	*Proteobacteria*, *Bacteroidetes*	tet31, sul2, cml_e3, cml, qnrs, dfra 1	Wang et al. (2018)

gyrase. Other reports have also successfully established the role of functional metagenomics by identifying new ARGs to β-lactam and dioxygenase through screening soil metagenomic libraries (Allen et al. 2015; dos Santos et al. 2015). Allen et al. (2015) demonstrated a response regulator gene encoded in a 5169-bp ORF which is being isolated from a resistant metagenomic clone of Alaskan soil along with a putative metallopeptidase gene. Through phenotypic experiments, it has been analyzed that the resistance to carbenicillin was due to the response regulator gene which usually alters the gene expression encoding porins and efflux pumps in *Escherichia coli*. Similarly, dos Santos et al. (2015) evaluated resistance toward nine β-lactam antibiotics through screening metagenomic library of Brazilian Cerrado soil.

However, the two abovementioned studies established the importance of metagenomics in ARG research which useful to not only reveal novel and unpredicted mechanisms of antimicrobial resistance but also to provide understanding about novel ARGs functions in natural environments (Allen et al. 2015; dos Santos et al.

2015). List of the antibiotic-resistant gene(s)/clone(s) reported from different sources is summarized (Table 1). However, functional metagenomics approach is more beneficial as it is not limited to earlier known sequences. It has also advantage of exploring some ARGs which have low or no expression (cryptic resistance genes).

6 Conclusions

Developing resistance against antibiotics in microbes is a serious concern to human health. Therefore, an improved understanding toward mechanisms conferring resistance is required to control clinically important multidrug-resistant strains. Although resistance toward antibiotics in nosocomial strains is relatively well established, natural environment comprises an immense and large ARGs diversity unexplored. To accomplish this genetic wealth, culture-independent metagenomic approaches have demonstrated to be a vital technique. This technique may provide easy way for bioprospecting new antimicrobial products and developing novel drugs through identifying and describing new mechanisms toward resistance. Apart from clinical background, roles of ARGs and antimicrobial resistance could be supported by adding new elements responsible for resistance to ARG databases such as CARD and ARDB. In the present chapter, we discussed the reports highlighting extensive diversity of ARGs in natural environments, particularly in soil. Furthermore, functional metagenomics could be utilized to characterize and classify new ARGs along with those not earlier related with antibiotic resistance. This approach will provide us the required influence to combat against multiple drug-resistant microbes of clinical importance and to unravel the ecological aspects of new ARGs present in the environment.

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Actinomycetes as Continued Source of New Antibacterial Leads

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Abstract

Early antibiotic discovery program has witnessed significant role of mainly *Streptomyces* in antibiotic/drug discovery program. Due to various constraints, both academic and industry levels, the discovery of new antibiotics with novel mode of action is drastically slowed down in the last three decades. Rapid development and spread of multidrug-resistant bacteria globally have reduced the utility and effectiveness of old antibiotics. Therefore, the discovery of novel antibacterial compounds is urgently needed to combat antimicrobial resistance. However, natural product-based academic research could prove to be a sustained mine of novel antimicrobial leads. According to an estimate among the bioactive compounds that have been obtained so far from microbes, 45% are produced by actinomycetes, 38% by fungi, and 17% by unicellular eubacteria. This has become possible because of great diversity of actinomycetes in different habitat and their extraordinary capacity to synthesize new antibiotics. The development in the screening strategies and the use of modern biochemical and molecular approaches have made possible to detect new compounds. In this chapter, we have focused on general characteristics of soil and marine actinomycetes and

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improved screening strategies adapted by various workers. A recent update is also provided to highlight the role of actinomycetes as the continued source of novel antibacterial lead compound and their future prospects.

Keywords
Actinomycetes · Antibacterial · Antibiotics · Screening strategy · Antibiotic resistance

1 Introduction to Actinomycetes

Actinomycetes belong to the order *Actinomycetales* (superkingdom, *Bacteria*; phylum, *Firmicutes*; class, *Actinobacteria*; subclass, *Actinobacteridae*) which are Gram-positive, spore-forming filamentous bacteria found on both terrestrial and marine habitats (Yuan et al. 2010). There are about 100 genera of actinomycetes in the soil. In addition to similar characteristics with bacteria, the morphological relationship of the actinomycetes with fungi is evidenced on the basis of three features:

(i) The higher actinomycetes mycelium has the extensive branching characteristic showing similarity with fungi.
(ii) Many actinomycetes, like fungi, form an aerial mycelium and also conidia.
(iii) Instead of unicellular bacteria, in liquid culture, the growth of most of the actinomycetes rarely results in turbidity although it occurs as distinct clumps or pellets.

On the other hand, the size of the hyphae and its morphology, conidia, and mycelium of individual fragments of species undergo segmentation similar to structures found in bacteria, and structural organization of prokaryotic cell is similar to Gram-positive bacteria (Alexander 1986; Okami et al. 1988; Chaudhary et al. 2013). Under taxonomic criteria, the class *Actinobacteria* is one of the largest taxonomic units among the major lineages currently recognized within the *Bacteria* domain. The majority of the *Actinobacteria* are free-living in nature and are widely distributed in both aquatic (involving marine) and terrestrial ecosystems (Macagnan et al. 2006). Majority of *Actinobacteria* requires oxygen, but some exceptions also exist. In addition, they can be chemoautotrophic or heterotrophic, but most of the populations are chemoheterotrophic and able to utilize a wide variety of nutritional sources, including complex nature of various polysaccharides (Zimmerman 1980; Ludwig et al. 2015).

Bacteria belonging to the order *Actinomycetales* are the predominant producer of secondary metabolites and account for 45% of the total bioactive metabolites (Bérdy 2005). As producers of antibiotics (80% of the world's antibiotics), actinomycetes occupy the highest position in the microbial world having numerous biotechnological applications (Kekuda et al. 2010; Alharbi et al. 2012). As degradation agents, actinomycetes are important in the degradation

of soil organic materials into humus and constitute important proportion of soil and aquatic ecosystem as well. Actinomycetes also secrete a range of enzymes that can completely degrade the complex lignocellulosic compounds (lignin, cellulose, and hemicellulose), while others may secrete narrow range of enzymes that can only partially carry out this degradation (Mason et al. 2001; Saini et al. 2015).

2 General Characteristics of Actinomycetes

Actinomycetes when grown on agar surface and on the undersurface, capable of growing in both the conditions, form a network of hyphae. Once they grow on the surface, they form aerial hyphae and the growth undersurface leads to substrate hyphae. The hyphae are divided by the septa into long cells (about 20 mm and longer) and comprise a large number of bacterial chromosomes or nucleoids. These hyphae make aerial hyphae capable of reproducing asexually and extend above the substratum. A generalized mode of reproduction in actinomycetes is represented in Fig. 1. Most commonly, the members of *Actinobacteria* do not have motility. But in some cases if motility is present, then it is limited to flagellated spores. Actinomycetes are organisms that are distributed in soil in the form of thread-like filaments in most parts of the world. The "earthy" or musty smell from healthy or fresh soil is the characteristic of actinomycetes occurrence in that habitat (Wink and Mohammadipanah 2015).

Actinomycetes are capable of degrading a wide range of complex substances and are especially important in decomposing recalcitrant (hard-to-degrade) compounds, such as cellulose and chitin, and perform their function at elevated level of pH. They are saprophytic, free-living bacteria and the prime source for

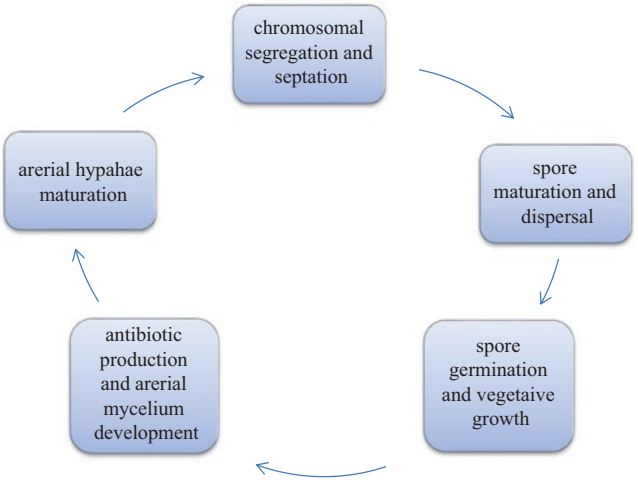

Fig. 1 Life cycle of sporulating actinomycetes

the antibiotic production (Atta and Ahmad 2009), extensively dispersed in natural as well as in synthetic environments, and play a vital role in organic matter decomposition (Naikpatil and Rathod 2011). They are capable of producing biologically active secondary metabolites, such as antitumor agents, antibiotics, enzymes, vitamins, cosmetics, nutritional materials, herbicides, pesticides, exhibiting economical and biotechnological importance; also an appreciable number of actinomycetes are the major source of antibiotic production (Ogunmwonyi et al. 2010; Naikpatil and Rathod 2011; Ahmed et al. 2016).

3 Diversity of Actinomycetes

A classification of any microbial order is a temporary and man-made arrangement in which similar individuals, sharing certain common features, are grouped together as taxonomic units at different levels in a taxonomic hierarchy (Alexander 1986). Bergey's *Manual of Systematic Bacteriology* divides the actinomycetes into seven families, primarily based on the properties such as cell wall type, conidia arrangement, and the presence or absence of a sporangium. The proposed classification of actinomycetes on morphological basis is given in Table 1.

The genus *Streptomyces* has long been recognized as a rich source of useful secondary metabolites and continues to be a major source of new bioactive molecules. As the frequency of novel bioactive compounds discovered from terrestrial actinomycetes decreases with time, much attention has been focused on screening of actinomycetes from diverse environments (Thenmozhi and Kannabiran 2010). Between 1950 and 1960, there was strong relation between microbiology and chemistry, and the "golden age of antibiotics discovery" was born. During this period half of the products which are commonly used today were isolated (Aminov 2010). Repeated rediscovery of streptomycetes' secondary metabolites gradually led to decrease in natural product-based drugs in pharmaceutical industries because of the belief that most of useful compounds had been rediscovered from soil microorganisms. In response to the increasing needs for new antimicrobial compounds in medicine, the interest in natural product discovery is shifted toward underexplored environments, especially marine and extreme environments. The discovery of antimicrobial compounds with different chemical moieties from rare actinomycetes isolated from such habitats confirmed that microbial natural products are promising source for new drug leads (Hozzein et al. 2011; Khan et al. 2011; Tiwari and Gupta 2012). It has been revealed that only 10% of the natural products are expressed under laboratory conditions, while the rest that are not expressed are cryptic gene clusters. For example, the actinomycete *Streptomyces coelicolor* has been known to produce only five secondary metabolites until its genome was identified in 2002. In-depth bioinformatics analysis of the genome has revealed that it produces about 32 secondary metabolites of diverse arrays covering 15 distinct families (Bentley et al. 2002; van Keulen and Dyson 2014).

Table 1 Generalized scheme for the classification of actinomycetes partially adapted from Alexander (1986)

S. no.	Family	Characteristics	Examples of genera
1.	***Actinomycetaceae***	Gram-positive, non-acid-fast, branched filaments fragmented into irregular rodlike or coccoid elements, non-motile cells neither form aerial hyphae nor spores	*Actinomyces*
2.	***Streptomycetaceae***	Non-fragmented hyphae, extensive aerial mycelium, and chains of spore along with 5–50 conidia/chain	*Streptomyces, Microellobosporia, Sporichthya*
3.	***Nocardiaceae***	Fragmented hyphae	*Nocardia, Pseudonocardia*
4.	***Micromonosporaceae***	Non-fragmented hyphae, no aerial mycelium, heat-sensitive spores (aleurospores) singly on substrate mycelium. Occurrence of conidia singly or in pairs or in short chains	*Micromonospora, Microbispora, Micropolyspora, Thermomonospora, Thermoactinomyces, Actinobifida*
5.	***Dermatophilaceae***	Branched filaments divided by longitudinal and transverse septa generating motile coccoid elements	*Geodermatophilus*
6.	***Frankiaceae***	Nonspore-forming obligate symbiotic actinomycetes, found in root nodules of nonleguminous plants to fix atmospheric N_2	*Frankia*
7.	***Actinoplanaceae***	Hyphae are septate, diameter is 0.2 or >0.2 μm, spore vesicles enclosed with either zoospore or aplanospores	*Streptosporangium, Actinoplanes, Planobispora, Dactylosporangium*

4 Common Habitat of Actinomycetes

Actinomycetes are distributed in both natural and artificial environments. The occurrence and distribution of actinomycetes in both well-explored terrestrial and underexplored marine habitats are described here.

4.1 Actinomycetes in Soil

The wide distribution of actinomycetes occur not only in soil but also in various other habitats such as compost, bagasse, fodders, grains, river muds, hay, and lake bottoms. Actinomycetes are also found at considerable depths of lower horizons in surface soil. According to their abundance and diversity they are placed next to bacteria. Environments having high pH ranges, supports the community

composition of Actinimycetes. (Anderson and Wellington 2001). They are heterotrophic (unable to make their own food), aerobic (needs oxygen), and mesophilic (25–30 °C) bacteria and some of the species present in manures and compost are thermophilic in nature and capable of growing at 55–65 °C (e.g., *Thermoactinomyces*, *Streptomyces*). In the order of their abundance, the most common genera of actinomycetes in soils are *Streptomyces* (nearly 70%), followed by *Nocardia* and *Micromonospora*, though *Actinoplanes*, *Actinomyces*, and *Streptosporangium* are also commonly encountered in soil ecosystem (Alexander 1986). The types and number of actinomycetes found in a particular type of soil will be greatly affected by geographical location, organic matter content, temperature of the soil, soil pH, soil type, cultivation, moisture content, and aeration (Alexander 1986; Paul 2014). Forest soils usually have a relatively low pH and contain a huge repertoire of *Streptomyces* that are tolerant to acidic conditions, while fewer numbers of *Streptomyces* and rare genera such as *Streptosporangium* and *Actinoplanes* are present in alkaline-arid soils (Williams and Cross 1971; Shade et al. 2012).

Streptomyces are widespread worldwide in soil ecosystem. Other genera appear to have a narrow distribution in nature; therefore, evaluation of their distribution plays a significant role in identifying the economic importance of actinomycetes (Krishnaraj and Mathivanan 2014).

4.2 Actinomycetes in Marine Environment

Biological diversity covers about 95% of the earth's total diversity, whereas oceans include more than 70% of the surface on the globe (Qasim 1999). Microbial diversity of oceans is unique including bacterial diversity. The occurrence and distribution of actinomycetes have been widely documented from seawater, intertidal zone, marine biota, and sediments (Ramesh and Mathivanan 2009; Sun et al. 2010; Thornburg et al. 2010). Actinomycetes of marine environment have been the continued source of new species discovery and isolation of novel bioactive compounds with potential therapeutic applications (Lam 2006; Liu et al. 2010). Interestingly the metabolites obtained from marine actinomycetes exhibit biological activities physiologically and phylogenetically distinct from terrestrial counterparts representing unique therapeutic potential (Subramani and Aalbersberg 2012; Xiong et al. 2013).

Although it is difficult to culture several of these extremophiles, culture-independent techniques have made it possible to discover and identify members of the genera such as *Aeromicrobium*, *Dietzia*, *Marinispora*, *Salinispora*, *Streptomyces*, *Rhodococcus*, and *Salinibacterium*. Some of these genera require seawater or 10% NaCl in the culture medium. Several successful efforts have been documented on the discovery of novel antibiotics and bioactive compounds from terrestrial actinomycetes (0.30%) as compared to marine counterparts including sediments (0.25%) and seawater (0.001–0.01) (Jones 1977; Baltz 2008). The reason behind this is variation due to difficulty in culturability of marine microorganisms especially in seawater. In the future, more innovative biotechnological applications are required for

continued exploration of actinomycetes as a source of bioactive compounds. Recently the bacterial diversity in the marine environment has been revealed by culture-independent approaches with the involvement of molecular techniques allowing in-depth assessment of their diversity in marine ecosystem and its possible exploitation (Duncan et al. 2015a, b).

5 Antibiotic Production by Actinomycetes

During the 1940s, Selman Waksman and his students isolated more than 15 antibiotics, the most famous of which was streptomycin, the first effective treatment for tuberculosis (Hopwood 2007). Antibiotics are truly referred as the "wonder drugs" for their virtual success against pathogenic microorganisms. This remarkable group of compounds forms a heterogeneous assemblage of biologically active molecules with different structures and modes of action. They attack virtually every type of microbial activity such as DNA, RNA, and protein synthesis, membrane function, electron transport, sporulation, germination, and many others. Members of the order *Actinomycetales* and filamentous fungi are responsible for producing secondary metabolites, about 90% of all known antibiotics. Of all antibiotics, two third are produced by actinomycetes, and the majority are produced by the genus *Streptomyces* (Newman and Cragg 2007). Among the antibiotics, targeting bacterial ribosome functions in treating respiratory infections, for example, in treating Legionnaires' disease, tetracycline and erythromycin are used. On the other hand, some antibiotics like vancomycin acts on deadly organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA) (multidrug-resistant) by inhibiting bacterial cell wall synthesis; rifamycins, useful for treating leprosy and tuberculosis, target bacterial RNA polymerase, and amphotericin attacks on fungal membranes (Bérdy 2005; Zhang et al. 2007). Recently, a variety of antifungal compounds like abequines, kocumarin, and hydrolytic enzymes have been reported from diverse sources of actinomycetes (Schulz et al. 2011; Ashokvardhan et al. 2014; Uzair et al. 2017).

In addition to antibiotics, some of the members are also capable of producing clinically useful antitumor drugs like anthracyclines (aclarubicin, daunomycin, and doxorubicin), enediynes (neocarzinostatin), peptides (bleomycin and actinomycin D), mitomycins, aureolic acids (mithramycin), carzinophilin, antimetabolites (pentostatin), and others. Actinomycetes products not only have the potential of therapeutic activities, but they also frequently possess the desirable pharmacokinetic properties that are required for proper dosing recommendation for the optimal usage in clinical and healthcare settings (Zotchev 2012). Although the metabolites produced by actinomycetes do not influence human cells, some of the metabolites, for example, Adriamycin, inhibits DNA replication (an agent used in cancer), while rapamycin is used to facilitate organ transplantation therapy (repress the immune system of host). Various well-known antibacterial compounds produced by actinomycetes are listed in Table 4.

6 New Approaches to Screen and Discover Novel Antibiotics from Actinomycetes

According to an estimate, microbial diversity is poorly explored and exploited for its industrial and therapeutic application since majority of bacteria are non-culturable. Even culturable microorganisms, including actinomycetes, of different habitats have not yet been fully understood and explored. It is presumed that screening of unexplored actinomycetes diversity with improved methods is the only approach through which entirely new class of compounds could be expected (Hozzein et al. 2011; Tiwari and Gupta 2012).

The efforts on the above have been pressing demands due to rapid development of resistance among pathogenic bacteria to almost all available antibacterial drugs. Yet, a greater number of new superbugs are reported. Therefore, there is a global concern to develop strategies to discover new antibiotics as well as to combat the multidrug resistance problem before it takes an epidemic scenario (van Duin and Paterson 2016).

As a result of increasing needs of new antimicrobials, the focus on discovery of natural products has been shifted toward underexplored habitats, particularly extreme and marine environments. There are a number of improved screening strategies developed in recurrent past. Most commonly, three different approaches have been applied for drug discovery programs, like bioactivity-guided screening, chemical screening, and target-based screening. In bioactivity-guided screening or forward pharmacology, without knowing the drug targets, crude extracts or purified chemicals have been screened for biological activity in whole cell assays. In chemical screening, chemical substances (from either chemical libraries or natural source) have been screened for biological activity by employing the usage of sophisticated analytic methods like high-performance liquid chromatography, nuclear magnetic resonance (NMR), or mass spectrometry (MS) to avoid repetition of already known active compounds, while in target-based screening, compounds are screened for their activity against particular molecular targets (Wohlleben et al. 2016). Some of the recently used screening strategies are briefly described here. See the review articles published on various approaches for details (Brown and Wright 2005; Abdelmohsen et al. 2015; Fedorenko et al. 2015; Zarins-Tutt et al. 2016; Kiran et al. 2018).

6.1 Culture-Based Isolation and Screening of New Antibacterial Compounds from Marine Actinomycetes

A huge number of rare actinomycetes are being isolated from diverse ecosystem worldwide from previously unexplored natural habitats with the use of selective isolation and screening methods. The efforts in this direction include the suppression of unwanted microorganisms (pretreatment) and enrichment methods for enhancing the growth of rare actinomycetes. For the discovery of novel marine microbial natural product, marine microorganisms are the major source. Marine

microorganisms do not form colonies on the nutrient-rich agar medium, and this low tendency of culturability may reflect the need of artificial conditions in culture media (Xiong et al. 2013). Therefore, new modifications in the culture media addressing specific requirement and conditions for incubation of marine microorganisms are needed that result in increase in the chance of culturability of marine bacteria. Sometimes pretreatment of marine sample is needed to recover bacteria which are present in low number (Tiwari and Gupta 2012; Janaki et al. 2014). Pretreatment methods include enrichment, physical, and chemical techniques (e.g., dry heat, exposure to 1–1.5% phenol, sucrose-gradient centrifugation, and filtration through cellulose membrane filters) are employed to favor the isolation of specific genera and improve the recovery of these microorganisms. These pretreatments eliminate or strongly reduce the risk of contamination, thereby facilitating the isolation of slow-growing marine microbes (Yamamura et al. 2003; Jensen et al. 2005; Bredholdt et al. 2007; Kjer et al. 2010; Xiong et al. 2013). Majority of nature's microbial biosynthetic potential still remains elusive, if the discovery of new antimicrobial leads relies on the classical culture-dependent platform. Therefore, efforts must be increased under laboratory conditions for the exploitation of majority of rare *Actinobacteria* that are still uncultured to date acting as a reservoir of new microbial molecules. For that purpose, some of the suitable methodologies and concepts have emerged (Vartoukian et al. 2010; Dhakal et al. 2017).

6.2 Designing New Culture Media

The three basic requirements for the growth of microorganisms are energy source, nutrients, and optimal physiological conditions. Most of the marine microbes need specific nutrient requirements for their proper growth like signal molecules, nontraditional electron donors, and electron acceptors (Abdelmohsen et al. 2010; D'Onofrio et al. 2010).

Another culture condition that influences their growth is ionic strength of the medium. The members of the genus *Salinispora* (marine actinomycete) is well known to produce desferrioxamine secondary metabolites. It was observed that three species of *Salinispora*, *S. tropica*, *S. pacifica*, and *S. arenicola*, require a high ionic strength for their optimal growth and metabolite production (Tsueng et al. 2008). Refinement of classical approaches is one of the common strategies to cultivate "not-yet-cultured" species. In enrichment culture technique in which nutrients may support the growth of faster-growing microbes retarding slow-growing species which thrive in nutrient limiting environments and may be inhibited by the enriched component of conventional media (Vartoukian et al. 2010). One of the strategies is diluting nutrient media, which is successfully applied in the cultivation of previously unculturable microbes from various marine environments. The uncultivated microorganisms can also be recovered by the culture medium that mimics the natural environmental conditions. For example, to culture previously uncultivated microbe SAR11 (a marine bacterioplankton clade), seawater has been used. In addition, certain microorganisms are capable of growing in the presence of helper strains

that release some of the growth-stimulating factors for the unculturable microbial strains. Therefore, cell-free extracts or extracellular material released from helper strains acts as growth stimulant for the unculturable species. Another culture designing strategy is to extend the incubation time of slow-growing marine microbiota at low substrate concentration in a nutrient-defined media that have the added benefits of retarding the growth of faster-growing species within mixed microbial populations (Wu et al. 2012; Jiang et al. 2016). Although many of the "not-yet-cultured" species can be cultivated by designing the composition of enriched culture media, still there is a need to identify mechanisms of uncultivability by molecular techniques, and developing new cultivation strategies is also an important task for the researchers in the identification and exploitation of new species.

6.3 Combinatorial Methods

Natural products are complex compounds that play an important role in drug discovery. Combinatorial biosynthesis is a useful tool to raise the chemical diversity of natural products by means of altering scaffold backbone through variations in biosynthetic enzymes, functional groups, and regiochemistry that otherwise would be difficult using other methods. It is a complement to traditional microbial drug development program that provides a robust platform for the diversity of natural products reinvigorating the drug discovery process (Sun et al. 2015). In comparison with the derived components of terrestrial sources, MMNPs' (Marine Microbial Natural Products) biosynthetic elements have their own unique aspects, such as halogenase and other types of novel enzymes. Unnatural products can be yielded by heterogonous expression of biosynthetic genes from diverse origins through combinatorial biosynthesis (Xiong et al. 2013).

Despite lot of success in combinatorial biosynthesis approach, the motivation for this field was high since the researcher had anticipated a variety of unnatural products with altered structures and derivatization of existing known compounds (Subramani and Aalbersberg 2012; Kim et al. 2015), although the productivity of the engineered compound is somewhat lower than that of the parent marine microbial natural products. This ultimately delays the analysis in laboratory settings or the clinical testing for the commercial utilization of derived compound with improved pharmacokinetic properties (Liu et al. 2017).

6.4 Molecular or Nonculture-Based Approaches for Cultivating Marine Actinomycetes

Culture-independent molecular approaches represent the existence of native actinomycetes in marine habitats (Jensen et al. 2005; Das et al. 2006). The culture-independent and metagenomic techniques are helpful tools to determine the uncultured microbial diversity and the biochemical synthesis pathways of compounds. Sequence-guided metagenomic investigations of the marine environment

through technological advances have proven to be useful in identifying new species of actinomycetes from various marine habitats. These methods basically employ direct extraction of nucleic acids followed by the amplification of DNA or cDNA from RNA by PCR, and with the subsequent analysis of the diversity of the amplified molecules, the amplified nucleic acids can be cloned and sequenced. The obtained sequences can be compared with already existing ones for the identification of new actinomycetes (Stach et al. 2003; Riedlinger et al. 2004). At present, no single technique or tool is employed to assess actinomycetes diversity. Therefore, a combination of techniques from microbiology, molecular biology, and geochemical techniques or microsensors must be used to obtain a better understanding of actinomycetes diversity from the unexplored sources that provide an important platform for obtaining biologically active compounds (Subramani and Aalbersberg 2012; Hughes and Karlén 2014).

Pyrosequencing is a next-generation sequencing methodology used for de novo microbial genome sequencing through metagenomics analysis of microbial communities. Next-generation sequencing technologies have also been utilized in uncovering the exceptional levels of determining bacterial diversity in various habitats like grassland and forest soils, marine invertebrates, and Arctic soils (Sunagawa et al. 2010; Chu et al. 2010; Nacke et al. 2011).

Another important parameter in natural product discovery is understanding the concepts of biogenesis of active molecule in the producer strains. Such studies can be revolutionized by mining the data and connecting it to the predicted biosynthetic gene clusters from the information obtained through whole genome architecture of respective strain (Weber and Kim 2016). The concept of genome mining has expanded to various marine *Actinobacteria* for getting insight into the mechanism of bioactive molecule synthesis. The concept of classical genome mining has been transitioned to culture-independent genome mining and comparative genome mining (Ziemert et al. 2014). Concurrently in support of genome mining approach, Duncan et al. (2015a, b) have isolated and characterized a non-ribosomal peptide, Ritimycin A, leading to the new avenues in natural product discovery from marine actinomycetes. Modulation and engineering in the precursor pathways of bioactive molecule lead to their enhancement and variation in natural products (Dhakal et al. 2016). Such type of manipulation in precursors can be attained chemically or biologically for structural diversification of compounds obtained from rare marine actinomycetes. Gene-based screening when combined with homology-based searches and phylogenetic analyses can prove as a powerful approach to predict the production of new secondary metabolites present within isolates or environments. The added advantage of this is to avoid repetition of compounds that are already known or to give new information about strains that comes within our requirements. Gene-guided screening plays a very important role to assess the secondary metabolite biosynthetic potential when there is lack of fully assembled pathways or genome sequences. The prediction of new secondary metabolites or known bioactive compounds in the newly identified strains can improve the process of novel compound discovery by prioritizing the strains for

chemical and fermentation studies (Subramani and Aalbersberg 2012; Xiong et al. 2013). Sometimes a large proportion of silent biosynthetic gene clusters in actinomycetes are not expressed for secondary metabolite production under normal fermentation condition. Accordingly, research has been done to de-silence the gene clusters involved in natural products production. Traditional approaches like cocultivation and changes in fermentation conditions (pH, temperature, and media composition) trigger significant changes in microbial metabolome (Marmann et al. 2014). Such changes in fermentation condition are called "one strain, many compounds" approach which is used as an effective strategy to express cryptic gene-associated metabolic pathways (Abdelmohsen et al. 2015).

More recently, the integrative approach based on bioactivity studies, taxonomic studies, and metabolite profiling for the characterization of rare marine actinomycetes and the biological function of metabolites produced by them has been elucidated (Betancur et al. 2017).

7 Significance of Marine Actinomycetes in Antibacterial Drug Development

The immense biotechnological potential of terrestrial actinomycetes has been extensively studied over the last few decades. This area of research has now become saturated and not many great results are expected anymore as thought by many researchers. Hence there is a need to give attention on a new source of chemical and genetic information. Sediments, oceans, or seawater provide a huge opportunity for exploration and isolation of new actinomycetes strains which are different from terrestrial bacteria both genetically and in chemical composition. They produce different secondary metabolites which can provide a huge repertoire of compounds having the potential to become antibacterial drugs (Williams 2009). In addition to various opportunities provided by marine actinomycetes to generate new drugs, some of the challenges for research still exist, which needs to be dealt with. First, all oceans are huge repertoires and sample collection from deep sea can be challenging. Second, somehow the samples are collected, another issue is low culturability of marine actinomycetes. Exploitation of the marine actinomycetes is a difficult task, but that does not mean to lose our efforts, since they are a promising source of new chemical drugs with altered targets playing an important role in modern medicine (Malve 2016). Modern pharmaceutical industries have hit a roadblock in the form of drug resistance. Various researches are ongoing to find new antibiotics which can be used in the combat against drug-resistant pathogens. In light of this, marine actinomycetes lead to a new direction to find new chemical entities which can help us to tackle the problem associated with drug resistance (Xiong et al. 2013; Böhringer et al. 2017).

8 Recent Development of New Chemical Entities Contributed by Actinomycetes

In the 1930s, the discovery of the sulfonamides and β-lactam has saved countless lives. Such breakthrough findings have initiated a 40-year "golden age" of antibiotic research. There are various approaches for discovering new antibiotic lead molecules, of which chemical modification of known antibiotics or already existing antibiotics is most widely used. A great number of compounds have been discovered between the 1950s and 1960s belonging to same antibiotic chemical classes. Even though the discovery of entirely new classes of antibacterial compounds is urgently required, the chemical alterations of antibiotics in already existing classes are still most commonly used to explore new antibiotics, resulting in a huge number of compounds in the discovery and clinical pipeline belonging to known classes. However, from the early 1970s to 1999, the initiative antibiotic pipeline dried up. All newly launched antibiotics were analogues of existing drugs except topical antibiotic Mupirocin, which was launched in 1985 (Butler et al. 2013).

At present, the emergence of antibiotic-resistant bacterial pathogens has become a serious alarm in clinical and healthcare settings that prompted the need of discovery of novel antibacterial drugs with new modes of action. Currently the most commonly employed approaches for the discovery of new antibiotics are:

1. Development of new compounds that belong to entirely differently chemical class that acts on new targets.
2. Development of new compounds that belong to new chemical classes and target on already established targets.
3. Development of compounds that belong to existing class of compounds that act on established target.

However, the process of discovering new classes of chemical compound is still difficult. Therefore, the chemical alterations of known antibiotics are still being pursued by many companies developing new antibiotics. There are usually two major stages in the process of developing a new drug: **(a)** the initial phase of discovery and preclinical testing in microorganisms or animals (safety pharmacology and in vitro and in vivo tests to determine efficacy) followed by regulatory approval and **(b)** the clinical trial stage (involving trials on humans) (Butler et al. 2013).

In 2010, America's Infectious Disease Society launched the 10 X 20 initiative to develop ten new systemically administered, safe, and effective antibiotics by 2020. This initiative focuses on the development of new antibiotics to treat problem groups of Gram-negative bacteria that are more challenging than Gram-positive bacteria because of the occurrence of an outer membrane permeability barrier, antibiotic target-modifying enzymes, and multiple efflux pumps. Some of the natural products obtained from actinomycetes are listed in Tables 2, 3 and 4.

Table 2 Some of the chemical classes of antibiotics produced by the members of *Streptomyces*

S. no.	Antibiotic	Producing organism	Chemical class
1	Chloramphenicol	*S. venezuelae*	N-Dichloroacyl phenyl propanoid
2	Chlortetracycline	*S. aureofaciens*	Tetracycline
3	Clavulanic acid	*S. clavuligerus*	β-Lactam
4	Daptomycin	*S. roseosporus*	Peptide
5	Erythromycin	*S. erythreus*	Macrolide
6	Gentamicin	*Micromonospora* sp.	Aminoglycoside
7	Kanamycin	*S. kanamyceticus*	Aminoglycoside
8	Lincomycin	*S. lincolnensis*	Sugar amide
9	Neomycin	*S. fradiae*	Aminoglycoside
10	Oxytetracycline	*S. rimosus*	Tetracycline
11	Streptomycin	*S. griseus*	Aminoglycoside

Table 3 Antibiotics of natural products (NP) or synthetic (S) origin launched since 2013 with antibiotic class, activity against Gram-positive and/or Gram-negative bacteria, lead source, and NP-lead source organism (Butler et al. 2013)

Year approved	Drug name	Class	Bacteria type	Lead source	NP-lead source organism
2001	Telithromycin	Macrolide	G +ve/G -ve	NP-derived	Actinomycetes
2002	Biapenem	Carbapenem	G +ve/G –ve	NP-derived	Actinomycetes
2002	Ertapenem	Carbapenem	G +ve/G –ve	NP-derived	Actinomycetes
2003	Daptomycin	Lipopeptide	G +ve	NP	Actinomycetes
2005	Doripenem	Carbapenem	G +ve/G –ve	NP-derived	Actinomycetes
2005	Tigecycline	Tetracycline	G +ve/G –ve	NP-derived	Actinomycetes
2009	Tebipenem pivoxil	Carbapenem	G +ve/G –ve	NP-derived	Actinomycetes
2009	Telavancin	Glycopeptide	G +ve	NP-derived	Actinomycetes

Table 4 Recent reports on the antibacterial products from actinomycetes

Compounds/extracts with antibacterial activity	Activity against	Producing organism and their source of isolation	References
Angiomycines D X 14881E	*Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*	*Streptomyces* sp. P294	Su et al. (2016)
Macrolides, terpenoids quinolones, and phenolics like 2,6-dibutyl phenol, alkaloid compound-1 H and quinolone compound-1,4-benzendiol	Antibacterial	*Streptomyces bacillaris* strain RAM25C4, Rameshwaram coastal region, Tamil Nadu	Wahaab and Subramaniam (2018)

(continued)

Table 4 (continued)

Compounds/extracts with antibacterial activity	Activity against	Producing organism and their source of isolation	References
1-Acetyl-b-carboline, indole-3-carbaldehyde, 3-hydroxy-acetaldehyde, brevianamide F	MRSA	*Streptomyces* sp. LGM B491, medicinal plant *Vochysia divergens*	Gos et al. (2017)
Actinomycin C1, C2, and C3 and actinophenol	M-tb strains and MRSA	*Streptomyces pratensis*	Shah et al. (2017)
Actinokineosin	*Micrococcus luteus*	*Actinokinespora spheciospongia*	Takasaka et al. (2017)
2-Amino-3,4-dihydroxy-5 methoxybenzamide, 4-hydroxy-3-methoxybenzoic acid, n-acetyl tyramine	Antimicrobial and antioxidant	Endophytic *Streptomyces* sp. YIM 67086	
Micro monohalamines A and B (diterpenoid)	MRSA	*Micromonospora* sp. from marine ascidian sample	Zhang et al. (2016)
3-Ethyl,3-methyl heptanes and diisodecyl ether	Antibacterial	Marine soil of Tamil Nadu *Streptomyces coelicolor* SU6 (JQ828940)	Nandhini and Selvam (2013)
Ethyl acetate extract	MDRSA	Coastal region of Andhra Pradesh	Rao (2012)
Crude extract	Activity against clinical pathogens	*Streptomyces* sp. ABTR112, Amirthi forest, Tamil Nadu	Naine et al. (2012)
Volatile compounds in crude extract	*Bacillus cereus*	VIT University, Vellore, Tamil Nadu	Naine et al. (2015)
	E.coli	*Streptomyces werraensis*	
	S. aureus		
	P. aeruginosa		
	S. typhi		
Different solvent extracts	MRSA, *Bacillus cereus*	Air (scientific and technological equipment building), Thailand	Lertcanawanichakul et al. (2015)
Crude extract	*S. epidermidis*	YXT131, tea cultivars Yunkang-10	Wei et al. (2018)
	Shigella flexneri		
	E. coli		
Fermentation broth extract	Antimicrobial	*Streptomyces* sp. ZLSD-24, bulrush rhizosphere, Zhalong Wetland, China	Li et al. (2018)

9 Role of Anti-pathogenic Compounds in Combating Drug-Resistant Bacteria

The problem of emergence and spread of drug-resistant pathogens has resulted in narrow treatment options and ultimately reduces the clinical efficacy of all known available drugs. The inhibition of pathogenicity and virulence of bacterial pathogens without affecting their survival by targeting the key regulatory system is a promising strategy to combat drug resistance. One of the anti-pathogenic drug targets is quorum sensing (QS), a global bacterial cell-to-cell communication system (Fuqua et al. 2001; Rumbaugh and Kaufmann 2012). Another important virulence factor related to pathogenicity of bacteria is the formation of biofilms that is also regulated through quorum sensing to a large extent.

Bacterial biofilms comprised sessile cells that are embedded in an extracellular polymeric material. At present, it has been recognized that the number of bacterial pathogens makes biofilm during colonization on host tissues and disease development. The development of biofilm is based on QS-mediated swarming and swimming motility as well as production of extracellular polymeric substances (Pompilio et al. 2008). In biofilm mode of growth, bacterial cells exhibit high level of drug tolerance (Alhede et al. 2009). Therefore, it becomes difficult to tackle biofilm-mediated bacterial infection inside the host. The compounds that interfere with the cell-to-cell communication system between bacteria, thereby suppressing the associated virulence factors, are termed as QS inhibitors (Brackman et al. 2012). Such QS inhibitors were first reported in a marine algae *Delisea pulchra* as halogenated furanones but was found to be unstable and toxic (Hentzer and Givskov 2003). However, scientists in the recent past demonstrated the occurrence of various natural products from both microorganisms and medicinal plants that can attenuate the virulence factors and formation of biofilms by targeting the quorum sensing. Such compounds are rising in progressive manner (Husain et al. 2015; Kalia 2015). Actinomycetes that produce anti-QS compounds have been isolated from the marine ecosystem tested against the reporter strain *Chromobacterium violaceum.* The main active compound has also been isolated from this actinomycete (Miao et al. 2017). Screening of marine actinomycetes provides a new platform for generating antibiofilm and anti-QS agents that may have therapeutic efficacy in both medical and environmental purposes. A proposed strategy for obtaining new anti-infective compounds from diverse sources of actinomycetes is represented in Fig. 2.

10 Conclusion and Future Prospects

Actinomycetes are ubiquitous organisms in both terrestrial and aquatic ecosystems. Soil and marine actinomycetes are diverse and have great capacity to synthesize antibacterial compounds and other products relevant for agriculture, environment, as well as human health and medicine. Advancement in molecular techniques helps in deeper understanding on molecular aspects of actinomycetes and improved screening methods allow the development of novel antimicrobial compounds that are capable of filling dry pipeline of novel antibacterial compounds in drug development for combatting the problem of multi

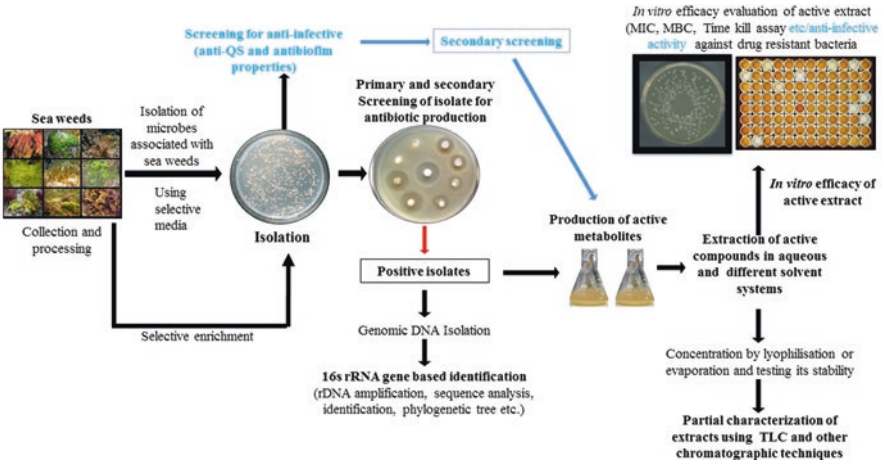

Fig. 2 Strategy to obtain new anti-infective compounds from diverse sources of actinomycetes

drug resistance. Furthermore, the information derived from rapidly accessing the genome of many microbial strains can provide new routes to natural product discovery as well as make more effective traditional, bioassay-based, and molecular screening efforts. However, no single technology will represent the magic bullet for discovery of antibiotics, but only the pains taking integration of a multidisciplinary teamwork with profound knowledge of chemistry, microbiology, molecular biology, and bioinformatics will ultimately lead to new antibacterial agents of medical significance and commercial success (Hughes and Karlén 2014). While screening for new antibacterial lead molecule from actinomycetes, new anti-infective compounds, such as anti-virulence, antibiofilm, and anti-QS, should be integrated to make effective use of screening program. Such anti-infective compounds might be very effective in future as combinational therapy for combating infections of MDR (Multi Drug Resistance) strains. Future research still needs the finding of structurally diverse and unique compounds with desired biological activities along with simultaneous application of recent approaches for the isolation and exploitation to recover more cultivated and as-yet-uncultivated huge microbial diversity (Gibbons and Gilbert 2015).

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Are Ancient Remedies the New Answer to Fighting Infections?

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Abstract

Although modern medicine has made great strides over the past decades, there still exists a struggle in the fight against microbial infections. As microbes continue to develop antimicrobial resistance, it is imperative that new treatment options be developed to overcome this hurdle. Bacteria can develop resistance to current antimicrobial agents through several methods, some requiring cell-to-cell contact through conjugation and other mechanisms that require no contact at all. As current treatments become less toxic to microbes, the need for new treatments is intensified. Throughout the history of human existence, plant and animal products have been used for various infectious diseases. As these products have been further analyzed, the phytochemicals, or active molecules involved, have begun to be uncovered. Discovering the mechanisms of action of the active molecules in these ancient remedies may lead to the development of new drugs to help fight infection.

Keywords

Antibiotic resistance · Alternative antibiotic · Anti-infective · Ancient remedy

1 Introduction

Throughout history, humans have fought infections caused by viruses, bacteria, parasites, and fungi that cause colds, sexually transmitted diseases, digestive disorders, and many more. Today, these diseases are typically treated with synthetic drugs, including various antimicrobials, but before modern medications were

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available, humans turned to their environment to fight off infections. These remedies were often plant-based, including leaves, flowers, stems, barks, roots, as well as animal-based substances, such as honey, venom, and mucus. Civilizations from all regions of the world expended their environment to fight infections throughout history. There are records dating back thousands of years within various cultures and regions around the world describing the use of environment-based medicines. For example, China, a country well-known for its ancient remedies, has a medicinal system approximately twenty-three centuries old (Chen 2001). Chinese medicine has focused on the balance of Yin (passive) and Yang (active); however, when this balance is disrupted, it is believed to cause illness (Chen 2001). One of the earliest archives of medicine includes the use of orally and externally administered medicinal wine to ward off disease, dating as far back as 2500 BC during the Shang dynasty and Zhou dynasty (Xia 2013). In fact, it is believed that the first record of using medicinal plants is found in the text of *The Divine Husbandman's Classic of Materia Medica (ShenNong Ben Cao Jing)*, written in the late Eastern Han Dynasty (25–220 AD) (Jaiswal et al. 2016).

China isn't the only country with a rich background in ancient remedies. India, for example, also has a long history of using natural remedies to treat illness, with the first record of using plants as medicine dating back to between 6000 and 4000 BCE (Pan et al. 2014). Ancient Indian remedies are based on approximately 25,000 plant formulations that have been used for almost 30 different types of human diseases (Sharma et al. 2007). Africa, having more than 5400 known species of plants used in traditional medicine, is another region of the world with a strong history of using environmental resources as medicine (Van Wyk 2015). For example, a well-known traditional plant dating back to prehistoric times, centella (*C. asiatica*), was utilized for wound healing, tuberculosis, lupus, inflammation, syphilis, diarrhea, and many more (van Wyk 2008). In 1500 BC, traditional medicinal practices were established in Mesoamerica and continued until the 1500s when the Spaniards arrived (Pena 1999). Two documents, the *Badianus codex* and the *Sahagun codices*, provide documentation of Aztec medicine before Columbian civilization (Guerra 1966). Native American medicinal practices frequently varied from tribe to tribe, but in many instances, healing required both rituals and botanical substances, ranging from over 200 different species of plants (Hershman and Campion 1985). However, medicinal practices quickly changed to treat infectious diseases like smallpox, influenza, measles, and other infections of European origin post-Columbus arrival. The *Old English Herbarium*, *Bald's Leechbook*, and *Lacnunga* are some of the earliest recordings of medicine in Europe, dating back to the ninth century. *Bald's Leechbook*, the most prominent of the three, was written around 950 AD. The chronicles examined remedies for various human diseases starting at the head and moving down toward the toes (Watkins et al. 2011). This series of records was composed of three books, the first two describing diseases of both external and internal complaints and the third providing a list of plant names and instructions (Watkins et al. 2011).

Within the last 100 years, modern medicine has implemented new ways of warding off infection through the use of medications, including antimicrobials

(Yazdankhah et al. 2013). Near the start of the antibiotic era, the Surgeon General of the United States stated, "It is time to close the book on infectious diseases, and declare the war against pestilence won" (Spellberg and Taylor-Blake 2013). Assuming the fight against infection was over, the emphasis for discovery of new antimicrobial agents was tapered. However, the capacity for pathogens to evolve resistance to antimicrobials was soon apparent. In a 2011 national survey, 60% of infectious disease specialists had seen a microbial infection that was resistant to the first line of antibiotics within the last year, further proving the fight against microbial infections is far from over (Ventola 2015). As antimicrobial resistance continues to rise, the need for new methods of fighting infection is becoming abundantly clear. Interestingly, the answer to much-needed new forms of antimicrobial drugs might be found by looking at traditional medicine. In this review, we discuss the need for new antimicrobial agents and explore the potential of the natural remedies that have been used in different regions of the world throughout history.

2 Antimicrobial Resistance

Antimicrobial-resistant infections are an increasing problem within the medical field, and one of the major causes of mortality and morbidity (Martinez and Baquero 2014). As antimicrobial drugs continue to be overly prescribed and abused by clinicians, patients, and livestock farmers, bacteria have been pressured by their environment for survival (Ventola 2015; Michael et al. 2015; Shay and Freifeld 1999; Fiore et al. 2017; Grigoryan et al. 2007). The majority of antibiotics in use today are secondary metabolites derived from actinomycete bacteria (Mak et al. 2014) and target essential cell processes, such as protein synthesis, cell wall synthesis, and DNA synthesis (Mahajan and Balachandran 2012). However, bacteria can become resistant to these metabolites through various mechanisms. One way bacteria resist killing by antimicrobials is by pumping the drug out of the cell before it can act. Multidrug resistance efflux pumps are frequently found in clinical strains of bacteria (Alcalde-Rico et al. 2016; Sun et al. 2014). Efflux pumps provide bacteria the capability to extrude antimicrobials, as well as heavy metals, organic pollutants, quorum sensing signals, and other substances (Blanco et al. 2016). For example, many strains of *Pseudomonas aeruginosa* express an efflux pump, PA1874-1877, which can be overly expressed during infection and result in biofilm-specific resistance to antimicrobials (Alcalde-Rico et al. 2016; Zhang and Mah 2008).

Bacteria can also develop resistance to antimicrobials through mutations or the acquisition of new genes that confer resistance (Martinez and Baquero 2014). Mutations in the genes that code for targets, transporters, or proteins that pre-antibiotics use for either activation or entrance into the targeted bacteria can lead to resistance (Baquero et al. 2009). The acquisition of new resistance determinants can occur through horizontal gene transfer in multiple ways. Through conjugation, plasmids, integrons, transposons, genetic islands, and integrative conjugative elements can carry genetic information among bacteria. It has been hypothesized that these resistance genes originate from either environmental microbiota or commensal

bacteria (Martinez and Baquero 2014; Sommer et al. 2009; Davies 1997). Frequently, the genetic information on these mobile genetic elements can insert themselves into the bacterial chromosomal DNA, which can be reversible or irreversible (Brown-Jaque et al. 2015). Without cell-cell contact, phages and genetic transfer agents can integrate genetic information into bacteria as well (Brown-Jaque et al. 2015).

Community-based resistance in the form of a biofilm promotes bacterial tolerance against antimicrobial agents. It is accepted that the majority of bacteria exist in a biofilm both in the environment and within the human body (Costerton et al. 1995). Biofilms are composed of a variety of microbes living in close proximity to each other and encased in a matrix that includes extracellular DNA (eDNA), exopolysaccharides, proteins, and various lipids (Donlan 2002). This matrix creates a barrier that can be exceptionally difficult for antimicrobial agents to penetrate and reach the bacteria within the biofilm. The bacteria in the biofilm generally live in an altered metabolic state in order to survive the low oxygen and nutrient-deplete environment (Penesyan et al. 2015). This slowed metabolism adversely impacts the capability of antimicrobials to enter the bacteria and work efficiently (Costerton et al. 1995). Persister cells, bacteria deep within the biofilm that have a slow or nongrowing phenotype, are highly resistant to antimicrobials (Conlon et al. 2015). Due to the mechanism of antibiotics, such as β-lactams that work by inhibiting growth factors, persister cells will oftentimes survive antimicrobial treatment, while the remaining bacterial population is killed off. This phenomenon can lead to recurring infections initiated by bacteria that have strategies for tolerating antimicrobials, producing further infections that are even more problematic (Cho et al. 2014). In conclusion, the vast variety and number of methods that bacteria can utilize to become resistant to antimicrobials further emphasize the need for new antimicrobial treatment agents.

3 Herbal Remedies

3.1 Africa

Africa, a continent of 54 countries, has one of the shortest life expectancies in the world at only 56 years (Kuate Defo 2014). One of the most virulent diseases in Africa is HIV/AIDS, with 5% of adults infected in Sub-Saharan Africa and 5.7% of adults in North Africa and the Middle East (Kilmarx 2009). Diarrheal diseases are another common infection seen in Africa, killing millions of children each year (Levine et al. 2013). Throughout the ages, people in Africa began utilizing various parts of plants and shrubs to help alleviate these diseases. Plants from the families of Guttiferae (Kuete et al. 2011), Apiaceae (El-Haci et al. 2014), Crassulaceae (Akinpelu 2000), Melastomataceae (Baba and Onanuga 2011), Bignoniaceae (Mbosso Teinkela et al. 2016), Fabaceae (Koffuor et al. 2014), Loranthaceae (Deeni and Sadiq 2002), and Balanophoraceae (Ohiri and Uzodinma 2000) were often used to treat diarrheal diseases and dysentery, and leaves from *Sutherlandia frutescens* (family: Fabaceae) were used to control HIV/AIDS (Koffuor et al. 2014). Many of

these families also include plants that were used to treat various infections and signs of disease, such as tuberculosis (Baba and Onanuga 2011), colds (Sonibare et al. 2016; Selles et al. 2013), coughs (El-Haci et al. 2014; Akinpelu 2000; Baba and Onanuga 2011), headaches (Akinpelu 2000; Bisignano et al. 2000), and chest pain (Ohiri and Uzodinma 2000; Sonibare et al. 2016; Viljoen et al. 2003).

Interestingly, the majority of the plants that have been studied from Africa are either shrubs or flowering plants. Different elements of these plants have been tested for active molecules or phytochemicals to determine their antimicrobial mechanism(s) of action (MOA). Some of the most common phytochemicals found in this region include tannins (Baba and Onanuga 2011; Koffuor et al. 2014; Chah et al. 2000), saponins (Baba and Onanuga 2011; Koffuor et al. 2014; Chah et al. 2000), and alkaloids (Baba and Onanuga 2011; Chah et al. 2000; Lohombo-Ekomba et al. 2004). It is hypothesized that tannins inhibit biofilm formation, as they are bacteriostatic, and can damage bacterial membranes and negatively impact matrix production (Trentin et al. 2013). Saponins are molecules that become "soaplike" in water. Their antimicrobial MOA is thought to be disruption of the bacterial cell membrane, which leads to cell lysis (Arabski et al. 2012). The proposed MOAs of alkaloids, on the other hand, are to inhibit cell division and/or nucleic acid synthesis or to disturb the Z-ring at the site of division (Cushnie et al. 2014; Lutkenhaus and Addinall 1997). Table 1 details 29 different species of plants, with the majority native to central, western, or southern Africa.

Along with plants, the mucus from *Achatina fulica* (giant African land snail) (Pitt et al. 2015), venom from *Androctonus amoreuxi* (African fat-tailed scorpion) (Almaaytah et al. 2012; Du et al. 2015), and propolis from bees (Suleman et al. 2015) have also been exploited in Africa for their antimicrobial properties.

3.2 Asia

For the context of this review, Asia includes Russia, China, India, the Middle East, Japan, and North and South Korea. Table 2 details 44 species of plants, royal jelly from honeybees (Fratini et al. 2016), and scorpion venom (Ahmed et al. 2012) that have been used medicinally as antimicrobials within this region of the world. Among plant-based medicines, herbs (Karuppiah and Rajaram 2012; Reiter et al. 2017; Ooi et al. 2006; Hong et al. 2014), mushrooms (Chowdhury et al. 2015), flowers (Hong et al. 2014; Ozusaglam et al. 2013; Yang et al. 2012; Sun et al. 2017), and fruits/berries (Shukla et al. 2016; Li et al. 2012; Paudel et al. 2014; Li et al. 2014) have been at the forefront. *Bletilla ochracea* (Chinese butterfly orchid), found throughout Vietnam and China, is particularly interesting due to the orchid's history of being used to treat vampirism. It is fascinating that in parts of India and Thailand, *Heterometrus xanthopus* (giant forest scorpion) venom has been utilized as an antimicrobial agent. Meanwhile, venom from the African fat-tailed scorpion in North Africa exhibits similar antimicrobial properties. The majority of agents tested for their antimicrobial properties within this region have also served as treatments for diabetes (Ooi et al. 2006; Shukla et al. 2016; Paudel et al. 2014; Zeng et al. 2011;

Table 1 African remedies

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals (if known)	Uses	References
Plant based						
1.	Subtropical Africa	*Albertisia villosa* Root, bark	Flowering plant (Menispermaceae)	Alkaloids	Malaria, other infectious diseases, dandruff control, antimicrobial against *K. pneumoniae*, *B. subtilis*, *C. diphtheria*, *P. aeruginosa*, *S. typhi*, *S. pyogenes*, *T. longiformis*, *C. albicans*, *A. flavis*, *M. canis*, *F. solani*, *E.* spp.	Lohombo-Ekomba et al. (2004)
2.	Tropical Forest Africa	*Allanblackia floribunda Oliver* Root, bark	Tree or shrub (Guttiferae)	Phenolics	Toothache, dysentery, anticancer, antimicrobial against *M. smegmatis*, *M. tuberculosis*, *T. rubrum*	Kuete et al. (2011)
3.	Northern Africa	*Ammodaucus leucotrichus* Fruit	Flowering plant (Apiaceae)	Perilla aldehyde, monoterpenes	Stomach pain, indigestion, diarrhea, vomiting, intestinal worms, allergy symptoms, coughing, antimicrobial against *E. coli*, *S. aureus*, *E. cloacae*, *B. cereus*, *S. typhimurium*, *F. oxysporum*, *A. flavus*	El-Haci et al. (2014)
4.	Central and Southern Africa	*Anacardium occidentale* Bark	"Cashew tree" (Anacardiaceae)	–	Antibacterial against *B. cereus*, *B. stearothermophilus*, *B. subtilis*, *C. sporogenes*, *C. pyogenes*, *K. pneumoniae*, *M. luteus*, *P. vulgaris*, *P. aeruginosa*, *P. fluorescens*, *S. dysenteriae*, *S. aureus*, *S. faecalis*	Akinpelu (2001)
5.	Northern Africa	*Anacyclus pyrethrum* Floral bud	"Mount Atlas daisy" (Asteraceae)	Sesquiterpenes	Common cold, toothache, pyorrhea, antimicrobial against *S. aureus*, *C. albicans*	Selles et al. (2013)
6.	Western and Tropical Africa	*Bridelia ferruginea* Bark extracts	Woody shrub (Euphorbiaceae)	–	Gonorrhea infections, candida oral thrush, and antimicrobial against *C. albicans*, *E. coli*, *P. mirabilis*, *P. vulgaris*, *Klebsiella* sp.	Irobi et al. (1994)

7.	Tropical and Subtropical Africa	*Bryophyllum pinnatum* Leaves	"Miracle leaf plant" (Crassulaceae)	–	Ear infections, cough, dysentery, headaches, and antimicrobial against *B. subtilis*, *E. coli*, *P. vulgaris*, *S. dysenteriae*, *S. aureus*	Akinpelu (2000)
8.	Tropical Africa	*Carica papaya* Fruit	Fruit tree (Caricaceae)	–	Eczema, dermatitis, psoriasis, antimicrobial against *E. faecalis*, *S. saprophyticus*, *C. albicans*	Mbosso Teinkela et al. (2016)
9.	South Africa	*Carpobrotus muirii* Whole plant	Flowering plant (Aizoaceae)	–	Antimicrobial against *S. aureus and M. smegmatis*	Springfield et al. (2003)
10.	South Africa	*Carpobrotus quadrifidus* Whole plant	Flowering plant (Aizoaceae)	–	Antimicrobial against *S. aureus*, *M. smegmatis*	Springfield et al. (2003)
11.	Semiarid savanna regions of Africa	*Combretum apiculatum* Leaves	Flowering plant (Combretaceae)	Bibenzyls (including combretastatin)	Abdominal disorders, conjunctivitis, infertility, and venereal diseases, antimicrobial against *S. aureus*, *C. albicans*	Katerere et al. (2012)
12.	Tropical Africa	*Combretum collinum* Leaves	Flowering plant (Combretaceae)	Phenanthrenes	Antimicrobial against *M. fortuitum*, *S. aureus*	Katerere et al. (2012)
13.	Eastern and Southern Africa	*Combretum hereroense* Fruit	Shrub (Combretaceae)	Phenanthrenes	Abdominal disorders, conjunctivitis, infertility, and venereal diseases, antimicrobial against *M. fortuitum*, *S. aureus*	Katerere et al. (2012)
14.	Western Africa	*Costus lucanusianus* Herb	Herbaceous plant (Costaceae)	Glycosides, tannins, saponins	Anti-abortive, weak antimicrobial against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*	Baba and Onanuga (2011)

(continued)

Table 1 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals (if known)	Uses	References
15.	Western Africa	*Dissotis rotundifolia* Herb	"Pink Lady" (Melastomataceae)	Alkaloids, glycosides, saponins	Rheumatism, painful swellings, stomachache, diarrhea, cough, conjunctivitis, venereal diseases, bilharzias (East Africa), weak antimicrobial against *B. subtilis*, *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae*	Baba and Onanuga (2011)
16.	Southern Africa	*Ficus bubu* Stem bark, leaves, latex	Terrestrial tree (Moraceae)	–	Anticancer, antimicrobial against *C. albicans*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *S. saprophyticus*	Mbosso Teinkela et al. (2016)
17.	Tropical Africa	*Mitracarpus scaber* Whole plant	Flowering plant (Rubiaceae)	–	Headaches, toothache, amenorrhea, dyspepsia, hepatic disease, leprosy, antimicrobial against *S. aureus*, *C. albicans*	Bisignano et al. (2000)
18.	South Africa	*Osmitopsis asteriscoides* Essential oil	Flowering plant (Asteraceae)	Sesquiterpene, lactones	Chest complaints, cuts, swelling, antimicrobial against *S. aureus*, *B. subtilis*, *C. neoformans*, *P. aeruginosa*	Viljoen et al. (2003)
19.	Western Africa	*Pavetta crassipes* Leaves	Low shrub (Rubiaceae)	PcF3.4	Antimicrobial against *E. coli*, *C. albicans*, *T. cruzi*, *L. infantum*, *T. brucei*, *P. falciparum*	Balde et al. (2010)
20.	Africa	*Paullinia pinnata* Saponin	"African woody vine" (Sapindaceae)	Methylinositol, steroidal terpenoids, oleanane, triterpenoids	Malaria, erectile dysfunction, antimicrobial against *S. typhi*, *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *E. faecalis*, *E. aeroginese*, *P. smartii*, *C. albicans*, *C. guilliermondii*, *C. lusitaniae*, *C. parapsilosis*, *T. equinum*, *M. audouinii*, *T. mentagrophytes*, *M. gypseum*, *E. floccosum*	Lunga et al. (2014)
21.	Tropical and Subtropical Africa	*Phyllanthus muellerianus* Stem, bark	Evergreen shrub (Euphorbiaceae)	–	Antimicrobial against *C. sporogenes*, *S. pyogenes*	Brusotti et al. (2011)

22.	Tropical Africa	*Solanum torvum* Fruit	"Turkey berry shrub" (Solanaceae)	Steroidal alkaloids, tannins, saponins	Abscesses, jigger wounds, ringworm, athlete's foot, and antimicrobial against *A. pyogenes*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *S. typhimurium*, *S. aureus*, *S. pyogenes*, *A. niger*, *C. albicans*	Chah et al. (2000)
23.	Western and Central Africa	*Solenostemon monostachyus* Leaves	Herbaceous plant (Lamiaceae)	Glycosides, flavonoids, tannins, saponins, anthraquinones	Type 2 diabetes, tuberculosis, anti-abortive, antimicrobial against *B. subtilis*, *S. aureus*	Baba and Onanuga (2011)
24.	Tropical dry forests of Africa	*Spathodea campanulata* Flowers	"African tulip tree" (Bignoniaceae)	–	Kidney disease, animal poisoning, wound healing, herpes, diarrhea, anticancer, antimicrobial against *E. faecalis*, *K. pneumoniae*, *S. saprophyticus*, *C. albicans*	Mbosso Teinkela et al. (2016)
25.	Southern Africa	*Sutherlandia frutescens* Leaves	"Cancer bush" (Fabaceae)	Saponins, sterols, tannins, coumarins	HIV/AIDS, fever, flu, chicken pox, rheumatism, hemorrhoids, cancer, diarrhea, antimicrobial against *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *E. faecalis*, *S. aureus*, *S. pneumoniae*, *B. subtilis*, *C. albicans*	Koffuor et al. (2014)
26.	Northern Africa	*Tapinanthus dodoneifolius* Leaves	"Kauchi or African mistletoe" (Lorenthaceae)	–	Anticancer, anti-parasite, stomachache, diarrhea, dysentery, and antimicrobial against *Bacillus* sp., *E. coli*, *Proteus* sp., *Pseudomonas* sp., *Salmonella* sp., *A. tumefaciens*, *A. niger*, *Candida* sp.	Deeni and Sadiq (2002)
27.	Southern and Western Africa	*Thonningia sanguinea* Root extract	"Pineapple of the bush" (Balanophoraceae)	Phenolics	Hemorrhoids, anal lesions, bronchial asthma, dysentery, sore throat, skin diseases, antimicrobial against *C. albicans*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*	Ohiri and Uzodinma (2000)

(continued)

Table 1 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals (if known)	Uses	References
28.	Western Africa	*Vernonia cinerea* Whole plant	"Ironweed" (Asteraceae)	Phenolics, flavonoids	Common cold, asthma, bronchitis, fever, filariasis, blisters, vaginal discharge, and antimicrobial against *S. aureus*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris*, *C. albicans*	Sonibare et al. (2016)
29.	Western and Tropical Africa	*Xylopia parviflora* Volatile oil	"Striped African pepper" (Annonaceae)		Anticancer, anti-inflammatory, and antimicrobial against *S. aureus*, *E. coli*, *P. aeruginosa*, *E. faecalis*, *C. albicans*	Woguem et al. (2014)
Animal based:						
30.	Mild climates in Africa	*Achatina fulica* Mucus	"Giant African land snail" (Achatinidae)		Antibacterial against *S. aureus*	Pitt et al. (2015)
31.	Northern Africa	*Androctonus amoreuxi* Venom	"African fat-tailed scorpion" (Buthidae)	AamAP1, AamAP2(Almaaytah et al. 2012) AaeAP1, AaeAP2 (Du et al. 2015)	Antimicrobial against *S. aureus*, *E. coli*, *and C. albicans*	Almaaytah et al. (2012) and Du et al. (2015)
32.	South Africa	Propolis	Bee product	Flavonoids	Antibacterial against *S. aureus*	Suleman et al. (2015)

Table 2 Asian remedies

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/phytochemicals	Uses	References
Plant based						
1.	Manchuria and Korea	*Aconitum macrorhynchus* Whole plant	"Friar's cap" (Ranunculaceae)	–	Fever, pain, anti-inflammatory, sedation, antimicrobial against *S. aureus*	Paudel et al. (2014)
2.	Central Asia	*Allium sativum* Clove	"Garlic" (Liliaceae)	Allicin	Common colds, flu, heart diseases, fever, antimicrobial against *E. coli*, *Enterobacter* sp., *P. aeruginosa*, *Proteus* sp., *Klebsiella* sp., *S. aureus*, *Bacillus* sp., *S. pneumoniae*, *K. pneumoniae*, *A. baumannii*, *S. pyogenes*	Karuppiah and Rajaram (2012) and Reiter et al. (2017)
3.	Turkey and Western Asia	*Allium scabriflorum* Flower	Flowering bulb (Liliaceae)	–	Antimicrobial against *E. coli*, *S. aureus*, *L. monocytogenes*, *B. cereus*, *P. aeruginosa*, *M. luteus*, *S. sonnei*, *Y. entercolitica*, *C. albicans*, *S. cerevisae*, *S. enteritidis*	Ozusaglam et al. (2013)
4.	Turkey and Western Asia	*Allium tchihatschewii* Flower	Flowering bulb (Liliaceae)	–	Antimicrobial against *E. coli*, *S. aureus*, *L. monocytogenes*, *B. cereus*, *P. aeruginosa*, *M. luteus*, *S. sonnei*, *Y. entercolitica*, *C. albicans*, *S. cerevisae*, *S. enteritidis*	Ozusaglam et al. (2013)
5.	Northeast Russia and Arctic	*Alopecurus roshevitzianus* Whole plant	"Foxtail grass" (Poaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)

(continued)

Table 2 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/phytochemicals	Uses	References
6.	Northern Asia and Europe	*Aquilegia oxysepala* Whole plant	"Granny's bonnet" (Ranunculaceae)	Genkwanin, apigenin, magnoflorine, berberine	Gynopathy, antimicrobial against *S. aureus*	Yu et al. (2007)
7.	China	*Aristolochia delavayi* Aerial parts	"Birthwort" (Aristolochiaceae)	Dodecanal, heptanal, decanal, (E)-dec-2-enal, (E)-dodec-2-enal	Gout, eczema, arthritis, pain, digestive disorder, snakebite, antimicrobial against *P. aeruginosa*, *E. coli*, *E. aerogenes*, *P. stuartii*, *S. typhi*, *E. faecalis*, *S. aureus*, *C. albicans*, *C. glabrata*, *C. guilliermondii*, *C. neoformans*	Li et al. (2013a)
8.	China and Russia	*Artemisia lagocephala* Whole plant	Flowering plant (Compositae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
9.	Japan and Korea	*Astragalus schelichovii* Whole plant	Legume (Fabaceae)	–	Diabetes, nausea, vomiting, diarrhea, antimicrobial against *S. aureus*	Paudel et al. (2014)
10.	China and Southwest Asia	*Betula alba* Leaves	"European white birch" (Betulaceae)	Saponins, coumarins	HIV/AIDS, eczema, gout, rheumatism, edema, antimicrobial against *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *E. faecalis*, *S. aureus*, *S. pneumoniae*, *B. subtilis*, *C. albicans*	Koffuor et al. (2014)
11.	Vietnam and China	*Bletilla ochracea* Roots	"Chinese butterfly orchid" (Orchidaeceae)	Phenanthrenes	Vampirism disease, antimicrobial against *S. aureus*, *S. epidermidis*, *B. subtilis*	Yang et al. (2012)

12.	Western Himalayas and India	*Cedrus deodara* Pine needle	"Deodar cedar" (Pinaceae)	Phenols, flavonoids, tyrosinase	Fevers, diarrhea, dysentery, tuberculosis, diabetes, insomnia, antimicrobial against *E. coli*, *P. vulgaris*, *S. aureus*, *B. subtilis*, *B. cereus*, *S. thermophilus*, *S. lutea*, *P. citrinum*, *A. niger*, *A. flavus*, *C. krusei*	Zeng et al. (2011)
13.	Korea and Eastern Russia	*Chosenia arbutifolia* Whole plant	Flowering plant (Salicaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
14.	Southern China	*Cinnamomum cassia* Bark	"Chinese cinnamon" (Lauraceae)	–	Erectile dysfunction, diabetes, high blood pressure, digestive disorders, cramps, menstrual problems, cancer, abortion inducing, depression, antimicrobial against *S. aureus*, *E. coli*, *E. aerogenes*, *P. vulgaris*, *P. aeruginosa*, *V. cholerae*, *S. typhimurium*, *V. parahaemolyticus*, *Candida* spp., *Aspergillus* spp.	Ooi et al. (2006)
15.	Northern Russia and Arctic	*Cladonia stellaris* Whole plant	"Star reindeer lichen" (Cladoniaceae)	Usnic acid	Antimicrobial against *S. aureus*	Paudel et al. (2014)
16.	Southwestern Asia and Europe	*Daucus carota* Root juice	"Wild carrot" (Apiaceae)	Carotol, sabinene, B-caryophyllene, a-pinene	Eyesight, digestive disorders, contraceptive, tonsillitis, mouth infections, anthelmintic, antimicrobial against *L. monocytogenes*, *S. aureus*, *S. enteritidis*, *S. typhimurium*, *E. coli*, *S. dysenteriae*, *A. niger*	Ma et al. (2015)

(continued)

Table 2 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/phytochemicals	Uses	References
17.	Eastern Russia	*Dracocephalum palmatum* Whole plant	Flowering plant (Lamiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
18.	Asia	*Dryopteris fragrans* Whole plant	"Fragrant fern" (Aspidiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
19.	Southern China	*Ecdysanthera rosea* Stem		–	Sore throat, chronic nephritis, antimicrobial against *S. aureus*, *E. faecalis*, *P. smartii*, *C. albicans*, *C. neoformans*, *C. guilliermondii*	Song et al. (2014)
20.	China, Korea, and Japan	*Edgeworthia tomentosa* Flower	"Nakai flower" (Thymelaeaceae)	Monoterpenes, sesquiterpenes, terpenoids	Anti-inflammatory, analgesic, antimicrobial against *D. pneumonia*	Sun et al. (2017)
21.	Central Asia	*Ephedra sinica* Stem	"Ephedrae herba" (Ephedraceae)	Ephedrine, pseudoephedrine, N-methylephedrine, N-methylpseudoephedrine, norephedrine, norpseudoephedrine	Asthma, cough, diaphoretic, antimicrobial against *P. aeruginosa*, *MRSA*, *C. albicans*, *B. subtilis*, *S. aureus*, *E. coli*	Zang et al. (2013)
22.	Asia and Europe	*Galium verum* Whole plant	"Lady's bedstraw" (Rubiaceae)	Terpenoid, benzopyrone	Urinary disease, epilepsy, anti-inflammatory, antimicrobial against *S. aureus*	Paudel et al. (2014)
23.	India	*Grewia asiatica* Fruit	"Falsa" (Grewioideae)	Phenols, saponins, flavonoids, tannins	Anti-inflammatory, urological disorders, diabetes	Shukla et al. (2016)

24.	Bangladesh and Southern Asia	*Hypsizygus tessulatus* Mushroom	"Shimeji mushroom" (Lyophyllaceae)	Glucans, niacin, vitamin B, vitamin D, sterols, ergosterol, phenols, flavonoids, ascorbic acid	Immunity, weight loss, osteoporosis, antiaging, antimicrobial against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *C. albicans*, *S. cerevisiae*	Chowdhury et al. (2015)
25.	Southern Asia, India, and Bangladesh	*Impatiens balsamina* Stem	"Rose balsam" (Balsaminaceae)	Quinones, flavonoids, coumarins	Anticancer, rheumatoid arthritis, bruises, antimicrobial against *P. italicum*, *P. digitatum*, *A. niger*, *A. oryzae*, *S. cerevisiae*, *C. utilis*, *B. subtilis*, *S. aureus*, *E. coli*, *S. boydii*	Su et al. (2012)
26.	Bangladesh and Southern Asia	*Lentinula edodes* Mushroom	"Shiitake mushroom" (Marasmiaceae)	Lentinan, phenols, flavonoids, ascorbic acid	Longevity, gastric cancer, antimicrobial against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *C. albicans*, *S. cerevisiae*	Chowdhury et al. (2015)
27.	Eastern Asia and South-Central China	*Myrica rubra* Fruit	"Chinese bayberry" (Myricaceae)	–	Digestive disorders, cholera, cardiovascular disease, arsenic poisoning, antimicrobial against *S. marcescens*, *P. aeruginosa*, *S. aureus*, *E. coli*, *M. luteus*, *H. anomala*	Li et al. (2012)
28.	China, India, Japan, and Vietnam	*Ophiopogon japonicas* Stem, roots	"Mai Men Dong" (Asparagaceae)	–	Digestive disorders, cardiovascular disease, sedative, fever, cough, diabetes, antimicrobial against *S. aureus*, *C. neoformans*	Liang et al. (2012)

(continued)

Table 2 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/phytochemicals	Uses	References
29.	Northeastern Asia	*Oxytropis adamsiana* Whole plant	Flowering plant (Leguminosae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
30.	China	*Panax notoginseng* Flower	"Chinese ginseng" (Araliaceae)	Flavonoids	Arthritis, liver disease, cardiovascular disease, energy, anti-inflammatory, antimicrobial against *S. aureus*, *A. hydrophila*, *P. aeruginosa*	Hong et al. (2014)
31.	Northern and Central Asia and Northern Europe	*Pentaphylloides fruticosa* Whole plant	"Shrubby cinquefoil" (Rosaceae)	Flavonoids	Digestive disorders, astringent, antimicrobial against *S. aureus*	Paudel et al. (2014)
32.	Northeastern Asia and Japan	*Pinus pumila* Whole plant	"Dwarf Siberian pine" (Pinaceae)	–	Rheumatoid arthritis, diuretic, urogenital infections, respiratory infections, sores, burns, boils, colds, coughs, influenza, antimicrobial against *S. aureus*	Paudel et al. (2014)
33.	Bangladesh and South Asia	*Pleurotus ostreatus* Mushroom	"Oyster mushroom" (Pleurotaceae)	Statins, phenols, flavonoids, ascorbic acid	Cardiovascular, antimicrobial against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *C. albicans*, *S. cerevisiae*	Chowdhury et al. (2015)
34.	Central Asia and Eastern Europe	*Ptarmica salicifolia* Whole plant	"Bloodwort" (Compositae)	–	Cardiovascular disease, diaphoretic, digestive disorder, antimicrobial against *S. aureus*	Paudel et al. (2014)

35.	Eastern Asia	*Ribes fragrans* Whole plant	Wildflower (Grossulariaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
36.	Eastern Russia, Northern China, Japan, and Korea	*Sorbaria sorbifolia* Whole plant	"False goat's beard" (Rosaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
37.	Asia	*Thymus pavlovii* Whole plant	Flowering plant (Lamiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
38.	Iran	*Tragopogon graminifolius* Aerial parts	"Sheng" (Compositae)	–	Gastrointestinal and hepatic ailments, antimicrobial against *S. dysenteriae*, *P. vulgaris*	Farzaei et al. (2014)
39.	Russia	*Vaccinium vitis-idaea* Whole plant	"Lingonberry" (Ericaceae)	–	Astringent, diuretic, gonorrhea, arthritis, diabetes, diarrhea, antimicrobial against *S. aureus*	Paudel et al. (2014)
40.	Western Asia and Europe	*Veratrum lobelianum* Whole plant	Flowering herb (Melanthiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
41.	Russia and Eastern Europe	*Veronica incana* Leaves	"Silver speedwell" (Plantaginaceae)	Flavonoids, iridoids, tannins	Astringent, antimicrobial against *S. aureus*, *E. coli*	Paudel et al. (2014) and Nemereshina et al. (2015)
42.	Tropical and temperate areas of East Asia	*Zanthoxylum myriacanthum* Fruit	"Maqian" (Rutaceae)	Sabinene, limonene, 1,8-cineole	Digestive disorders, detoxification, swelling, pain, antimicrobial against *S. aureus*, *A. baumannii*, *K. pneumoniae*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*	Li R. et al. 2014 (Li et al. 2014)

(continued)

Table 2 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/phytochemicals	Uses	References
43.	Tropical and temperate areas of East Asia	*Zanthoxylum schinifolium* Fruit	"Huajiao" Chinese prickly ash (Rutaceae)	Linalool, limonene, sabinene	Detoxification, vomiting, stomachache, antimicrobial against *S. aureus*, *A. baumannii*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*	Li et al. (2014)
44.	Southern Asia	*Zingiber officinale* Root	"Ginger" (Zingiberaceae)		Nausea, asthma, cough, colic, heart palpitation, swelling, loss of appetite, rheumatism, antimicrobial against *E. coli*, *Enterobacter* sp., *P. aeruginosa*, *Proteus* sp., *Klebsiella* sp., *S. aureus*, *Bacillus* sp.	Karuppiah and Rajaram (2012)
Animal based						
45.	India, Thailand, and Indonesia	*Heterometrus xanthopus* Venom	"Giant forest scorpion" (Scorpionidae)	Hadrurin, scorpine, pandinin 1, pandinin 2	Antimicrobial against *B. subtilis*, *E. faecalis*, *P. aeruginosa*, *S. typhimurium*	Ahmed et al. (2012)
46.	China	Royal Jelly	"Ambrosia" Honeybee hypopharyngeal gland secretion		Longevity, overall health, and antimicrobial against *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S. aureus*	Fratini et al. (2016)

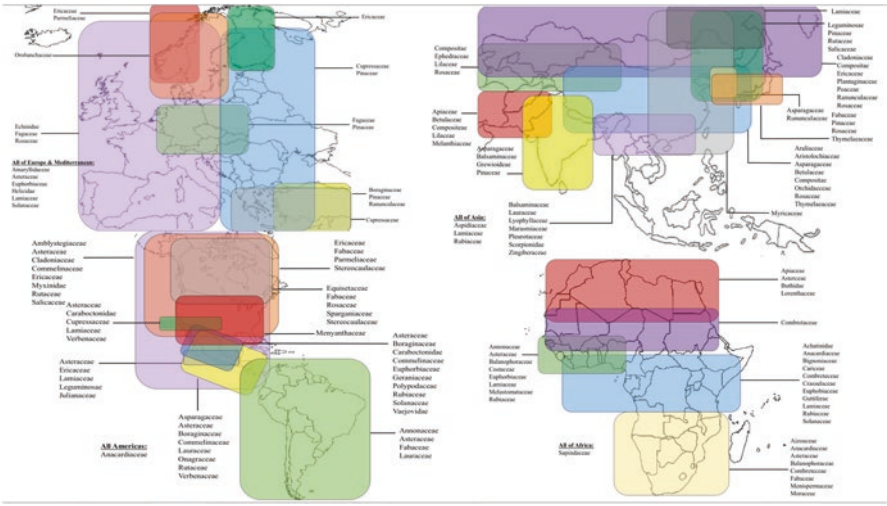

Fig. 1 Geographical distribution of ancient remedies by family classification focusing on Africa, Asia, Americas, and European and Mediterranean regions

Liang et al. 2012) and digestive disorders (Ooi et al. 2006; Li et al. 2012; Paudel et al. 2014; Li et al. 2014; Zeng et al. 2011; Liang et al. 2012; Li et al. 2013a; Ma et al. 2015; Farzaei et al. 2014). Asian herbal remedies are depicted by their geographic location of use in the western, eastern, southern, or northern regions of Asia or as being exclusive to regions in India, Russia, or East Asian countries as seen in Fig. 1. Similar to the African traditional remedies, the most common phytochemicals found in Asia include tannins (Shukla et al. 2016; Paudel et al. 2014; Nemereshina et al. 2015), flavonoids (Hong et al. 2014; Chowdhury et al. 2015; Shukla et al. 2016; Zeng et al. 2011; Nemereshina et al. 2015; Su et al. 2012), and saponins (Koffuor et al. 2014; Shukla et al. 2016). Flavonoids can be found in vegetables, nuts, seeds, tea, wine, honey, stems, and flowers and have a long history of use for their antimicrobial effects. They have the ability to inhibit fungal spore germination and prevent infection and replication of viruses; however, the mechanism of their antibacterial properties is yet to be understood (Cushnie and Lamb 2005).

3.3 Americas

Within the Americas, ancient remedies can be further subdivided by either Canada/the USA, Mexico, Central America, or South America. Table 3 details 48 plant-based remedies and 3 animal-based remedies originating from these regions. Two of the animal-based remedies include venom from *Hadrurus aztecus* (Torres-Larios et al. 2000) and *Vaejovis punctatus* (Ramirez-Carreto et al. 2015), both scorpions found in Mexico. Intriguingly, Asia and northern Africa have also utilized venom from scorpions (*Heterometrus xanthopus* and *Androctonus amoreuxi*). *H.*

Table 3 Americas

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
Plant based						
1.	Mexico and Central America	*Aguue* ssp. Sap	"Maguey sap" (Asparagaceae)	Saponins, polysaccharides	Digestive disorders, anti-inflammatory, tuberculosis, jaundice, syphilis, menstrual problems, antimicrobial against *P. aeruginosa*, *E. coli*, *S. paratyphi*, *S. sonnei*, *S. lutea*, *S. aureus*	Davidson and Ortiz de Montellano (1983)
2.	Canada, USA, Sweden, and Norway	*Alectoria ochroleuca* Whole plant	"Witch's hair lichen" (Parmeliaceae)	–	Antimicrobial against *S. aureus*, *E. coli*	Paudel et al. (2014)
3.	Central and Southern Mexico	*Amphipterygium adstringens* Bark	"Cuachalalate" (Julianaceae)	–	Digestive disorders, fever, hypocholesterolemia, antifungal, antiprotozoal, anticancer, antimicrobial against *S. mutans*, *P. gingivalis*, *A. actinomycetemcomitans*, *C. albicans*, *C. dubliniensis*, *E. coli*	Rodriguez-Garcia et al. (2015)
4.	Canada and USA	*Astragalus frigidus* Whole plant	"American milk-vetch" (Fabaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
5.	Latin America and Brazil	*Bowdichia virgilioides* Stem bark	"Alcornoco" (Fabaceae)	Alkaloid	Anti-hematic, astringent, antimicrobial against *S. aureus*	Agra et al. (2013)
6.	Mexico	*Callisia fragrans* Leaves	"Basket plant" (Commelinaceae)	Flavonoids, phytosteroids	Burns, arthritis, skin diseases, tuberculosis, asthma, antimicrobial against *B. cereus*, *B. subtilis*, *M. luteus*, *MRSA*, *S. epidermidis*, *E. faecalis*, *A. hydrophila*, *P. vulgaris*	Tan et al. (2014)

7.	North America	*Cassiope ericoides* Whole plant	Flowering plant (Ericaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
8.	North America	*Cladonia arbuscula* Whole plant	"Reindeer lichen" (Cladoniaceae)	–	Anti-diarrheic, scurvy, chest pains, antimicrobial against *S. aureus*	Paudel et al. (2014)
9.	Mexico	*Cnidoscolus tehuacanensis* Whole plant	Flowering plant (Euphorbiaceae)	–	Antimicrobial against *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
10.	Central America and Caribbean Islands	*Cordia curassavica* Aerial parts	"Barredor" (Boraginaceae)	–	Gastrointestinal disorders, respiratory infections, dermatological disorders, malaria, antimicrobial against *A. salina*, *S. aureus*, *S. epidermidis*, *B. subtilis*, *S. lutea*, *V. cholerae*, *A. niger*, *T. mentagrophytes*, *F. sporotrichum*, *F. moniliforme*	Hernandez et al. (2007)
11.	Southwestern North America	*Cupressus macrocarpa* Leaves	"Monterey cypress" (Cupressaceae)	–	Rheumatism, abortion inducing, antifungal against *T. rubrum*, *T. mentagrophytes*, *T. soudanense*, *T. violaceum*, *T. tonsurans*	Fahed et al. (2017)
12.	Mexico	*Datura lanosa* Leaves	Flowering plant (Solanaceae)	–	Antimicrobial against *S. aureus*, *B. subtilis*, *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
13.	USA, Mexico, and South America	*Dyssodia papposa* Whole plant	"Fetid marigold, dogweed" (Asteraceae)	Flavonoids, acetylenic thiophenes, monoterpenes	Digestive disorders, upset babies, indigestion, antimicrobial against *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
15.	Northern North America	*Equisetum fluviatile* Whole plant	"Water horsetail" (Equisetaceae)	Silica, nicotine	Arthritis, kidney infections, repair broken bones, antimicrobial against *S. aureus*	Paudel et al. (2014)

(continued)

Table 3 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
16.	Mexico	*Galium mexicanum* Aerial parts	"Mexican bedstraw" (Rubiaceae)	Triterpenes, saponins, flavonoids, sesquiterpene, lactones, glucosides	Digestive disorders, chest pain, skin diseases, antimicrobial *B. subtilis*, *MRSA*, *S. aureus*, *S pyogenes*, *C. albicans*, *C. neoformans*, *T. rubrum*	Bolivar et al. (2011)
17.	Mexico	*Geranium niveum* Flower	Flowering plant (Geraniaceae)	Proanthocyanidins, phenolics	Anticancer, epilepsy, digestive disorders, hormonal disorders, kidney dysfunction, antimicrobial against *S. aureus*, *T. mentagrophytes*	Gutierrez-Lugo et al. (1996) and Maldonado et al. (2005)
18.	Mexico and Southwestern USA	*Hadrurus aztecus* Venom	"Mexican scorpion" (Caraboctonidae)	Hadrurin	Antimicrobial against *S. typhi*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *E. coli*, *S. marcescens*	Torres-Larios et al. (2000)
19.	SoutheasternMexico	*Haematoxylon brasiletto* Bark	"Palo de brasil" (Leguminosae)	Caffeic acid, methyl gallate, gallic acid, phloroglucinol	Hypertension, digestive disorders, diabetes, antimicrobial against *S. aureus*, *E. faecium*, *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *C. albicans*, *S. mutans*, *P. gingivalis*	Rivero-Cruz (2008)
20.	Northern USA and Canada	*Hedysarum alpinum* Whole plant	"Alpine sweetvetch" (Fabaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
21.	Western USA and Northern Mexico	*Helianthella quinquenervis* Root	"Little sunflower" (Asteraceae)	–	Antimicrobial against *T. mentagrophytes*, *C. albicans*	Gutierrez-Lugo et al. (1996)
22.	North-Central Mexico	*Heliopsis longipes* Root	"Chilcuague" (Asteraceae)	–	Anesthetic, pain, dry mouth, cough suppressant, antimicrobial against *S. aureus*, *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
23.	Mexico	*Hofmeisteria schaffneri* Essential oil	Flowering plant (Asteraceae)	Thymol	Skin infections, antimicrobial against *S. aureus*, *B. subtilis*, *C. albicans*	Perez-Vasquez et al. (2011)

24.	Northern USA	*Juncus alpinoarticulatus* Whole plant	"Northern Green Rush" (Juncaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
25.	Mexico	*Laennecia confusa* Aerial parts	"Horseweed" (Asteraceae)	Flavonoids, cyanogenic glycosides, saponins, sesquiterpene, lactones, triterpenes	Sedative, alcoholism, antimicrobial against *E. coli*, *K. pneumoniae*, *MRSA*, *P. aeruginosa*, *S. aureus*, *C. albicans*, *C. neoformans*, *T. rubrum*	Martinez Ruiz et al. (2012)
26.	Southwestern USA, Mexico, and Central America	*Lippia graveolens* Leaves, flower	"Oregano" (Verbenaceae)	2-Methyl-butanoic acid ethyl ester, benzaldehyde, B-pinene, D-limonene, eugenol, copaene, B-bisabolene	Respiratory infections, menstruation problems, diabetes, digestive disorders, antimicrobial against *Salmonella* sp., *P. fragi*, *L. plantarum*, *M. luteus*, *B. subtilis*, *S. aureus*, *E. coli*, *S. typhi*	Hernandez-Hernandez et al. (2014) and Rivero-Cruz et al. (2011)
27.	Mexico, El Salvador, and Guatemala	*Lopezia racemosa* Aerial parts	"Mosquito flower" (Onagraceae)	Oenothein B, flavonoids, sterols	Digestive disorders, anginas, infection, stomach cancer, urinary tract infection, incontinence, antimicrobial against *B. subtilis*, *A. baumannii*, *MRSA*, *M. smegmatis*, *S. aureus*, *S. pyogenes*, *C. albicans*, *C. neoformans*, *T. rubrum*	Cruz Paredes et al. (2013)
28.	Latin America	*Malmea depressa* Wood	"Elemuy" (Annonaceae)	–	Fever, digestive disorders, insomnia, pain, diabetes, antimicrobial against *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
29.	USA	*Menyanthes trifoliata* Whole plant	"Bog Bean" (Menyanthaceae)	Coumarin derivatives, aucubin	Rheumatism, rheumatoid arthritis, digestive disorders, loss of appetite, antimicrobial against *S. aureus*, *S. epidermidis*, *S. lugdunensis*, *S. pyogenes*, *M. morganii*, *P. maltophilia*, *C. amycolatum*, *C. pseudodiphteriticum*	Weckesser et al. (2007)

(continued)

Table 3 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
30.	North America, South America, and Caribbean	*Metopium brownei* Wood	"Black poisonwood" (Anacardiaceae)	–	Healing/recovery, antimicrobial against *S. aureus*	Gutierrez-Lugo et al. (1996)
31.	Central America, South America, and Southern China	*Mikania micrantha* Leaves	"Mile-a-minute weed" (Asteraceae)	Mikanolide, dihydromikanolide, sesquiterpene lactone	Dermatitis, wound dressings, digestive disorder, malarial fever, antimicrobial against *S. aureus*, *B. subtilis*, *M. luteus*, *B. cereus*, *R. dolaanacearum*, *X. oryzae*, *F. solani*, *R. solani*, *P. aphanidermatum*, *P. parasitica*	Li et al. (2013b)
32.	Mexico, Central America, and South America	*Persea Americana* Seeds	"Avocado" (Lauraceae)	–	Diarrhea, dysentery, rheumatism, asthma, antimicrobial against *M. tuberculosis*	Jimenez-Arellanes et al. (2013)
33.	Mexico	*Pleopeltis polylepis* Aerial parts	"Redscale scaly polypody" (Polypodiaceae)	–	Fever, typhoid, cough, pertussis, chest pain, hepatic diseases, antimicrobial against *A. baumannii*, *B. subtilis*, *E. coli*, *MRSA*, *P. aeruginosa*, *S. aureus*, *C. albicans*, *C. neoformans*, *T. mentagrophytes*	Contreras Cardenas et al. (2016)
34.	Southwestern USA and Northern Mexico	*Poliomintha longiflora* Aerial parts	"Mexican Oregano" (Lamiaceae)	a-Pinene, B-phellandrene, B-caryophyllene, thymol, eugenol	Respiratory disease, digestive disorders, liver obstruction, menstruation, diabetes, infections, antimicrobial against *B. subtilis*, *S. aureus*, *E. coli*, *S. typhi*, *P. aeruginosa*	Rivero-Cruz et al. (2011)

35.	Northern and Central America	*Ptelea trifoliata* Stem bark	"Common hoptree" (Rutaceae)	–	Anthelmintic, digestive disorders, fever, antimicrobial against *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
36.	Mexico	*Rhoeo bermudensis* Leaves	"Boat lily" (Commelinaceae)	Phenol, tannin, flavonoid	Anticancer, whooping cough, dysentery, tuberculosis, cough, bronchitis, pain, antimicrobial against *B. cereus*, *B. subtilis*, *M. luteus*, *MRSA*, *S. epidermidis*, *E. faecalis*, *A. hydrophila*, *P. vulgaris*	Tan et al. (2014)
37.	Southern Mexico, Guatemala, and Belize	*Rhoeo spathacea* Leaves	"Hawaiian dwarf" (Commelinaceae)	Phenol, tannin, flavonoid	Cancer treatment, anti-inflammatory, gonorrhea, diabetes mellitus, rheumatic arthritis, cardiovascular disease, antimicrobial against *B. cereus*, *B. subtilis*, *M. luteus*, *MRSA*, *S. epidermidis*, *E. faecalis*, *A. hydrophila*, *P. vulgaris*	Tan et al. (2014, 2015)
38.	New England and Canada	*Rubus chamaemorus* Leaves	"Cloudberry" (Rosaceae)	Flavonoids, ellagic acid, gallic acid	Antidiuretic, antimicrobial against *S. aureus*, *S. epidermidis*, *E. coli*, *B. subtilis*, *C. albicans*, *M. luteus*	Thiem and Goslinska (2004)
39.	North America and Canada	*Salix pulchra* Whole plant	"Tealeaf willow" (Salicaceae)	Salicin	Anesthetic, dry eyes, antimicrobial against *S. aureus*	Paudel et al. (2014)
51.	North America	*Scorpidium scorpioides* Whole plant	"Hooked scorpion-moss" (Amblystegiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
40.	Canada	*Sparganium hyperboreum* Whole plant	"Northern bur-reed" (Sparganiaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)

(continued)

Table 3 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
41.	Canada	*Stereocaulon botryosum* Whole plant	"Cauliflower foam lichen" (Stereocaulaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
42.	Canada and USA	*Stereocaulon paschale* Whole plant	"Cottontail foam lichen" (Stereocaulaceae)	–	Rheumatism/arthritis, diabetes, bleeding, dizziness, antimicrobial activity	Paudel et al. (2014)
43.	Mexico	*Tournefortia hirsutissima* Stem bark	"Chigger bush" (Boraginaceae)	–	Fever, diabetes, hypertension, urinary problems, antimicrobial against *T. mentagrophytes*	Gutierrez-Lugo et al. (1996)
44.	Mexico	*Tradescantia pallida* Leaves	"Purple-heart" (Commelinaceae)	Phenol, tannin, flavonoid	Sore eyes, circulation, antimicrobial against *B. cereus*, *B. subtilis*, *M. luteus*, *MRSA*, *S. epidermidis*, *E. faecalis*, *A. hydrophila*, *P. vulgaris*	Tan et al. (2014)
45.	Mexico, Central America, and Columbia	*Tradescantia zebrina* Leaves	"Wandering jew" (Commelinaceae)	Phenol, tannin, flavonoid	Anticancer, antiarrhythmic, kidney disease, digestive disorders, antimicrobial against *B. cereus*, *B. subtilis*, *M. luteus*, *MRSA*, *S. epidermidis*, *E. faecalis*, *A. hydrophila*, *P. vulgaris*	Tan et al. (2014)
46.	North America	*Vaccinium corymbosum* Berry	"Northern highbush blueberry" (Ericaceae)	Anthocyanin	Digestive disorders, circulation, anti-inflammatory, cardiovascular diseases, antimicrobial against *S. aureus*, *MRSA*, *Methicillin-sensitive Staphylococcus aureus MSSA*, *E. coli*, *P. aeruginosa*, *P. mirabilis*, *A. baumannii*	Silva et al. (2016)

47.	Eastern North America	*Vaccinium macrocarpon* Berry	"Cranberry" (Ericaceae)	Anthocyanins, proanthocyanidins, phenolics	Bladder infections, antimicrobial against *E. coli*	Lacombe et al. (2010)
48.	North America	*Vaccinium uliginosum* Whole plant	"Bog blueberry" (Ericaceae)	–	Hypnotic, hypoglycemic, carminative, astringent, antimicrobial against *S. aureus*	Paudel et al. (2014)
Animal based:						
49.	Pacific Ocean from Canada to Mexico	*Eptatretus stoutii* Mucus	"Hagfish" (Myxinidae)	–	Antimicrobial against *E. coli*, *S. enterica*, *S. epidermidis*, *P. aeruginosa*, *C. albicans*	Subramanian et al. (2008)
50.	Mexico and Southwestern USA	*Hadrurus aztecus* Venom	"Mexican scorpion" (Caraboctonidae)	Hadrurin	Antimicrobial against *S. typhi*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *E. coli*, *S. marcescens*	Torres-Larios et al. (2000)
52.	Mexico	*Vaejovis punctatus* Venom	Scorpion Venom (Vaejovidae)	VpAmp1.0, VpAmp2.0	Antimicrobial against *S. aureus*, *S. agalactiae*, *E. coli*, *P. aeruginosa*, *C. albicans*, *C. glabrata*, *M. tuberculosis*	Ramirez-Carreto et al. (2015)

xanthopus and *H. aztecus* both have the phytochemical hadrurin in common. Hadrurin is a known antimicrobial peptide that disrupts membrane organization in prokaryotic cells, leading to cell lysis, and it has been suggested that this peptide would be beneficial in treating Gram-negative bacterial infections (Sanchez-Vasquez et al. 2013). Although there are no other active molecules in common between the four venoms, there is overlap when looking at the microbial species that they have antimicrobial properties against. For example, *A. amoreuxi* and *V. punctatus* have an antifungal property active against *C. albicans*, *and A. amoreuxi*, *H. aztecus*, and *V. punctatus* exhibit antibacterial activity against *E. coli*, while *H. xanthopus*, *H. aztecus*, and *V. punctatus* are effective against *P. aeruginosa (*Sanchez-Vasquez et al. 2013*).*

Another animal-based remedy found within the Americas is mucus from *Eptatretus stoutii* (hagfish) (Subramanian et al. 2008). *E. stoutii* mucus has been shown to have antimicrobial properties against *E. coli*, *P. aeruginosa*, *C. albicans*, *Staphylococcus epidermidis*, and *Salmonella enterica*, but the active molecules responsible have yet to be determined. Remedies from the northern hemisphere also contain mucus from two fish, haddock and brook trout, suggesting the interesting possibility that fish mucus could have similar active compounds.

Within the Americas, there is a large overlap between remedies that have antimicrobial activity and those that were historically used for digestive disorders (Paudel et al. 2014; Davidson and Ortiz de Montellano 1983; Rodriguez-Garcia et al. 2015; Hernandez et al. 2007; Gutierrez-Lugo et al. 1996; Bolivar et al. 2011; Maldonado et al. 2005; Rivero-Cruz 2008; Hernandez-Hernandez et al. 2014; Cruz Paredes et al. 2013; Weckesser et al. 2007; Li et al. 2013b; Jimenez-Arellanes et al. 2013; Rivero-Cruz et al. 2011; Tan et al. 2014; Silva et al. 2016), rheumatism (Paudel et al. 2014; Weckesser et al. 2007; Jimenez-Arellanes et al. 2013; Tan et al. 2014; Fahed et al. 2017; Tan et al. 2015), and diabetes (Paudel et al. 2014; Gutierrez-Lugo et al. 1996; Rivero-Cruz 2008; Hernandez-Hernandez et al. 2014; Rivero-Cruz et al. 2011; Tan et al. 2014; Tan et al. 2015). Interestingly, there were also remedies commonly used for tuberculosis (Davidson and Ortiz de Montellano 1983; Rivero-Cruz et al. 2011; Tan et al. 2014). Tuberculosis was the leading cause of death in the early twentieth century; therefore, it is not surprising that herbal remedies were sought after to find a treatment for this disease (Abrams 2013). The most common phytochemicals utilized in the Americas include flavonoids (Gutierrez-Lugo et al. 1996; Bolivar et al. 2011; Cruz Paredes et al. 2013; Tan et al. 2014; Tan et al. 2015; Martinez Ruiz et al. 2012; Thiem and Goslinska 2004) and saponins (Davidson and Ortiz de Montellano 1983; Bolivar et al. 2011; Martinez Ruiz et al. 2012), which have already been discussed.

3.4 Northern Hemisphere

Throughout our review of the literature, there was a noticeable overlap of remedies between continents located in the northern hemisphere, including Asia, Europe, and North America. Table 4 details 22 plant-based remedies, as well as mucus from two

Table 4 Northern Hemisphere (Europe, Asia, North America)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
Plant based						
1.	Asia, Europe, and North America	*Achillea millefolium* Whole plant	"Yarrow" (Asteraceae)	–	Dysmenorrhea, oral mucositis, kidney disease, antimicrobial against *S. aureus*	Paudel et al. (2014)
2.	Canada, Alaska, Russia, Finland, and Sweden	*Arctophila fulva* Whole plant	"Pendant Grass" (Poaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
3.	Asia, Europe, and North America	*Artemisia jacutica* Whole plant	"Wormwoods" (Compositae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
4.	Asia, Europe, and North America	*Beckmannia syzigachne* Whole plant	"Slough grass" (Poaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
5.	Russia, Northern Europe, and Canada	*Cladonia amaurocraea* Whole plant	"Quill pixie lichen" (Cladoniaceae)	–	Headaches, dizziness, antimicrobial against *S. aureus*, *E. coli*	Paudel et al. (2014)
6.	Norway, North America, and Arctic	*Cladonia stygia* Whole plant	"Reindeer moss" (Cladoniaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
7.	Russia, Alaska, British Columbia, and Canada	*Cnidium cnidiifolium* Whole plant	"Hemlock-parsley" (Apiaceae)	–	Skin conditions, anticancer, infertility, erectile dysfunction, antimicrobial against *S. aureus*	Paudel et al. (2014)
8.	Asia, North America, and Europe	*Comarum palustre* Whole plant	"Purple marshlocks" (Rosaceae)	Proanthocyanidins	Antidiabetic, anti-arthritic, antimicrobial against *S. aureus*	Paudel et al. (2014)
9.	Eastern Russia, China, Europe, and Alaska	*Dianthus repens* Whole plant	"Northern pink" (Caryophyllaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)

(continued)

Table 4 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
10.	Asia, Europe, North America, and Northeastern South America	*Eleocharis acicularis* Whole plant	"Needle spikerush" (Cyperaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
11.	Asia, North America, and Europe	*Empetrum nigrum* Whole plant	"Crowberry" (Ericaceae)	–	Urine problems, diarrhea, cough, cold, antimicrobial against *S. aureus*, *C. albicans*	Paudel et al. (2014)
12.	Northern hemisphere	*Equisetum arvense* Whole plant	"Horsetail" (Equisetaceae)	–	Edema, kidney and bladder stones, urinary tract infections, incontinence, antimicrobial against *S. aureus*	Paudel et al. (2014)
13.	Northern Asia, Europe, and USA	*Juniperus communis* Berry, branch	"Common juniper" (Cupressaceae)	Phenolics, flavonoids, tannins	Diuretic, antiseptic, gastrointestinal issues, antimicrobial against *S. aureus*, *S. epidermidis*, *E. hirae*	Paudel et al. (2014) and Taviano et al. (2011)
14.	Northern Asia, Northern Europe, and Northern North America	*Oxycoccus microcarpus* Berry	"Small cranberry" (Ericaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
15.	Northern and Central Asia, North America, Europe, and Northern Africa	*Parnassia palustris* Herb	"Flowering plant" (Saxifragaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
16.	Asia, Canada, and Northern USA	*Ribes triste* Berry	"Swamp red currant" (Grossulariaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
17.	Asia, Europe, and North America	*Rosa acicularis* Whole plant	"Prickly wild rose" (Rosaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)

18.	Europe and North America	*Rubus fruticosus* Fruit, Leaves, Root, Stem	"European blackberry" (Rosaceae)	Tannins, gallic acid, villosin, iron, niacin, pectin, anthocyanins	Anticancer, digestive disorders, antidiabetic, antimicrobial against *E. coli*, *S. typhi*, *S. aureus*, *P. mirabilis*, *M. luteus*, *Citrobacter*, *B. subtilis*, *P. aeruginosa*	Verma et al. (2014)
19.	Asia, Europe, and North America	*Rubus matsumuranus* Berry	"Ku ye xuan gou zi" (Rosaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
20.	Asia, Europe, and North America	*Sanguisorba officinalis* Whole plant	"Great burnet" (Rosaceae)	–	Antimicrobial against *S. aureus*, *MRSA*	Paudel et al. (2014) and Chen et al. (2015)
21.	North America	*Vaccinium microcarpon* Berry	"European cranberry" (Ericaceae)	Proanthocyanidins, anthocyanins, hydroxycinnamic acid, hydroxybenzoic acid, flavonols	Digestive disorders, scurvy, urinary tract infections, *H. pylori* infections, antimicrobial against *S. aureus*	Kylli et al. (2011)
22.	Boreal forest and Arctic tundra	*Vaccinium vitis-idaea* Berry	"Lingonberry" (Ericaceae)	Proanthocyanidins, anthocyanins, hydroxycinnamic acid, hydroxybenzoic acid, flavonols	Arthritis, diabetes, diarrhea, gonorrhea, fever, antimicrobial against *S. aureus*	Kylli et al. (2011)
Animal based						
23.	North Atlantic Ocean	*Melanogrammus aeglefinus* Mucus	"Haddock" (Gadidae)	–	Antimicrobial against *E. coli*, *S. enterica*, *S. epidermis*, *P. aeruginosa*, *C. albicans*	Subramanian et al. (2008)
24.	Iceland, Europe, Asia, and North America	*Salvelinus fontinalis* Mucus	"Brook trout" (Salmonidae)	–	Antimicrobial against *E. coli*, *S. enterica*, *S. epidermis*, *P. aeruginosa*, *C. albicans*	S. Subramanian et al. (2008)

fish, *Salvelinus fontinalis* (brook trout) found in freshwater and *Melanogrammus aeglefinus* (haddock) inhabiting saltwater. As a result of the cold climate in the northern latitudes, the majority of herbal remedies were derived from berries (Paudel et al. 2014; Taviano et al. 2011; Verma et al. 2014; Kylli et al. 2011), grass (Paudel et al. 2014), lichen (Paudel et al. 2014), or moss (Paudel et al. 2014), all of which can withstand the extreme temperatures in this region. The most common dual purpose for these herbal remedies overlaps between treatments for infection and urinary disorders, such as kidney and bladder stones, urinary tract infections, and incontinence (Paudel et al. 2014; Taviano et al. 2011; Kylli et al. 2011). The two most common phytochemicals from the northern hemisphere are tannins (Paudel et al. 2014; Taviano et al. 2011; Verma et al. 2014), similarly to those found in African and Asian remedies, and proanthocyanidins (Paudel et al. 2014; Kylli et al. 2011), condensed tannins that, as mentioned earlier, can impact biofilm formation.

3.5 Other

Other regions include the European, Mediterranean, and Arctic regions. Table 5 includes 21 plant-based remedies from the European and Mediterranean regions, along with 2 animal-based remedies. One animal-based remedy is coelomic fluid from *Echinus esculentus*, the European edible sea urchin, that exhibits antimicrobial effects on a variety of microorganisms including *E. coli*, *S. aureus*, *P. aeruginosa*, and others (Solstad et al. 2016). Another animal-based remedy is mucus from *Helix aspersa*, the brown garden snail. This mucus has been used for skin regeneration and exhibited an antimicrobial effect against *S. aureus* and *P. aeruginosa (*Pitt et al. 2015*)*. Allantoin, a compound found in the mucus, is valuable for healing as it promotes keratolysis and impacts cell proliferation (Tsoutsos et al. 2009). The mucus from the giant African land snail also exhibits similar antimicrobial properties (Pitt et al. 2015). The majority of plant-based remedies were derived from the leaves (Hernandez-Hernandez et al. 2014; Fahed et al. 2017; Pavlovic et al. 2017; Quave et al. 2015; Antunes Viegas et al. 2014; Bouyahyaoui et al. 2016) or the bark/twigs (Fahed et al. 2017; Taviano et al. 2011; Apetrei et al. 2011). The most common phytochemicals within this region are flavonoids (Taviano et al. 2011; Pavlovic et al. 2017; Antunes Viegas et al. 2014; Apetrei et al. 2011; Tadic et al. 2008), also one of Asia's top active molecules. Only three antimicrobial remedies, as seen in Table 6, were found in the Arctic regions of the world, all of which were classified as a lichen (Paudel et al. 2014), but there have been no published studies analyzing the phytochemicals of these plants. In fact, there is lack of reports on the history of lichens being used for any form of ancient remedy.

Table 5 European and Mediterranean remedies

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
Plant based						
1.	Lebanon, Syria, and Turkey	*Abies cilicica* Leaves	"Cilician fir" (Pinaceae)	α-pinene, camphene, δ-3-carene, β-phellandrene, bornyl acetate, α-longipinene, β-caryophyllene, himachala-2,4-diene	Diuretic, anti-helminthic, hair growth, antimicrobial against *T. rubrum*, *T. mentagrophytes*, *T. soudanense*, *T. violaceum*, *T. tonsurans*	Fahed et al. (2017)
2.	Europe and Northern Asia	*Allium ursinum* Leaves	"European wild garlic" (Amaryllidaceae)	Organosulfur, polyphenols, tannins, flavonoids	Cardiovascular disease, respiratory disorders, gastrointestinal disorders, skin diseases, antimicrobial against *E. coli*, *P. aeruginosa*, *E. aerogenes*, *P. mirabilis*, *S. enteritidis*, *S. aureus*, *E. faecalis*, *C. albicans*	Pavlovic et al. (2017)
3.	Persia	*Arnebia* spp. Roots	Wildflower (Boraginaceae)	Naphthoquinones such as alkannins, shikonins	Diarrhea, amenorrhea, gout, kidney stone, jaundice, chronic fever, burn wound, antimicrobial against *S. aureus*, *E. faecalis*, *C. krusei*, *S. cerevisiae*, *C. glabrata*	Hosseini et al. (2018)
4.	Norway	*Asahina chrysantha* Whole plant	"Golden asahinea lichen" (Parmeliaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
5.	Norway and Arctic	*Cassiope tetragona* Whole plant	"Arctic bell-heather" (Ericaceae)	–	Antimicrobial against *S. aureus*, *C. albicans*	Paudel et al. (2014)
6.	Western and Central Europe and Northern Iran	*Castanea sativa* Leaves	"European chestnut" (Fagaceae)	–	Respiratory infections, digestive disorders, fever, pain, sclerosis, tuberculosis, sore throat, antimicrobial against *S. aureus*, *MRSA*	Quave et al. (2015)

(continued)

Table 5 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
7.	Eastern Mediterranean basin	*Cedrus libani* Cones	"Cedar of Lebanon" (Pinaceae)	α-Pinene, β-pinene, sclarene, abieta-8,11,13-triene, abieta-7,13-diene	Skin diseases, allergies, rash, respiratory infections, antimicrobial against *S. aureus*, *T. rubrum*	Fahed et al. (2017)
8.	Europe, Western Asia, and Northern Africa	*Crataegus monogyna and Crataegus oxyacantha (1:1)* Berry	"Hawthorn" (Rosaceae)	Flavonoids, procyanidins, flavone, flavonol, Phenolics	Heart failure, high blood pressure, digestive disorders, antimicrobial against *E. coli*, *S. aureus*, *S. epidermidis*, *B. subtilis*, *M. luteus*, *M. flavus*, *P. aeruginosa*, *L. monocytogenes*, *C. albicans*	Tadic et al. (2008)
9.	Europe, Asia, and Northern Africa	*Euphorbia helioscopia* Whole plant	"Madwoman's milk" (Euphorbiaceae)	–	Anthelmintic, anticancer, cholera, dermatitis, antimicrobial against *S. aureus*, *E. coli*, *A. flavus*	Lone et al. (2013)
10.	Norway and Sweden	*Euphrasia hyperborea* Whole plant	Parasitic plant (Orobanchaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
11.	Europe, Mediterranean, and Northern Africa	*Helichrysum italicum* Flowers, leaves	"Strawflower, immortelle" (Asteraceae)	Flavonoids, terpenes, acetophenones, phloroglucinols, terpenoids	Allergies, colds, cough, liver disorders, sleeplessness, antimicrobial against *S. aureus*, *C. albicans*, *HSV*, *HIV*	Antunes Viegas et al. (2014)
12.	Southern Greece, Southern Turkey, Western Syria, and Lebanon	*Juniperus drupacea* Branch	"Syrian juniper" (Cupressaceae)	Phenolics, flavonoids, tannins	Anthelmintic, antimicrobial against *S. aureus*, *S. epidermidis*, *E. hirae*	Taviano et al. (2011)
13.	Eastern Mediterranean	*Juniperus excelsa* Twigs	"Greek juniper" (Cupressaceae)	α-Pinene, δ-3-carene, α-cedrol	Antimicrobial against *S. aureus*, *T. rubrum*	Fahed et al. (2017)

14.	Morocco, Portugal, France, Iran, Lebanon, and Israel	*Juniperus oxycedrus* Branch	"Cade juniper" (Cupressaceae)	α-Pinene, α-cubebene, germacrene D, τ-cadinol	Hyperglycemia, obesity, tuberculosis, bronchitis, pneumonia, parasitic disease, antimicrobial against *S. aureus*, *S. epidermidis*, *E. hirae*, *T. rubrum*	Taviano et al. (2011) and Fahed et al. (2017)
15.	Morocco, Portugal, Italy, Turkey, Egypt, Lebanon, Israel, Jordan, Saudi Arabia, and Algeria	*Juniperus phoenicea* Leaves	"Phoenician juniper" (Cupressaceae)	a-Pinene, sesquiterpenes B-caryophyllene, germacrene	Antimicrobial against *P. aeruginosa*, *E. coli*, *C. albicans*	Bouyahyaoui et al. (2016)
16.	Mediterranean and Eurasia	*Origanum vulgare* Leaves	"Oregano" (Lamiaceae)	a-Pinene, thymol, carvacrol, germacrene D, alloaromadendrene	Digestive disorders, fever, respiratory infections, anthelmintic, antimicrobial against *Salmonella* sp., *B. thermosphacta*, *P. fragi*, *L. plantarum*, *M. luteus*	Hernandez-Hernandez et al. (2014)
17.	Europe and Northern Asia	*Physalis alkekengi* Aerial parts	"Winter cherry" (Solanaceae)	Alkaloids, glucocorticoids, lycopene, ethanolic compounds, vitamin C	Gout, urinary diseases, rheumatism, digestive disorders, malaria, antimicrobial against *S. aureus*, *S. epidermidis*, *E. faecalis*, *B. subtilis*, *B. cereus*, *E. coli*, *P. aeruginosa*, *E. cloacae*, *K. pneumoniae*, *P. vulgaris*, *C. albicans*, *C. krusei*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*	Helvaci et al. (2010)
18.	Northern and Central Europe	*Picea abies* Rosin	"Norway spruce" (Pinaceae)	–	Cough, asthma, gout, bronchitis, whooping cough, antimicrobial against *S. aureus*, *MRSA*, *E. coli*, *P. aeruginosa*, *B. subtilis*, *C. albicans*	Sipponen and Laitinen (2011)

(continued)

Table 5 (continued)

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
19.	Central Europe	*Pinus cembra L.* Bark, needles	"Swiss stone pine" (Pinaceae)	Phenol, flavonoid, proanthocyanidin	Antimicrobial against *S. aureus*, *S. lutea*, *B. cereus*, *E. coli*, *P. aeruginosa*, *C. albicans*	Apetrei et al. (2011)
20.	Finland	*Rhododendron lapponicum* Whole plant	Flowering plant (Ericaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
21.	Grasslands in Europe	*Thalictrum foetidum* Whole plant	"Yellow meadow rue" (Ranunculaceae)	Alkaloids	Hypertension, antimicrobial against *S. aureus*	Paudel et al. (2014)
Animal based:						
22.	Western Europe	*Echinus esculentus* Coelomic fluid	"European edible sea urchin" (Echinidae)	EeCentrocins 1,2, EeStrongylocin 2 peptides	Food, antimicrobial against *B. subtilis*, *C. glutamicum*, *S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*, *A. pullulans*, *C. albicans*, *Cladosporium* sp., *Rhodotorula* sp., *S. cerevisiae*	Solstad et al. (2016)
23.	Europe and Mediterranean	*Helix aspersa* Mucus	"Brown garden snail" (Helicidae)	–	Skin regeneration, antibacterial against *P. aeruginosa and S. aureus*	Pitt et al. (2015)

Table 6 Arctic

#	Region	*Scientific name* Element	Laymen name (family)	Active molecule/ phytochemicals	Uses	References
1.	Antarctic	*Cladonia verticillata* Whole plant	"British lichens" (Cladoniaceae)	–	Antimicrobial against *S. aureus*, *E. coli*	Paudel et al. (2014)
2.	Arctic-alpine	*Flavocetraria cucullata* Whole plant	"Curled snow lichen" (Parmeliaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)
3.	Arctic-alpine	*Flavocetraria nivalis* Whole plant	"Crinkled snow lichen" (Parmeliaceae)	–	Antimicrobial against *S. aureus*	Paudel et al. (2014)

4 Conclusions

Various segments of plants and animal products have been used by humans for thousands of years to ward off disease and infection. In some instances, concentrated oils and topical application of various plant and animal agents have shown enhanced antimicrobial effects in comparison to the current marketed antimicrobials and wound dressings. Although phytochemicals within these agents need to be further identified and understood, these active molecules could potentially increase our armamentarium of agents to combat antimicrobial-resistant infections. As these compounds continue to be studied, they could be of therapeutic value, and as modern medicine continues to lose the battle against antimicrobial resistance, the answer to fight back could be discovered by looking into the past. A notable example of this was a recent study that sought to reconstruct a recipe that was used in medieval times by Anglo-Saxons to treat eye infections. The recipe from *Bald's Leechbook* was translated, made in the laboratory using medieval techniques, and then tested in mice with methicillin-resistant *S. aureus* (MRSA) infections (Harrison et al. 2015). The investigators demonstrated that the medieval recipe was more efficacious at treating this modern-day, drug-resistant infection than the current last line therapy, vancomycin. Thus, with the combination of ancient antimicrobial agents and a modern understanding of medicine, new remedies could impact our battle against persistent microbial infections.

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Part III

Alternative Antibiotic Resistance Treatment Strategies

Pre- and Probiotics: Using Functional Foods in the Fight Against Microbial Resistance to Antibiotics

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Abstract

Functional foods such as prebiotics, dietary fibers, and probiotic microorganisms have several beneficial effects on the human body. Probiotic microorganisms are reported to produce and enhance the absorption of vitamins and minerals, short-chain fatty acids, amino acids, and organic acids, resulting in the enhancement of the host immune system. Generally, lactic acid bacteria and yeasts are used as probiotics. Prebiotics are nonabsorbable polysaccharides/oligosaccharides such as fructooligosaccharides, inulin, and human milk oligosaccharides and have positive effects on host health, maintaining the balance of the gut microbiome, as well as stimulating immunomodulatory activity. Prebiotics are not metabolized by digestive enzymes, allowing them to reach the colon unaltered, where they can be fermented by probiotics. They also promote mineral absorption and act as a fertilizer for gut microflora. These prebiotics can act in synergy with probiotics (synbiotics) and can thus be even more effective if used wisely, selectively stimulating the growth of specific microorganisms. As these synbiotics can directly inhibit the growth and colonization of pathogens and regulate the immune system, they can be developed as an alternative strategy for combating antibiotic resistance in pathogens.

Keywords

Probiotic · Prebiotic · Synbiotic · Antibiotic resistance · Gut microflora

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1 Introduction

The health benefits of the indigenous microflora of the human body are evident, and the effects on mucosal immunology have recently received considerable attention. This has led to a resurgence of focus on maintaining the gut microbial balance, and in the use of prebiotics and probiotics. Probiotics are live microbial supplements that beneficially affect colon health by improving microbial colonization. Prebiotics, on the other hand, are indigestible food ingredients, such as oligosaccharides, which act by selectively increasing the growth of beneficial microorganisms in the colon, such as *Bifidobacteria* and *Lactobacilli*, eventually improving host health. A synbiotic is a combination of prebiotics and probiotics that work synergistically by improving the colonization and survival of beneficial microflora inside the GI tract. The intestinal mucosa forms a first line of defense, acting as a barrier to pathogens and toxins. Further inhibition of pathogens by the intestinal microbiota occurs due to their barrier effect, microbial interference, antagonism, colonization resistance, and competitive exclusion of harmful microorganisms. As gut immunity is directly affected by available nutrients and the resident microbial community, it can be targeted by therapeutic approaches in order to treat various diseases.

2 Probiotics

Probiotics (meaning "for life") are microorganisms which have health-promoting effects in humans and animals (Marteau et al. 2001). Schrezenmeir and de Vrese (2001) defined the term "probiotic" as "a preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host." These beneficial microorganisms are utilized to alter the indigenous microflora, "the usually complex mixture of bacterial population that colonizes (establishes in size over time without the need for periodic reintroduction of the bacteria by repeated oral doses or other means) a given area in the host that has not been affected by medical or experimental intervention, or by disease" (Schrezenmeir and de Vrese 2001). It should be noted that probiotics do not solely influence the GI tract, but they also influence other organs as well via by regulation of intestinal permeability, bacterial translocation, and immunomodulatory activities.

When considering probiotic usage, it must be taken into consideration that the GI tract contains a mixture of surfaces that are primarily colonized by differing types of microorganisms. For example, several indigenous, pathogenic, or beneficial microorganisms colonize the surface of the gut epithelium via adhesion mediated by special organelles, such as fimbriae (Beachey and Courtney 1987; Gibbons and Houte 1975), while the mucosal crypts are colonized by motile, spiral bacteria, such as *Borrelia, Treponema, Spirillum*, and *H. pylori* (Lee 1985).

The most commonly used probiotic food supplements include *Lactobacillus, Bifidobacterium, Enterococcus, Bacillus, Escherichia, Streptococcus,* and *Saccharomyces* (Jin et al. 2000; Alvarez-Olmos and Oberhelman 2001; Reid et al. 2003). The first probiotic, *Lactobacillus rhamnosus* GG (LGG), was first utilized in 1995, and has since shown beneficial health effect including improvement of intestinal immunity (Brestoff and Artis 2013). However, the concept of ameliorating microbial imbalance for longevity and health is almost a century old. In the early 1900s, Elie Metchnikoff, a Nobel laureate, also called the grandfather of modern probiotics, hypothesized about the consumption of fermented milk products in human health and longevity in his book *The Prolongation of Life*. Although his concept was not taken seriously until 80 years, now modern-day research has proven the importance of his hypothesis culminating in our increased understanding of mechanisms and the potential benefits of healthy gut flora (Anukam and Reid 2007).

3 Mechanism of Action

Probiotic microorganisms have several beneficial effects on the human body (Hemarajata and Versalovic 2013). Some are natural producers of vitamin B complex, can enhance the absorption of vitamins and minerals, and can trigger the generation of short-chain fatty acids, amino acids, and organic acids, resulting in enhancement of the host immune system (Sanders et al. 2007; Nova et al. 2007; Ouwehand et al. 1999; Mishra and Lambert 1996). They also have a direct and indirect influence on pathogenic bacteria, such as *Staphylococcus aureus* (Sikorska and Smoragiewicz 2013), *Clostridium perfringens* (Schoster et al. 2013), *Salmonella* Enteritidis (Carter et al. 2017), *Shigella* spp. (Hussain et al. 2017), *Escherichia coli* (Chingwaru and Vidmar 2017), and *Campylobacter jejuni* (Saint-Cyr et al. 2017). Probiotics can also suppress pathogens by stimulation, proliferation, and differentiation of the epithelial cell as well as fortification of the intestinal barrier (Thomas and Versalovic 2010). In addition to balancing the gut microflora, probiotics can be used to counter food poisoning, candidiasis (Kumar et al. 2013), dental caries (Näse et al. 2001) and as a treatment for food allergies (Thomas and Greer 2010; Markowiak and Śliżewska 2017), among many other applications. In terms of disease resistance, they operate by several mechanisms (Fig. 1), listed as follows:

1. **Generation and synthesis of vitamins, amino acids, and fatty acids.**
   - Probiotic microorganisms such as *Lactobacillus reuteri* (Gu et al. 2015), *L. plantarum* (Li and Gu 2016), *Bifidobacterium adolescentis* (Pompei et al. 2007), and *B. pseudocatenulatum* are known producers of vitamin B complex (B1, B2, B3, B5, B6, B7, B9, and B12) and can provide several essential amino acids and fatty acids to the host. Moreover, *Lactobacillus* can enhance the absorption of vitamins and mineral compounds.
2. **Competition with pathogens for adhesion to the epithelium and for nutrients and maintaining the balance of the host's intestinal microbiota** (Elli et al. 2000; Weinberg 1997).

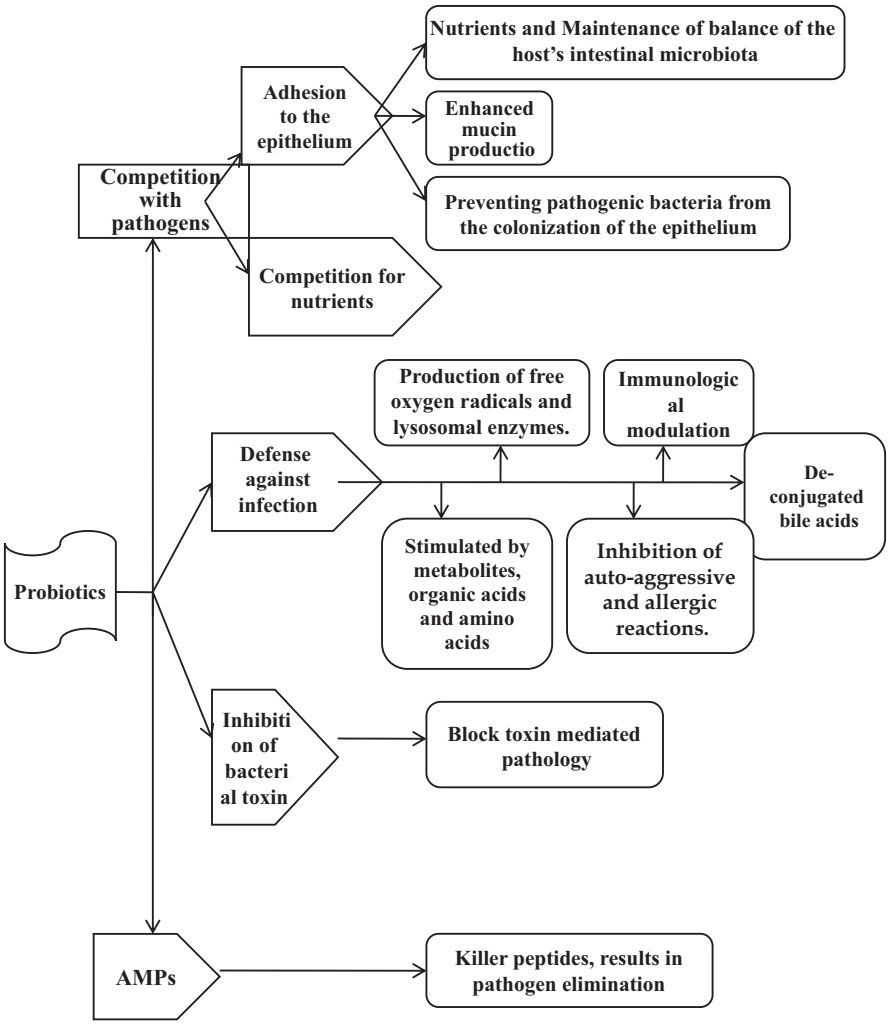

Fig. 1 Mechanisms of action of probiotics

- Lactic acid bacteria produce organic acids, predominantly lactate and acetate, creating an acidic environment that is inhibitory to pathogens.
- *Lactobacillus delbrueckii* inhibits the growth of other microbes by binding iron hydroxide to its cell surface, making it unavailable to other microbes The bacterium does not need iron in their natural environment, hence it becomes an advantage over other microorganisms.
- Lactobacilli coaggregate, leading to the formation of a protective physical barrier, preventing pathogenic bacteria from colonizing the epithelium.
- Probiotics often have better ability to adhere to epithelial cells.

3. **Direct defense against infection by antagonism through the production of antimicrobial substances** (Oelschlaeger 2010; Begley et al. 2006).
   - Probiotics synthesize proteins or peptides capable of inhibiting specific pathogenic strains. These antimicrobial compounds have potential applications as food preservatives or prophylactic agents against enteric infections.
   - Lactic acid bacteria produce many inhibitory peptides such as lantibiotics (class I), low-molecular-weight bacteriocins (class II) (LMWB), antibacterial peptides, high-molecular-weight (class III) bacteriocins, and antibiotics (acidophilin, lactacin).
   - Low-molecular-weight substances produced by probiotic microorganisms (e.g., hydroperoxide and short-chain fatty acids) can inhibit the replication of pathogens.
   - Deconjugated bile acids (derivatives of bile acids), like those produced by *Lactobacillus* and *Bifidobacterium* spp., result in stronger antibacterial bile salts as compared to those produced by their host.
4. **Enhancement of the host defense system against pathogens** (Guillot 2003; Brestoff and Artis 2013).
   - The mucosal epithelial cell barrier is the first line of defense against pathogen attack. The adhesion of probiotic microorganisms to epithelial cells may also trigger a signaling cascade, leading to immunological modulation.
   - Enhanced mucin production, as well as the reduction of gut permeability, prevents penetration of pathogenic organisms.
   - Probiotics can stimulate the activity of macrophages via production of free oxygen radicals and lysosomal enzymes.
   - The acquired immune system can be stimulated by metabolites or components of the cellular wall or DNA, which can trigger a signaling cascade, leading to immunological modulation.
   - Induction and maintenance of immunological tolerance to environmental antigens (nutritional and inhalatory), and induction and control of immunological reactions against pathogens of bacterial and viral origin.
   - Inhibition of auto-aggressive and allergic reactions.
   - Enhanced activity of macrophages and lymphocytes, and stimulation of γ-interferon production.
   - Generation of organic acids and amino acids resulting in regulation of host metabolism and immunological modulation.
   - Production of enzymes, such as esterase, lipase, and coenzymes A, Q, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP).
5. **Inhibition of bacterial toxin production** (Brandão et al. 1998)
   - Mucin production and reduction of gut permeability prevent the penetration of toxic substances.
   - Some lactobacilli use enzymatic mechanisms to modify toxin receptors and can block toxin-mediated pathology.

4 Probiotic Microorganisms and AMPs

Considering the recent alarming rise in antibiotic resistance among pathogens, there is an urgent need for the discovery of novel antimicrobials. A potential dual therapy to fight against infectious diseases is the use of probiotics and antimicrobial peptides (AMPs) as novel strategies in the control of multidrug-resistant (MDR) pathogens. This strategy provides additional advantages by combining the benefits of probiotics with the antimicrobial activity of AMPs (Candido et al. 2014). Therefore, many researchers are searching for novel AMPs (Silva et al. 2011), including a group of innate immune effectors which can sufficiently control MDR pathogens (Silva et al. 2011). Furthermore, various probiotic microorganisms have the ability to produce their own AMPs. A limitation of AMPs is that they cannot be taken orally, due to their quick degradation before reaching their targets (Candido et al. 2014). Therefore, probiotic microorganisms having capability to produce AMPs are good alternative sources of antimicrobial agents currently attracting keen interest as health supplements. Table 1 describes some examples of AMP, their bacterial sources, and their activities.

5 Probiotic Microorganisms

The majority of probiotic microorganisms belong to the genera *Bifidobacterium* and *Lactobacillus*, as they are normal gut inhabitants in humans and animals (Anukam and Reid 2007). However, some yeasts and other bacteria, such as *Bacilli*, also exhibit exceptional probiotic properties (Anukam and Reid 2007). Lactobacilli are Gram-positive lactic acid-producing bacteria found in an array of habitats that are rich with carbohydrate-containing substrates, such as animal and human mucosal membranes, spoiling food/plant materials, sewage, and fermented milk products.

Table 1 Examples of AMPs, their bacterial sources, and their activities

Bacterial source	AMP	Activity
Lactobacillus acidophilus *Lactobacillus amylovorus* *Lactobacillus brevis* *Lactobacillus bulgaricus* *Lactobacillus casei* *Lactobacillus curvatus*	Acidolin, acidophilin, lactacin B Lactobin A Lactobacillin, lactobrevin Bulgarin Lactocin 705 Curvacin A	- Control of enteropathogenic organisms and spore formers - Effects of organic acids and hydrogen peroxide - Antimicrobial activity - Control of *Listeria monocytogenes* and mainly foodborne pathogens - Control of *Listeria monocytogenes* and *Enterococcus faecalis*
Leuconostoc gelidum	Leucocin A	Bactericidal
Enterococcus faecium CTC492	Enterocin A	Anti-listerial activity
Pediococcus acidilactici	Pediocin AcH, pediocin F	Inhibit foodborne pathogens

Bifidobacteria are nonmotile, nonsporulating rods and are mostly composed of strict anaerobes. Regardless of the genera, in general, probiotic microorganisms are selected from lactic acid-producing bacterial strains or other microorganisms known to impart health benefits (Brestoff and Artis 2013). Some of the most commonly used probiotic microbes are listed here:

Bacteria

- ***Lactobacillus* spp.:** *L. amylovorus, L. acidophilus, L. casei, L. gasseri, L. helveticus, L. johnsonii, L. pentosus, L. plantarum, L. crispatus, L. reuteri, L. rhamnosus, Lactococcus lactis*

***Bifidobacterium* spp.:** *B. adolescentis, B. breve, B. animalis, B. bifidum, B. infantis, B. longum,*

Other bacterial strains: *Enterococcus faecium, Streptococcus thermophilus, Bacillus clausii, Bacillus cereus, Escherichia coli, Propionibacterium freudenreichii*

Yeasts:

- *Saccharomyces cerevisiae, Saccharomyces boulardii*

6 Probiotic Selection Criteria

The microorganisms selected as candidate probiotics should be readily associated with gastrointestinal tract of healthy individuals, nonpathogenic and safe. They should have high cell viability and should be resistant to bile, hydrochloric acid, and pancreatic juices in order to survive the adverse acidic and alkaline conditions of the abdomen and duodenum. They should be highly competitive to gut microflora for effective colonization to be possible. Microorganisms having immunomodulatory or anticancerous ability are also preferred. Safety considerations are essential, and probiotics are subject to regulations of global food safety agencies, according to which they should be proven safe for human and animal health, or should be classified as GRAS (Generally Regarded As Safe), as determined by the Food and Drug Administration (FDA) in the United States (Anadón et al. 2006; Gaggìa et al. 2010). Additionally, probiotic microorganisms should be genetically stable, with no adverse genotypes, and have a low potential for antibiotic resistance development. They should also exhibit weak competition with regard to beneficial microbiota inhabiting the intestinal ecosystem, and their production should be simple and practical, producing highly viable and stable cell counts that are resistant to bacteriophages, and with a high storage survival rate in finished products. Finally, the finished product should have desirable sensory properties and palatability.

7 Commercially Available Probiotics

While any microorganism can have probiotic properties, most are bacterial, with lactic acid bacteria being the most common. Probiotics can be differentially categorized as natural health products (Canada), dietary supplements, drugs, live biotherapeutic agents, medical foods (USA), functional foods (Japan, China, Malaysia, and India), food supplements (Sweden, Denmark, and Finland), or biotherapeutic/pharmaceuticals (Belgium, Germany). According to the market analysis on the probiotic industry, the European market is the highest ranked, while Japan's is the second highest. Probiotics can be commercially available in various delivery forms, including powder, liquid, gel, paste, granules, capsules, injections, and sachets. Probiotic microorganisms may also be present in pharmaceutical products as food additives and may contain one or more selected microbial strains (Gilliland and Speck 1977). For example, VSL3 is a probiotic comprised of eight different strains of live, lyophilized lactic acid bacteria including *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus*. Regardless of the formulation, commercial probiotic products should have an extended shelf life and should be able to deliver live active probiotic cells, even after prolonged storage, in inadequate quantity to the lower gastrointestinal tract. Some commercial probiotic products are enlisted in Table 2.

8 Status of Probiotics in India

Since the awareness about probiotics and their health benefits has grown tremendously among the Indian population, the demand for probiotic foods has greatly increased. Indian and multinational companies have multiplied rapidly since they first entered the Indian food industry in 2007, with milk and fermented milk products comprising 62% of the market share. In fact, the Indian probiotic market, valued at just $2 million USD in 2010, increased to nearly $310 million by 2011, and the value is estimated to increase to as high as $522.8 million by 2018.

Major pharmaceutical companies have become active in the probiotic market, and are attempting to formulate newer, more effective, drugs and more desirable products, such as probiotic-based nutritional supplements. In India, Amul, Nestle, and Mother Dairy are contributing significantly to the production and distribution of probiotic dairy products, and acceptance among the urban population is helping to grow the industry [Raja and Arunachalam 2011]. Several probiotics-based pharmaceutical products are already available on the market, some of the most prevalent of which are listed in Table 3.

Table 2 Commercially available probiotics

Strains	Probiotic product	Company	Country
Lactobacillus rhamnosus GG	Leporanta	Valio Dairy, Helsinki (www.valio.com)	Finland
Lactobacillus casei Shirota, *Bifidobacterium breve*	Yakult	Yakult, Tokyo (www.yakult.co.in)	Japan
Lactobacillus johnsonii Lal		Nestle, Lausanne (www.nestleinstitutehealthsciences.com)	Switzerland
Lactobacillus acidophilus NCFM		Rhodia, Madison	USA
Lactobacillus casei CRL-43i Gilliland (La-Mo)		Chr. Hansen, Wisconsin (www.chr-hansen.com)	USA
Lactobacillus reuteri SD 2112	Protectis	BioGaia, North Carolina (www.biogaia.com/research/lactobacillus-reuteri-strains/)	USA
Lactobacillus plantarum 299V	ProbiDigestis	Probi, Lund (https://probi.com)	Sweden
Lactobacillus casei DN 014001	Actimel	Danone, Paris (www.actimel.com)	France
Streptococcus thermophilus 1131		Meiji Milk Products, Tokyo (www.meiji.com/global/)	Japan
Bifidobacterium longum SBT-2928		Snow Brand Milk Products, Tokyo (www.meg-snow.com/english)	Japan
Saccharomyces boulardii CNCM I-745	Enterol	Biocodex, Seattle (http://ua.biocodex.com/en/Product/577/)	USA
Bifidobacterium longum BB536		Morinaga Milk Industry (www.probiotaamericas.com/morinaga-milk/)	Japan

9 Prebiotics

The term "prebiotic" was first coined by Gibson and Roberfroid (1995) and has generally been applied to carbohydrates which are metabolized by gut microorganisms, providing nutrition to intestinal epithelial cells (IECs), eventually improving overall gut health. In 2004, the definition was updated, and "prebiotics" were defined as "selectively fermented components allowing specific changes in the composition and/or activity of microorganisms in the gastro-intestinal tract, beneficial for the host's health and well-being" (Gibson et al., 2004). Finally, in 2007, the FAO/WHO described prebiotics as a "nonviable food component that confers a health benefit on the host's health by selectively stimulating the growth and activity of some genera of microorganisms in the colon, generally Lactobacilli and Bifidobacteria" (De Vrese and Schrezenmeir 2008). Prebiotics are generally nonabsorbable polysaccharides having positive effects on host health, increasing diversity

Table 3 Commercially available probiotics in India

Product	Company	Microorganisms	Formulation	Used for treatment of
Eubioz	Lupin (www.lupinpharmaceuticals.com)	*Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Bifidobacterium bifidum* *Bifidobacterium longum* *Streptococcus thermophilus* *Saccharomyces boulardii*	Oral (tablet)	Constipation, acute diarrhea Immunity, bacterial infection, antibiotic-associated diarrhea, AIDS, bacterial infections, traveler's diarrhea, high cholesterol levels, depression
Econova	Glenmark (www.glenmarkpharma.com)	*Lactobacillus reuteri* *Lactobacillus rhamnosus*	Oral (capsule)	Antidiarrheal
Bifilac	Tablets, Tamil Nadu, India (www.tabletsindia.com)	*Lactobacillus sporogenes* *Streptococcus faecalis* *Clostridium butyricum* *Bacillus mesentericus*	Oral (tablet)	Diarrhea as well as the associated bloating, flatulence, and constipation

Product	Company	Microorganisms	Formulation	Used for treatment of
Actigut	Alembic (www.alembicpharmaceuticals.com)	*Lactobacillus acidophilus* *Lactobacillus rhamnosus* *Bifidobacterium longum* *Bifidobacterium bifidum* *Saccharomyces boulardii* *Streptococcus thermophilus*	Oral (capsule)	Diarrhea, traveler's diarrhea, rotavirus diarrhea in children, diarrhea associated with using antibiotics
Becelac	Dr. Reddy Lab (www.drreddys.com)	*Lactobacillus acidophilus*	Oral (capsule)	Vitamin B12 deficiency, wound healing, diarrhea, Alzheimer disease, high cholesterol, anemia, treatment of megaloblastic anemias due to a deficiency of folic acid
Vi Bact	Unique Biotech Ltd (www.uniquebiotech.com)	*Streptococcus faecalis* *Clostridium butyricum* *Bacillus mesentericus* *Lactobacillus sporogenes*	Oral (sachet)	Irritable bowel syndrome, antibiotic treatments, diarrhea, long-lasting inflammation in the digestive tract, vaginosis, ulcers in the digestive tract, sugar intolerance, irritable bowel disease
Lactisyn	Franco-Indian (www.francoindian.com)	*Lactobacillus acidophilus* *Lactobacillus lactis* *Streptococcus thermophilus* *Streptococcus lactis*	Injection	Bacterial infections, gastrointestinal disorders, diarrhea, rotavirus diarrhea in children, traveler's diarrhea, diarrhea associated with using antibiotics, immunity

(continued)

Table 3 (continued)

Product	Company	Microorganisms	Formulation	Used for treatment of
Binifit	Rexcel (www.sunpharma.com)	*Clostridium butyricum* *Streptococcus faecalis* *Bacillus mesentericus* *Lactobacillus sporogenes*	Oral (capsule)	Immunity, gastrointestinal disorders, bacterial infections
Ecoflora	Tablets India Ltd Allianz Biosciences (P) Ltd (www.abpl.co.in)	*Lactobacillus rhamnosus* *Lactobacillus reuteri*	Oral (capsule)	Vaginosis, antibiotic treatments, sugar intolerance, diarrhea, irritable bowel syndrome, irritable bowel disease, skin diseases in infants and children who are allergic to cow's milk
Sporlac	Sanzyme Ltd (www.sanzyme.com)	*Lactobacillus sporogenes*	Oral (capsules and sachet)	Diarrhea, irritable bowel syndrome, long-lasting inflammation in the digestive tract, antibiotic treatments, vaginosis, ulcers in the digestive tract
Vizyl	Unichem (unichemlabs.com)	*Bacillus mesentericus* *Clostridium butyricum* *Lactobacillus sporogenes* *Streptococcus faecalis*	Oral (capsules)	Bacterial infections, diarrhea in young children, irritable bowel syndrome, immunity, vaginosis
Darolac	Aristo Pharmaceuticals Pvt Ltd (aristopharma.org)	Lactic acid bacteria	Oral (sachet)	Bowel problems, acute diarrhea, indigestion, dyspepsia, antibiotic-associated diarrhea, inflammatory bowel disease, urinary tract infections, eczema, AIDS

in human gut microbiome, and stimulating immunomodulatory activity. They also promote mineral absorption and can act as a fertilizer for healthy gut microflora. Since the human body is not capable of digesting these plant fibers, they are directly used to boost the expansion of various desirable microorganisms within the gut. Prebiotics can be readily fermentable dietary fibers, oligosaccharides such as fructooligosaccharides (FOS), inulin, galactooligosaccharides (GOS), mannan-oligosaccharides (MOS), xylooligosaccharides (XOS), and human milk oligosaccharides. They also include conjugated linoleic acid (CLA), polyunsaturated fatty acids (PUFA), and some phytochemicals. Fruit, vegetables, cereals, and various edible plants are other sources of carbohydrates, making them potential prebiotics (Markowiak and Śliżewska 2017). Some other synthetic prebiotics include lactulose, cyclodextrins, and lactosaccharose. Fructans, like inulin and oligofructose, are believed to be the most used and most effective, in regard to several species of probiotics (Jakubczyk and Kosikowska 2000).

10 Health Benefits of Prebiotics

Prebiotics have a huge potential for modifying the gut microbiota. This potential is directly influenced by the nature of the individual strain and species, and by the gut atmosphere, especially in terms of pH, as it plays a key role in deciding the end result of interspecific competition and colonization of the gut lining (Chung et al. 2016). Prebiotics also have many health advantages, such as decreasing the prevalence and duration of diarrhea, relief from inflammation and different symptoms related to intestinal bowel disorder, and protection against colon cancer (Peña 2007). Additionally, prebiotics are also involved in enhancing the bioavailability and uptake of minerals, lowering of some risk factors of cardiovascular disease, and promoting repletion and weight loss, thereby preventing obesity (Pokusaeva et al. 2011).

11 Types of Prebiotics

Prebiotics can be grouped into various types based on their chemical nature, as follows:

1. **Polysaccharides**
   (a) **Starch and polyfructans** are good sources of prebiotics and are currently available in the market. Starch is insoluble polyglucan linked by α-(1→4) and α-(1→6) bonds, and synthesized in chloroplasts, while soluble polyfructan is stored in vacuoles (Heldt 2005). Both polysaccharides are hydrolyzed enzymatically into prebiotic oligosaccharides.
   (b) **Pectin** is a complex, galacturonic acid-rich polysaccharide and is one of the most important components of plant cell walls (Ridley et al. 2001). It is made up of covalently linked homogalacturonan (HGA), rhamnogalacturo-

nan-I (RG-I), and rhamnogalacturonan-II (RG-II) (Ridley et al. 2001), which can be used to synthesize pectic oligosaccharides by enzymatic hydrolysis or physical methods. For example, oligosaccharides of 3–4 kDa can be produced in membrane reactors by enzymatic hydrolysis of citrus and apple pectins (Olano-Martin et al. 2001), while low atomic weight arabinose-based oligosaccharides can be produced by nitric acid hydrolysis of citrus peels (Fishman et al. 1999).

2. **Oligosaccharides**
   (a) **Isomaltooligosaccharides (IMOs)** are found in fermented foods, such as miso and soy sauce, sake, and honey and can be made from starch via a two-stage enzymatic process. In the first stage, starch is converted to maltooligosaccharides by treating with α-amylase and β-amylase. Thereafter, transglycosylation of α-(1-4) linkages into α-(1-6) linkages is performed by α-glucosidase (Yoo et al. 2012). Basically, IMOs have only α-(1-6) linkages with a DP (degree of polymerization) range of 2–6. Panose, a glucose trisaccharide, has both α-(1-4) and α-(1-6) linkages. It was observed that these IMOs can be utilized by many probiotic bacteria, such as bifidobacteria and *Bacteroides fragilis*, promoting their growth (Sarao and Arora 2017).
   (b) **Gentiooligosaccharides** include α-(1-6) linked glucoses having DP range of 2–6. They are made from the hydrolysis of starch by enzymatic transglucosylation (Wichienchot et al. 2009). GOS are not digested in the stomach and small intestine therefore reaching the colon intact (Yoo et al. 2012).
   (c) **Fructooligosaccharides (FOSs)** are inulin and the structurally related FOSs are nondigestible oligosaccharides (NDO) which are commonly consumed in the human diet. Since they are not digested in the upper human gastrointestinal tract, they reach the colon intact, where they can be metabolized as dietary fibers by the resident microbiota. Inulin is widely distributed in nature as plant storage carbohydrates, being present in more than 36,000 plant species (Sarao, and Arora 2017). Good sources of inulin include garlic, onion, asparagus, chicory, artichoke, and wheat. Chemically, inulin is considered as oligosaccharide and polysaccharide having the structure GFn (where G = glucose, F = fructose, and n = the number of fructose residues linked to one another). The fructose residues are arranged in a linear form by β-(2,1) bonds, however, a single glucose molecule is linked to the end of the polysaccharide by an α-(1, 2) bond. The DP length of chicory fructans ranges from 2 to 60, with an average DP of 10 (Flickinger et al. 2003). In fact, the highest number of linked fructose residues, in general (~60), has been reported in chicory. Other types of fructans that are structurally similar to inulin have both GFn and FFn molecules, where the number of fructose residues can range from 2 to more than 70 units. These FOS, having a lower molecular weight as compared to inulin, have a positive effect on intestinal *Bifidobacteria* and are categorized as important prebiotics. Inulin extracted from chicory roots can be hydrolyzed by the enzyme inulinase under controlled conditions to produce short-chain FOSs as Glu-α1-2(β-D-Fru 1-2)n, where n = 2–9. Additionally, the

other FOS product, called "neosugar" or "meioligo," is a combination of oligosaccharides of varying lengths, including 1-ketose (Glu-Fru2) and 1F-β-fructosylnystose (Glu-Fru4). These oligosaccharides are enzymatically synthesized from sucrose by the transfructosylation of β-fructosidase.

Moreover, the β configuration of anomeric carbon in fructose is thought to make FOS resistant to digestion (Desai et al. 2004). In vitro, they selectively stimulate the growth of *Bifidobacterium* (Sarao and Arora 2017). Other bacteria, such as *Klebsiella pneumoniae, Bifidobacteria*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis, Enterococcus faecium*, *Bacteroides vulgatus, Bacteroides thetaiotaomicron, Bacteroides ovatus, Bacteroides fragilis, Lactobacillus acidophilus,* and *Clostridium*, can utilize inulin and other FOSs.

(d) **Galactooligosaccharides (GOS)** are one of the most common and well-studied types of prebiotic oligosaccharide. Lactose is transformed in GOS via β-galactosidase-mediated transgalactosylation reactions (Fai and Pastore 2015; Vera et al. 2016), namely, β-galactosidase-mediated hydrolysis of lactose followed by polymerization of β-linked sugars (Yoo et al. 2012). First, covalent bonding of the lactose molecule through its galactosyl moiety occurs with the enzyme, enabling a catalysis reaction. Thereafter, the reaction can be diverted into various paths based on the selectivity degree between the lactose concentration and enzyme, affecting the type of the galactosyl acceptor. For example, if water molecule is the acceptor, hydrolysis takes place, and as a result, a galactose-free molecule is formed. However, if the acceptor is a sugar molecule (lactose, GOS, glucose, or galactose), it acts as both the donor and the acceptor of the galactosyl moiety. The resulting oligosaccharides produce mixtures of GOS having a DP of up to 10 (Muñiz-Marquez et al. 2015). These GOSs are similar in structure and prebiotic characteristics to the oligosaccharides found in human milk (Sharon and Ofek 2000).

(e) **Xylooligosaccharides (XOSs)** are oligomers having xylose residues linked by β(1 → 4) xylosidic bonds with normal DP ranges from 2 to 6 (and up to 20) (Samanta et al. 2015). They are NDOs obtained by the hydrolysis of xylans and categorized by the number of monomers among them (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexose) (Kumar and Satyanarayana, 2011) and can be formed by chemical, enzymatic, and autohydrolysis processes (Xue et al. 2016). XOSs are considered to be ideal prebiotics, as they are soluble fibers stable over a wide range of temperatures (up to 100 °C) and pH conditions (2.5–8.0). The best sources of XOSs are various food products, such as fruits, vegetables, honey, milk, sugarcane bagasse, bamboo, corncobs, barley straw, wheat bran, and cotton stalk (Carvalho et al. 2013; Alice et al. 2012; Singh et al. 2015). In comparison to FOSs, XOSs are more stable during several food-processing techniques, such as pasteurization and autoclave sterilization at low pH characteristics (Courtin et al. 2009; Wang et al. 2009).

Health benefits of XOS are due to its selective growth of gut microbiota; increasing number of probiotic microorganisms, such as *Bifidobacteria* and

lactobacilli; immunomodulation; regulation of insulin secretion; reduction of blood cholesterol levels; enhanced mineral absorption; antioxidant activity; and anticancerous and anti-inflammatory effects (Mäkeläinen et al. 2009; Chapla et al. 2012; Samanta et al. 2015; Bian et al. 2013; Kallel et al. 2015a, b; Kyoji et al. 2006).

(f) **Mannan-oligosaccharides (MOSs)** belonging to hemicellulose groups are present in plant cell walls, and as storage carbohydrates in plant seeds (Mikkelson et al. 2013). Their nomenclature is based on the main sugar constituent. For example, mannan contains only mannopyranosyl units linked by β-1,4 bonds, and glucomannan consists of mannopyranosyl and glucopyranosyl units linked by β-1,4 bonds, and they may also have α-1,6 galactopyranosy l residues as side groups, known as galactomannans and galactoglucomannans, respectively (Mikkelson et al. 2013). The main constituent of hemicellulose is glucomannan/galactoglucomannan and galactomannan, mainly found in seeds (Moreira 2008). MOSs are less explored, but valuable, prebiotic compounds, as they stimulate the growth of probiotic microorganisms while inhibiting pathogenic microorganisms (Patel and Goyal 2012). Additionally, MOSs can be used in food, feed, and pharmaceutical fields, as these compounds exhibit a positive effect on immune-pharmacological, therapeutic, and biomedical properties (Ferreira et al. 2012; Yamabhai et al. 2016; Srivastava and Kapoor 2017).

3. Long-Chain Beta-Glucans

Cereal beta-glucans pass undigested through the GI tract, ultimately acting as substrates for probiotic microflora (Gibson et al. 2004), and thus can also be used as prebiotics (Bigliardi and Galati 2013). Pleuran, beta-glucans isolated from the fruiting body of mushroom, *Pleurotus*, are also used as food supplements and are known for their prebiotic and immunosuppressive properties (Patel and Goyal 2012). Some commercially available beta-glucan products are Ceapro from oats (Tomasik and Tomasik, 2003), Glucan Elite, a mixture of grain, yeast, and mushroom β1,3-D-Glucan (by Pro Formulations Md), Beta-1,3/1,6-D-Glucan (Now Foods), Glucagel from barley (Lam and Cheung 2013), and Betamune from Yeast (Vetvicka et al. 2008).

4. Short-Chain Fatty Acids (SCFAs)

SCFAs are produced as end-products of the metabolism of prebiotics. These volatile fatty acids have fewer than six carbons arranged in straight and branched-chain conformation, such as acetic acid, carboxylic acid, and butyric acid. They are made within the large intestine as fermentation products of unabsorbed and undigested food elements by gut microbiota. SCFAs also stimulate the synthesis of hepatic triacylglycerols. The major sources of SCFA are carbohydrates, but, amino acids, such as isoleucine, leucine, and valine, can also be transformed into isobutyrate, isovalerate, and 2-methylbutyrate, which are known as branched-chain SCFAs (BSCFAs) (Vitali et al. 2010).

12 Some Examples of Novel Prebiotics

(a) **Acacia gum (AG)** (Fibregum™) is an example of a natural prebiotic, having high gut tolerance. Due to its low viscosity and its resistance to processing, it can be used to formulate a wide range of food products with nutritional and health benefits. It is not metabolized in the upper GI tract, as it is resistant to various digestive enzymes, such as galactanases or arabinases. In the colon, AG represents an extra carbon source, providing fuel for microbial fermentation, resulting in SCFAs stimulating the growth of probiotics (Meance 2004) (Fig. 2).

(b) **Human milk oligosaccharides (HMO)** are considered to be "the first prebiotics in humans" (Coppa et al. 2004). An array of human milk oligosaccharides have been discovered recently for pediatric uses, such as lacto-N-neotetraose (LNnT) and lacto-N-biose I. Both tetrasaccharides are highly specific, natural prebiotics for *Bifidobacteria*. Lacto-N-biose I and Lacto-N-neotetraose have been artificially synthesized using N-acetylglucosamine (GlcNAc) by adding sucrose and lactose, respectively.

(c) ***L. barbarum* polysaccharides (LBP)**, isolated from *Lycium barbarum* (goji berries), contain arabinose, rhamnose, xylose, mannose, galactose, and glucose residues and have been reported to promote the proliferation of lactic acid bacteria strains, especially *Bifidobacterium longum subsp. infantis* Bi-26 and *Lactobacillus acidophilus* NCFM. It promotes the bacterial biosynthetic and metabolic processes, gene expression, transcription, and transmembrane transport. Furthermore, LBP improves cell vitality during freeze-drying and tolerance of the gastrointestinal environment. LBP can be used as a potential prebiotic for *Bifidobacterium* and *Lactobacillus* (Sohail et al. 2010)

(d) **Mushroom polysaccharides**: The polysaccharides obtained from mushrooms, e.g., *Lentinula edodes* stipe, *Pleurotus eryngii* base, and *Flammulina velutipes* base, can enhance the survival rate of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium longum subsp. longum* in simulated gastric and bile juice conditions to achieve beneficial effects in the host. These results show that mushroom wastes, which are cheaper than most other sources, could be an important, new, alternative source of prebiotics (Singdevsachan et al. 2016).

13 Mechanism of Action of Prebiotics

Although prebiotics are naturally present in various food products, they may also be used as additives to improve the nutritional and health value of foods. Given that prebiotics are unmetabolized by digestive enzymes, they reach the colon unaltered, where they can be used as a substrate for probiotics, and can stimulate their growth, often leading to a dramatic increase in the numbers of beneficial bacteria (Schiffrin et al. 2007; Vulevic et al. 2008). Table 4 presents the mechanism of action of prebiotics on different diseases. Prebiotics modulate the intestinal microbiota and its metabolic activity by altering lipid metabolism, mineral absorption, immune system activity, and bowel function (Van Loo et al. 2005). There are many proposed models

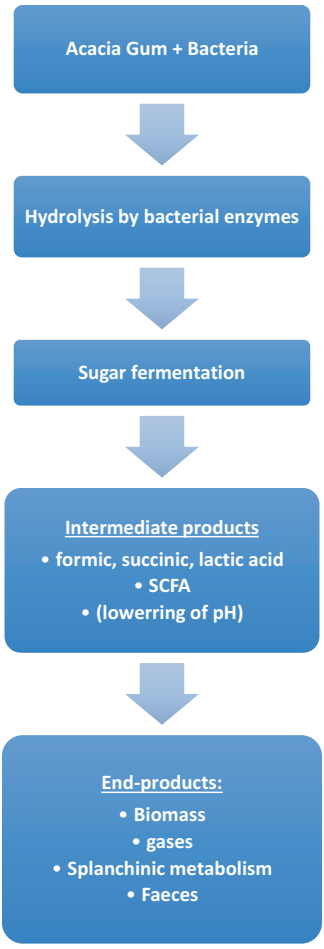

Fig. 2 Fermentation of acacia gum in the colon resulting in beneficial effects on host health through both the improvement of the composition of the large intestine microflora and SCFA formation. Yacon (*Smallanthus sonchifolius*) contains beta-1, 2-oligofructans as the main saccharides, and its roots are consumed in various South American countries. Traditionally, yacon roots and infusions from dried leaves were consumed by people suffering from diabetes or from various digestive disorders in countries such as Brazil. The percentage of FOS in yacon is 70–80% of its dry weight. Thus, yacon could be a potential prebiotic and has been found to exert an effect on the intestinal ecosystem. Yacon root flour has an immunomodulatory effect, and this effect may be indirect, being that the prebiotic stimulates the growth of *Bifidobacteria* and *Lactobacilli*. (Gibson and Roberfroid 1995)

to describe the beneficial effect of prebiotics on immunomodulation, which are as follows (Schley and Field 2002):

(a) By increased production of SCFAs, such as propionic acid, prebiotics are able to regulate the action of hepatic lipogenic enzymes.

Table 4 Prebiotics and their mechanism of action on different diseases

Prebiotic used	Disease name	Mechanism of action	References
Inulin	Crohn's disease	Enhancement of immune response	Hijová et al. (2013)
	Colitis	Effect on innate immunity	Macfarlane et al. (2008)
	Constipation	Modification of microbiota and increase in *Bifidobacteria*	Hopping et al. (2009)
FOS (fructooligosaccharide)	Crohn's disease	Increase in *Bifidobacteria*	Scholtens et al. (2006)
	Colitis	Decrease in colon pH	Benjamin et al. (2011)
	Constipation	Secretion of anti-inflammatory substances	Cummings et al. (2001a, b)
	Traveler's diarrhea	Local induction of Reactive oxygen species (ROS)	Arslanoglu et al. (2008)
GOS (galactooligosaccharide)	Crohn's disease	Improvement of growth performance and immune responses	Saavedra and Tschernia, (2002)
	Colitis	Diminishment of intestinal bacterial growth	Macfarlane et al. (2008)
Soluble fiber (guar gum, pectin)	Crohn's disease	Enhancement of short-chain fatty acid production, and mainly acetate	Peng et al. (2013)
	Colitis	Effect on epithelial permeability	Chen et al. (2013)
	Celiac disease	Normalization of intestinal microbiota	Slavin (2013)
	Metabolic syndrome	Anti-inflammatory effect	Cao et al. (2011)

(b) The produced SCFAs, such as butyric acid, can modulate histone acetylation, resulting in increased transcription.
(c) An increase in mucin production.
(d) FOS and several other prebiotics cause an increase in the number of lymphocytes and/or leukocytes in gut-associated lymphoid tissues (GALTs).
(e) The phagocytic function of intra-inflammatory macrophages has also been reported to increase the secretion of IgA by GALTs.

14 Prebiotic Selection Criteria

Any food element possessing the following properties can be considered as a prebiotic:

(i) It should be resistant to the action of extreme pH and hydrolyzing enzymes within the intestine, and should not be absorbed in the upper GI tract, instead targeting the distal colon.

(ii) It should be simply fermentable and selectively stimulate the growth and activity of beneficial intestinal microflora (Kuo 2013).

Some other desired properties of prebiotics are:

- The fermentation of prebiotic compounds results in the enhanced production or modification of various SCFAs, the reduction of pH, and an overall improvement of the immune system (Lee and Salminen 2009).
- Have low dosage requirements and low calorific value.
- Have multifarious properties with no undesired side effects.
- Should be easily added into food and possess various types of glycosidic bonds and sugar residues.
- Should have varying molecular weight and viscosity.

15 Synbiotics

The term "synbiotic" was first introduced by Gibson and Roberfroid in 1995 to describe a combination of synergistically acting probiotics and prebiotics, such as a product containing oligofructose and probiotic *Bifidobacteria*. Prebiotics are used to selectively stimulate and enhance the survival, as well as the colonization, of probiotic microorganisms in the intestine. Although more studies are required to elucidate the mechanisms of action, the health benefits of synbiotics are found to be associated with the individual combination of prebiotic and probiotic (De Vrese et al. 2008). As there can be a large number of possible combinations, the scope of application of synbiotics is very wide (Scavuzzi et al. 2014). Some commonly used examples of synbiotics are listed in Table 5.

16 Mechanism of Action

Prebiotics are used as substrates for the growth of probiotic microorganisms, and these microorganisms can flourish in the intestine (Sekhon and Jairath 2010). Synbiotics create viable dietary supplements for microorganisms and also build a suitable environment, resulting in a positive impact on the host's health. Two modes of action of synbiotics have been devised (Manigandan et al. 2012), including improving the viability of probiotic microorganisms, and providing positive health effects. They do not let the pathogen to colonize as prebiotics help probiotic microbes in colonizing the gut and give the pathogens tough competition for growth factors, nutrients and for adhesion sites (Biofilm formation) and coaggregation. The combination of pre- and probiotics strengthens host health by increased nutrient absorption. They influence the activity of certain enzymes so as to modify toxin receptors and block toxin-mediated pathology. They may be directly affecting the growth of pathogens due to their antimicrobial activity as production of AMPs as well as inhibitory compounds as hydrogen peroxide, bacteriocin, lactic acid, and

Table 5 Examples of synbiotics

Synbiotic		References
Probiotics	**Prebiotics**	
Lactobacillus casei strain *Shirota*	Oligomate 55™	Figueroa-González et al. (2010)
Bifidobacterium longum	Oligofructose	Sarao and Arora (2017)
Bifidobacterium lactis Lafti™B94	Resistant starch	Crittenden et al. (2001)
Bifidobacterium breve strain *Yakult*	Galactooligosaccharide	Kano et al. (2013)
Lactobacillus acidophilus ATCC 4962	Mannitol, fructooligosaccharide, and inulin	Liong and Shah (2005)
Lactobacillus sakei JCM	Fructooligosaccharide and trehalose	Yanagida et al. (2005)
Lactobacillus plantarum and *L. acidophilus*	Xylo- and fructooligosaccharide	Olveira and González-Molero (2016), and Sáez-Lara et al. (2016)
Lactobacillus	Inulin	Crittenden et al. (2006), Olveira and González-Molero (2016), and Sáez-Lara et al. (2016)
Lactobacillus, Streptococcus, and Bifidobacterium	FOS	Crittenden et al. (2006), Olveira and González-Molero (2016), and Sáez-Lara et al. (2016)
Lactobacillus and *Bifidobacterium*	Lactulose	Crittenden et al. (2006), Olveira and González-Molero (2016), and Sáez-Lara et al. (2016)
Lactobacillus and *Bifidobacterium*	Lactosucrose	Crittenden et al. (2006), Olveira and González-Molero (2016), and Sáez-Lara et al. (2016)
Lactobacillus sporogenes	Arabinogalactan	Dixit et al. (2016)
Lactobacillus casei or *rhamnosus*	Tagatose	Dixit et al. (2016)
Bifidobacterium and *Streptococcus* *Enterococcus* and *Lactococcus* *Staphylococcus* and *Peptostreptococcus* *Lactobacillus, Saccharomyces* *Propionibacterium*	High amylose carbohydrate Amylose-resistant starch product Fructooligosaccharide Potato Protein	Dixit et al. (2016)

ammonia by probiotics. Furthermore, synbiotics can support the immune system of the host by stimulating IgA and cytokines (TNF-α, IFN-γ, and IL-10), having an adjuvant effect, stimulating phagocytes, decreasing MMP production, immunomodulation via increased production of mucin, and producing SCFAs (propionic acid and butyric acid). The various mechanisms of synbiotic action, based on the

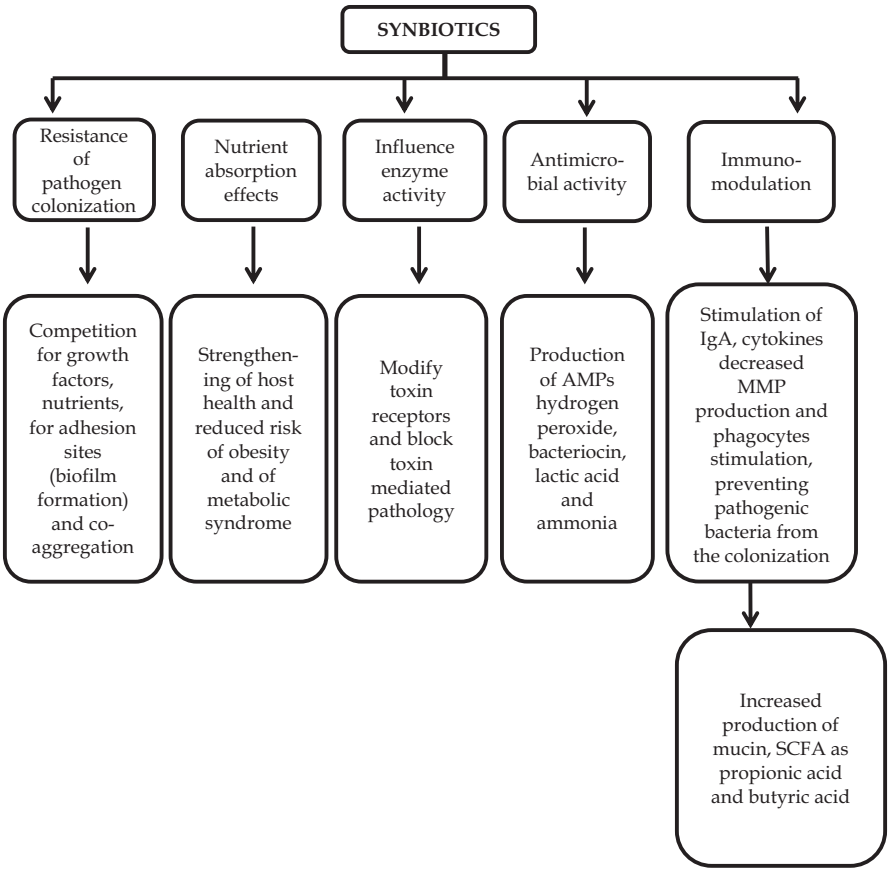

Fig. 3 Mechanism of action of synbiotics

modification of intestinal microbiota with probiotic microorganisms and appropriately selected prebiotics as their substrates, are presented in Fig. 3.

17 Beneficial Effects on Human Health

Probiotic microorganisms are stimulated by the presence of prebiotics, thereby regulating metabolic activity in the intestine. The ideal synbiotics include those with antibacterial, anti-oncogenic, and anti-allergic effects. They are helpful for the treatment of inflammatory diseases, such as inflammatory bowel disease and other syndromes, and are generally recommended along with antibiotic therapy in order to maintain the microbial balance of the intestinal tract. Synbiotics reduce the concentrations of toxic metabolites and oncogenic substances, and inhibit potential pathogens present in the GI tract (De Vrese et al. 2008). The use of synbiotics also causes significant increases in the level of SCFAs, carbon disulfides, ketones, and methyl

acetates, thus providing health benefits to the host (Markowiak and Śliżewska 2017). They also regulate the immune system, can prevent osteoporosis, and can reduce blood fat and sugar levels. Synbiotics have various other positive effects on humans including enhanced *Lactobacillus* and *Bifidobacterium* counts and the maintenance of a balanced intestinal microbiome and inhibition of bacterial translocation and reduced incidence of nosocomial infections in postsurgical procedures and similar interventions (Zhang et al. 2010). Synbiotics have also been recently shown to aid in the treatment of neurological disorders linked with abnormal liver function cirrhotic patients (Pandey et al. 2015) and can aid in the treatment of skin ailments, such as atopy. In addition to these works, countless other applications of synbiotics are currently ongoing, such as in the treatment of chronic kidney disease.

18 Synbiotic Selection Criteria

During composition of a synbiotic formula, the first feature to be taken into account is the selection of a suitable probiotic and prebiotic that each has a positive impact on the host's health when used individually. A selected prebiotic should selectively stimulate the growth of subject microorganisms, while other microorganisms remain unaffected. In addition to this, probiotics should be able to metabolize the prebiotic compounds in the environment of the GI tract and synbiotics should be in a position to inhibit the growth of pathogenic microorganisms.

19 Conclusion

As the problem of antibiotic resistance among pathogens is increasing, there is an urgent need for the discovery of novel alternative strategies. The direct and indirect beneficial effects of probiotic microorganisms can help in the fight against infectious diseases. Additionally, they can be used along with specific prebiotics as a dual therapy for the management of multidrug-resistant (MDR) pathogens. As their combined action can possibly balance the dysbiosis and these synbiotics can be further developed to directly inhibit the growth and colonization of pathogens as well as regulate the immune system, for combating antibiotic resistance in pathogens.

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Combination of Drugs: An Effective Approach for Enhancing the Efficacy of Antibiotics to Combat Drug Resistance

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Abstract

Currently available antibiotics have been effective in treating infectious diseases; however, the development of resistance to these drugs has led to the emergence of new and the re-emergence of old, infectious diseases. Therefore, newer antibiotic approaches with mechanistic differences are needed to combat antimicrobial resistance. Combining antibiotics is an encouraging strategy for increasing treatment efficacy and for controlling resistance evolution. This approach may include the combination of one antibiotic with another antibiotic and the development of adjuvants that either directly target resistance mechanisms, like inhibition of β-lactamase enzymes, or indirectly target resistance by interrupting the bacterial signaling pathways, such as two-component systems. Other natural products, like essential oils, plant extracts, and nanoparticles, can also be combined synergistically with antibiotics. The aim of this chapter is to highlight the strategy of treating infections with arrays of drugs rather than discrete drugs. We have addressed here three categories of approaches being used in combination therapy: the inhibition of targets in different pathways, the inhibition of distinct nodes in the same pathway, and the inhibition of the same target in different ways. Here, we have described the most recent developments toward combination therapies for the treatment of infectious diseases caused by multidrug-resistant bacteria.

Keywords

Antibiotic · Combination therapy · Drug resistance · Synergy

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1 Introduction

In recent years, bacterial infections have become a global health challenge due to the emergence of multidrug resistance in pathogenic strains. The indiscriminate use of antibiotics has led to an alarming increase in resistance among microorganisms, and also opened the door for re-emergence of old infectious diseases (Fair and Tor 2014). Majority of infectious diseases are caused by biofilm-forming strains that are several 1000-fold tolerant to antibiotics (Hoiby et al. 2010). As a result, the existing antibacterial drugs are becoming less effective and it has forced the investigators to develop newer varieties (Khameneh et al. 2016; Zaman et al. 2017). Various approaches have been developed and employed by researchers to eliminate antibiotic resistance, though understanding the underlying mechanisms. These include the removal of antibiotics from the bacterial cell through efflux pumps, enzymatic modification or degradation of the antibiotics, and modification of the antibiotic targets (Kalan and Wright 2011). Thus, overcoming resistance requires the use of various approaches, like inhibiting the enzymes that degrade or modify the antibiotic to a non-active form, hindering antibiotic efflux, enhancing antibiotic entry into the cell, and changing the physiology of the cells to render them more sensitive to antibiotic killing (Kalan and Wright 2011; Zaman et al. 2017).

However, the higher prevalence of resistant strains is making the solution as extremely difficult and necessitates newer approaches. Therefore, novel antimicrobial discovery and drug combinations are being explored in order to combat the multidrug-resistant (MDR) phenotype. The toxic effects of antibiotics are lowered in drug combinations and the potency of antimicrobial compounds also get increased against resistant strains (Khameneh et al. 2016). Application of synergistic activity between antibiotics and non-antibiotics is also exploited. Such efforts include the combination of an antibiotic with a non-antibiotic adjuvant compound to directly target resistance mechanisms or by interfering with the bacterial signaling pathways (Worthington and Melander 2013a). One such strategy is the coupling of β-lactam antibiotics with β-lactamase inhibitors (Worthington and Melander 2013b). Several plant extracts, essential oils, phytocompounds, and nanoparticles have also exhibited synergistic interactions with various classes of antibiotics against microorganisms, including drug-resistant strains (Hemaiswarya et al. 2008; Allahverdiyev et al. 2011; Khan and Ahmad 2011; Khan et al. 2012). In clinical settings, two or more antimicrobial drugs are often combined to treat MDR infections (Worthington and Melander 2013a), including those caused by bacteria and fungi. For example, a combination of four drugs is being used for the treatment of *Mycobacterium tuberculosis* infections (Mitchison and Davies 2012). The emergence of many new MDR pathogens has indicated that monotherapy is no longer satisfactory to treat these infections, and instead, combination therapy should be utilized (Tamma et al. 2012).

The success of combination therapy against microbial infections depends on its ability to combat the infection, avoid resistance, minimize host toxicity, and leave the natural microflora intact. To further boost the efficacy of combination therapy while minimizing drug concentrations, local drug delivery is also necessary. Overall, the key features of a combination treatment include (i) enhancement of antibiotic

activity by synergistic effect, (ii) prevention of resistance emergence, (iii) possession of anti-biofilm activity, (iv) improvement of antibiotic penetration to cell and tissues, and (v) inhibition of virulence factors, such as toxin or enzyme production in pathogens (Hagihara et al. 2012). When drugs are combined, their individual effects on cells may be augmented or weakened, resulting in either synergistic, antagonistic, or no interactions (Khameneh et al. 2016). High-throughput studies have resulted in the identification of drugs based on their interactions with established antibiotics, thereby enabling the prediction of drug interactions (Bollenbach 2015). It has also been found that the mechanism of action of drugs in combination therapy can significantly differ from that of the single drugs. Therefore, the selection of proper combinations is critical, and necessitates an understanding of the potential interactions between the antimicrobial compounds (Yeh et al. 2009; Hamoud et al. 2014). This approach is not only restricted to the use of biologically active compounds; the use of smart controlled delivery strategies could also be considered. Overall, the conceptual and technical establishment for the rational design of effective drug combinations is quickly developing (Bollenbach 2015; Khameneh et al. 2016).

In this chapter, we aimed to summarize recent approaches used in combining antibiotics based on their mechanisms of action. We have briefly considered examples of combination therapies that pair antibiotics with other naturally occurring antibacterial agents, such as plant products and nanoparticles, to formulate new prospects for future studies. We have also addressed the opportunities and challenges in making influential use of drug combinations.

2 Combination Approaches of Antibiotics

The combination approach can be divided into three categories based on the drug target: (1) combining antibiotics that target different pathways (e.g., treatment of *Mycobacterium tuberculosis* infections with a combination of isoniazid, rifampicin, ethambutol, and pyrazinamide), (2) combining antibiotics that target different parts of the same pathway (e.g., sulfamethoxazole and trimethoprim), and (3) combining antibiotics that attack the same target by multiple mechanisms (e.g., streptogramins and virginamycin) (Fischbach 2011; Worthington and Melander 2013b; Hamoud et al. 2014).

2.1 Combination Approaches that Target Different Pathways

Utilizing drugs that target multiple pathways is one of the most successful approaches to combat antibiotic resistance. A prime example of this is DOTS chemotherapy, used in treating *Mycobacterium tuberculosis*, which employs a combination of four drugs: isoniazid, an inhibitor of the enoylreductase subunit of fatty acid synthase; rifampicin, an RNA polymerase inhibitor; ethambutol, an inhibitor of arabinosyl transferases involved in cell wall biosynthesis; and pyrazinamide, whose mechanism of action is not well understood (Fischbach 2011). Thus with this therapy, at least three pathways are inhibited at once, meaning that even if a strain of *M.*

tuberculosis manages to protect one of the pathways, other crucial pathways will be obstructed.

The incredible improvements in survival for HIV-infected patients have been made possible because of combination approaches (Richman 2001). Treatment for such patients includes the combination of two nucleoside reverse transcriptase inhibitors, emtricitabine and tenofivir. One of them adds raltegravir, an integrase inhibitor; the second adds the non-nucleoside reverse transcriptase inhibitor, efavirenz; and a third adds a mixture of ritonavir and darunavir, both protease inhibitors (Lennox et al. 2009). With this therapy, HIV is not completely eradicated, but instead becomes a manageable chronic illness.

It should be noted that many effective combinations of drugs targeting different pathways are not only limited to antibiotics, but also include pairings with non-antibiotic adjuvants as well (Smith et al. 2013; Hamoud et al. 2014).

2.1.1 Combinations with Non-antibiotic Adjuvants

One of the prominent strategies for the treatment of MDR bacterial infections is to combine an antibiotic with a compound that is non-antimicrobial alone, but that assists in the enhancement of drug activity. For example, the compound may act by blocking the mechanism of resistance to the antibiotic. Such an approach is particularly attractive as resistance development is minimized (Worthington and Melander 2013a). Three common adjuvant types with clinical achievements are antiseptics, inhibitors, and biological (bacteriophage) or natural (phyto-compounds, nanoparticles) compounds. Additionally, several other known pharmaceutical compounds, such as antihistamines, antihypertensives, antispasmodics, anti-inflammatory drugs, and tranquilizers, are now being discovered as antibiotic adjuvants (Ejim et al. 2011; Smith et al. 2013).

2.1.1.1 Antiseptic Adjuvants

Antiseptics or biocides are the most commonly used adjuvants. For example, chlorhexidine, a bisbiguanide, is used to either kill or inhibit the growth of pathogens and is reported to show multiple sites of targets (Muller and Kramer 2008). Their ability to permeate and disrupt the membrane or inactivate ATPase has made them a very effective choice in combination (McDonnell and Russell 1999). For example, the coating of catheters with an antibiotic/antiseptic combination has shown significant efficacy against a variety of pathogens (Wu and Grainger 2006). It should be noted that despite their success, the development of resistance has been reported for in vitro combinations of chlorhexidine or silver sulfadiazine (antiseptics) and minocycline or rifampicin (antibiotics) (Lewis 2005).

2.1.1.2 Inhibitor Adjuvants

Inhibitor adjuvants augment the bactericidal treatments by targeting the applicable mechanisms of resistance (Roemer et al. 2013; Drawz et al. 2014). Multiple adjuvants are used to counter enzymatic degradation of antibiotics. Augmentin, for example, is a combination of β-lactam antibiotic (amoxicillin) and β-lactamase inhibitor (clavulanic acid). In this combination, the in vivo β-lactamase production

in bacteria is inhibited by clavulanic acid, facilitating inhibition of cell wall biosynthesis by amoxicillin. In this case, the addition of the adjuvant has allowed for the continued use of amoxicillin to treat infections caused by pathogens that may develop resistance to β-lactam antibiotics (Ball 2007).

Compounds inhibiting efflux pumps have also been exploited in several antibiotic combinations in order to reduce the prevalence of a resistant phenotype. For example, reserpine, a well-known mammalian MDR pump inhibitor, when used in combination with ciprofloxacin, has resulted in suppression of resistance in *Staphylococcus aureus* and *Streptococcus pneumoniae* strains (Lomovskaya et al. 2001). Similarly, celecoxib, a nonsteroidal anti-inflammatory drug (NSAID) that inhibits the MDR1 efflux pump, when combined with antibiotics like ampicillin, kanamycin, chloramphenicol, and ciprofloxacin, results in improved sensitivity of *S. aureus* to these antibiotics (Kalle and Rizvi 2011). The use of inhibitor as adjuvant is advantageous compared to antiseptic adjuvants for two reasons: (i) the antiseptic adjuvants are antimicrobial in nature, whereas inhibitor adjuvants being non-antimicrobial, can avoid the evolution of resistance against them (Worthington and Melander 2013a), and (ii) the complementary act of an inhibitor adjuvant toward the action of its antimicrobial counterpart does not promote the development of new mechanisms of resistance (Hamoud et al. 2014).

2.1.1.3 Biological and Natural Adjuvants

Use of natural and biological adjuvants with antibiotics is a very encouraging approach and has shown its extensive application. Many investigators have demonstrated that the combination of an antibiotic with a bacteriophage adjuvant can lead to a more effective therapy than either agent alone (Petty et al. 2007; Ghannad and Mohammadi 2012). In Georgia, for example, a company named PhagoBioDerm is using a combination of a lytic phage and ciprofloxacin in a biodegradable polymer matrix (Markoishvili et al. 2002). Biological adjuvants in drug combinations naturally enhance antimicrobial efficacy as they target multiple sites of action in pathogens that will not allow to develop resistance easily. In a study by Barekzi et al. (2002), IgG antibodies were used as adjuvants to promote a host immune response, while also suppressing any additional development of resistance, as the bacteria do not experience any direct selective pressure against them.

There are many reports on the use of natural compounds as synergistic adjuvants, especially plant-derived essential oils, extracts, and phytocompounds, such as eugenol, cinnamaldehyde, geraniol, and thymol, in combination with antifungals, like azoles, and antibiotics, like vancomycin (Khan and Ahmad 2011; Hamoud et al. 2014). The use of biosurfactants, such as sophorolipid, has also shown synergistic interaction with many antibiotics. Importantly, the use of phytocompounds and biosurfactants is considered safe, and has been approved by the FDA for use in pharmaceuticals and food (Navare and Prabhune 2013).

Due to their possession of antimicrobial activities, metallic nanoparticles, such as silver, zinc, and gold, represent an effective class of agents for overcoming bacterial resistance. Unfortunately, metallic nanoparticles are considered toxic at pharmacological doses, which cause restrictions in their use. However, studies have

revealed that the combination of various nanoparticles with antibiotics lessens the toxicity of both agents toward human cells by reducing the dosage required while at the same time increasing their bactericidal efficacy (Allahverdiyev et al. 2011).

2.1.1.4 Screening of Previously Approved Drugs as Adjuvants

Discovery of newer antibiotic adjuvants could be achieved by screening the drugs approved previously for other medications. It is an interesting approach, as these drugs are well known for their toxicology and pharmacology profiles. Systematic screenings of approved non-antibiotic compounds for antimicrobial potential have uncovered various compounds from many drug classes, including antihistamines, antihypertensives, antispasmodics, anti-inflammatory drugs, and tranquilizers. These drugs display activity against a broad spectrum of Gram-positive and Gram-negative bacteria (Worthington and Melander 2013a).

2.2 Combination Approaches that Target the Same Pathway

Combining antimicrobial compounds with different targets in the same pathway is a more specified strategy than targeting different pathways. If the proper pathway is chosen, this could result in a very effective strategy. There are two points of consideration for selecting this approach: (i) The targeted pathway must be essential to the survival of the pathogen, such as a requirement for folate to synthesize dTMP, or a precursor for DNA synthesis. (ii) The pathway chosen should not be non-meaningful, as it may lead to resistance (Fischbach 2011). Targeting two steps in the same pathway offers a more perilous strategy than attacking two or more separate pathways, as it may lead to an increase in antibiotic resistance. Despite this, in most cases it is still more effective than monotherapies, which are comparatively less potent at inhibiting a single pathway (Payne et al. 2007; Read and Huijben 2009; Pena-Miller et al. 2013).

2.3 Combination Approaches that Act on the Same Target

If the drugs in combination have the same target, then the approach becomes very less diversified, such as is the case for a combination of antibiotics that act on the bacterial ribosome. However, in a study by Fischbach (2011), synercid, which is a semisynthetic combination of two drugs, it was shown that both of the components bind to adjacent regions in the 50S ribosomal subunits, resulting in 10–100-fold more efficacy than either drug alone. As the target was of a critical and conserved nature, the authors were able to achieve enhanced antimicrobial efficacy. Therefore, the selection of an appropriate, vital target is a critical prerequisite for this strategy and may help counter the inherent risk, in terms of resistance generation, of attacking a single target.

3 Combination Approaches in Combating Polymicrobial Infections

In addition to being useful in treating single species, combination approaches are quite successful and crucial for the treatment of polymicrobial infections (Ahmed et al. 2013). The majority of infectious diseases are associated with medical devices, which often harbor more than one pathogen, resulting in more antimicrobial tolerant, mixed species infections (Moran et al. 2007; Marculescu and Cantey 2008; Aggarwal et al. 2013). Therefore, the ability of combinational drugs to target multiple pathogens, including multispecies biofilm communities, is emerging as a valuable tool in fighting infections. For example, combinations of three antibiotics, such as a β-lactam, a glycopeptide, and an aminoglycoside, have demonstrated highly improved activity against multidrug-resistant *S. aureus* (MRSA) strains when compared to two antibiotic combinations (Wood et al. 2012). Furthermore, combinations of antibiotics showing a varied range of mechanisms of action are very effective in suppressing the development of resistance. Due to diverse modes of action, antibiotics in combinations, such as protein synthesis inhibitors (macrolides, aminoglycosides, tetracyclines, lincosamides, and chloramphenicol), DNA synthesis inhibitors (fluoroquinolones and quinolones), folic acid synthesis inhibitors (sulfonamides and diaminopyrimidines), and cell wall synthesis inhibitors (polypeptide antibiotics, preservatives, and analgesics), is very effective in combating polymicrobial infections (Wood et al. 2012; Ahmed et al. 2013).

4 Consequences of Drug Combinations

Initially, it appears that the use of drug combinations would address multiple resistance development, but in fact, it may actually promote the evolution of drug resistance (Hegreness et al. 2008; Yeh et al. 2009; Pena-Miller et al. 2013). As indicated through in vitro studies, resistance to aminoglycosides can lead to increased sensitivity to other antimicrobials (Lazar et al. 2013). The use of many drugs in combination carries with it the danger of evolving "super-pathogens" due to the co-evolution of multidrug-resistant variants and desensitization to other antibiotics of the same class (Ahmed et al. 2013). In order to overcome this challenge, combination therapies should be designed to reduce the emergence of multidrug-resistant bacteria while increasing the efficacy of the treatment.

The drug combinations must also be crafted while considering the effect of drug–drug interactions, drug metabolism, compound ratios, the doses required for drug adsorption, and also the rate of excretion for each drug in the treatment (Kalan and Wright 2011; Goldberg et al. 2012; Roemer et al. 2013). The administration of two or more drugs in synergy may alter the pharmacokinetics of drug delivery, and could be toxic to the host cells and valuable natural microflora. Novel synergistic drug therapies may cope with some of these common challenges via multiple methods, including the use of a hybrid single antibiotic with two distinct functions, such as lantibiotic and nisin (Walsh 2000; Hasper et al. 2006). Remarkably, some

combinations of antimicrobial agents can actually alleviate the toxicity of single agents alone, e.g., when nanoparticles are combined with antibiotics. This happens due to a decrease in amount required for activity in combination when compared to individual use (Allahverdiyev et al. 2011). Also, the mechanisms of action of the individual drugs must be considered to avoid any antagonistic interactions, such as with the combination of certain DNA synthesis inhibitors with protein synthesis inhibitors (Bollenbach et al. 2009). Finally, combinations of drugs should be done only after understanding the mechanism of action in combination to obtain innovative combinations, as with the combination of an antibiotic with non-antibiotic adjuvant or inhibitor of quorum sensing.

5 Use of Antibiotics in Combination with Plant Products and Nanoparticles

Another promising approach in managing antibiotic resistance is the use of natural antimicrobial substances, such as plant extracts, essential oils, or their active compounds. These products possess high antimicrobial activity and have also demonstrated antioxidant, anti-inflammatory, immune modulatory, regenerative, and other beneficial properties (Chao et al. 2008; Sadlon and Lamson 2010; Miguel 2010; Silva and Fernandes Jr 2010). The drug synergism between antimicrobial agents and bioactive plant products is a new concept, and in order to control a particular disease, in vitro experimentation should be carried out with various antibiotics in combination with plant products. This way, a proper combination may be administered to the patient for early and safe recovery from a specific ailment. In general, plant products are safer and cheaper, and their use can reduce the administration doses of antibiotics. A few examples of such combinations are summarized in Table 1.

Further, it has been found that when certain nanoparticles are combined with antibiotics, the bactericidal activity of drug is restored against resistant strains (Li et al. 2005; Fayaz et al. 2009). Also, when antibiotics are tagged with nanoparticles that can also act as efficient drug delivery agents (Chaloupka et al. 2010), the concentration of antibiotics at the site of drug-bacterial interaction is increased. This facilitates the binding of antibiotics to bacteria, resulting in increased efficacy, and the overcoming of bacterial resistance to antibiotics, such as vancomycin (Gu et al. 2003; Allahverdiyev et al. 2011). Because they possess substantial antibacterial properties, the nanoparticles of copper, gold, iron, silver, titanium, and zinc are being investigated in combination with other antibiotics. Some of the key studies are summarized in Table 1.

5.1 Promising Combinations: Essential Oils and Nanoparticles

Some investigators have also studied the interactions of essential oil components with polymeric nanoparticles for delivering oil-active compounds into the site of microbe-host interaction. Chen et al. (2009) prepared nanoparticles by grafting two

Table 1 Examples of synergistic combinations of various antibiotics with plant products or nanoparticles

Plant products	Antibiotics	Strains	References
Methanol extract of *Euphorbia hirta* leaves	Erythromycin	*S. aureus*	Adikwu et al. (2010)
Ethanol extract of *Mangifera indica* L. peel	Tetracycline and erythromycin	*S. aureus*	Souto de Oliveira et al. (2011)
Spices and herbs like *Coriandrum sativum*, *Cumium cyminum*, *Mentha piperita*, *Micromeria fruticosa* L., and *Rosmarinus officinalis*	Cephalothin, ceftriaxone, gentamicin, and nystatin	Gram-positive and gram-negative bacteria	Toroglu (2011)
Ethanolic extracts of *Rhus coriaria* (seed)	Oxytetracycline HCl, penicillin G, cephalexin, sulfadimethoxine, and enrofloxacin	Multidrug-resistant *Pseudomonas aeruginosa*	Adwan et al. (2010)
Ethanol extracts from the leaf and stem of *Salvadora persica*	Tetracycline	*S. aureus*	Ahmed et al. (2009)
Essential oils of *Cinnamomum verum*, *Cymbopogon citratus*, *Thymus vulgaris*, and *Syzygium aromaticum* and their active compounds, including cinnamaldehyde, eugenol, thymol, geraniol, and citratus	Azole drugs	*Candida albicans* and filamentous fungi	Khan and Ahmad (2011), Khan et al. (2012), and Khan and Ahmad (2013)
Nanoparticles			
Silver nanoparticles	Amoxicillin, ampicillin, erythromycin, kanamycin, and chloramphenicol	*E. coli*, *S. aureus*, *Micrococcus luteus*, *E. coli*, and *Salmonella typhi*	Li et al. (2005) and Fayaz et al. (2009)
TiO_2 nanoparticles	Penicillins, cephalosporins, and aminoglycosides	MRSA	Roy et al. (2010)
ZnO nanoparticles	Aminoglycosides, cephalosporins, glycopeptides, lincosamides, macrolides, penicillins, and tetracyclines	*S. aureus*	Thati et al. (2010)
Chitosan-capped gold nanoparticles	Ampicillin	Gram-positive and gram-negative bacteria	Chamundeeswari et al. (2010)

active compounds of essential oils, namely eugenol and carvacrol, on chitosan nanoparticles. The authors found stronger antibacterial activity of grafted eugenol and carvacrol against *E. coli* and *S. aureus* compared to the original chitosan nanoparticles. Similarly, Hu et al. (2009) synthesized chitosan nanoparticles embedded with thymol, and studied their antibacterial activity against *S. aureus*, *Bacillus subtilis*, and some species of fungi. They observed that this combination was more potent than thymol alone. However, it should be taken into consideration that the combination of nanoparticles and essential oils could not be applicable in coatings of medical devices, due to the volatile characteristics of essential oils. However, such combinations could be exploited for the treatment of topical infections, due to the presence of antimicrobial properties in both agents, as well as the various healing aptitudes of essential oils (Allahverdiyev et al. 2011).

6 Conclusion

The emergence of multidrug resistance among microbial pathogens is a global problem that requires improvements in the present methods of, and novel discovery of, antimicrobial strategies. The use of single drugs is no longer a satisfactory approach to combatting this problem. Though the "one drug–one target" approach is no longer optimal, drugs administered in combination provide multiple targets, resulting in greater efficacy and a reduction in resistance. Treatment of many life-threatening diseases, such as cancers, HIV, and tuberculosis is dependent on combination therapy, and similarly, drug couplings, such as antibiotic/adjuvant combinations, for the treatment of infectious diseases caused by MDR pathogens has attracted considerable attention. Furthermore, the use of high-throughput screening of drug compounds that have been previously approved for other applications has led to the discovery of many potential adjuvants. In addition, the combination of nanoparticles or phyto-products with antibiotics results in reduced toxicity of drugs toward human cells. Due to this combinatorial approach, the efficacy of many previously effective antibiotics is restored and can once again be utilized to combat emerging resistance. However, the pitfall of drugs in combination should be taken under consideration. There have been reports on issues with drug–drug interactions, optimized drug ratios, and dosing amounts for pharmacokinetics properties of each compound, and thus the combination therapies with specific dosages and synergistically active drugs are of utmost importance for their ability to increase the efficacy of antibiotics and decrease resistance generation.

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Targeted Delivery of Antibiotics Using Microparticles to Combat Multidrug-Resistant Tuberculosis

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Abstract

Tuberculosis (TB) continues to be a major global challenge, claiming about two million deaths each year. The emergence of drug resistance due to high incidence of poor patient compliance has further worsened the situation. Targeted delivery of drugs to macrophage, the site of *Mycobacterium tuberculosis* infection and replication, has been shown to have implications as a promising option in TB treatment. A variety of biocompatible and biodegradable polymer-based carrier-based delivery systems have emerged as potential drug delivery systems (DDS). Such targeted delivery systems have been shown to have significant merits over free drug, including improved drug bioavailability, limiting adverse drug effects and requiring less frequent administration regimes and lowering drug doses. The pulmonary administration of inhalable dry powders incorporating multiple drugs has particularly exhibited encouraging results against MDR-TB, and is expected to shorten the treatment duration, thereby improving patient compliance. Recently, the administration of pulmonary drug delivery as an adjunct to existing oral treatment regimens has been shown to achieve sufficient drug concentrations

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in certain systemic compartments and thus further enhance treatment effectiveness. The present chapter discusses the recent research updates on carriers used in preclinical or clinical studies against TB, the challenges associated, and future perspectives.

Keywords
Tuberculosis · MDR-TB · Microparticles · Pulmonary delivery · Dry powder

1 Introduction

Tuberculosis (TB) is a lifelong, devastating, chronic granulomatous disease caused by the inhalation of airborne droplet nuclei containing *Mycobacterium tuberculosis (M.tb.)*, and it is one of the leading killers of humans worldwide. The use of antibiotics against tuberculosis (TB) in the middle of the twentieth century heralded a new era in our fight against this age-old disease. We observed a significant decrease in TB cases in the half century since the anti-TB drugs were first introduced; however, the success of the antibiotics was short-lived, with the emergence of drug-resistant strains of *M.tb.* In 1993, the World Health Organization (WHO) declared TB a global emergency (Zumla et al. 2014), and by 2015, 480,000 people developed multidrug-resistant TB (MDR-TB) annually (Kendall et al. 2017; Petersen et al. 2017). This staggering number that also includes individuals harboring the extensively drug-resistant TB (XDR-TB) strains known to be even more difficult to treat than MDR-TB, where in some cases, no effective treatment regimen exists. Recently, the WHO has reported 10.4 million incident cases of TB across the world in 2016, of which ~two million people die annually due to this potentially curable disease (Global TB Control 2017 Report). The only available pediatric vaccine, using *Mycobacterium bovis* BCG, is protective against severe forms of childhood TB, but is ineffective against TB in adults. TB has, therefore, become a global pandemic and is a priority concern across the world.

2 Pathogenesis and Immunology of TB

2.1 Progression of Disease

2.1.1 Early Events Following Infection

Infection is initiated by inhalation of aerosol droplets containing *M.tb.* that are expectorated by active pulmonary TB patients. These inhaled tiny droplet nuclei find their way into terminal alveoli and are readily phagocytosed by alveolar macrophages and dendritic cells. Subsequently, the mycobacteria are also reported to bind, invade, and infect non-phagocytic cells, including pneumocytes and alveolar endothelial cells (Ahmad 2010; Fernstrom and Goldblatt 2013).

M.tb. pathogenicity lies in its ability to survive and multiply within host macrophages, for which they specifically use mannose receptor (MR) and complement receptor (CR) for uptake. The bacteria engage with vesicular trafficking machinery, inhibiting phagosome–lysosome fusion (phagosomal maturation arrest) and depleting H^+- ATPases from vacuolar membranes, thereby inhibiting phagosome acidification (Flannagan et al. 2012). That is, though the initial trafficking pattern of the *M.tb.*-containing phagosomes is normal, the arrest is marked by the absence of Rab7 in later stages, thereby inhibiting progression to phagolysosomes (Deretic et al. 1997) and promoting the intracellular survival and growth of the pathogen.

2.1.2 Disease Progression

In immunocompetent hosts, an effective immune response develops 2–8 weeks post-infection that arrests further multiplication of the bacteria. In most cases, acquired responses result in the containment of bacteria within well-organized granuloma, marked by the presence of a large number of activated macrophages, which infiltrate the region and enclose the infected cells in tubercles. These macrophages later differentiate into epithelioid cells, so called due to the resemblance of clustered macrophages to epithelial cells. Activated macrophage secretes large amounts of lytic enzyme, leading to the formation of spheroidal regions of necrotic tissue, known as the "Ghon focus." Tubercle formation leads to hypoxic conditions within it, which, together with acidosis, kills most of the bacteria. Granuloma formation in most cases, therefore, limits infection, but does not totally eliminate it (Rom and Garay 2003). Thus, in "susceptible" persons, such as HIV patients and other immunosuppressed individuals, *M.tb.* can potentially become reactivated.

2.1.3 Immune Responses

Protective responses to *M.tb.* are complex and involve both arms of immunity: innate and acquired. *M.tb.* phagocytosis is typically followed by innate immune responses, including an increase in oxidative burst, reactive nitrogen intermediates (nitric oxide), phagosome acidification, and proinflammatory cytokines (Rom and Garay 2003; O'Garra et al. 2013). The classical pathway of interferon-gamma-dependent activation of macrophages by T helper 1 (Th1)-type responses is a well-established feature of cellular immunity to intracellular *M.tb.* infection. Virulent *M.tb.* strains subvert this immune response for its own survival, inducing what is known as "alternative activation" within host macrophages. MR-associated phagocytosis has been shown to be linked with activation of anti-inflammatory activity marked by "alternative activation" of macrophage, and non-activation of NADPH oxidase (Guirado et al. 2013). In susceptible individuals, the infected macrophage displays an "alternative" phenotype with diminished NO production, suppressing apoptosis (Verma et al. 2011). Such macrophages exhibit increased Th2 cytokine (IL-4, IL-13, and IL-10) production (Kahnert et al. 2006), thereby inhibiting the protective Th1 response by antagonizing IFN-γ and decreasing IL-6 and TNF-α (O'Garra et al. 2013). A great deal of work has also implicated the inhibition of autophagy as a survival strategy of *M.tb.* within host macrophage.

3 Different Forms of TB

While about one-third of the world population is infected with *M.tb.*, only 10% develop active disease (Raviglione and Sulis 2016). In "susceptible" individuals, innate and acquired immune responses are insufficient to eliminate or contain the bacteria, leading to bacterial proliferation inside macrophages. In a limited number of cases, risk factors are identifiable, which include HIV/AIDS, diabetes, age, alcohol, smoking, and corticosteroid therapy. In other individuals, the immune response is sufficient to clear the infection, or arrests it in a latent state. The different forms of TB are:

Active TB Active TB patients have rapidly multiplying bacteria and the typical symptoms of active TB, including coughing, phlegm, chest pain, weakness, weight loss, fever, chills, and night sweats. Persons with active pulmonary TB disease are the main source of disease spread via the aerosol route (Rom and Garay 2003).

Miliary TB Unchecked multiplication of TB bacteria leads to bacterial dissemination throughout the body via the blood. Miliary TB is a rare form of active disease in which bacteria quickly spread all over the body and affect multiple organs at once. This form of TB can be rapidly fatal.

Latent TB In some individuals, the host T cell immune response confines the pathogen to a hostile environment where it may become latent, though viable. People with latent tuberculosis infections are asymptomatic and not infectious, but can develop active TB disease in the future due to "reactivation" of latent mycobacteria.

Thus, the inhalation of mycobacteria may ultimately lead to four different infection outcomes (Rom and Garay 2003):

(i) Innate response is sufficient to clear the bacteria.
(ii) Active disease develops soon after infection, known as *primary infection.*
(iii) Asymptomatic, *latent infection* develops.
(iv) Active disease may develop many years after infection, known as *reactivation, or secondary TB.*

4 Drug Regimens and Current Anti-TB Therapy

When the bactericidal mechanisms of the macrophage fail to clear the intracellular bacilli, the TB patient requires treatment by the intervention of chemotherapy. In India, patients presenting with TB are treated as per the guidelines of the Revised National Tuberculosis Programme (RNTCP) (Verma et al. 2013b), with similar

guidelines for other countries. The "directly observed therapy (short course)" or DOTS regimen has been demonstrated to be highly efficacious for TB treatment in various clinical settings in India. The RNTCP recommends a therapeutic regimen of a combination of drugs and dosage schedules in which two or more first-line drugs are administered for a period of no less than 6 months. The *first-line drugs* used in TB treatment include isoniazid [INH], rifampicin [RIF], ethambutol [EMB], and pyrazinamide [PYZ] (Tiberi et al. 2017). The *second-line drugs* are used for infections with tubercle bacilli resistant to first-line drugs. These include aminoglycosides [AMG], such as amikacin and kanamycin; polypeptides, such as capreomycin [CPR], viomycin, and enviomycin; fluoroquinolones, such as ciprofloxacin, ofloxacin [OFX], levofloxacin, and moxifloxacin; and thioamides, such as ethionamide, prothionamide, and cycloserine.

To overcome the rise in multidrug resistance (against first- and second-line drugs), the WHO included third-line drugs for TB treatment. These include linezolid, thioridazine, and macrolides, such as clarithromycin and thioacetazone, selected on the basis of drug susceptibility testing (DST). This further complicates treatment, requiring even longer regimens with drugs that are more expensive, less effective, and often more toxic. Rifabutin (another rifamycin) is added to the regimen if rifampicin resistance is detected with rifabutin susceptibility.

4.1 Existing TB Treatment Regimen

According to the WHO, the standard treatment regimen for drug-susceptible TB includes daily oral administration of INH, RIF, PZA, and EMB for 2 months (Pai et al. 2016). This is followed by daily administration of INH and RIF for another 4 months (WHO 2017). Daily dosing is recommended, although the 3-times weekly dosing can be used during the continuation phase under DOTS, as well as fixed-dose combinations.

The WHO strongly recommends DST (rapid and/or conventional) in all cases, and particularly for those previously treated for TB disease. While awaiting DST results, in settings with a medium or low probability of MDR-TB, retreatment cases could initially be treated with an empiric regimen, INH-RIF-PYZ-EMB-STR, for 2 months, followed by INH-RIF-PYZ-EMB for 1 month, and INH-RIF-EMB for 5 months.

For patients whose DST results are not available (a rather common phenomenon in many developing countries), a third-line regimen is recommended by the WHO. This regimen contains four drugs (an AMG, ethionamide (ETD), PYZ, and OFX) during initial phase and two drugs (ETD and OFX) during the continuation phase (Sotgiu et al. 2015).

5 Drug-Resistant Tuberculosis (MDR, XDR, and TDR-TB)

The current antibiotic treatment regimen requires a minimum of 6 months therapy that causes severe and prolonged side effects, and subsequently leads to nonadherence to the treatment regimen. Non-compliance with treatment ultimately leads to the generation of drug-resistant strains of *M.tb.* that not only increase the treatment regimen up to 2 years, but are also more costly to treat.

The first drug-resistant case of TB was observed in 1947 against streptomycin, and lead to the development of a regimen using multiple drugs for treatment (Kerantzas and Jacobs 2017). ***Multidrug-resistant (MDR) TB*** with resistance to INH and RIF was first reported in the 1990s (Matteelli et al. 2014). The WHO definition of ***extensively drug-resistant*** (***XDR) TB*** involves resistance to at least RIF and INH, in addition to any fluoroquinolone, and to at least one of the three injectable second-line drugs, CPR, kanamycin, and amikacin, used in anti-TB treatment (Prasad et al. 2017). A 6-month course of standard first-line medication is ineffective in MDR-TB cases. Different combinations of second-line drugs, supported by selected first-line TB drugs, are used in patients with RIF-resistant or MDR-TB for 18 months or longer of treatment. Regardless, the success rate of XDR-TB treatment is very low, with mortality as high as >30%, as reported from treatment cohorts (WHO Global TB report 2017; Kvasnovsky et al. 2016).

Totally drug-resistant (TDR) TB is caused by *M.tb.* clinical strains which are resistant to all first- and all second-line drugs. It is extremely difficult to treat, but not totally impossible to treat, and thus is also referred to as extremely drug-resistant TB (XXDR-TB). Italy reported the first TDR-TB case in two patients in 2003, and India first reported four cases in 2012 (Ahmed et al. 2016). Further, in recent decades, very few anti-TB antibiotics have been approved for human use, including the newest drugs, bedaquiline and delamanid, that may be used for TDR-TB (D'Ambrosio et al. 2017). Alarmingly, both bedaquiline and delamanid have recently encountered resistant strains (Hoffmann et al. 2016), meaning that resistance has developed against every current TB antibiotic.

6 Novel Drug Delivery Systems for TB

In the last few decades, we have seen the advent of micronized carrier systems as an alternative to the conventional form of TB therapy. Several studies have shown that carrier systems incorporating single or multiple anti-TB drugs for the targeted delivery of antibiotics form an effective therapeutic approach against TB.

One of the main advantages of such particulate systems is the "intracellular delivery" of the bolus of loaded drug to macrophages, thereby directly targeting the sites of mycobacterial replication. Since such particles are rapidly phagocytosed by macrophage, they build up high intracellular drug concentrations and result in significant enhancement in antimicrobial efficacy (Sharma et al. 2001; Sen et al. 2003). These particulate drug delivery systems (DDS) are developed using biocompatible and biodegradable polymers, and have been shown to reduce the dosing frequency

and days of treatment. Targeted drug delivery allows controlled release of drugs, and results in minimal host toxicity as compared to the conventional oral dosage. Therefore, while free antibiotics need to be administered daily, new formulations such as nano- or microparticles have been seen to be effective, even when administered after every few days.

6.1 Microparticles

Microparticles are spherical particles with sizes ranging from 50 nm to 2 mm, and containing a core substance.

A great deal of literature reports the entrapment of anti-TB drugs in microparticles composed of polymers, such as poly DL-lactide-coglycolic acid (PLGA) and poly DL-lactic acid (PLA) (Sharma et al. 2001), by various methods, such as solvent evaporation-double emulsification and spray-drying (Hirota and Terada 2014), leading to formation of particles containing antibiotic(s) embedded in a polymer matrix. Alternatively, the drug may be attached to the particle surface by physical adsorption or chemical reactions. The therapeutic advantages of microparticles include the following**:**

1. Macrophages, which harbor the *M.tb. cells,* readily phagocytose such microparticles and thus significantly improve the uptake of the loaded drug (as compared to that by diffusion).
2. The simultaneous intake of multiple drugs is recommended for TB therapy in order to prevent the emergence of drug resistance. Drugs can be easily incorporated with relatively high efficiency, and various drug release rates can be achieved via manipulations in the preparation procedure. The spray drying technology, in particular, has been widely utilized to formulate microparticles incorporating multiple hydrophilic and/or hydrophobic anti-TB drugs (Hirota and Terada 2014).
3. The embedded drugs have slower release rates, and thereby longer durations of action.
4. Therefore, such drug-loaded microparticles exhibit significantly greater in vitro and in vivo (in infected animal models) anti-TB activity than that observed by administration of an equivalent amount of drug(s) in soluble form (Hirota and Terada 2014).
5. Such particles are more physiochemically stable, both in vitro and in vivo.
6. A number of biodegradable microspheres have proved to be nontoxic, biocompatible, and non-immunogenic.
7. Recent studies have shown that uptake of microparticles induces classical activation within *M.tb.*-infected macrophages (Verma et al. 2011).

Apart from synthetic polymers, several natural polymers, particularly saccharides, such as alginate, chitosan, and lactose, have been used to develop drug delivery systems for entrapping and delivering various therapeutic agents. Sodium

alginate, a salt of alginic acid (linear copolymer of α-guluronic acid and α-mannuronic acid), has the ability to form a gel/meshwork in the presence of divalent cations, such as $CaCl_2$. This gel is mucoadhesive, and is likely to adhere to mucosa for prolonged periods of time, thereby having the potential to release the drug in a sustained and controlled manner. Thus, alginate microparticles have been prepared as anti-TB drug carriers and studied as oral delivery systems for TB treatment (Qurrat-ul-Ain et al. 2003). In addition, leucine-containing microparticles have also found application for delivery of anti-TB drugs (Verma et al. 2013a; Garcia-Contreras et al. 2017).

Recently, the 2–4 μm hollow, porous, yeast-derived β-1, 3/1, 6 glucan particles (GPs) have been utilized for targeted payload delivery of anti-TB drugs to macrophages (Soto et al. 2010; Upadhyay et al. 2017). Particulate glucan is biodegradable and biocompatible polysaccharide that has been consumed for thousands of years, and has the GRAS (*g*enerally *r*egarded *a*s *s*afe) status granted by FDA. The *β*-1,3-D glucan surface composition also permits its recognition by cell surface receptors on macrophages (via dectin-1 and Complement Receptor 3) and other phagocytic cells (Legentil et al. 2015). Such particles have been shown to incorporate "nanoprecipitates" or "nanoparticles" of anti-TB rifamycin drugs (RIF and RFB) within internal pore spaces, thus functioning as "nano-in-micro" particulate formulations. These particles are prepared by alkaline and acidic extraction of the cell wall of baker's yeast (*Saccharomyces cerevisiae*) and have showed adequate thermal stability for pharmaceutical application.

Recently, novel carrier-free microparticles of anti-TB drugs, such as rifampicin (Parikh et al. 2014), have been developed and successfully evaluated as macrophage delivery systems against TB.

6.2 Nanotechnology for TB Treatment

Nanoparticles are colloidal structures composed of synthetic or semi-synthetic polymers. These can be formulated as monolithic nanoparticles (nanospheres) that embed the drug in the polymeric matrix, or nanocapsules, where the drug is confined within a hydrophobic or hydrophilic core surrounded by a definitive "capsule." Nanoformulations of antibiotics enclosed within polymers, such as PLGA (Ahmad et al. 2008) and alginate (Ahmad et al. 2007) have been shown to be successful for the administration of anti-TB drugs within experimental, in vivo (animal) TB models. The use of nanotechnology in TB treatment has been expertly researched and reviewed in detail (Ahmad et al. 2007; Ahmad et al. 2011; Nasiruddin et al. 2017).

Other Nanotechnology-Based Formulations

Liposomes are concentric nano- to micro-sized vesicles in which an aqueous volume is enclosed by one or multiple phospholipid bilayers. The hydrophobic domain is utilized to entrap insoluble agents, and the core enables encapsulation of water-soluble drugs. Liposomal formulations have been developed with first- and/or second-line antibiotics and are proposed as alternative to current therapy (Shegokar

et al. 2011). Intravenous administration of INH and RIF encapsulated in lung-specific stealth liposomes (liposomes with a polyethylene glycol-coated surface) showed enhanced affinity toward the lung tissue of mice and thus allowed the targeted delivery of these anti-TB drugs to lungs (Deol and Khuller 1997).

One caveat for liposome use is that, since the vesicles are degraded by intestinal lipases, they cannot be administered by oral route (Pinheiro et al. 2011). To circumvent this limitation, solid lipid nanoparticles (SLNs) and niosomes have been proposed as novel anti-TB drug delivery vehicles (Nasiruddin et al. 2017) that can be administered orally. SLNs are aqueous suspensions of nanocrystalline lipids, which have better encapsulation effectivity and increased ability to entrap hydrophobic or hydrophilic drugs when compared to liposomes and polymeric nanoparticles. While free drugs are rapidly cleared from circulation, SLN-loaded anti-TB drug(s) have been shown to have improved bioavailability and thus, are effective at reduced dosages and dosing frequency. Pandey et al. (2005) showed that 5 oral doses of drug-loaded SLNs on every tenth day were able to completely eradicate *M.tb.* H37Rv from the lungs and spleens of infected mice, whereas free drug needed the administration of 46 daily oral doses to achieve the same result.

Niosomes are composed of a surfactant bilayer, and are thus thermodynamically stable, colloidal, uni- or multi-lamellar (liposome-like) particles, formed by self-assembly of non-ionic surfactants and a hydrating mixture of cholesterol (Nasiruddin et al. 2017). Three polymers, Brij-35, Tween 80, and Span-80, have been used for pyrazinamide niosome formulation, of which span-80-based formulation exhibited the highest release (Thomas and Bagyalakshmi 2013).

Despite the fact that nano- and micro-particles are rapidly taken up by host macrophages, their appropriate delivery for sufficient in vivo efficacy is yet another challenge. Thus, the success of delivery vehicles is by and large dependent on their route of administration. In the following section, we will discuss the various routes of drug delivery used against TB, along with their advantages and disadvantages. We will also discuss how the pulmonary route of drug delivery could potentially minimize the evolution of resistant *M.tb.* strains and reduce the long treatment duration currently employed, thus aligning with the recent WHO guidance on having a shorter treatment regimen (Grace et al. 2018).

7 Drug Delivery Routes Explored in Humans

The oral route of anti-TB drug delivery is currently prescribed for long-term treatment, whereas the intravenous (IV) route is utilized where aggressive therapy is needed.

7.1 Oral Delivery

The oral route of drug administration is the most common route of anti-TB drug delivery, arising from the feasibility of long-term oral administration with high drug

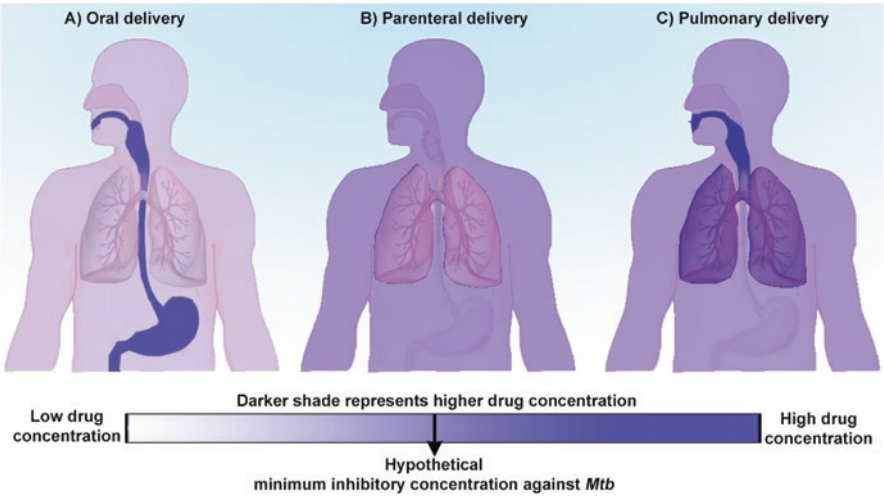

Fig. 1 Drug concentration in various body compartments after (**a**) Oral delivery; (**b**) Parenteral delivery; and (**c**) Pulmonary delivery. Darker shade represents higher drug concentration. Note that the highest drug concentration in the lung is achieved by pulmonary delivery, followed by parenteral and oral delivery

doses in TB-endemic regions. That being said, it becomes extremely challenging to adopt another administration route without compromising patient compliance. The prolonged treatment with multiple antibiotics leads to severe side effects, due to high systemic drug exposure and, paradoxically, sub-therapeutic drug levels in the host tissue where *M.tb.* resides. This is especially true for pulmonary TB, where the *M.tb.* hides in hard-to-penetrate lung granulomas (Muttil et al. 2009; Hickey et al. 2016) (Fig. 1a). While the second-line drugs are used in potential MDR-TB patients who do not achieve sputum conversion with first-line therapy, these antibiotics are less effective and more toxic than first-line therapy, resulting in poor patient compliance; non-compliance to the treatment regimen is regarded as the main reason for the generation of resistant *M.tb.* strains.

7.2 Parenteral Delivery

Injectable anti-TB drugs are usually administered parenterally for second-line therapy, and include amikacin, kanamycin, and capreomycin. These agents are administered either by the intramuscular or the intravenous route. When compared to oral administration, the parenteral route achieves higher systemic drug levels (Fig. 1b), avoids first pass metabolism, and prevents GI toxicity. However, this route is also associated with painful daily injections, unacceptably high rates of ototoxicity, and renal toxicity, and requires the presence of a health care worker for drug administration. These concerns lead to poor patient compliance. In addition, parenteral

administration generates needles that have the potential to be re-used if not properly disposed of (Mitragotri 2005).

7.3 Pulmonary Delivery

The pulmonary route of anti-TB drug delivery was first reported in the middle of the twentieth century, during the time when streptomycin resistance first developed (Miller et al. 1950; Hickey et al. 2016). Aerosol delivery allows for higher drug concentration in the lung, the organ that is first exposed to airborne *M.tb.* Since 80% of all TB cases manifested as pulmonary TB, direct lung delivery of anti-TB drugs could be a more systematic treatment strategy since it allows the drug to follow the path of *M.tb.* and achieve therapeutic drug levels in the niches where *mycobacteria* reside. As shown in Fig. 1c, aerosol delivery can potentially achieve therapeutic drug levels in the respiratory system (above MIC), and the large surface area of the lung, coupled with the thin alveolar epithelium and extensive pulmonary vascularization, allows for an overall increased systemic bioavailability of the drug (Dharmadhikari et al. 2013). The other advantages of pulmonary drug delivery include the noninvasive nature of the delivery route when compared to injection, the lower required dosages, and the potentially minimized *M.tb.* transmission from active TB patients to healthy individuals (Hickey et al. 2016; Pham et al. 2015). High drug concentration in the lung can also minimize *M.tb.* spreading to other extra-pulmonary organs from the lung. Taken together, these advantages are expected to result in better treatment outcomes, and a reduction in overall drug toxicity, when compared to oral and parenteral routes of administration. However, aerosol drug delivery alone may not be able to achieve sufficient drug concentrations in certain systemic compartments. This can be overcome by including aerosol drug delivery as an adjunct to existing oral or parenteral treatment regimens (Muttil et al. 2009; Hickey et al. 2016).

Particles below ~0.5 μm diameter are reported to be exhaled un-deposited in the lung, while those larger than 5 μm will get entangled in the upper airway and shut out by the lung's defenses (Malcomson and Embleton 1998). Microparticles having an ideal size, specifically the median mass aerodynamic diameter (MMAD) (generally 0.5–5 μm), deposit into the deep alveoli of lungs, and can be then taken up by alveolar macrophage.

There have been occasional reports where inhaled anti-TB drugs were administered to TB patients or healthy individuals. Inhaled kanamycin was administered along with other anti-TB drugs to treat five patients with MDR-TB, and was well-tolerated, with sputum conversion reported in all patients within 60 days of treatment initiation (Turner et al. 1998). In another study, Sacks et al. treated patients harboring MDR-TB (12 patients) and drug-susceptible TB (7 patients) with inhaled aminoglycosides as an adjunct to conventional therapy. The results demonstrated that 13 of the 19 patients (6 of 7 with drug-susceptible TB, and 7 of 12 with MDR-TB) converted to smear-negative within a month of inhaled therapy (Sacks et al. 2001). These patients were smear and culture-positive after many months of

treatment with conventional anti-TB therapy, demonstrating the importance of pulmonary drug delivery in achieving *M.tb.* clearance, especially in the lungs. In 2013, Dharmadhikari et al. conducted a phase I trial with inhaled, dry powder capreomycin as a treatment strategy against MDR-TB. Healthy adults were asked to self-administer 25, 75, 150, or 300 mg of capreomycin dry powder using a marketed dry powder inhaler (DPI) (Dharmadhikari et al. 2013). Dry powder capreomycin was well-tolerated by all subjects, with no changes in lung function observed. Further, peak and mean plasma drug concentrations were dose-proportional, and the systemic concentrations were achieved immediately after pulmonary delivery. Dry powder microparticles were thus delivered for the first time in humans as a possible treatment strategy against MDR-TB. Furthermore, dry powder microparticles, when delivered by the pulmonary route, have been studied and expertly reviewed for preclinical models (Muttil et al. 2007; Kaur et al. 2008; Muttil et al. 2009; Sharma et al. 2011; Verma et al. 2013a; Hickey et al. 2016; Garcia-Contreras et al. 2017; Parumasivam et al. 2016).

8 Challenges

Studies in the last half century have shown that the challenge of successful prolonged TB treatment with multiple high dose drugs with toxic side effects can be met by micro-particulate formulations containing multiple drugs. While pulmonary drug delivery has been shown to be a particularly effective therapeutic approach, it has some challenges that need to be overcome before it can become a mainstream treatment strategy against TB in humans, especially in resource-poor countries. The pulmonary route of drug delivery is more complex than the oral and parenteral routes, and certain considerations, such as the patient's age, general health conditions, breathing capacity and lung physiology, formulation characteristics, inhaler performance, and the reliability of the patient to correctly and consistently use the inhaler device, are some of the concerns that need to be addressed before an inhalation product can become successful for the treatment of TB. Since TB treatment requires chronic use of multiple drugs for at least 6 months, the ability of the patient to adhere to daily inhaler use is vital. Patient compliance can be initially implemented under the guidance of a health care provider who oversees their inhaler use, thereby training and preparing the patient for good compliance in the remaining treatment duration. Further, the expectation to treat TB using pulmonary drug delivery is to shorten the treatment regimen that would further improve patient compliance (Uplekar et al. 2015).

The cost and availability of inhalers in low- and middle-income countries is another hurdle that must be overcome by governmental agencies and pharmaceutical companies. Aerosol treatment against TB has been delivered using both nebulizers and DPIs in patients. Nebulizers, usually air-jet or ultrasonic, emit micron-sized droplets of solutions or suspensions. However, the ability to formulate multiple anti-TB drugs in a single solution or suspension, the challenges of keeping them stable for at least 6 months without refrigeration, and the high cost of the devices

themselves, limit their applicability for effective TB therapy in resource-poor countries (Hanif and Garcia-Contreras 2012). DPIs, on the other hand, include the dry powder formulation, usually in a capsule, and the inhaler device. In the case of DPIs, the powder formulation and device are evaluated together in clinical trials, and are considered drug products by regulatory authorities (Price et al. 2018). DPIs have the advantage of product stability over liquid formulations, given that the drug is powdered. This becomes critical in regions of the world where the temperature-controlled supply chain is inadequate (Kunda et al. 2016; Parumasivam et al. 2016). Further, dry powder formulations can potentially incorporate multiple anti-TB drugs in a single formulation, and DPI devices have increased portability over nebulizers (Chan et al. 2014; Hou et al. 2015).

Lastly, regulatory agencies around the world need to be on board for pulmonary drug delivery to become a common treatment modality against TB. The highly controlled regulatory environment, especially encountered in developed countries, has been a hindrance for the widespread use of inhaled therapy against TB. Thus, pharmaceutical companies and device manufacturers should consider the regional patient population before developing the final product. This requires sufficient clinical data to be generated using the device in the regions of the world where the treatment will ultimately be implemented. Another obstacle is that approximately 20% of TB cases are diagnosed as extrapulmonary pathologies (Lee 2015), and with inhaled therapy alone, there is a risk of inducing resistance due to sub-therapeutic drug concentrations outside of the lungs. Therefore, inhalation therapy will require the standardization of inhaled doses and the proof of systemic drug concentrations above the MIC for *M.tb.* (Fig. 1c) in order to convince the regulatory agencies that this novel route of drug delivery will not exacerbate drug-resistant *M.tb.* strains. This will require the use of multiple anti-TB drugs in a single formulation, the possible use concurrent conventional therapy, and the proper optimization of the pharmacokinetic and pharmacodynamic parameters of all delivered drugs that could potentially lead to better clinical outcomes with pulmonary administration (Hickey et al. 2016).

9 Conclusions and Future Perspective

The increasing resistance to the existing drugs, coupled with the recent acquisition of resistance to the two newest anti-TB drugs approved by the FDA for MDR-TB and XDR-TB treatment (Zumla et al. 2014), poses a serious threat to TB control across the world. The ultimate hope with pulmonary drug delivery is to implement a more-optimal treatment strategy against TB in comparison to the status quo, which has been unsuccessful in controlling *M.tb.* antibiotic resistance development. The highly stable, inhalable dry powder microparticles, containing multiple drugs in a single formulation, have exhibited encouraging results and possess the significant potential to address MDR-TB. Furthermore, future studies are expected to provide evidence that combining pulmonary drug delivery with conventional oral treatment could ensure that therapeutic drug concentrations are achieved in different

biological compartments within the patient in order to treat both pulmonary and extra-pulmonary TB. Therefore, inhalation therapy needs to be granted the appropriate amount of attention, possibly as an adjunct therapy to conventional oral therapy, for successful translation of these preclinical studies for the effective control of TB.

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Practical Applications of Bacteriophage Therapy: Biofilms to Bedside

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Abstract

As the golden age of antibiotics crumbles away in the face of untreatable bacterial infections arising globally, novel, safe, and adaptable therapies are essential. Bacteriophages, co-discovered over 100 years ago by Félix d'Herelle, were widely utilized before the antibiotic era. Unable to compete with antibiotics in terms of price, manufacturing ease, and safety, phage use was largely terminated in the West, though clinical use has continued in the Eastern bloc. With rampant fears of a post-antibiotic era, phage has gained traction in the West and appears the ideal weapon to employ alongside and in conjunction with antibiotics. Up to 80% of human infections are caused by bacterial biofilms, and select phages have been reported to break up these bacterial cities via polysaccharide depolymerases and lysins, though quorum sensing can reduce phage receptors and increase resistance. Phage antibiotic synergy has been observed with specific antibiotic classes, where low levels of antibiotics cause bacterial filamentation and increased bacterial killing by phage. What has arisen from numerous animal infection models is that early treatment (post-infection) is critical to phage efficacy. With phage now being recognized as part of the human microbiome, the anti-inflammatory and apparent tolerized immune response to bacteriophage is fitting, though there are inflammatory concerns with increased endotoxin levels remaining following phage purification. Recent clinical studies using phage against a vast array of infections highlight the translational promise of this therapy.

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1 Introduction

Bacteriophages (phages) are viruses that specifically infect bacteria. Phages are ubiquitous in nature, and are the most abundant entity on the plant, numbering over 10^{30} phage particles. Phages infect bacteria by binding to a specific receptor on the bacterial cell surface and injecting their genetic material into the bacterial cytoplasm. Lytic bacteriophages will then replicate using the bacterial host machinery and lyse the bacterial host, resulting in the release of phage progeny into the milieu to infect surrounding bacteria.

In the early 1900s, phages were first studied and used as a therapeutic against bacterial infections in humans (reviewed in El-Shibiny and El-Sahhar 2017). Following the advent of antibiotics in the 1930s, phage therapy fell out of popularity in the USA, although phages have continued to be used in Eastern Europe to this day. With the rise in multidrug-resistant bacterial pathogens, the need for novel or alternative antimicrobials has increased, bolstering the USA interest in phage therapeutics. Indeed, in 2014, the US National Institute of Allergy and Infectious Diseases included phage therapy as one of seven strategies to address antibiotic resistance (NIAID 2014).

While phages have been studied for decades outside the USA, many questions still exist on their safety, efficacy, and how phage therapies might be regulated by the US Food and Drug Administration. Phage have many potential benefits over standard small molecule antibiotics: Phage prevalence in nature make isolation of phages relatively rapid, and once at the site of infection, phage will multiply exponentially, potentially reducing the frequency of doses needed for successful treatment compared to antibiotics (El-Shibiny and El-Sahhar 2017). Phages are narrow spectrum in their activity, typically infecting only a subset of a species, thus treatment with phage is very specific and should not perturb the human microbiome. Additionally, phages are believed to be safe because of their ubiquitous nature and the fact that they can be found in and on humans at any given time; phase I clinical trials support this notion (Rhoads et al. 2009).

There are potential pitfalls to developing phage therapeutics. While a narrow spectrum of activity can be beneficial, it can also make it difficult to find phages that can be widely used to treat infection, even for a single bacterial species. Issues with resistance also exist – a single mutation could lead to a change or loss of the receptor for the phage, rendering the phage useless against the bacteria. One solution to these problems is the use of a mixture of multiple phages, termed a phage cocktail. Cocktails are ideally comprised of phages that bind to different bacterial cell receptors and have distinct but overlapping host ranges. Thus, if the bacterial host

becomes resistant to one of the phages via loss or mutation of the cell surface receptor, the remaining phages in the cocktail will still be active against the bacteria. Recently, several publications have described the creation of "intelligent" cocktails that are compounded in a multi-step process. First, the bacterial host is incubated with a monophage preparation to select for bacterial mutants that are resistant to the phage. Then, additional phages are isolated that target the mutant strains. All of these phages are subsequently mixed with the original phage. Intelligent cocktails are primed to lyse not only the parent bacterial strain, but also any mutant strains that are selected for during treatment. In the published studies, these cocktails were bactericidal and also decreased the phage-resistance frequency in vitro (Yu et al. 2018; Regeimbal et al. 2016).

Another issue in the development of phage therapies is the translatability of in vitro activity to in vivo efficacy. It was recently discovered that phages were better at lysing *C. difficile* in the presence of human colon cells than in a standard planktonic in vitro assay (Shan et al. 2018). Other studies have found that phage activity in vitro did not necessarily result in phage efficacy in vivo (El-Shibiny and El-Sahhar 2017; Sabouri et al. 2017). This could be due to various reasons, including inactivation or destruction of phages in the human or animal host, the pharmacokinetics of specific phages preventing them from reaching their intended destination at a concentration high enough to be efficacious, or a dearth of in vitro assays to assess phage activity that are representative of in vivo conditions.

Addressing these issues must be the main focus of phage therapy research moving forward. The selection criteria used to include phages in a therapeutic cocktail must be extensive and stringent. Phages should be selected based not only on the ability to lyse bacteria of interest, but also on the biofilm penetration and destruction attributes, decreased immunogenicity, and synergistic potential. Additionally, the assays developed to characterize these phages must be assessed for translatability to in vivo efficacy.

This chapter will describe the ongoing research and development of bacteriophage therapeutics focusing on phage activity against biofilms, phages in concert with antibiotics, the host immune response to phages, and how phages are being applied clinically.

2 Phage Against Biofilms

Biofilms are a major roadblock in the treatment of bacterial infections. A biofilm is formed when bacteria adhere to a surface, and produce a three-dimensional matrix consisting of extracelluar polysaccharides (EPS), proteins, and DNA. Biofilms comprise a community of bacteria, in different metabolic phases, due to an inherent nutrient gradient within the biofilm. Biofilms can be found in the natural environment, and also in industrial and hospital settings; they are recalcitrant to antibiotic therapy, making it difficult to eradicate biofilm-mediated bacterial infections. Biofilms are responsible for more benign issues, such as tooth plaque and decay, and more severe problems, such as non-healing chronic wounds that can lead to amputation and death.

Phage interactions with biofilms are highly dependent on the bacterial host strain, the phage characteristics, and the biofilm structure and composition. In vivo biofilms infections are often polymicrobial and include human host cell components (proteins, nucleic acids, and cell debris), which will impact phage activity in comparison with a biofilm grown in vitro under standard laboratory growth conditions (Pires et al. 2017). The EPS matrix can act as a barrier to limit diffusion of phages into the lower layers of the biofilm. Biofilms may also contain microbial enzymes that inactivate phages, and dead bacterial cells which can bind phages, preventing them from reaching a living host on which they can replicate (Pires et al. 2017). Biofilms also have a metabolic gradient, in which there are areas of the biofilm where bacteria are not rapidly dividing. As phage depends on the bacterial host machinery to replicate, metabolically slow or inactive bacteria will prevent rapid phage expansion (Pires et al. 2017). Quorum sensing in biofilms can also hinder phage activity by reducing the number of phage-receptors present on the bacterial cell surface (Pires et al. 2017). Specifically, it was shown that *E. coli* decreases the number of λ receptors on the cell surface due to signaling of *N*-acyl-L-homoserine lactone (AHL), a quorum-sensing molecule (Gupta et al. 2014). The reduction of phage receptors directly reduces the phage adsorption rate, thus helping to increase the population density of uninfected survivor cells after phage exposure. This phenomenon may be true for other phages; AHLs also help enteric bacteria like *Escherichia*, *Salmonella*, and *Serratia* to survive infection by phage χ (Hoyland-Kroghsbo et al. 2013).

In vitro studies have shown that bacteriophage can penetrate mature biofilms and cause bacterial cell lysis (Carson et al. 2010; Khalifa et al. 2015; Hanlon et al. 2001). In order to access their specific bacterial cell surface receptors and inject phage DNA into the host bacterial cell, phages may have to penetrate various barriers, including the bacterial capsule or peptidoglycan. To deal with these carbohydrate barriers, phages express polysaccharide depolymerases and lysins (Latka et al. 2017); these enzymes could prove helpful in penetrating biofilms to access the bacteria within. Identifying phages that express these enzymes, and combining them into a cocktail with phages with strong lytic activity against the bacterial host of interest, could result in strong anti-biofilm activity; further research is needed in this area.

3 Potential of Phage and Antibiotic Combinations

Combinatorial therapy utilizes multiple selective pressures that attack the organism/disease from distinctive angles, and has been useful in treating malaria, HIV, MDR-bacterial infections, and cancer (Mulet et al. 2018). The use of phages in combination with other treatments (e.g., antibiotics) has been investigated as a means to reduce resistance and target recalcitrant infections. The probability of resistant mutants arising from dual-armed treatments is significantly decreased, and often the treatment dose can be lowered to achieve an even greater effect (Soudeiha et al. 2017). Resistance to combination therapies does occur, but evolutionarily the mutants are less fit, as they have to expend increased genetic resources to survive the treatment,

and thus are ill-equipped to compete with wild-type strains in the population (Zhang and Buckling 2012). Combining phage and antibiotics can lead to a variety of effects against pathogens ranging from antagonistic, additive, or synergistic efficacy, respectively, describing the combination as less than the sum, the total sum, or greater than the sum of individual treatment activities (Piggott et al. 2015). For example, when a continuous culture of *S. aureus* was treated with phage or gentamicin alone, the bacterial density stays relatively stable over 96 h at ~10^9 colony-forming units per milliliter (CFU/ml), but when these treatments are combined, the population is reduced to ~10^3 CFU/ml, with increasing phage density (Kirby 2012). In another study, a diarrhea-inducing strain of *E. coli* and corresponding phage were isolated from piglet feces, and tested in combination with 100 μg/μl of ampicillin, kanamycin, penicillin, or tetracycline (Easwaran et al. 2015). The combination was tested against planktonic *E. coli* and displayed additive efficacy as the phage alone inhibited growth at 54%, while combined antibiotics inhibited up to 68%. Additionally, phages have been reported to sensitize multidrug-resistant bacterial strains to antibiotics. Specifically, Benjamin et al. discovered that tetracycline-resistant *P. aeruginosa* could be rendered tetracycline-sensitive after exposure to phage OMKO1, which utilizes the multidrug efflux pump systems MexAB and MexXY of *P. aeruginosa* as phage receptors Emergent phage-resistant *P. aeruginosa* had an altered efflux pump that increased sensitivity to different antibiotic classes (Chan et al. 2016).

3.1 Phage Antibiotic Synergy

Another curious phenomenon observed with phage–antibiotic amalgamations is that a low dose of certain antibiotics can enhance the production of bacteriophage. This effect of combining sublethal antibiotic concentrations with phage is called phage antibiotic synergy (PAS) and was first described in 2007 (Comeau et al. 2007). *E. coli* phages were combined with low doses of antibiotic classes that disrupted cell division: quinolones or β-lactams; when phages were applied with low doses of these antibiotics, significantly larger plaques were observed. This phenomenon was attributed to SOS-induced filamentation (cell stress-mediated elongation but no division), specifically leading to faster phage assembly and increased lysis. Recently we evaluated the synergistic effects of antibiotics and phage on several clinical isolates (data not published) using our modified OmniLog™-based system (Henry et al. 2012). This in vitro study clearly indicated that phage K combined with several different antibiotics resulted in synergistic effects on methicillin-resistant *S. aureus* (MRSA) (Fig. 1a). Additionally we observed that low multiplicity of phage infection along with antibiotics was also very effective against meropenem-resistant *Klebsiella pneumoniae* (Fig. 1a–d). Another group tested a novel *S. aureus* phage with sublethal concentrations of quinolones, β-lactams, or inhibitors of protein synthesis (linezolid, tetracycline, ketolides) against MRSA (Kaur et al. 2012). Unlike the prior study, only inhibitors of protein synthesis enhanced plaque size (3x enlargement) of the test phage and seven other *S. aureus* phages.

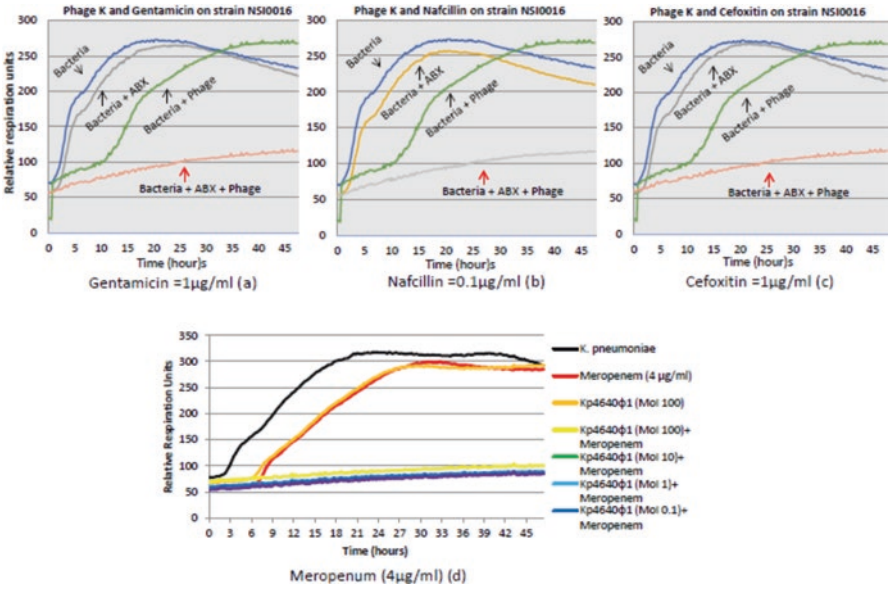

Fig. 1 Phage antibiotic synergy on MDR *S. aureus* and *K. pneumoniae* clinical isolates. The combination effects of phage K (10 moi) and various antibiotics (gentamicin, nafcillin, and cefoxitin) produced a synergistic effect on the growth inhibition of a *S. aureus* (**a**–**c**). Similarly, a combination of various concentrations of *K. pneumoniae*-specific phage (Kp4640φ1) and meropenem (4 µg/ml) produced a synergistic effect on the growth inhibition of a meropenem-resistant *Klebsiella* (**d**)

PAS has been observed against *P. aeruginosa* when subinhibitory concentrations of the β-lactam ceftriaxone were applied with *Siphoviraidae* phage (Knezevic et al. 2013). In this study the PAS was very limited as this phenomenon was not observed for gentamicin, ciprofloxacin, or polymyxin B, nor with *Podoviridae* phages; the mechanism of action (MOA) was again attributed to elongated filamentation. Another gram-negative pathogen, *Burkholderia cenocepacia*, displayed increased sensitivity to PAS when exposed to phage plus meropenem, ciprofloxacin, or tetracycline (Kamal and Dennis 2015). In this study PAS dramatically improved the survival rates of the wax worm *Galleria mellonella*, specifically low doses of meropenem with phage (78%) as compared to meropenem (20%) or phage (33%) alone. Using transmission electron microscopy, *B. cenocepacia* was imaged when exposed to subinhibitory concentrations of ciprofloxacin, meropenem, or tetracycline and, respectively, displayed filamentation, elongation, or clusters, suggesting multiple MOAs for PAS. Alternatively, the Ref endonuclease from *E. coli* lytic phage, which disrupts DNA metabolism and degrades the bacterial genome, was shown to induce filamentation, and be critical to PAS against *E. coli* (Ronayne et al. 2016). In addition to filamentation, another plausible explanation for phage–antibiotic synergy is the massive reduction of bacterial biomass after initial phage exposure and loss of fitness of phage-resistant bacteria against antibiotics.

3.2 Phage–Antibiotic Dual Treatment of Biofilms

As described in the previous section, there are many challenges to overcome when trying to eradicate biofilms. While it is known that specific phage strains can degrade the EPS matrix to get to the bacterial cells, no substantial data exists to support this. This highlights the need for increased treatment regimens/therapies against these recalcitrant "bacterial cities." Much of the work on phage and antibiotic combinations against in vitro and in vivo biofilms has been conducted by Dr. Sanjay Chhibber's laboratory. Starting in 2009 this group showed that treating 1–8-day-old *K. pneumoniae* biofilms over 24 h combining ampicillin (512 μg/ml) with phage treatments (MOI 0.01) resulted only in additive killing, but significant biofilm populations remained (Bedi et al. 2009). This group went on to test another *K. pneumoniae* phage with ciprofloxacin (at the MIC: 0.625 μg/ml) against 0.5-day-old *K. pneumoniae* biofilms, and after 3 h, the combination treatment killed fewer cells (3 log decrease) than phage alone (4 log decrease) (Verma et al. 2009). Although the combination therapy against *K. pneumoniae* biofilms was antagonistic, it did dramatically decrease the selection of resistant mutants as compared to phage or antibiotics alone. Further testing of ciprofloxacin and phage with or without depolymerase activity against 1–7-day-old *K. pneumoniae* biofilms resulted in additive, neutral, or antagonistic effects depending on the age of the biofilm (Verma et al. 2010). A separate study reported synergistic killing of *E. coli* biofilms when T4 phage was combined with cephalosporin or cefotaximine, reducing the MBC of cefotaxime one- to fourfold depending on the phage concentration (Ryan et al. 2012). Tobramycin (0.5–2 μg/ml) was combined with phage (MOI 0.01) against 48-h *E. coli* or *P. aeruginosa* biofilms, and additive killing of bacterial cells was observed over a 24-h treatment time (Coulter et al. 2014). Lastly, when phage was engineered to inhibit the bacterial SOS system and combined with ofloxacin, a robust increase in eradication of persister cells and biofilm cells was observed, as compared to either treatment alone (Lu and Collins 2009). Overall the in vitro results of phage in combination with antibiotics against biofilm are quite varied and of unknown biological importance. As combinatorial therapy of phage and antibiotics can lead to antagonism in some instances, it begs the questions, does treatment order matter, and what are the optimal conditions to combine these treatments?

3.3 A Question of Treatment Order: Phage Before Antibiotics?

While many of the studies in this section apply phage and antibiotic treatments simultaneously, increasing attention is focused on how to optimize bacterial killing by adjusting treatment order and intervals. This is logical as phages often are ineffective against the less metabolically active cell populations in biofilms, and thus by using an antibiotic first, this could create a similar or unhospitable cellular state for phage replication (Payne and Jansen 2001; Abedon 2016). Studies in the 1980s highlighted this phenomenon, where antibiotics disrupted phage production, specifically DNA gyrase inhibitors (aminocoumarins like novobiocin and quinolones

like nalidixic acid/ciprofloxacin) (Hamatake et al. 1981; Alonso et al. 1981; Constantinou et al. 1986). A study focused on a rifampicin-resistant *Pseudomonas fluorescens* strain found that phage alone decreased cultures ~0.75 log, while applying phage to planktonic cells before rifampicin caused ~1.5 log drop (Escobar-Paramo et al. 2012). Interestingly, when applied simultaneously, there was no decrease in planktonic cells compared to the untreated control. In another study, *P. aeruginosa* planktonic cells were treated with 10^7 PFU/ml phage for 0, 12, or 24 h and then treated with streptomycin (100–240 μg/ml) (Torres-Barcelo et al. 2014). Applying phage treatment for 12 h, before streptomycin at either concentration resulted in the optimal eradication of planktonic cells as compared to 0 or 24 h. Recently, 48-h *P. aeruginosa* biofilms were treated with an 8× MIC of ceftazidime, ciprofloxacin, colistin, gentamicin, or tobramycin combined with 10^6 PFU/ml of a two-phage cocktail with the antibiotics added: (1) no phage, antibiotic alone control, (2) concurrently with phage, (3) 4 h post phage, and (4) 24 h post phage (Chaudhry et al. 2017). Following 48 h of treatment, the authors observed synergistic killing of biofilm cells with gentamicin (~3 log decrease) and tobramycin (~5 log decrease) post-24-h phage treatment compared to simultaneous phage. Treating with phage for 24 h before antibiotics also increased phage densities 4 log PFUs/ml for gentamicin, tobramycin, and ciprofloxacin when compared to concurrent phage treatment. Synergistic activity against *P. aeruginosa* was observed for ceftazidime (3–4 log decrease) and colistin (2 log decrease) regardless of when phage was added, while ciprofloxacin alone significantly reduced biofilm cells to ~10^3 CFUs/ml. These data suggest that aminoglycosides especially benefit from phage treatment first, which can then replicate, before protein synthesis becomes disabled.

A similar study was published determining the optimal treatment order of phage and antibiotics for MRSA biofilms (Kumaran et al. 2018). Similarly the maximal additive or synergistic activity against MRSA biofilms was observed for all five antibiotics tested when added 24 h post-phage treatment. Cefazolin and vancomycin at 32 μg/ml displayed optimal synergy when added post-24-h phage. Initial treatment with phage for 12–24 h before administering antibiotics has been shown to optimize the synergistic potential of these therapies against planktonic and biofilm cells of multiple pathogens. One possible explanation for this is that pre-exposure to phage and resulting phage resistance was shown to make *P. aeruginosa* more sensitive to multiple antibiotics because of alterations to efflux membrane proteins (Chan et al. 2016).

3.4 In Vivo Phage–Antibiotic Combination Studies

Although the efficacy of phage and antibiotic dual treatments varies in vitro by the pathogen, phage, treatment order, and antibiotic MOA, the limited studies in vivo reveal strong clinical efficacy. One of the initial studies combining phage and antibiotic therapy used male broiler chicks with *E. coli* sepsis, administering oral treatments for 7 days of antibiotic enrofloxacin (50 ppm), a two-phage cocktail (10^9 PFU), or a combination of the two. Treatment improved survival in all groups (enrofloxacin 97%, phage 85%, combined 100%) versus 32% survival in untreated, and combination treatment was additive (Huff et al. 2004). A murine (CD-1) model of *E.*

coli sepsis was used to evaluate phage engineered to cripple the bacterial SOS response with an SOS repressor *lexA3* (Lu and Collins 2009). Treatment was applied once at 1 h post-infection, and the survival profiles quantified over 5 days: (1) untreated (10%), (2) IV ofloxacin 0.2 mg/kg (20%), (3) IV phage-10^9 + ofloxacin (50%), and (4) IV phage^{lexA3}–10^9 + ofloxacin (80%). This study shows that suppression of the SOS response in combination with phage and antibiotic had a synergistic effect; as stated earlier, this is most likely due to SOS causing bacterial filamentation, which results in rapid phage assembly and increased lysis (Comeau et al. 2007).

A 2013 study established MRSA or *P. aeruginosa* biofilm-implant tibial infections in rats, and treatment was applied to four subgroups: (1) untreated, (2) phage alone (50 μL locally over 3 days), (3) antibiotic alone (IP over 14 days), (4) or combination of treatments (Yilmaz et al. 2013). The MRSA group receiving dual treatments displayed synergistic killing of bacteria with 1 log decrease (5.0×10^4 untreated, 3.0×10^4 phage, 1.7×10^4 antibiotic, vs. 5.0×10^3 combo) and no biofilm formation compared to all other groups. The combination treatment had additive activity against *P. aeruginosa* (1.4×10^4 untreated, 6.4×10^3 phage, 2.6×10^3 antibiotic, vs. 1.7×10^3 combo). That same year Chhibber et al. evaluated the ability of the *S. aureus* MR-10 phage alone (single, local dose 10^8 PFUs/mL), linezolid alone (oral, 25 mg/kg), or in combination with clear MRSA hindpaw infections in diabetic BALB/c mice over 12 days (Chhibber et al. 2013). Overall, when both therapies were combined, an additive effect was observed along with decreased inflammation, while all three treatments cleared the MRSA infection by day 7; the MRSA in the untreated group persisted until day 12. The phage remained present at the infection site until day 9, with 10^2 PFU/mL isolated at day 7. Chhibber et al. went on to develop a BALB/c mouse model for prosthetic joint MRSA infections persisting over 20 days and evaluated the therapeutic activity of slow-release K-wire coatings: phage (10^9 PFU/mL), linezolid (5% w/w), or a combination (Kaur et al. 2016). Overall additive activity was observed for the combination group, and at day 10 MRSA was eliminated from the joint tissue in all treated groups versus 10^5 CFU/mL in the untreated controls.

Most recently, a rat *P. aeruginosa* endocarditis infection model was treated 18 h post-infection with a single IV dose of Pherecydes 12-phage cocktail (1 ml in 1 min at 10^{10} PFU/ml), Pherecydes 12-phage cocktail (100 μl in 1 h at 10^{10} PFU/ml), ciprofloxacin (1 ml in 1 min at 20 mg/kg), or a combination of phage cocktail and antibiotic (Oechslin et al. 2017). Following 6 h of treatment, the log CFU/gram of tissue for controls was ~10^9, versus phage or antibiotics alone at ~10^6, while synergistic activity was observed with combination therapy at ~10^2. While phage-resistant mutants were isolated from in vitro samples, surprisingly no phage-resistant mutants were isolated from in vivo samples before or after treatment, suggesting that the demands to adapt to the host environment disfavors phage resistance. These limited animal studies are encouraging as phage treatment was applied concomitantly with antibiotics, and yet additive or synergistic activity was still observed, without any treatment antagonism as observed in vitro. Many questions remain to be addressed in vivo for phage and antibiotic combinations including the following: Does treatment time matter? Will systemic vs. local phage delivery impact efficacy? Is antibiotic MOA still important to synergy?

4 Immune Responses to Bacteriophage

4.1 Phage as Part of the Microbiome

Investigations of the microbiome have taken off since the low-cost development of next-generation sequencing platforms leading to characterization of the human microbiome (Human Microbiome Project Consortium 2012) and many follow-on studies about the effects of diet and environmental exposures on the bacterial diversity. Of the microorganisms that inhabit the skin, gut, and mucosal surfaces are phage and their interactions with the bacteria and host cells from infancy throughout life (Mirzaei and Maurice 2017). This "phageome" is the most diverse of any microflora, has an extensive distribution throughout the body, and is subsequently turned over with lysis of its bacterial hosts (Breitbart et al. 2003; Colomer-Lluch et al. 2011; Minot et al. 2011; Virgin 2014; Oh et al. 2014). Estimates of the number of phages in the gut are in the 10^{12} range (Sender et al. 2016). Moreover, translocation of phages have been noted to occur when orally ingested (Duerr et al. 2004; Hamzeh-Mivehroud et al. 2008), as well as from systemic blood to fetal tissues in pregnant mice (Srivastava et al. 2004a). Demonstration that phage can translocate across host cells yields potential systemic exposure to 10^{10} phages per day (Nguyen et al. 2017). Accordingly, it is reasonable to consider that phage had evolved to coexist with their host in nature without adverse effects (Gomez and Buckling 2011; Waterbury and Valois 1993). This concept is derived from a number of factors, such as fluctuating selection, costs of defense, the loss of immunity due to impaired host defense mechanisms (Weissman et al. 2018), and costs that arise due to trying to balance host growth and immunity vs host range and immune evasion of phage (Chao et al. 1977; Levin et al. 1977; Jover et al. 2013; Meyer et al. 2016).

Although studies have demonstrated that phage can coexist with their host, there is plenty of evidence that supports the notion that phage and/or phage lysate are able to be immunogenic and elicit specific immune responses, as well as have immunomodulatory activity on a variety of different immune cell populations in both innate and adaptive immune responses (Gorski et al. 2012). Some of these immunomodulatory effects include the generation of antibodies and cytokines, shifts in T-cell-mediated immunity, or changes in free radical production. It is important to note that it is sometimes difficult to interpret the results of studies involving phage due to varying methods of preparation and purification. As a result, remnants of bacteria, lipopolysaccharide (LPS) and endotoxins, or components of the bacterial cell wall may be present with the phage and may cause a false-positive detection or boost the immune reaction with no relevance to bacteriophage activity.

4.2 Innate Immune Responses to Phage

Phage therapy, like antibiotics, is designed with the intent to eliminate bacterial infections. However, despite rational design to broaden the host range and/or counter bacterial resistance to phage infection, complete elimination of the bacterial

cause of infection by phage therapy must consider the role of the host immune system. Specifically how the phage and both humoral and cellular immune components work in tandem, and eventual clearance of bacterial structures and components by the immune cells, is important to resolve the inflammatory process and return to homeostasis. Using a combination of mathematical modeling, supplemented with experimental validation, Roach and colleagues (Roach et al. 2017) demonstrated that host innate immunity was necessary for bacterial clearance and also, interestingly, for the prevention of bacterial resistance to phage. These findings along with the more frequent use of phage to display peptides for vaccines (Aghebati-Maleki et al. 2016) suggest that there is an interplay between the human immune system and bacteriophages that is both safe and can potentially be leveraged for therapeutic purposes. This section will discuss what is currently known about how the immune system senses and responds to phage and how phage and immune cells cooperate in bacterial clearance.

Pattern recognition receptors (PRRs) are molecular sensors expressed on host cells that selectively bind to conserved structures on pathogens and foreign materials, eliciting expression of inflammatory cytokines that signal recruitment and activation of both cellular and humoral immune components to counter the microbial threat. The best known PRRs include the Toll-like Receptors (TLRs); the best characterized is TLR-4, which binds to the bacterial lipopolysaccharide (LPS) (Kumar et al. 2009). Signal transduction following PRR binding to its cognate antigen cascades to nuclear factor kappa beta (NF-kB)-mediated transcription of cytokines and chemokines, which are released by the sentinel cells to initiate the immune response. The critical role of PRR detection is apparent in mice with a knockout of the MyD88 gene that lack the ability to respond through PRR/TLR binding. The morbidity and mortality in these mice to pathogens of lower virulence and/or lower numbers of inoculated organisms is significantly increased compared to immunocompetent mice (Warner and Nunez 2013).

There is little known about PRR recognition of purified phage preparations and which if any of these bind to phage proteins and the subsequent effect on cellular activation. In a mouse model, intraperitoneal administration of an M13 phage induced an IgG-based antibody response that was dependent on MyD88 signaling, but no reduction of activity was noticeable in TLR-2/4/7-deficient mice (Hashiguchi et al. 2010). This is interesting, because it suggests that these TLRs are not involved in phage recognition, but carryover of bacterial antigens is also not important for phage-dependent immune activation. However, TLR-9 may be involved in a tolerogenic response to phage, as TLR-9-deficient mice had an augmented IgG1 response relative to wild-type mice (Hashiguchi et al. 2010). Phage genomes may be methylated similar to bacterial genomic sequences and thus recognition of CpG motifs by TLR-9 may account for the immunomodulatory and adjuvant activity noticeable in vaccine applications (Aghebati-Maleki et al. 2016). Further findings regarding the role of TLRs (or lack thereof) in the immune responses to T4 phage were seen when TLR-2/TLR-4 expression was unaltered in unstimulated and LPS-activated monocytes, in contrast to T4 lysate-stimulated cells with a slight increased TLR4 expression profile (Gorski et al. 2012). Additionally, human peripheral blood monocytes

downregulated CD14 expression when stimulated with *P. aeruginosa* phage PNM, further indicating a lack of TLR-4 association and a potential mechanism of immune suppression (Van Belleghem et al. 2017). Additional work on recognition by host innate PRRs across the TLR range and additional PRRs such as the intracellular Nod-like receptors (NLRs) and surface receptors, such as the mannose-binding lectins (MBLs), remains to be determined. As foreign antigens, there are likely host receptors that recognize phage, so determining the baseline recognition for the different phage families is important for safety profiling of therapeutic phage cocktails and monitoring these purified preparations for carryover of bacterial antigens that may induce a toxigenic response.

4.3 Cytokine Responses to Phage

Phages have been shown to influence inflammatory responses both in a stimulatory and inhibitory manner, depending on the phage preparation/purity, species, dose, and route of administration (Gorski et al. 2012). For example, purified T4 phages were able to increase serum interferon levels when administered intravenously to mice, independently of LPS (Kleinschmidt et al. 1970). However, Miernikiewicz et al. found that phage T4 and its head surface proteins gp23∗, gp24∗, Hoc, and Soc did not alter production of inflammatory cytokines or ROS production (Miernikiewicz et al. 2013). Further studies also observed that purified T4 was not able to induce significant intracellular IL-6 and TNF-α synthesis in human monocytes (Bocian et al. 2016). In human mononuclear cells, however, purified T4 were shown to inhibit mitogen-induced IL-2 production (Przerma et al. 2005). In a recent study, purified *S. aureus* phages were compared against phage lysate or the *S. aureus* host strain. When co-cultured with PBMCs for six normal donors, titration effects indicated that sufficiently high phage titers (i.e., $\geq 10^3$ PFU/PBMC) were required in order to induce an immune response (Van Belleghem et al. 2017). Similarly, there was a lack of a pro-inflammatory response to T4 and its capsid proteins when delivered to human PBMCs and in vivo to mice (Miernikiewicz et al. 2013) and similar findings reported from patients who received phage therapy (Gorski et al. 2016).

Alternatively, studies have demonstrated that phage can also have anti-inflammatory effects. T4 phages were able to inhibit activation of NF-κB, a key transcription factor regulating pro-inflammatory cytokines (Gorski et al. 2006). Additional studies show that purified phages can suppress reactive oxygen species (ROS) production (Gorski et al. 2012). Weber-Dabrowska et al. demonstrated that phage therapy was able to not only normalize TNF-α levels in serum, but also the production of TNF-α and IL-6 in blood cell cultures (Weber-Dabrowska et al. 2000a). In 2017, Van Belleghem et al. found that four different *P. aeruginosa* phages were all able to induce IL-6 and the anti-inflammatory genes *IL1RN*, *IL10*, and *SOCS3*, as well as the pro-inflammatory genes *CXCL1*, *CXCL5*, *IL1A*, and *IL1B* (Van Belleghem et al. 2017). Other studies found that purified *S. aureus* phages were able to promote IL-6 production in unstimulated and ConA-stimulated splenocyte cultures (Zimecki et al. 2003).

4.4 Complement Deposition and Activation

Very little is known about the role of complement deposition and activation in regards to natural phage. Phages are inactivated and removed from the blood by potentially both the reticuloendothelial system (Pincus et al. 2015), filtering organs (e.g., liver and spleen) (Hodyra-Stefaniak et al. 2015), and serum proteins (Sokoloff et al. 2000). The inhibitory effect is at least partially dependent on antibody binding (IgM and IgG) that may subsequently trigger the complement pathway (Hodyra-Stefaniak et al. 2015), but the mechanism of complement in phage clearance remains undetermined to date.

4.5 Phagocyte Interactions with Phage

The interactions of bacteriophage and phagocytic cells were reviewed recently and highlighted several key characteristics with implications toward use of phage as therapeutics (Jonczyk-Matysiak et al. 2017). While data is growing that phages are part of the human microbiome and that frequent environmental exposure to phage throughout the lifetime does not cause adverse effects, the sudden high dose of phage given systemically for therapeutic application could present unexpected immune activation, which could be altered in immunocompromised patients. As presented by multiple groups, bacteriophages are ingested by phagocytes, polymorphonuclear neutrophils, macrophages, dendritic cells, and splenocytes, after which they are localized in phagosomes or lysosomes and inactivated through degradation in these compartments (Jonczyk-Matysiak et al. 2017). Interestingly, it has been speculated that phages may act as opsonins (facilitators of phagocytosis) when bound to bacteria, increasing bacterial uptake (Jonczyk-Matysiak et al. 2017) and may act synergistically to augment intracellular killing by phagocytes (Jonczyk-Matysiak et al. 2015; Kurzepa-Skaradzinska et al. 2013; Tiwari et al. 2011). Through clinical experiences, phage therapy may be beneficial to patients with deficiencies in phagocyte functions, thus boosting host cell uptake and clearance of invading bacterial pathogens (Gorski et al. 2016).

One of the primary mechanisms phagocytes use to kill engulfed bacteria is through a respiratory burst resulting in production of reactive oxygen species (ROS) (Kobayashi et al. 2005). In contrast to potential enhancement of bacterial killing and inactivation of phages in phagolysosomes, it seems that purified phages do not stimulate a respiratory burst in phagocytic cells (Jonczyk-Matysiak et al. 2017), despite some conflicting results between low ROS stimulation and no stimulation (Miernikiewicz et al. 2013; Borysowski et al. 2010; Miedzybrodzki et al. 2008). Considering the context of intravenous delivery of phage cocktails, this is as positive as a safety profile, because phages are unlikely to cause ROS release among peripheral blood-circulating phagocytes that could lead to endothelial and organ damage. Similarly, experiments have demonstrated that neutrophils do not degranulate to release antimicrobial proteins and enzymes when stimulated with *Staphylococus aureus* A3R phages (Borysowski et al. 2017), further confirming the safety of phage therapy.

As summarized by Jonczyk-Matysiak (Jonczyk-Matysiak et al. 2017), bacteriophages seem to have a much different effect on the innate immune system than eukaryotic viruses across the spectrum of signaling and functions. This may be due to exposure early in life when the host immune system is developing at the same time the host microbiome is populated with both bacteria and bacteriophage, thus generating a tolerant immune response versus rapid activation by pathogenic viruses not normally seen by the host immune system (Nguyen et al. 2017). Further studies are needed to outline these differences between viral sensing and also in how the immune system responds to bacteria lysed by phages.

4.6 Adaptive Immune Responses to Phage

Several studies have implicated the immunomodulatory effects of phage to not just innate immunity but the adaptive as well. In 1974, Mankiewicz et al. reported that mycobacteriophages could inhibit the phytohemagglutinin (PHA)-induced activation of lymphocytes in a dose-dependent manner in culture (Mankiewicz et al. 1974). In addition, phage phi X174 has been extensively shown to be a potent T-cell-dependent neoantigen IV, which has enabled it to be used as a standard antigen to evaluate humoral immunity in patients with primary and secondary immunodeficiencies for over 30 years (Bearden et al. 2005a). Meanwhile, some studies have shown that purified T4 phage preparation inhibits human T-cell proliferation (Gorski et al. 2006), whereas purified *S. aureus* phage provoked costimulatory effects on splenocytes that has been activated by a suboptimal concentration of ConA (Zimecki et al. 2003).

Antibodies, also called immunoglobulins (Ig), are produced by B-cells that have been activated and matured into plasma cells. Their primary function is to identify and neutralize pathogens and foreign elements that invade the host. Studies suggest that the generation of antibodies targeted against bacteriophage is dependent on route and frequency of administration, dosage, and the individual features that comprise the bacteriophage (Dabrowska 2018). As early as the 1950s, scientists were able to detect the presence of antibodies against bacteriophages in serum, albeit at low levels (Jerne 1956). These antibodies, coined "natural antibodies," were generally found to be of the IgM class and exhibited broad cross-reactivity and low affinity (Jerne 1956; Kamme 1973; Kucharewicz-Krukowska and Slopek 1987). More recent studies have confirmed the natural occurrence of bacteriophage-specific antibodies in humans (Dabrowska et al. 2014). The presence of these antibodies is most likely due to the continual presence of phages in human biomes (Gorski et al. 2006; Gorski and Weber-Dabrowska 2005), and homologies shared by phage, whether due to capsid composition or other structural components (Tikhonenko et al. 1976).

In the case of pathogenic viruses, or in this case bacteriophage, these antibodies can be categorized by four main activities: virus neutralization, antibody-dependent cellular cytotoxicity, antibody-dependent cell-mediated virus inhibition, and phagocytosis (Forthal and Moog 2009). In regard to therapeutic efficacy of phages in vivo,

specific antibodies are considered to be one of the most important limiting factors. This notion was supported by the fact that the clearance of T7 phage from the blood of mice is slower in B-cell-deficient animals compared with wild-type mice (Srivastava et al. 2004b). However, it is important to note phage–antibody interactions do not necessarily mean phage inactivation. For example, some phage–antibody complexes led to the loss of phage antibacterial activity, whereas others did not display any visible effect on phage viability (Jerne and Avegno 1956). Also, there appears to be a vast range of anti-phage humoral response as some phages are very weak immunogens and require repeated injection coupled with administration of adjuvant to induce detectable antibody titers (Sulakvelidze and Barrow 2005). In a study examining anti-phage-neutralizing antibodies in sera of patients with bacterial infection, no significant increase in antibody activity was noted in patients who received phage lysates orally (Gorski et al. 2012). Similarly, Majewska et al. reported that orally applied phage T4 was only found to induce anti-phage antibodies following a combination of long exposure times and high doses (Majewska et al. 2015). These findings further emphasize the importance of route and degree of exposure to phage to elicit antibody production.

4.7 Implications for Phage Therapy in Regard to Host Immunity

In a therapeutic setting, the integrity of the host immune system plays a significant role in both susceptibility to pathogen infection and the effectiveness of antimicrobial therapeutic agents, such as phages, especially because the dynamics between phage and bacteria further compound the pressures on mammalian host immunity. Taken in context, the anti-inflammatory and apparent tolerized immune response to bacteriophage is fitting. However, not all bacteriophages are the same. Although phages of common commensal bacteria (e.g., *E. coli*, *S. aureus*) may be commonly seen by the host immune system and thus not induce an inflammatory response, phages of environmental opportunistic pathogens may be less commonly seen by the host immune system (e.g., *A. baumannii*, *P. aeruginosa*) and thus may induce an entirely different immune response. Further, there is a distinct difference between purified phage preparations and phage lysates (mix of phage and bacterial components). Additionally, differences in the volume of these particles seen during normal homeostasis or natural infection versus high doses given in phage therapy resulting in massive lysis of an overwhelming infection may result in a robust inflammatory response. There is a possibility of carryover of bacterial components following laboratory preparation of phage cocktails, even in highly purified phage preparations. Thus, these therapeutic preparations must be evaluated systematically for their ability to stimulate PRRs and immune cells in tissue culture, ex vivo, and animal studies prior to delivery to human patients.

5 Phage Efficacy in Animal Infection Models

With the resurgence of interest in phage therapy to combat multidrug-resistant bacteria, there has been a desire to test phage therapy in animal models that closely mimic clinical conditions. Animal models provide an ideal platform to measure the efficacy of phage therapy in regard to safety, infection prevention, toxicity, and pharmacokinetics. Relative to clinical trials, animal models are also significantly cheaper, numbers can be scaled up, and critical variables such as biofilm formation, use of single phage or multiple phage cocktails, dosage, and method of delivery can all be evaluated to determine optimal parameters. In addition, phage therapy can be utilized in well-established animal models that mimic clinically relevant pathologies, and data from these animal studies can be used to move forward with human trials. Table 1 is a list of animal models, predominately mice, showing the efficacy of phage therapy against MDR pathogens, detailing important variables such as pathogen targeted, dosage, route of delivery, time course of phage therapy, and results.

One issue of concern with phage therapy in animal models is the time course of phage delivery. In many of the studies, phage was given close to bacterial inoculation. Not surprisingly, success of phage therapy was highest when given early. Furthermore, phage given prior to bacterial inoculation was also protective, indicating the possibility that phage can be used prophylactically. It should be noted that in clinical cases, phage therapy is given days or weeks after infection has been established. Some investigators have given phage therapy after lengthy delays of up to 16 days. In the rabbit osteomyelitis model, even after a 16-day delay, phage therapy was able to eradicate the staphylococcal infection (Kishor et al. 2016).

While investigators try to mimic physiological conditions, animal models are not perfect representations of human disease due to a number of factors. Patients most likely to undergo phage therapy often suffer from chronic infections with the presence of biofilm, a major factor in antibiotic resistance. Resistance to phage therapy is another complication that is often encountered in patients and has been studied by several investigators. Duerkop et al. showed a significant drop in *E. faecalis* with the first 24 h but quickly rebounded 24 h later (Duerkop et al. 2016). Fecal analysis identified the presence of phage-resistant *E. faecalis* only. In many studies, single phages were utilized rather than a cocktail of multiple phages.

A review of phage therapy in various animals has produced a consistent result; there was no evidence of toxicity when phage therapy was administered to the animal. Even at the highest doses, phage was tolerated. One issue of concern with phage therapy in the animal models was the lack of endotoxin level determination in these studies. Phage preparations that are used in patients require extensive endotoxin level assessments to minimize the level of exposure.

Pharmacokinetic analysis was performed in some of the studies, indicating that the mechanism of delivery can have a significant impact on the success of phage therapy. Not surprisingly, distribution of phage throughout the body (liver, spleen, blood) was most effective when given i.p. rather than topically.

Table 1 In vivo phage efficacy

Pathogen	Animal	Pathology	Dose	Delay*	Route	Outcome	REF
Staphylococcus aureus	Mouse	Bacteremia: 2 X 10^8 CFU/i.p.	1 phage 1 dose 10^{10} PFU	4 hours	i.p.	90% cure Diabetic 100% cure Non-diabetic	[107]
	Rabbit	Osteomyelitis: 5 X 10^4 CFU/i.med	7 phage cocktail 1 dose/48 hours 4 doses total 5.25 X 10^{11} PFU	*16 days* *6 weeks* *(chronic)*	Localized Soft tissue	Negative culture after 4th dose 100% Cured	[108]
	Mouse	Flank Abscess: 10^8 CFU/s.c.	1 phage 1 dose (or 4 doses) 10^9 PFU	0 hours 4 days 4 days (4 doses)	Localized s.c.	0 hr = 0 mg abscess 4 day single = 34 mg abscess 4 day multiple = 15 mg abscess	[109]
	Mouse	Bacteremia: 10^8 CFU/i.v.	1 phage 1 dose 10^7; 10^8; or 10^9 PFU	0 hours	i.v.	10^7 PFU = 0% Cured 10^8 PFU = 40% Cured 10^9 PFU = 100% Cured	[109]
Klebsiella pneumoniae	Mouse	3rd degree burn: 10^8 CFU/topical	5 X 10^{10}	0.5 hours	Topical	~64% Survival	[110]
	Mouse	Liver abscess: 2 X 10^8/lavage	1 phage 1 dose 10^5; 10^6; or 10^7 PFU	0.5 hours	i.p.	10^5 PFU = 30% Cured 10^6 PFU = 90% Cured 10^7 PFU = 100% Cured 10^8 PFU = 100% Cured ***10^9 PFU = 0% Cured***	[111]
	Mouse	Liver abscess: 2 X 10^8/lavage	1 phage 1 dose 10^5; 10^6; or 10^7 PFU	0.5 hours	Oral lavage	10^5 PFU = 44% Cured 10^6 PFU = 100% Cured 10^7 PFU = 100% Cured 10^8 PFU = 100% Cured	[111]
	Mouse	Liver abscess: 2 X 10^8/lavage	1 phage 1 dose 10^5; 10^6; or 10^7 PFU	6 hours 24 hours	i.p. or Oral lavage	10^5 PFU i.p.6 hr = 88% Cured 10^6 PFU i.p. 24 hr = 75% Cured 10^7 PFU oral 6 hr = 75% Cured 10^8 PFU oral 24 hr = 56% Cured	[111]
	Mouse	3rd degree burn: 10^8 CFU/s.c.	1 phage 2 doses 10^5; 10^6; or 10^7 PFU	0.5 hours 6 hours	i.p. or s.c.	100% Survival at 24 hours	[112]
	Mouse	Pneumonia 2 X 10^8 CFU	1 phage 1 dose 10^7; 10^8; or 10^9 PFU	2 hours	intranasal	10^7 PFU = 30% Cured 10^8 PFU = 60% Cured 10^9 PFU = 80% Cured	[113]
Acinetobacter baumannii	Mouse	Dermal Wound: 2 X 10^4 CFU/topical	5 phage cocktail 3 doses 4 X 10^9 PFU	4, 24, 48 hours	i.p.	100% survival Bacterial Clearance on Day 5	[5]
	Mouse	Dermal Wound: 2 X 10^4 CFU/topical	5 phage cocktail 3 doses 4 X 10^9 PFU	4, 24, 48 hours	topical	100% survival Bacterial Clearance on Day 5	[5]
	Mouse	Keratitis and Cornea perforation	1 phage Single dose 5 x 10^9 PFU	0.5 hours	Topical	100% survival Cornea fully cleared by day 5	[114]
	Mouse	Lung infection 1-2 x 10^7 i.n.	2 phage cocktail Single dose 2-5 x 10^9 PFU	2 hours	I.n.	Reduction in bacterial levels of 3-4 logs within 6 hours	[115]

(continued)

Table 1 (continued)

Pseudomonas aeruginosa	Mouse	Bacteremia 10^8 CFU/ml in drinking water	Single phage Continuous dose 10^{10} PFU orally administered	96, 240 hours	Oral gavage	None: 0% Survival 96 hour delay: 66% Survival 240 hour delay: 10% Survival	[116]
	Mouse	Subcutaneous wounds 10^5 CFU/ml on biofilm laden catheters inserted s.c.	Single phage 10 doses/ 1 per day 10^7 PFU/ml	0 hours	s.c.	In phage treated mice, significant drop in bacterial counts, from 3.87 x 10^6 CFU to 3.52 x 10^4 CFU, even with biofilm present	[117]
	Mouse	Lung infection 3 x 10^6 CFU intranasal	Single phage Single dose 3 x 10^{6-7} PFU (2 hours after) 3 x 10^{7-8} PFU 4 days before	2 hours 4 days prior	i.n.	Results show significant protection and survival with phage therapy, with the higher dose most protective. Prophylactic administration was also protective.	[118]
	Mouse	Bacteremia 5 x 10^6 CFU i.p. CP mice: 5 x 10^3 CFU	Single phage Single dose 5 x 10^{6-8} PFU CP mice: 5 x 10^4 PFU	0 hours	i.p.	Results show 80-100% survival rates, dose dependent. In CP treated mice (Neutropenic), phage therapy was not protective, although mice survived longer (48 hours vs 36 hours in control mice)	[88]
	Mouse	Lung infection 1 x 10^7 CFU i.n.	Single phage Single dose 1 x 10^{6-8} PFU	2 hours 24 hours prior	i.n.	Completely protective at 10^7 PFU, with significant reduction in bioluminescent bacteria 4 hours after phage therapy. Complete protection when given prophylactically 24 hours prior to infection. Live phage required for protection	[119]
	Mouse Fruit flies	Peritonitis 2 x 10^6 CFU Fruit flies: 50-200 CFU thorax	Single phage Single dose 2 x 10^{6-7} PFU 5 x 10^7 PFU in corn medium	6-12 hours	i.p. i.m.	While phage delivered i.m. had better pharmacokinetics in blood, liver, lungs, phage delivered i.p. was more protective. Drosophila phage therapy was completely protective	[120]
Pseudomonas aeruginosa	Mouse	Burn wound 2-3 x 10^8 CFU s.c.	3 phage cocktail Single dose 3 x 10^8 PFU	0 hours	i.m. s.c. i.p.	Phage therapy was protective, from 6% survival to 22-87% survival, with the best survival using i.p. delivery. Levels of phage in the liver, spleen, and blood was highest after i.p., lowest with s.c.	[121]
	Mouse	Pneumonia 1 x 10^7 CFU i.n.	Single phage Single dose Post infection: 1 x 10^8 PFU Before infection: 1 x 10^9 PFU	2 hours post infection 4 days before infection	i.n.	Phages well tolerated and not neutralized in lungs by immune system, MyD88 KO mice: Phage therapy increased survival rates by only 15% relative to saline treated, both significantly lower than WT, Rag2/IL2 KO mice: Phage therapy had 90% survival rate, Neutrophil depletion: Phage therapy or saline both had 0% survival, Prophylactic phage therapy protected mice for 4 days from fatal pneumonia	[122]
Escherichia coli	Mouse	DSS-induced colitis 1 x 10^9 CFU AIEC orally	3 phage cocktail 2 doses 3 x 10^7 PFU	24 hours Second dose 7 hours after first 24 hours prior	Oral gavage	2-log reduction in AIEC levels in the gut lumen. Significant decrease in DSS-induced colitis symptoms with phage therapy delivered 24 hours prior or 24 hours after AIEC infection.	[123]

(continued)

Table 1 (continued)

Enterococcus spp.	Mouse	Bacteremia *Enterococcus faecium (VRE)* 10^9 CFU i.p.	Single phage Single dose 10^8 PFU	45 minutes 24 hours	i.p.	100% rescue of mice when phage given 45 minutes after infection. 50% rescue when given 24 hours after infection. Live phage required for protection	[124]
	Mouse	Bacteremia *Enterococcus faecalis (VRE)* 2 x 10^9 CFU i.p.	Single phage Single dose 4 x 10^5 PFU	1 hour	i.p.	100% protection with phage therapy. Also prevented colonization in the GI tract	[125]
	Gnotobiotic mice	*Enterococcus faecalis* 5 x 10^7 CFU oral inoculation	Single phage Oral gavage: 1 x 10^{10} PFU then Continuous inoculation 5 x 10^8 PFU/ml	6 hours	Oral	Development of resistance to phage therapy started at 48 hours even after continuous administration in drinking water. Resistance to phage therapy was through mutations in the integral membrane protein, PIP_{EF}, which is required for phage infection. Identified the molecular basis for resistance.	[126]
Aeromonas hydrophila	Striped Catfish	Motile Aeromonas Septicemia 3.2 x 10^6 CFU i.p.	2 phage cocktail Single dose 3.2 x 10^4 PFU, 3.2 x 10^6 PFU, 3.2 x 10^8 PFU	0 hours	i.p.	*Aeromonas* resistant to multiple antibiotics 81% mortality rate in negative control 68.33% mortality: 3.2 x 10^4 PFU 45% mortality: 3.2 x 10^6 PFU 0% mortality: 3.2 x 10^8 PFU	[127]

6 Clinical Cases Involving Phage Therapy

6.1 Safety Rationale for Utilizing Bacteriophage Therapy Clinically

Bacteriophages appear to be intrinsically safe in humans, as they are exquisitely specific in targeting and infecting bacterial cells, and are devoid of any capacity to infect plant or animal cells (Furfaro et al. 2017). Indirect evidence attests to the safety of exploiting bacteriophages for clinical therapeutics: phages comprise an appreciable percentage of our microbiome (confirmed by electron microscopy and metagenomics) and are ubiquitously present in food and water sources. The human gut "phageome" comprises an estimated 10^{15} bacteriophages, and fecal metagenomic studies suggest that phages far exceed eukaryotic viruses both in number and diversity (Dalmasso et al. 2014). For example, *Escherichia coli* phage (coliphage) titers up to 10^9 pfu/gram have been detected in human feces subsumed under 10^3 virotypes. The ubiquitous phage composition of the microbiomes undoubtedly imparts unique and significant influence upon the structure and function of the microbiome (Furfaro et al. 2017). As intimated earlier, lysogenic phages may introduce genes for virulence or antibiotic resistance into their bacterial host via transduction (and to a lesser extent, lytic phages can do this via generalized transduction); therefore any phage considered for clinical therapeutics requires genomic sequencing to confirm the absence of genes encoding lysogeny or deleterious factors. Advances in bioinformatics and high-throughput genomic sequencing allow the ad hoc screening of phages for genes associated with transduction or virulence (Atterbury 2009).

A literature review of the utilization of bacteriophages in recent clinical therapeutics is delineated below, covering clinical cases using phage since 2000, along with older seminal clinical studies. To be reiterated in subsequent passages, despite a dearth of rigorous methodological trials meeting contemporary standards of reporting, the myriad of observational studies almost universally identified no safety issues associated with phage administration. Perhaps best attesting to phage safety as an adjunctive clinical therapeutic, phages have been safely administered intravenously (IV) [as a diagnostic antigen to signal immune reconstitution] to tens of thousands of immunosuppressed patients over four decades without noting any adverse events including both primary and secondary immunodeficiencies, including adenosine deaminase deficiency, X-linked agammaglobulinemia, X-linked hyper-IgM syndrome, major histocompatibility complex class II deficiency, Wiskott–Aldrich syndrome, AIDS, and the immunodeficiency state in allograft recipients and after bone marrow transplantation (Bearden et al. 2005b; Weber-Dabrowska et al. 2000b). For example, the ØX174 coliphage, which is currently a standard antigen for the evaluation of humoral immunity in clinical immunology has been safely administered intravenously for over four decades (Bearden et al. 2005b; Ochs et al. 1971). Finally, attesting to our confidence in securing FDA regulatory approvals, phages targeting the foodborne pathogen *Listeria* spp. were approved by the FDA in 2006 for use in packaged meats and cheeses and have been given the designation generally recognized as safe (GRAS) (Kutter et al. 2015).

6.2 Topical Bacteriophage Therapy

Phages to be applied topically may be prepared in several vehicles including ointments, gels, and creams to promote durable residence on the skin surface. For applications involving chronic ulcers, phages have been successfully impregnated into dressings or bandages. An exciting area of research involves optimizing dressings impregnated with antibiotics, bacteriophages, and a host of novel compounds which promote an optimal balance of pro and anti-inflammatory mediators in the local wound milieu, angiogenesis, and expedited wound healing. Topical (cutaneous) phage delivery may shield it from the host immune system.

Seventy percent (67 of 96) of patients afflicted by infected venous stasis ulcers refractory to antibiotic treatment from 1999 to 2000 in Tbilisi, Georgia, were successfully treated with a novel topically applied wound healing dressing (PhagoBioDerm). PhagoBioDerm is a novel biodegradable polymer impregnated with lytic bacteriophages (“pyophage cocktail”), antibiotics (ciprofloxacin), benzocaine, and α-chymotrypsin purported to promote expedited wound healing and currently licensed in the country of Georgia (Markoishvili et al. 2002). Of 22 cases in which microbiologic data were available, healing was associated with concomitant elimination of the infecting pathogen in the ulcer.

A case series involving two lumberjacks reports the successful application of PhagoBioDerm therapy applied to refractory *S. aureus*-infected ulcers [stemming from

complications of radiation (strontium-90)-induced injury]. After failing 1 month of antibiotic therapy, a 7-day course of PhagoBioDerm treatment (executed in Tbilisi, Georgia) yielded microbiological eradication, reduced purulent draining, and clinical healing over a course of 2–7 days (Jikia et al. 2005). The authors confirmed that the infecting *S. aureus* isolates were sensitive to phages represented within the PhagoBioDerm preparation.

In a compendium report of experience in Georgia and the Phage Institute in Wroclaw, Poland, phage therapy successfully treated 80% of secondarily infected post-burn injuries attributed to *Enterococcus* and achieved 90% efficacy against *S. aureus*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* (Ahmad 2002). A case report describes the successful decolonization of *P. aeruginosa* from the burn surface and skin grafts utilizing topical application of filter paper discs impregnated with PsA targeting bacteriophages potentiating successful grafting (Pires et al. 2015). A fixed three-phage cocktail (targeting *P. aeruginosa* and *S. aureus*) called BFC-1 was safely applied to nine colonized burn patients (via a single "spray" application) without establishing any inferences on efficacy (Rose et al. 2014). The phage composition was selected to encompass strains known to be represented within the burn unit microbiome. No adverse events, clinical abnormalities, or changes in laboratory test results were observed that could be related to the application of phages. The authors noted that the spray application often resulted in drainage from the wound bed; this prompted research into alternative delivery vehicles promoting local residence (gels, creams, ointments, or dressings).

Diabetic foot ulcers occur as a common complication of diabetes, whose healing is delayed by concomitant infection. Antibiotic treatment of these complicated infected ulcers is undermined by the polymicrobial nature of the infection, poor tissue vascularization, immune depression, and the formation of microbial biofilms (Morozova et al. 2018). A contemporaneous case series (six patients) was recently published describing the efficacy of weekly topical administration (per methods of Morozova et al. 2018) of a fully sequenced *S. aureus* targeting phage (Sb-1, the Eliava *S. aureus* phage employed commercially). Application of the Sb-1 cocktail achieved healing of complicated diabetic toe ulcers with proven *S. aureus* colonization, some associated with underlying osteomyelitis, refractory to antecedent antimicrobial therapy and otherwise resigned to amputation as the only remaining viable treatment (Fish et al. 2016, 2018). All ulcers exhibited healing after an average of 7 weeks of therapy. Acknowledging the polymicrobial nature of most diabetic wound infections, this investigation provides support that eradication of a dominant pathogen may promote healing. Further improvements (including expedited healing) may be garnered by administering targeted polyvalent phage cocktails upon identifying the polymicrobial infectious isolates. These results spawn intense interest to pursue further investigations assessing the efficacy of adjunctive bacteriophage treatment in complex infected cutaneous ulcers/wounds including decubitus ulcers, diabetic ulcers, post-burn, post-trauma, and post-surgical wound infections.

Three patients suffering from chronic bacterial prostatitis secondary to *E. faecalis* refractory to antibiotic courses (and autovaccines and biostimulation methods) were successfully treated with rectally applied phage therapy (administered at the Phage Therapy Unit in Wrocław Poland) (Letkiewicz et al. 2009). The phage preparations were confirmed active against the *E. faecalis* isolates acquired from expressed prostatic secretions (EPS) and delivered topically (10^9 pfu/ml) "rectally" in a 10 ml preparation twice daily for 28–33 days, achieving confirmed microbiological eradication (negative cultures from EPS conducted 7 to 17 weeks apart) and clinical improvement.

6.3 (Aerosolized/Nebulized) Phage Therapy

The lungs of cystic fibrosis (CF) patients are colonized very early in life by multiple bacterial species (typically commencing with *Pseudomonas aeruginosa* as the canonical opportunistic bacterial pathogen) often sequestered within a biofilm, lending to antibacterial and bacteriophage resistance complicating treatment. It appears that bacteria adopt a greater resistance to antibiotics as opposed to bacteriophages within the sequestered biofilms (Essoh et al. 2013). Intuitively, therapeutic administration of bacteriophage cocktails for treatment of pulmonary infections could be optimized if administered both IV and intratracheally via aerosolization (nebulization) of the phage treatment (mirroring the concomitant use of nebulized antibiotic coupled to its IV counterpart). Viable phages exhibiting ideal particle sizes <5 μm for lower alveoli deposition were recovered from two nebulizers (1% for the Omron and 12% for an AeroEclipse nebulizer) (Sahota et al. 2015).

A recent case study of import describes a 5-year-old child failing multiple antibiotic therapies for chronic airway colonization by *P. aeruginosa*. Aerosolized bacteriophages derived from the "pyophage" cocktail (acquired from the Eliava Institute in Tbilisi, Georgia) administered three times daily, 6–10 days per month during 3 consecutive months, fostered clinical improvement and microbiologic eradication (Hraiech et al. 2015). Similarly, between 2007 and 2010 in Tbilisi, Georgia, eight cystic fibrosis patients experienced clinical improvement (reduced frequency of infection-mediated exacerbations) and reduced bacterial colonization burden upon administration of aerosolized phages administered for 6–10 days (coupled to standard of care therapy encompassing antibiotics and vitamins).

6.4 Oral Bacteriophage Therapy

Successful oral delivery of numerous medications including phages must reconcile retaining stability transiting the low pH environment of the stomach. Toward this end, a seminal study took place during the Soviet era where a dry tableted pectin-coated polyvalent mixture of phages showed efficacy in a controlled large-scale (1000 s) trial for prophylaxis of *Shigella* dysentery (manufactured in Russia at the Gorky Production Facility with quality control at the Tarasevich Control Institute

(Solodovnikov Iu et al. 1971)). More recently, this has been overcome by employing microencapsulation techniques or neutralizing stomach acid via temporal antecedent administration of sodium bicarbonate (Furfaro et al. 2017; Miedzybrodzki et al. 2017). Intuitively, similar procedures should be embraced if administering bacteriophages orally, which has been adopted by investigators delineated below. However, further research is required to assess phage stability in the acidic environments and their stability in transit throughout the gastrointestinal (GI) tract.

Bacteriophage therapy was safely administered orally three times daily over 3 weeks to 37 patients experiencing varied infections (bronchitis, urinary tract infections, rhinitis, furunculosis, perianal abscess, otitis) successfully treating 19 patients (Drulis-Kawa et al. 2002). Additionally, readdressing the Eastern European literature, we emphasize that the observational results reported by Weber-Dabrowska (2001) and Weber-Dabrowska (2000) employed predominantly bacteriophage cocktails delivered orally to over 1300 patients presenting with varied clinical syndromes stemming from numerous MDR pathogenic species (strains) (Weber-Dabrowska et al. 2000b; Dabrowska et al. 2001). Their non-controlled results revealed safety and efficacy.

6.5 Emergency Investigational New Drug (eIND) Applications

Emergency INDs have been executed and provide ample support for the potential efficacy of bacteriophages as salvage (if not first-line) therapy for MDR infections.

A seminal eIND revealed that successive bacteriophage cocktails targeting MDR *Acinetobacter baumannii* successfully treated a recalcitrant and complicated intra-abdominal infection refractory to several antibiotic courses in a 68-year-old male (Schooley et al. 2017). The success achieved with this case inspired and spawned further eIND efforts (in parallel with clinical trial designs) delineated below.

An eIND to provide phage cocktails targeting *P. aeruginosa* in a 2-year-old with DiGeorge syndrome status post-multiple surgeries is complicated by presumptive infectious mycotic aneurysms and infected mediastinal fluid collections (Duplessis et al. 2017). The patient had sustained bacteremia (positive cultures for over 28 days) due to MDR *P. aeruginosa* failing all antibiotic cocktails and not amenable to continued surgical interventions (fluid collections unamenable to aspirations) as the cardiothoracic surgeons concluded any intervention (given the scar tissues within the mediastinum) would result in excessive risk. The introduction of bacteriophage achieved sterile blood cultures after administration of six doses at a 6-h frequency. The treatment was interrupted given cardiac decompensation resulting in repeated bacteremia. However, the reintroduction of phage cocktails quickly and reproducibly achieved sterile blood cultures. Regrettably, the child died due to cardiac complications prior to achieving clinical resolution.

Two recent complex patients experiencing chronic MDR *P. aeruginosa* infections were successfully treated with protracted and multiple bacteriophage cocktails under eINDs. Adjunctive phage therapy successfully achieved microbiological eradication

and clinical resolution in these two cases representing the most complicated infectious disease scenarios, unlikely to have responded to protracted antibiotic courses.

7 FDA Phage Clinical Trials

Currently bacteriophage therapy is used to prevent and treat bacterial infections in some former Soviet Union and European countries, with the most active phage centers located in Georgia and Poland. Numerous clinical studies have been conducted in the eastern bloc, and in the USA, case reports have been published with outcomes suggestive of efficacy. However, randomized, well-controlled clinical trials evaluating phage were scarce before the twenty-first century and ones conducted were limited in number, primarily focused on safety and mainly conducted overseas. As a result, these efforts have had limited impact on the adoption of bacteriophage therapy in the USA. This section will focus on modern FDA-regulated clinical trials evaluating phage for antimicrobial clinical indications (Table 2).

Topical and Local Application A large fraction of the trials conducted to date have tested topical or local applications of bacteriophage. This approach is understandable given the novelty of phage therapeutics: the concerns about safety with these routes of administration are significantly lessened.

One of the first bacteriophage trials in the USA (ClinicalTrials.gov Identifier: NCT00663091), concluded in 2008, was a phase I trial examining the safety of an eight-phage cocktail against *S. aureus*, *P. aeruginosa*, and *Escherichia coli*, in patients with venous leg ulcers. Twenty-one patients were treated, and 18 patients received a saline control treatment, over the course of 12 weeks via an ultrasonic debridement device. The phage preparation was shown to be safe. The trial was not designed to test efficacy, and no effort was made to match the bacteriophage treatment to the organisms present in the wounds; having said that, no difference in healing frequency or rate was observed (Rhoads et al. 2009).

Another recent trial in the US (NCT02757755) assessed the safety of topically applied bacteriophage on intact skin. In this trial, a three-phage *S. aureus* cocktail at a titer of 10^8 or 10^9 per bacteriophage was applied to intact skin on a gauze and covered with Tegaderm; no safety issues were observed.

The French firm Pherecydes Pharma has two notable clinical trials of topically applied experimental bacteriophage products. Their PhagoBurn study (NCT02116010) was initiated in 2014 at seven different military and civilian European sites, focused on treating *P. aeruginosa* and *E. coli* burn infections. The relatively low frequency of *E. coli* burn infections led to an emphasis on *P. aeruginosa*, and overall low enrollments have made the study outcome a proof of concept. Detailed results have not yet been reported. This was nonetheless a significant study for its establishment of a regulatory framework and manufacturing practices.

Table 2 FDA clinical trials utilizing bacteriophage therapy

Study title	Start date	Phase	Patient number	Trial details: indication, pathogen, treatment, primary and secondary outcomes, phage manufacturing company	Host country/ies	ID
Individual Patient Expanded Access for AB-PA01, an Investigational Anti-*Pseudomonas aeruginosa* Bacteriophage Therapeutic	January 1, 2018	**Expanded access**	**Unspecified**	Indication: serious or immediately life-threatening *P. aeruginosa* infections Pathogen: *P. aeruginosa* Treatment: AB-PA01, phage product Primary outcome: unspecified Secondary outcomes: unspecified Phage manufacturing company: AmpliPhi	**United States of America**	NCT03395769
Individual Patient Expanded Access for AB-SA01, an Investigational Anti-*Staphylococcus aureus* Bacteriophage Therapeutic	January 1, 2018	**Expanded access**	**Unspecified**	Indication: serious or immediately life-threatening *S. aureus* infections Pathogen: *S. aureus* Treatment: AB-SA01, phage product Primary outcome: unspecified Secondary outcomes: unspecified Phage manufacturing company: AmpliPhi	**United States of America**	NCT03395743

(continued)

Table 2 (continued)

Study title	Start date	Phase	Patient number	Trial details: indication, pathogen, treatment, primary and secondary outcomes, phage manufacturing company	Host country/ies	ID
A Prospective, Randomized Double-Blind Controlled Study of WPP-201 for the Safety and Efficacy of Treatment of Venous Leg Ulcers	September 1, 2006	**I**	**64**	Indication: venous leg ulcers (wound infections) Pathogen: *E. coli. P. aeruginosa, S. aureus* Treatment: local application of 10^9 phage cocktail, WPP-201, Georgian product composed of 8 phages Primary outcome: safety Secondary outcomes: unspecified Phage manufacturing company: commercial product of the country of Georgia	**United States of America**	NCT00663091
Ascending Dose Study of the Safety of AB-SA01 When Topically Applied to Intact Skin of Healthy Adults	May 1, 2016	**I**	**12**	Indication: topical skin safety Pathogen: *S. aureus* Treatment: local forearm application of $10^{8\text{-}9}$ 3-phage cocktail, AB-SA01, compared to placebo Primary outcome: safety (# of adverse events, blood chemistry, urinalysis, skin reaction) Secondary outcomes: unspecified Phage manufacturing company: AmpliPhi	**United States of America**	NCT02757755

PHAGE Study: Bacteriophages as Novel Prebiotics	October 1, 2016	**I**	**43**	Indication: gut inflammation Pathogen: unspecified Treatment: consumption of 4-phage cocktail, PreforPro, or placebo once a day for 28 days (x2 txt arms) Primary outcome: microbiome modulation at day 0, and 4 weeks after each treatment arm Secondary outcomes: local (fecal calprotectin) and systemic inflammation (CRP, circulating cytokines) Phage manufacturing company: Deerland Enzymes	**United States of America**	NCT03269617
Evaluation of Phage Therapy for the Treatment of *Escherichia coli* and *Pseudomonas aeruginosa* Wound Infections in Burned Patients *(PhagoBurn)*	July 1, 2015	**I/II**	**Unspecified**	Indication: Burn wound infections Pathogen: *E. coli*, *P. aeruginosa* Treatment: Local application of phages cocktail vs. silver sulfadiazine Primary outcome: bacterial load 1 week after treatment Secondary outcomes: safety, wound improvement, bacterial clearance Phage manufacturing company: Pherecydes Pharma	**Belgium, France, Switzerland**	NCT02116010

(continued)

Table 2 (continued)

Study title	Start date	Phase	Patient number	Trial details: indication, pathogen, treatment, primary and secondary outcomes, phage manufacturing company	Host country/ies	ID
Standard Treatment Associated With Phage Therapy vs. Placebo for Diabetic Foot Ulcers Infected by *S. aureus (PhagoPied)*	January 1, 2018	**I/II**	**60**	Indication: diabetic foot ulcers (wound infections) Pathogen: methicillin sensitive/ resistant *S. aureus* Treatment: 10^7 PFU/mL phage or placebo control soaked dressings applied at days 0, 7, and 14 Primary outcome: wound healing over 12 weeks Secondary outcomes: safety, wound microbiome and bioburden, antibiotic resistance, phage antibodies Phage manufacturing company: Pherecydes Pharma	**France**	NCT02664740
Randomized, Double Blind Placebo-controlled Studies to Evaluate the Effect of an Orally-fed *Escherichia coli* (*E. coli*) Phage in the Management of ETEC and EPEC Induced Diarrhea in Children	August 1, 2009	**I/ II**	**120**	Indication: bacterial diarrhea Pathogen: *E. coli:* enteropathogenic (EPEC) and enterotoxigenic *E. coli* (ETEC) Treatment: 2 distinct oral phage cocktails 10^6 PFU/ ml up to 5 days compared to rehydration Primary outcomes: safety, tolerability, and efficacy Secondary outcomes: clinical evaluation, hematology, morbidity, hospitalization length Phage manufacturing company: Microgen	**Bangladesh**	NCT00937274

Bacteriophages for Treating Urinary Tract Infections in Patients Undergoing Transurethral Resection of the Prostate: A Randomized, Placebo-controlled, Double-blind Clinical Trial	November 1, 2015	**II/III**	**81**	Indication: urinary tract infections Pathogens: *Enterococcus, E. coli, P. mirabilis, P. aeruginosa, Staphylococcus,* and *Streptococcus* Treatment: intravesical delivery of phage compared to intravesical placebo or systemic antibiotics Primary outcome: bacterial clearance of urine culture 1 week after treatment Secondary outcomes: urine culture, bladder diary, pain diary, IPSS Questionnaire Phage manufacturing company: Pyo phage, commercial product of the country of Georgia	**Georgia**	NCT03140085
Experimental Phage Therapy of Drug-resistant Bacterial Infections, Including MRSA Infections	December 1, 2005	**Unspecified**	**Unspecified**	Pathogen: *Staphylococcus, Enterococcus, Pseudomonas, Escherichia, Klebsiella, Proteus, Citrobacter, Acinetobacter, Serratia, Shigella, Salmonella, Enterobacter, Stenotrophomonas,* or *Burkholderia* Treatment: oral, topical, or rectal application of phage or phage lysate Primary outcome: unspecified Secondary outcomes: unspecified Phage manufacturing company: unspecified	**Poland**	NCT00945087

Pherecydes Pharma's PhagoPied trial is a pending phase I/II effort (NCT02664740) to evaluate topical *S. aureus* cocktails as treatments of diabetic foot ulcers. As described, that study will compare standard of care plus wound dressing with 10^7 pfu bacteriophage to standard of care plus placebo for wound healing and microbiological changes out to 12 weeks posttreatment.

Two other trials listed in ClinicalTrials.gov include topical or local application of bacteriophage at bacterial infection sites. At the National Center of Urology in Tbilisi, Georgia, the commercially produced therapeutic PYO Phage is being used in a Phase II/III trial to treat post-surgical urinary tract infections (NCT03140085). The PYO Phage product is a complex bacteriophage mixture with phages against *Streptococcus pyogenes*, *S. aureus*, *E. coli*, *P. aeruginosa*, *Proteus vulgaris*, and *Proteus mirabilis*. In this study, candidate patients are screened for presence of these species, and bacterial isolates are tested for susceptibility to the phage preparation, as conditions for enrollment (Leitner et al. 2017). Results from this study have not yet been reported.

A clinical trial in Poland (NCT00945087) was initiated in 2005 to treat a broad array of infections (wounds, osteomyelitis, urinary, and pulmonary), by ESKAPE and other pathogens, where antibiotic therapy has failed. Given the wide range of pathogens and indications, this trial uses several routes of administration including topical and rectal as well as oral. Notably, this trial uses personalized treatments where each patient receives a selected set of phages from the Bacteriophage Collection of the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences in Wrocław, Poland.

Finally, a single topical application of a cocktail of six anti-*P. aeruginosa* targeting phages (commercially available as Biophage-PA) into the external ear canal was found safe and efficacious in the adjunctive treatment of chronic otitis externa secondary to antibiotic-resistant *P. aeruginosa*. Twelve patients, age ranging from 2 to 58 years, and harboring MDR *P. aeruginosa* strains sensitive to at least 1 member of the cocktail, received the single instillation, relative to 12 placebo control subjects (experiencing a similar affliction) all participating in a formal phase I/II clinical trial conducted in the UK (Wright et al. 2009). The phage recipients experienced both clinical and microbiological improvement relative to placebo. The trial wasn't designed to continue phage administration until microbiological eradication and clinical resolution, and therefore no further inferences may be rendered.

Oral Administration In addition to the oral component of NCT00945087 just mentioned, some other registered trials address a range of indications via oral administration of bacteriophages.

The coliphage clinical trial NCT00937274, completed in 2013, examined the safety and efficacy of 2 different oral *E. coli* phage cocktails to treat acute diarrhea in 120 patients in Bangladesh. While oral administration of either cocktail did not affect patient welfare as monitored by vital signs, weight, and temperature, or relief of diarrhea, it was observed that the phage did transit intact through the gut (Sarker et al. 2016). Possible reasons suggested for therapeutic failure included inadequate phage coverage, and *E.*

coli host cell presence too low to support phage amplification. The observation that phages did not increase in number is consistent with this interpretation.

The "prebiotic" trial NCT03269617 uses a bacteriophage mixture administered orally to test a different concept: can consumption of the phage mixture targeting detrimental gut bacteria including *E. coli* improve gut bacteria profiles in individuals relative to a placebo control, and is it associated with reduced incidence and severity of gastrointestinal distress? At present no results have been reported for this study.

Surveillance and In Vitro Assessments Several surveillance-oriented observational trials have been registered. One exploring the potential for bacteriophage to influence the gut microbiome is the "PHAGO-BMR" (NCT03231267) trial, determining the relationship between the presence of intestinal phage against Enterobacteriaceae and the presence or acquisition of MDR *E. coli* and *K. pneumoniae*. This trial is projected to begin in 2017/2018. The MetaKids gut microbiome surveillance study in infancy and early childhood (NCT03296631) will follow the dynamics of gut bacteria and phages over the course of early development and associated with perturbations such as immunizations and antimicrobial treatment. Another multicenter outpatient trial (NCT03009903) will evaluate the presence of both *Propionibacterium acnes* and its phages on skin, in subjects with and without acne.

A non-interventional approach oriented toward development of an antimicrobial treatment is the Mucophages trial (NCT01818206), in which a cocktail of 10 bacteriophages against *P. aeruginosa* is evaluated against clinical isolates in the sputa of cystic fibrosis patients. In that study, the phage mixture was capable of antimicrobial activity and phage amplification in the biological matrix of sputum, across the spectrum of clinical presentation tested (Saussereau et al. 2014).

8 Conclusions

The Way Forward for Clinical Evaluation of Bacteriophage Therapeutics In recent years the perception and expectations for bacteriophage antimicrobial therapy have shifted significantly. While there is not yet an FDA-licensed bacteriophage therapeutic on the market, there is now a steady demand for emergency access to phage therapy, and a growing body of case histories in which significant improvement followed phage therapy.

The regulatory environment is likewise changing. In 2015 and 2017, the FDA and the National Institute of Allergy and Infectious Diseases have hosted two workshops for the consideration of bacteriophage as an alternative to antibiotics. The first event, "Bacteriophage Therapy: An Alternative Strategy to Combat Drug Resistance," was largely an overview of the field (July 20–21, 2015) (Atterbury 2009). By the second event, "Bacteriophage Therapy: Scientific and Regulatory Issues" (July 10–11, 2017) (Bearden et al. 2005b), the FDA communicated its readiness to regulate bacteriophage therapeutic products and identified genetic characterization and manufacturing quality as major components of the pathway to licensure of bacteriophage products.

In contrast to the registered trials already described, the next generation of bacteriophage clinical trials will prominently feature parenteral administration of bacteriophage to treat serious bacterial infections. AmpliPhi Biosciences has registered two expanded access protocols for use of intravenous (IV) phage against *P. aeruginosa* (NCT03395769) and *S. aureus* (NCT03395743). These and similar protocols from other developers of phage therapies will maximize the knowledge gained from emergency/compassionate use of phages.

While there are challenges to designing and executing a pivotal trial for antimicrobial bacteriophage therapeutics, there is also significant momentum in the field. We can expect continuing progress toward the established use of bacteriophages to treat antibiotic-resistant bacterial infections.

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Strategies for the Eradication of Biofilm-Based Bacterial Infections

Roberta J. Melander and Christian Melander

Abstract

Biofilm-based bacterial infections are a significant health threat due to their chronic nature and lack of susceptibility to both the host immune response and to treatment with conventional antibiotics. There are numerous complex and interrelated mechanisms underling this tolerance, and strategies to overcome them are required in order to combat the considerable threat posed by biofilm-based bacterial infections. Several such strategies that have been explored toward the eradication of biofilm-based bacterial infections are discussed in this chapter. One strategy involves developing new antibiotics that are active against biofilm cells, while other approaches center on enhancing the activity of conventional antibiotics against biofilm cells with compounds that interfere with quorum sensing and other bacterial signaling and communication pathways, target biofilm-specific genes, or target the biofilm matrix.

Keywords

Biofilm · Antibiotic Tolerance · Matrix · Adjuvant

1 Introduction

Biofilm formation is often considered to be a significant factor in the failure of bacterial infections to respond to antibiotic treatment, with an estimated 65–80% of all infections thought to be biofilm-related (Van Acker et al. 2014). Biofilm-based infections are recognized as a significant health threat, with an estimated 17 million

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new biofilm infections arising each year in the U.S., resulting in up to 550,000 fatalities (Quave et al. 2012). This has led the CDC to declare that biofilms are one of the most pressing clinical obstacles of this century (Donlan 2002; Davies 2003). A significant economic burden is placed upon healthcare systems both as a result of protracted hospital stays and increased fatalities, and also due to persistent colonization of hospital facilities by bacteria within a biofilm state (Smith and Hunter 2008).

The discovery and development of therapeutic agents that are active against biofilm-based bacteria is of vital importance. In this chapter, we discuss the various strategies that have been explored toward the development of therapeutics for the eradication of biofilm-based infections, and give examples of compounds that act via these strategies. We do not cover empirical screening for compounds with anti-biofilm activity, which has been extensively reviewed elsewhere (Worthington et al. 2012; Rabin et al. 2015).

1.1 Overview of Bacterial Biofilms

The development of strategies to eradicate biofilm-based infections requires an understanding of many aspects of the biofilm, including structure, the biofilm life cycle, matrix composition, and the signaling and regulatory networks that mediate the biofilm lifestyle. Bacterial biofilms are highly complex surface-associated communities comprising microcolonies of bacterial cells that are embedded in a matrix of extracellular polymeric substances (EPS) (see below) and separated from one another by interstitial voids that act as channels for the transport of water, nutrients, and waste (Donlan 2002). Biofilms are pervasive, occurring across almost all bacterial species and across many environmental settings, including within the human body in both normal and pathogenic processes. Biofilm formation is an adaptive response that is initiated in response to environmental triggers such as nutrient depletion, pH change, and the presence of antibiotics, to enhance survival under these conditions (Webb et al. 2003; Jefferson 2004). Biofilms are heterogeneous and highly structured, and although each biofilm is unique and dependent upon the constituent microorganisms and specific environment, some structural features are common.

The highly structured and cooperative nature of biofilms has led them to be likened to multicellular organisms (Nikolaev Iu and Plakunov 2007). This multicellular existence confers distinct advantages to the constituent cells that include better access to resources, improved ability to colonize new territories, increased survival in intermicrobial conflicts, and a markedly increased tolerance to antimicrobial agents and the host immune response (Lyons and Kolter 2015). Additionally, multicellularity allows for specialization of different cells, analogous to cellular differentiation seen in multicellular organisms (Jefferson 2004). This differentiation allows a division of labor among cells and optimizes population survival. It has even been reported that there exists an altruistic component to biofilm behavior, one theory being that programmed cell death of a subpopulation of the biofilm is activated to benefit surviving cells (Webb et al. 2003), while another theory centers on

the adoption of a persister state by a fraction of the biofilm population to enable survival in the occurrence of unfavorable environmental conditions (Lewis 2001).

1.1.1 Biofilm Life Cycle

Biofilms are dynamic entities, both spatially and temporally, and while specific changes occur in response to external and internal processes, the overall lifecycle of a biofilm can be generally described as being made up of five stages that are controlled by a complex developmental cascade of signaling and regulatory molecules. Stage 1 involves the initial attachment of cells to a substratum, mediated by bacterial adhesins (Hancock et al. 2011). During this stage, only a small amount of EPS is present, the cells remain capable of independent movement, and attachment at this stage is thus reversible. A subsequent increase in EPS production marks stage 2, in which cells begin to adhere irreversibly. In stage 3, the biofilm matures and complex architecture such as water channels develops. This architecture continues to develop in stage 4, during which time individual microcolonies may begin to detach to leave hollow remnants that become part of the mature water channels. Finally, stage 5 encompasses dispersion of single cells from the biofilm that become planktonic revertants and are able to colonize additional sites (Stoodley et al. 2002).

1.1.2 Matrix

As mentioned above, bacterial cells within a biofilm are encased in a self-produced matrix. The matrix forms the three-dimensional architecture of the biofilm, provides mechanical stability, mediates adhesion, and allows for transport within the biofilm. The matrix also affords protection against desiccation, ultraviolet radiation, some predators, metallic cations, attack from host immune defenses, and certain biocides and antibiotics (Flemming and Wingender 2010). Water accounts for up to 97% of the biofilm matrix, while the three-dimensional structure of the matrix is afforded by the EPS. Biofilm formation and maintenance is critically dependent upon EPS production, which can account for up to 90% of the dry mass of the biofilm. The composition and therefore the properties of the EPS vary considerably depending on the bacterial species and the environment; however, the major constituent is typically polysaccharides, which are indispensible for biofilm formation in many bacteria. The polysaccharides that comprise the EPS vary in nature and can be anionic, such as the widely studied alginate produced by *Pseudomonas aeruginosa*, cationic such as the partially deacetylated β-1,6-linked *N*-acetylglucosamine (PNAG) produced by *Staphylococcus aureus*, or neutral (Flemming and Wingender 2010). Other macromolecules that constitute the EPS include proteins, extracellular DNA (eDNA), and lipids. Extracellular enzymes in the biofilm matrix have several functions: to act as an external digestive system by degrading biopolymers to low molecular weight compounds that can be taken up by cells and used as energy sources, to effect structural degradation of parts of the EPS to promote detachment of cells from the biofilm, and finally, some extracellular enzymes serve as virulence factors during biofilm-based infections. Non-enzymatic proteins, such as lectins and amyloids, play a role in the formation and stabilization of the matrix, forming a link between bacterial cell surfaces and exopolysaccharides, abiotic surfaces and host cells. eDNA is

also an integral part of the biofilm matrix, and has roles as an adhesin and as an intercellular connector. Extracellular surfactants produced by some bacteria play a role in attachment, and also function to render hydrophobic materials bioavailable.

Not surprisingly, nutrient availability affects EPS production, with levels increased in response to the availability of excess carbon and to low levels of other nutrients such as nitrogen, potassium, or phosphate. Slow growth rates also enhance EPS production (Sutherland 2001). The immobilization of cells within the biofilm matrix keeps them proximal to one another and facilitates cell—cell communication (see below) and horizontal gene transfer via conjugation, while DNA from lysed cells present in the matrix can also act as a reservoir of genes for horizontal gene transfer.

1.1.3 Signaling and Communication Within a Biofilm

Bacterial biofilm formation and maintenance is mediated by a complex system of signaling and regulatory networks that include quorum sensing (QS), second messenger signaling, indole signaling, and two-component signal transduction systems (TCS).

Perhaps, the best-studied bacterial communication system with respect to biofilm formation and maintenance is QS. QS allows a bacterial community to coordinate gene expression, and therefore behaviors including biofilm formation, in response to changes in population density (Camilli and Bassler 2006). This is achieved by the production, release, and detection of small diffusible signaling molecules known as autoinducers. QS is an extremely complex process, but at a basic level can be described as comprising two primary proteins: a synthase that produces the autoinducer in response to population changes and environmental stresses, and a receptor protein that binds and responds to the autoinducer. The autoinducer must accumulate above a threshold level to effect an alteration in gene expression, thus ensuring that QS-regulated genes are only expressed when the ensuing phenotypes would be most beneficial. Several classes of signaling molecules exist, some of which are species specific and some of which are universal signaling molecules produced by, and regulating behaviors of, multiple bacterial species.

Gram-negative bacteria utilize a class of signaling molecules known as acyl homoserine lactones (AHLs). In *P. aeruginosa*, which has become a model organism for QS research, two major QS systems involved in biofilm regulation include the related *las* and *rhl* systems, which employ 3-oxo-C12-AHL **1** and *N*-butyrylhomoserine lactone (C4-AHL) **2** (Fig. 1), respectively, as signaling molecules (Parsek and Greenberg 2000). These systems influence biofilm formation in multiple ways including rhamnolipid production, which is required for maintaining the open spaces between biofilm aggregates, and also affects swarming, which is important in biofilm development. Additionally, these systems regulate production of lectins and siderophores that are important for biofilm formation. Another QS system that plays a role in biofilm regulation in *P. aeruginosa* is the PQS system, which utilizes 2-alkyl-4-quinolones (AQs), including **3** as signaling molecules, and regulates production of the matrix component eDNA, which is important in generating the initial scaffold of the biofilm (Passos da Silva et al. 2017).

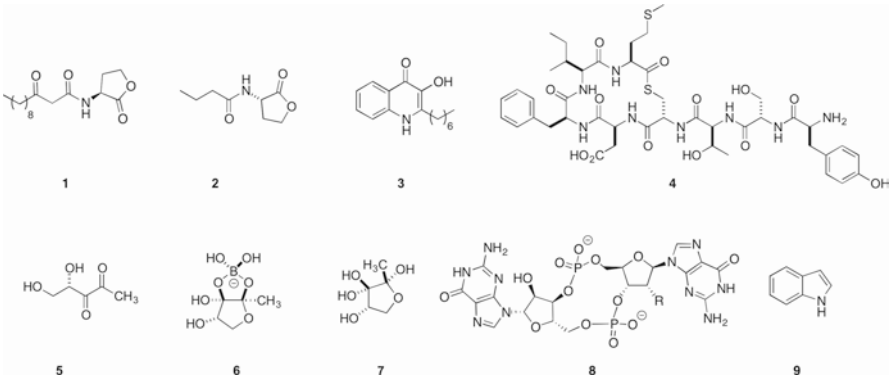

Fig. 1 Structures of compounds used by bacteria for signaling and communication

In Gram-positive bacteria, the predominant QS systems utilize autoinducing peptides (AIPs) as signaling molecules. *S. aureus* produces at least four classes of AIPs, including **4** (Fig. 1) (Baldry et al. 2016), which, upon reaching a critical concentration, activate the Agr QS system (Malone et al. 2007). This system plays a role in biofilm regulation by driving expression of a small non-coding RNA (RNA-III) upon binding of the AIP to the histidine kinase AgrC. RNA-III downregulates the expression of surface adhesins including protein A and the fibronectin-binding protein (Yarwood et al. 2004), and repression of the Agr system is necessary for biofilm formation to occur, while reactivation of the system in established biofilms has been shown to trigger biofilm dispersal (Boles and Horswill 2008).

A third class of autoinducer molecules is the autoinducer-2 (AI-2) class, which is employed by both Gram-negative and Gram-positive bacteria, and as such has been termed the universal QS system. AI-2 molecules are derived from a common precursor; (S)-4,5-dihydroxy-2,3-pentanedione (DPD) **5** (Fig. 1), which is a by-product of the activated methyl cycle and is synthesized by LuxS during the conversion of S-ribosylhomocysteine to homocysteine (Vendeville et al. 2005). AI-2 molecules are cyclic derivatives of DPD that are generated spontaneously and vary between bacterial species, such as the *Vibrio harveyi* AI-2 **6** and the *Salmonella typhimurium* AI-2 **7**. In *Escherichia coli*, AI-2 is phosphorylated by LsrK following uptake into the cell, and the phospho-AI-2 binds the repressor LsrR. Upon sensing AI-2, LsrK and LsrR alter the expression of genes involved in the regulation of aggregation, attachment, and biofilm formation (Jani et al. 2017).

In addition to QS systems, other signaling pathways also play a role in the complex process of biofilm regulation. The ubiquitous second messenger bis-(3′5’)-cyclic di-guanylic acid (c-di-GMP) **8** (Fig. 1) is synthesized and metabolized by diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), respectively, in response to both environmental and intercellular signals (Yan and Chen 2010). c-Di-GMP allosterically regulates downstream proteins, known as effectors, which respond by regulating phenotype changes and cellular functions, one of which is the transition from a planktonic to a biofilm

state. For example, in *Pseudomonas fluorescens* Pf0–1, when cellular levels of c-di-GMP are high, the messenger binds the effector LapD, which is a transmembrane protein involved in the export of the adhesin LapA to the outer membrane. Allosteric changes in LapD upon binding of c-di-GMP result in a conformation that allows export of LapA, and subsequent adoption of a biofilm phenotype, while at low cellular levels of c-di-GMP, during which the messenger is not bound, LapA is not exported and the bacteria remain in a planktonic state (Yan and Chen 2010).

Another proposed class of universal signaling molecules that play a role in numerous bacterial behaviors including biofilm regulation is the indole class (Melander et al. 2014). Indole itself **9** (Fig. 1) is known to be produced by at least 85 bacterial species, and even species that are not themselves indole producers have been shown to respond to the presence of indole (Lee and Lee 2010). Indole has been reported to affect biofilm formation in *E. coli* by inducing the transcription regulator SdiA, which in turn leads to repression of motility and biofilm formation (Lee et al. 2007b).

Finally, TCS also mediate biofilm formation and regulation across many bacterial species. TCS are regulatory systems found predominantly in prokaryotes that allow the organism to sense and respond to changes in their environment, and regulate many bacterial phenotypes. TCS consist of a sensor histidine kinase and a DNA-binding response regulator. In response to an extracellular stimulus, the histidine kinase undergoes autophosphorylation and in turn transfers the phosphate group to a conserved aspartate residue on the cognate response regulator. This induces a conformational change that results in up- or downregulation of gene expression, leading to changes in biofilm formation (Worthington et al. 2013).

1.1.4 Role of Biofilms in Chronic Infections and Tolerance to Antibiotics and the Host Immune Response

Biofilms are implicated in many chronic infections, and these infections are often characterized by persisting and progressive pathology (Hoiby et al. 2015). Biofilm-based infections include lung infections in cystic fibrosis (CF) patients, chronic otitis media, chronic wound infections, bacterial endocarditis, tooth osteomyelitis, and infections of indwelling medical devices (IMDs).

The chronic nature of biofilm-based infections is in part due to their ability to evade the host immune response. Furthermore, cells residing within a biofilm exhibit high levels of antibiotic tolerance, which renders antibiotics ineffective even against bacterial strains that do not harbor genetic resistance determinants. Tolerance, by definition, involves a reduction in the rate of antibiotic-induced killing of a whole bacterial population by comparison with the behavior of cultures of the strain from which the tolerant strain was derived (Tuomanen et al. 1986). In contrast to acute infections caused by planktonic bacteria, which (outside of drug-resistant strains) can typically be successfully treated with antibiotics, biofilm-based infections are often recalcitrant to antibiotic treatment, exacerbating the development into a chronic state. Importantly, cells exhibiting antibiotic tolerance as a result of the adoption of a biofilm phenotype will exhibit susceptibility to the antibiotic upon dissociation from the biofilm

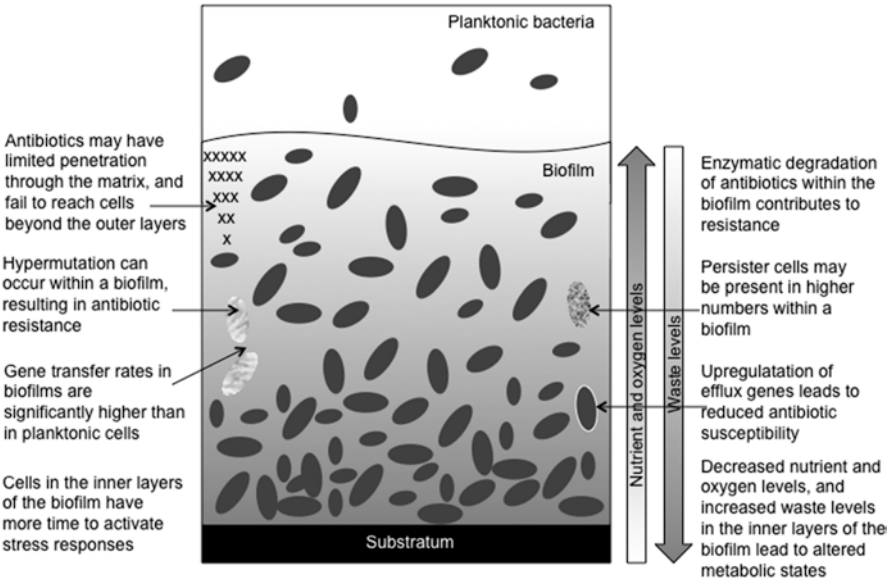

Fig. 2 Mechanisms of antibiotic tolerance within a biofilm

(Bjarnsholt 2013). Both tolerance and genetic resistance mechanisms contribute to the ability of bacteria within a biofilm to withstand antimicrobial challenge.

The impervious nature of biofilms can be attributed to numerous complex and interrelated mechanisms (Fig. 2), and an in-depth review of these mechanisms was recently published by Hall and Mah (2017). Understanding the mechanisms that govern antibiotic tolerance in biofilms is necessary for the development of strategies to overcome them, which may ultimately provide a means to eradicate biofilm-based infections.

One important factor is the matrix, which contributes to the tolerance of bacterial cells within a biofilm to antibiotics and immune effectors, primarily by limiting access to the bacterial cells. This may be a physical effect whereby the antimicrobial is unable to diffuse through the EPS, or may be a result of degradation or inactivation of the antibacterial agent within the matrix (Van Acker et al. 2014). For example, it has been shown that the penetration of ciprofloxacin and tobramycin is significantly impeded in *P. aeruginosa* biofilms, while *Klebsiella pneumoniae* has been shown to secrete a ß-lactamase into the matrix that degrades ampicillin before it is able to reach the cells (Hall and Mah 2017).

As mentioned earlier, biofilms are heterogeneous entities with nutrient and oxygen levels varying in different areas of the biofilm, which leads to altered bacterial growth rates in different areas of the biofilm (Mah and O'Toole 2001). Cells in nutrient and oxygen poor areas exhibit reduced metabolic activity and exist in a stationary phase-like state, leading to decreased susceptibility to most conventional antibiotics that predominantly target one of five biosynthetic processes that occur only in actively growing bacteria: the biosynthesis of proteins, RNA, DNA, peptidoglycan,

and folic acid (Hurdle et al. 2011). Most antibiotics that effectively kill or inhibit the growth of dividing bacterial cells tend to be very inefficient at killing non-multiplying bacteria and are therefore inactive against biofilm cells (Coates and Hu 2008).

Biofilm cells exhibit significantly altered gene expression profiles, both compared to planktonic cells and as a function of their location within the biofilm. For example, a comparison of the transcriptome profiles of planktonic and biofilm cells of *Acinetobacter baumannii* ATCC 17978 showed that 1621 genes were overexpressed in biofilm cells relative to stationary phase planktonic cells, and 55 genes were expressed only in biofilm cells (Rumbo-Feal et al. 2013). Some of these changes directly contribute to antibiotic tolerance in addition to having biofilm-specific functions; for example, general efflux pump expression is often upregulated in biofilm cells, and it has been postulated that functional efflux systems are required for full biofilm formation, possibly due to their role in effecting waste removal under cramped biofilm conditions (Van Acker and Coenye 2016). Indeed, inactivation of any of the efflux systems of *Salmonella enterica* serovar Typhimurium has been shown to result in the inability of the bacterium to form a biofilm (Baugh et al. 2012). Some antibiotic-specific efflux systems are also upregulated in biofilm cells, for example, in *P. aeruginosa*, the MexCD-OprJ and MexAB-OprM efflux pumps are thought to be biofilm-specific defense mechanisms against azithromycin and colistin, respectively (Van Acker and Coenye 2016).

Horizontal gene transfer is a major driver in bacterial evolution and rapid adaptation, and the transfer of plasmids by conjugation is one of the best-understood mechanisms for dissemination of genetic information (Davey and O'Toole 2000). Many antibiotic resistance determinants are spread by horizontal gene transfer (Barlow 2009), and the high population density and proximal nature of cells within a biofilm provide a favorable environment for the efficient transfer of genetic material, with rates of 1000–16,000-fold higher than those found in planktonic cells reported(Hausner and Wuertz 1999; Savage et al. 2013), though rates have been shown to be dependent on location within the biofilm and rate of cell growth (Stalder and Top 2016). Thus, facile transfer of antibiotic resistance genes in biofilms is another mechanism contributing to the lack of efficacy of antibiotics against biofilm cells.

Another contributory factor to antibiotic recalcitrance within a biofilm is the phenomenon of hypermutation. Hypermutable strains mutate at higher rates than the general population, and environmental stresses such as antibiotic challenge select for (and in some cases promote the generation of) these strains (Blazquez 2003). The production of reactive oxygen and nitrogen intermediates, formed as a result of the vast oxygen and nutrient gradients found with biofilm microcolonies, and the presence of oxidative stress in these environments have been linked to the occurrence of hypermutation in *P. aeruginosa* (Conibear et al. 2009). One study that examined 128 *P. aeruginosa* isolates from 30 cystic fibrosis patients reported that 36% of patients were colonized by a hypermutable strain that persisted for years in most patients, while such strains were not present in non-cystic fibrosis patients presenting with acute *P. aeruginosa* infections (Oliver et al. 2000).

It has also been suggested that biofilm cells exhibit a greater tolerance to oxidative stress, such as the antibiotic-induced generation of reactive oxygen species (ROS), which have been hypothesized to contribute to the killing of bacterial cells by bactericidal antibiotics. The molecular basis of this increased tolerance is not yet fully understood, but enzymes such as the catalase KatA, which detoxifies hydrogen peroxide, are thought to play a role, and it has been shown that KatA expression is higher under anaerobic conditions (Su et al. 2014) such as those experienced by cells located in oxygen-limited areas of a biofilm. Another way in which biofilm cells combat oxidative stress is via the production of polyamines that reduce intracellular ROS levels, a process controlled by two major transcriptional regulators, OxyR and SoxRS (Slachmuylders et al. 2018).

The stringent response is also thought to contribute to antibiotic tolerance in biofilms. The stringent response is a signaling pathway controlled by the alarmone (p)ppGpp, a second messenger that is activated under nutrient-limiting conditions, as experienced by cells in certain areas of a biofilm, and involves a comprehensive restructuring of the metabolic gene expression profile from one that facilitates growth to one allowing for prolonged survival in the stationary phase (Traxler et al. 2008). It has been shown that nutrient supplementation increases the antibiotic susceptibility of biofilm cells and that biofilms formed by a *P. aeruginosa* mutant deficient in (p)ppGpp exhibit significantly increased susceptibility to several antibiotics with diverse mechanisms of action compared to wild-type biofilms (Hall and Mah 2017).

Finally, the presence of persister cells has also been posited to contribute to antibiotic tolerance in biofilms and the chronic nature of biofilm-based infections. Persister cells comprise a subpopulation of cells (about 1% in biofilms) that are genetically homogeneous to the rest of the population and appear susceptible to antibiotic exposure, yet are phenotypically heterogeneous and are able to withstand the antibiotic, ultimately ensuring the survival of the population (Poole 2012). The mechanisms of persister cell formation and tolerance are not well understood and have been discussed in depth by Wood et al. (Wood et al. 2013) and Conlon et al. (Conlon et al. 2015). It is generally accepted that persister cells arise as a result of entering a state of dormancy in which they become metabolically inactive and thus impervious to the effects of antibiotics.

1.1.5 Current Clinical Approaches to Treating Biofilm-Based Infections

Current clinical approaches for the treatment of biofilm-based infections are limited, and the nature of the treatment is dependent upon the location of the infection, and the causative agent. In the case of colonization of IMDs or other foreign bodies, treatment involves removal of the foreign body, surgical debridement, and aggressive antibiotic therapy (Stewart and Costerton 2001; Wu et al. 2015). When the infection results in the formation of an abscess, which has been likened to a biofilm state (May et al. 2014), drainage of the abscess is necessary. In the absence of a foreign body or abscess, treatment encompasses long-term high-dose antibiotic regimens, often involving combinations of antibiotics with different mechanisms of

action. If treatment is not started early when the biofilm is immature, this may only result in a reduction of the biofilm, and subsequent chronic biofilm suppressive treatment will likely be necessary (Wu et al. 2015). In the case of chronic biofilm-based lung infections in CF patients, treatment involves long-term suppressive therapy with both topical (nebulized) and systemic antibiotics; both routes of administration are necessary in order to achieve adequate concentrations in the respiratory and conductive compartments of the lungs (Hoiby et al. 2015).

2 Strategies to Eradicate Biofilms

Alternative strategies to the traditional antibacterial approach of developing compounds that kill or inhibit the growth of metabolically active bacteria are necessary to effectively treat biofilm-based infections. Such strategies include: developing compounds that exhibit bactericidal activity against biofilm cells and the more widely taken approach of developing compounds that counteract biofilm tolerance mechanisms and enhance the activity of conventional antibiotics against the biofilm.

2.1 The Development of Antimicrobial Agents that Kill Biofilm Cells

As described above, most clinically approved antibiotics have limited efficacy against bacteria within a biofilm, and are effective only against bacteria that are actively growing and dividing. The development of antibiotics that are active against bacteria within a biofilm would have a significant impact on the outcome of treating biofilm-based infections.

One class of antimicrobial agents that has demonstrated microbicidal activity against biofilm cells is antimicrobial peptides (AMPs). AMPs are short cationic amphipathic peptides that comprise part of the innate immune response of many eukaryotic and prokaryotic organisms (Reddy et al. 2004). AMPs exhibit broad-spectrum activity against most bacterial pathogens, and importantly some AMPs exhibit activity against metabolically inactive cells, including biofilm cells (Di Luca et al. 2014). AMPs act upon biofilms via multiple mechanisms of action in addition to simply killing, including preventing biofilm formation by targeting the stringent response (Pletzer et al. 2016), which will be discussed later. One example of an AMP that is active against biofilm cells is human ß-defensin 3 (hBD-3) **10** (Fig. 3), which exhibits potent broad-spectrum antibacterial activity and has been shown to kill biofilm cells within a 3-week-old polymicrobial biofilm composed of *Actinomyces naeslundii*, *Lactobacillus salivarius*, *Streptococcus mutans,* and *Enterococcus faecalis* (Di Luca et al. 2014).

Carolacton **11** (Fig. 3), a secondary metabolite produced by the myxobacterium *Sorangium cellulosum*, demonstrates selective killing of biofilm cells of *S. mutans*, exhibiting only minor effects on planktonic cell growth (Kunze et al.

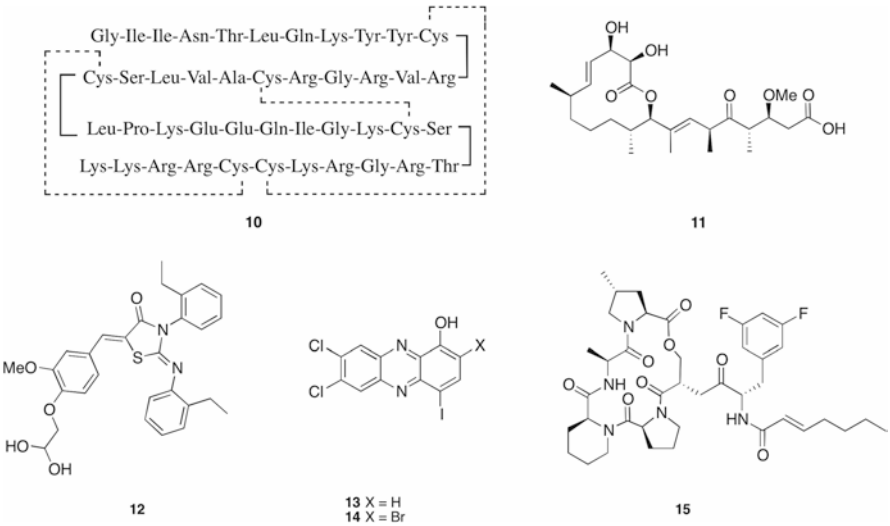

Fig. 3 Compounds that kill bacterial biofilm cells

2010). Carolacton affects expression of several TCS including VicKR, which is required for the response to oxidative stress (Banu et al. 2010) likely mediated through the serine/threonine protein kinase PknB (Reck et al. 2011). Similarly, the thiazolidinone **12**, which was identified from a structure-based virtual screen (SBVS) of 85,000 potential drug-like molecules for inhibitors of YycG in *Staphylococcus epidermidis*, also exhibits bactericidal effects on biofilm cells (though this compound is also bactericidal toward planktonic cells) and was shown to bind to YycG and inhibit autophosphorylation in vitro (Qin et al. 2006). A class of halogenated phenazines based upon a marine phenazine antibiotic has been reported to possess antibacterial activity against methicillin-resistant *S. aureus* (MRSA) biofilm cells, with lead compounds from this class **13** and **14**, exhibiting near equipotent killing of biofilm and planktonic cells. Compounds **13** and **14** also eradicated biofilms of other Gram-positive species including methicillin-resistant *S. epidermidis* (MRSE), and vancomycin-resistant enterococci (VRE), and interestingly some members of this class were also able to kill non-biofilm persister cells of MRSA (Garrison et al. 2015).

The acyldepsipeptide antibiotic (ADEP 4) **15** (Fig. 3), which targets the ClpP protease, is known to be active against growing *S. aureus* cells (Brotz-Oesterhelt et al. 2005) and has recently been shown to also kill biofilm and persister cells. ADEP4 works by activating ClpP leading it to become a non-specific protease that degrades over 400 intracellular targets, and also removes the ATP requirement for ClpP explaining the activity observed against dormant biofilm and persister cells. ADEP4 effected considerable killing of biofilm cells following 24 hours of treatment, and although the population rebounded after 72 hours, due to the rapid emergence of *clpP* mutants as a result of the non-essential nature of ClpP in *S. aureus*, these *clpP* mutants displayed increased susceptibility to several conventional antibiotics compared to persister or biofilm

non-mutant cells, and remarkably a combination of ADEP4 with rifampicin was able to eradicate living cells in a biofilm below the limit of detection (Conlon et al. 2013).

2.2 The Development of Non-antibiotic Compounds that Reduce Biofilm Tolerance to Antibiotics and Immune Response

While the identification of compounds that are able to directly kill biofilm cells is one avenue for the development of therapeutics to eradicate biofilm-based infections, perhaps the more widely explored approach is that of identifying compounds that circumvent the tolerance mechanisms exhibited by bacteria within a biofilm, rendering them susceptible to conventional antibiotics and the host immune response. Such compounds have the potential to be utilized as adjuvants, and co-administered with conventional antibiotics. Several approaches have been explored to this end, including compounds that interfere with the bacterial signaling and communication systems that regulate the biofilm and compounds that degrade the biofilm matrix. One potential advantage of this approach is the fact that compounds that do not elicit direct microbicidal activity will not exert the same pressure on bacteria to evolve resistance as compared to conventional bactericidal therapeutics.

2.2.1 Compounds that Target Bacterial Signaling and Communication

The numerous signaling and communication pathways described earlier that are used by bacteria to regulate biofilm formation and maintenance represent a myriad of potential targets for the development of anti-biofilm therapeutics.

QS Inhibitors

Early approaches in this field centered on the development of QS inhibitors, particularly inhibitors of AHL QS pathways. The use of native AHLs is impeded by their instability and immunomodulatory activity, making them unsuitable for use as a therapeutic (Yates et al. 2002). Several classes of synthetic analogues that are not beset by these issues have been developed as potential anti-biofilm agents, in particular against *P. aeruginosa* biofilms (Mattmann and Blackwell 2010). The non-native AHL analogues **16** and **17** (Fig. 4) inhibit biofilm formation by *P. aeruginosa* PAO1 at low micromolar concentrations, by acting as potent antagonists of LasR (Geske et al. 2005), while analogues **18** and **19** inhibit biofilm formation by *A. baumannii* via antagonism of the LuxR-type receptor, AbaR (Stacy et al. 2012). Taking a slightly different approach, Amara et al. developed isothiocyanate **20**, which acts as a covalent inhibitor of LasR in *P. aeruginosa*, selectively binding Cys 79 in the AHL-binding pocket and inhibiting QS and biofilm formation (Amara et al. 2009). Replacement of the lactone moiety of native AHLs with a thiolactone isostere has led to the identification of a series of analogues including compound **21**, which inhibits biofilm formation by *P. aeruginosa* in vitro and exhibits in vivo activity in a *Caenorhabditis elegans P. aeruginosa* infection model. (O'Loughlin et al. 2013).

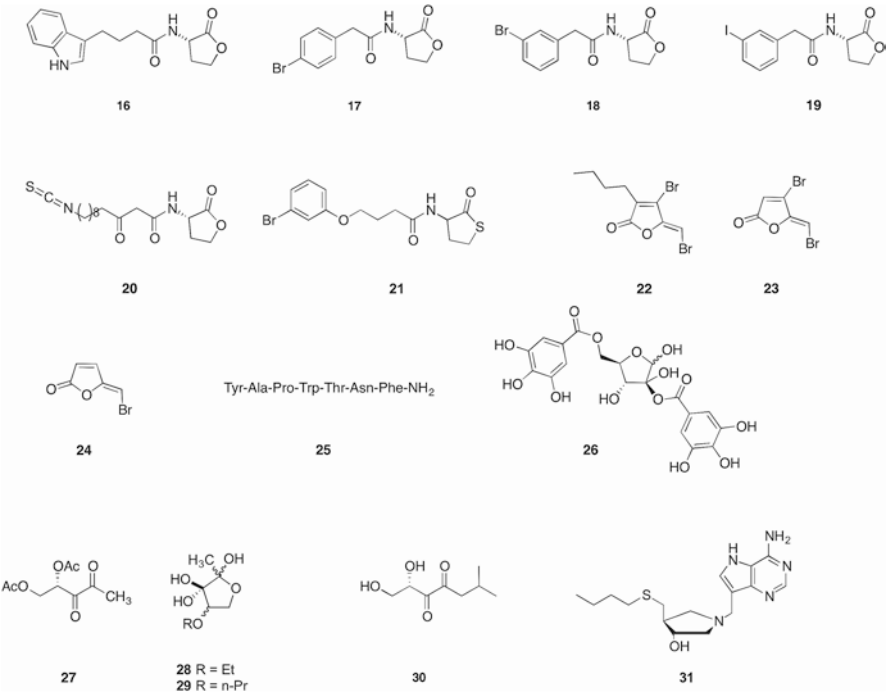

Fig. 4 Compounds that exhibit anti-biofilm activity by targeting QS pathways

Non-lactone compounds that target AHL-based QS pathways and interfere with biofilm formation and regulation include the brominated furanone class of marine algae secondary metabolites. The naturally occurring (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone **22** (Fig. 4) inhibits biofilm formation by *E. coli* and *Bacillus subtilis* (Ren et al. 2001; 2002), while the synthetic furanone C-30 **23** exhibits a host of activities, increasing the susceptibility of *P. aeruginosa* biofilms to tobramycin, promoting clearance in a mouse pulmonary *P. aeruginosa* infection model (Hentzer et al. 2003), and inhibiting biofilm formation by the Gram-positive *Streptococcus intermedius* and *S. mutans* (Lonn-Stensrud et al. 2007). Transcriptome analysis of furanone C-30-treated *P. aeruginosa* showed that this compound specifically targeted QS systems, while another synthetic furanone **24** was shown to penetrate microcolonies and inhibit QS in *P. aeruginosa* biofilm cells; compound **24** did not prevent initial attachment, but rather affected biofilm architecture and enhanced bacterial detachment (Hentzer et al. 2002).

In addition to the development of small molecule QS antagonists, enzymatic degradation of native AHLs as a means of inhibiting QS pathways and interfering with biofilm regulation has also been investigated. This is a natural phenomenon known as quorum quenching and is utilized by numerous organisms, either as a means of clearing self-produced QS signals or as a competitive mechanism to degrade QS signals produced by other organisms (Grandclement et al. 2016). One

of the first examples of the potential of exploiting quorum quenching as an anti-biofilm strategy was the recombinant production of the hydrolase BpiB05, which had been reported to inhibit AHL activity in the plant pathogens *Erwinia carotovora* and *Agrobacterium tumefaciens,* and its inhibition of biofilm formation, motility, and pyocyanin synthesis in *P. aeruginosa* (Bijtenhoorn et al. 2011). Similarly, the lactonase SsoPox-I, an engineered variant of the *Sulfolobus solfataricus*, produced SsoPox, inhibits *P. aeruginosa* QS and biofilm formation in vitro and reduces mortality and histological lung damage in a rat respiratory *P. aeruginosa* infection model (Hraiech et al. 2014).

Synthetic analogues of the Gram-positive QS signals, AIPs, have also been developed as potential anti-biofilm agents (Gordon et al. 2013). FS3 **25** (Fig. 4), an analogue of the *S. aureus* RNA-III inhibiting peptide (RIP) that acts to inhibit biofilm formation, has been shown to enhance tigecycline efficacy in a rat model of staphylococcal vascular graft infection (Simonetti et al. 2013). The small molecule hamamelitannin **26**, which was identified from a virtual screen of an RIP-based pharmacophore against a database of commercially available compounds, has been shown to prevent *S. aureus*- and *S. epidermidis*-mediated device-associated infections in vivo (Kiran et al. 2008). Hamamelitannin **26** effects significantly increased killing of *S. aureus* biofilm cells by several classes of antibiotics, including glycopeptides, ß-lactams, lipopeptides, oxazolidinones, and aminoglycosides, and is thought to act through the TraP receptor, affecting cell wall synthesis and eDNA release (Brackman et al. 2016).

Inhibitors of AI-2 signaling have also shown promise as anti-biofilm agents, and while DPD itself is unstable at high concentrations, several synthetic AI-2 analogues have been investigated for their ability to interfere with AI-2 signaling and subsequently affect biofilm formation (Guo et al. 2013). One example is the acetylated DPD analogue Ac_2-DPD **27** (Fig. 4), which affects AI-2-mediated behaviors in *Bacillus cereus*, including the inhibition of biofilm formation, most likely a result of the release of DPD by in situ hydrolysis (Frezza et al. 2007). C4-Alkoxy-5-hydroxy-2,3-pentanediones **28** and **29** act as AI-2 system agonists and activate the AI-2 pathway in *V. harveyi* more potently than DPD (Tsuchikama et al. 2012), while isobutyl-DPD **30** acts as an AI-2 system antagonist and has been shown to both inhibit maturation of *E. coli* biofilms and to achieve near complete biofilm clearance when administered in combination with gentamicin (Roy et al. 2013).

In addition to developing compounds that directly intercept the AI-2 pathway, signaling can also be disrupted by inhibiting biosynthesis of the native AI-2 molecule. The nucleoside analogue **31** is an inhibitor of 5′-methylthioadenosine nucleosidase (MTAN), which is involved in AI-2 biosynthesis and as such inhibits AI-2 production and subsequently biofilm formation in both *E. coli* and *Vibrio cholerae* (Gutierrez et al. 2009).

Compounds that Interfere with TCS

Given the ubiquitous nature of TCS among bacteria, their conserved nature across multiple species, the numerous behaviors they regulate, and the lack of an analogous system in eukaryotes, they represent an appealing drug target and are attracting

increasing attention for the development of anti-biofilm compounds (Worthington et al. 2013). As mentioned above, some compounds that display bactericidal activity against biofilm cells have been shown to target TCS; in contrast, other compounds described below do not individually kill biofilm cells, but enhance susceptibility to conventional antibiotics. Early efforts at exploiting TCS, both for anti-biofilm and other applications, centered on compounds that target the histidine kinase; for example, walkmycin C **32** (Fig. 5) is a histidine kinase inhibitor isolated from *Streptomyces* sp. strain MK632-100F11. Walkmycin C inhibits the autophosphorylation activity of the *S. mutans* kinases VicK and CiaH, which play a role in sucrose-dependent biofilm formation and cause the formation of abnormal biofilms (Qi et al. 2004; Eguchi et al. 2011).

Alternatively, compounds that target the response regulator have been investigated as a means of controlling biofilm formation and maintenance. This approach confers some advantages over targeting the histidine kinase, predominantly the avoidance of issues with cross talk, in which a response regulator can be phosphorylated by non-cognate histidine kinases, and as such inhibition of the histidine kinase may not result in complete inhibition of function of the targeted TCS. Directly targeting the response regulator increases the likelihood of interference with only the TCS of interest. The 2-aminoimidazole (2-AI) class of small molecules, which are derived from the marine sponge alkaloids oroidin and bromoageliferin (Melander et al. 2016), are potent broad-spectrum anti-biofilm compounds, some of which have been shown to target response regulators. 2-AI **33** (Fig. 5), which belongs to the reverse amide class of 2-AI compounds, inhibits and disperse biofilms of both *P. aeruginosa* and *A. baumannii* (Ballard et al. 2008) and has been shown to bind the response regulator BfmR, which plays an important role in biofilm formation (Tomaras et al. 2008) in *A. baumannii* (Thompson et al. 2012). A series of aryl 2-AI compounds including **34** and **35** were also shown to bind both BfmR and the *Francisella* spp. response regulator QseB and to inhibit biofilm formation by both species, with a correlation between inhibition and binding observed across the series (Milton et al. 2018). Another 2-AI **36** inhibits biofilm formation by *S. mutans* and inhibits accumulation of *Porphyromonas gingivalis* on a substratum of *Streptococcus*

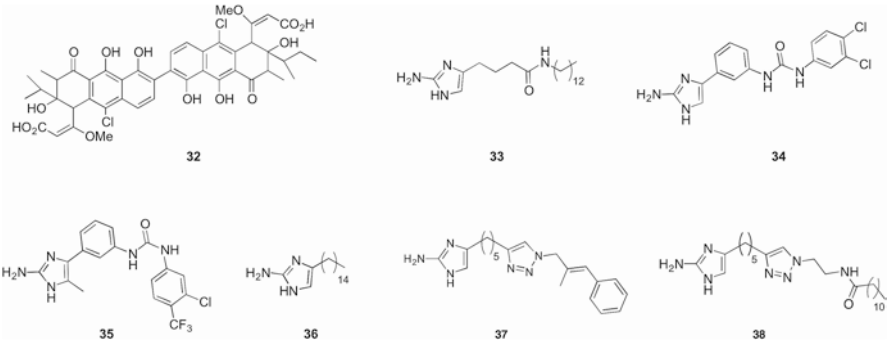

Fig. 5 Compounds that exhibit anti-biofilm activity by targeting TCS

gordonii, and downregulates the histidine kinase comD in *S. mutans* (Liu et al. 2014; Wright et al. 2014). The 2-aminoimidazole triazole compound **37** inhibits and disperses biofilms by a broad spectrum of bacteria including *P. aeruginosa*, *A. baumannii*, and *S. aureus* (Rogers and Melander 2008) and also exhibits synergy with novobiocin against *S. aureus* and *S. epidermidis,* with tobramycin against *P. aeruginosa* and with colistin against *A. baumannii* biofilms (Rogers et al. 2010). The related triazole-containing compound **38** inhibits biofilm formation by *S. mutans* and reduces bacterial colonization and the incidence of dental caries incidence in vivo in a rat infection model (Pan et al. 2015).

Compounds that Interfere with Other Signaling and Stress Response Systems

Compounds that interfere with signaling by second messengers such as c-di-GMP have also been explored to control biofilm formation. Examples include sulfathiazole **39** (Fig. 6), which acts as a DGC inhibitor and inhibits *E. coli* biofilm formation at low micromolar concentrations (Antoniani et al. 2010) and benzimidazole **40**, which has broad-spectrum anti-biofilm activity, including against *P. aeruginosa*, *K. pneumoniae*, *Shigella boydii*, *S. aureus,* and several other species (Sambanthamoorthy et al. 2011). The organoselenium compounds ebselen **41** and ebselen oxide **42** reduce DGC activity by covalently modifying cysteine residues within the protein, and have been shown to inhibit biofilm formation by *P. aeruginosa* (Lieberman et al. 2014). Benzamide **43** was identified from a high-throughput screen for

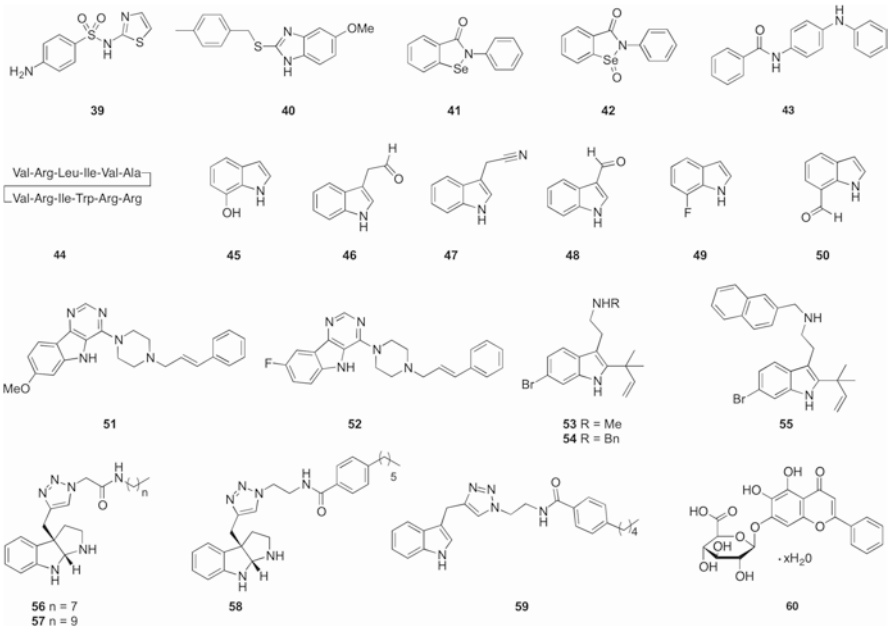

Fig. 6 Compounds that exhibit anti-biofilm activity by targeting other signaling and stress response systems

inhibitors of DGC enzymes and was shown to reduce c-di-GMP levels in *V. cholerae* and to inhibit biofilm formation by both *V. cholerae* and *P. aeruginosa* (Sambanthamoorthy et al. 2012).

Another second messenger, the alarmone (p)ppGpp, which plays a role in the stringent response as described earlier, has also been targeted as an anti-biofilm strategy. The immunomodulatory peptide IDR (innate defense regulator) 1018 **44** (Fig. 6), which exhibits broad-spectrum activity, preventing biofilm formation and eradicating pre-formed biofilms of *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, MRSA, and several other species (Mansour et al. 2015), exhibits synergy with ceftazidime against biofilms of *A. baumannii, S. enterica*, and MRSA, and with tobramycin against biofilms of *E. coli*, *A. baumannii*, and *K. pneumoniae* (Reffuveille et al. 2014), and is thought to bind to (p)ppGpp causing its degradation.

Exogenous indole has been used to perturb various aspects of indole signaling-mediated behavior, and various naturally occurring indole metabolites and derivatives have also been shown to influence bacterial behaviors including biofilm formation. For example, the oxidized metabolite 7-hydroxyindole **45** (Fig. 6) inhibits biofilm formation by enterohemorrhagic *E. coli* (EHEC) to a greater degree than indole at the same concentration (Lee et al. 2007a) and the plant metabolite indole-3-acetaldehyde, **46**, which is produced by the plant pathogen *Rhodococcus* sp. BFI 332, inhibits biofilm formation by EHEC (Wood et al. 2008). Other indole-derived plant metabolites that affect biofilm formation include 3-indolylacetonitrile (IAN) **47** and indole-3-carboxyaldehyde (I3CA) **48**, which inhibit biofilm formation by *E. coli* O157:H7 to a greater extent than indole and also weakly inhibit biofilm formation by *P. aeruginosa*, a phenotype that is enhanced by indole (Lee et al. 2011).

Numerous synthetic indole derivatives have been investigated in efforts to augment biological activity; these include simple synthetic derivatives such as 7-fluoroindole (7FI) **49** and 7-formylindole **50**, both of which inhibit biofilm formation by *P. aeruginosa* (Lee et al. 2012). These simple derivatives, along with indole itself and naturally occurring metabolites, however require high concentrations that are too high to be therapeutically useful, often up to 1 mM, in order to exert their effects. This has led to the construction of complex synthetic indole-containing compounds that act at much lower concentrations. The indole-containing compound **51** was reported to inhibit biofilm formation by *S. enterica* serovar Typhimurium, and a subsequent structure–activity relationship study led to the discovery of the more active 8-fluoro-4-[4-(3-phenyl-2-propen-1-yl)-1-piperazinyl]-5H-pyrimido[5,4-b] indole **52**, which inhibits biofilm formation at low micromolar concentrations (Robijns et al. 2012). Synthetic indole-containing compounds derived from the bryozan secondary metabolite desformylflustrabromine (dFBr) **53** (Peters et al. 2003) include compounds **54** and **55**, which potently inhibit biofilm formation by *E. coli* and *S. aureus*. Their activity in *E. coli* was shown to be dependent on the same factors as the activity of indole itself, i.e., temperature, SdiA, and tryptophanase (TnaA), suggesting that these compounds modulate bacterial behavior through the indole-signaling pathway (Bunders et al.

2011b; Minvielle et al. 2013b). Related flustramine-derived small molecules that have been reported to inhibit biofilm formation by various bacterial species include compound **56**, which inhibits biofilm formation by *A. baumannii*, compound **57**, which inhibits biofilm formation by *E. coli*, and the Gram-positive acting compounds **58** and **59**, which inhibit biofilm formation by *S. aureus* (Bunders et al. 2011a; Minvielle et al. 2013a).

The flavonoid baicalin hydrate **60** (Fig. 6) is known to increase the susceptibility of *Burkholderia cenocepacia* biofilms to tobramycin and was initially thought to act via QS inhibition (Brackman et al. 2011). Recently, however, it has been demonstrated that the mechanism of action of this compound involves modulation of the oxidative stress response, whereby baicalin hydrate affects multiple pathways including oxidative phosphorylation, glucarate metabolism and modulates biosynthesis of the polyamine putrescine. This leads to increased ROS production in the presence of tobramycin and a subsequent increase in tobramycin-mediated killing of biofilm cells (Slachmuylders et al. 2018).

2.2.2 Targeting the Biofilm Matrix

As discussed earlier, the matrix is vital to numerous aspects of biofilm integrity, and as such targeting the matrix has received much attention as a means to eradicate biofilms and treat biofilm-based infections. This has been predominantly pursued by the use of enzymes such as glycosidases, proteases, and DNases that degrade the major constituents of the matrix; indeed, the endogenous secretion of such enzymes is an innate phenomenon used by many bacteria to initiate dispersion from the biofilm (Kaplan 2010).

It has been demonstrated that biofilms formed in the presence of DNases exhibit reduced biomass and decreased antibiotic tolerance (Tetz and Tetz 2010), and recombinant human DNase I (rhDNase), also known as dornase alfa and marketed as Pulmozyme by Genentech, inhibits and disperses *S. aureus* biofilms in vitro, increases the susceptibility of *S. aureus* biofilm cells to several antimicrobials, and enhances the efficacy of tobramycin in *S. aureus*-infected *C. elegans* (Kaplan et al. 2012). Pulmozyme also increases the susceptibility of *P. aeruginosa* biofilm cells to aminoglycosides (Alipour et al. 2009; Kaplan et al. 2012) and is marketed for the treatment of pulmonary disease in cystic fibrosis (CF) patients, (Parsiegla et al. 2012) in whom it leads to reduced demand for antibiotics and improved lung function (Frederiksen et al. 2006). Other DNases that exhibit anti-biofilm activity include NucB, which is an extracellular DNase produced by *Bacillus licheniformis* that has been shown to cause rapid biofilm dispersal against a range of bacteria including *B. subtilis* and *E. coli* (Bayer et al. 1992).

Dispersin B is a soluble glycoside hydrolase that degrades poly-*N*-acetylglucosamine (PGA), which is a major matrix component of several bacterial biofilms (Ramasubbu et al. 2005). Dispersin B inhibits and disperses biofilms by several medically relevant bacterial species including *S. aureus*, *S. epidermidis*, and *E. coli* and also sensitizes *S. epidermidis* biofilm cells to the action of various antimicrobials and lowers the rate of catheter colonization by *S. aureus* in combination with triclosan in a rabbit model of infection (Kaplan et al. 2004; Itoh et al.

2005; Donelli et al. 2007; Izano et al. 2008; Darouiche et al. 2009). Alginate lyases catalyze the degradation of the matrix component alginate and have been shown to enhance the microbicidal activity of aminoglycosides against *P. aeruginosa* biofilms in vitro (Lamppa and Griswold 2013) and to augment the effectiveness of amikacin in clearing *P. aeruginosa* in a rabbit model of endocarditis (Bayer et al. 1992).

Proteases that have been investigated for this approach include the serine protease Esp from *S. epidermidis*, which inhibits and eradicates preformed biofilms of *S. aureus*, enhances the susceptibility of *S. aureus* biofilms to hBD2, and eliminates human nasal colonization by *S. aureus* in vivo (Iwase et al. 2010). Similarly, the metalloprotease serratopeptidase (SPEP), a widely used anti-inflammatory therapeutic, enhances the activity of ofloxacin against biofilms of *P. aeruginosa* and *S. epidermidis* (Selan et al. 1993).

Non-enzymatic approaches to targeting components of the biofilm matrix as a means of eradicating biofilms have also been investigated. A screen of multivalent fucosyl-peptide dendrimers for the inhibition of binding of the *P. aeruginosa* lectin LecB led to the identification of FD2 (C-Fuc-LysProLeu)$_4$(*Lys*PheLysIle)$_2$*Lys*HisIleNH$_2$, which was subsequently shown to inhibit *P. aeruginosa* biofilm formation and to induce complete dispersion of established biofilms in several *P. aeruginosa* clinical isolates (Johansson et al. 2008).

2.2.3 Compounds that Target Biofilm-Upregulated Genes

Efflux Pump Inhibitors

As mentioned earlier, efflux pump genes are often upregulated in biofilm cells, and it is thought that functional efflux systems are required for full biofilm formation, leading to investigation into the effect of efflux pump inhibitors on biofilm cells both in the absence and presence of antibiotics. The efflux inhibitors chlorpromazine **61**, cyanide 3-chlorophenylhydrazone (CCCP) **62**, and PAßN **63** (Fig. 7), all of which have different mechanisms of efflux inhibition, effectively prevent biofilm formation in *E. coli*, *P. aeruginosa*, and *S. aureus* (Baugh et al. 2014). Similarly, PaβN **63**, thioridazine **64**, and 1-(1-naphthylmethyl)-piperazine (NMP) **65** significantly repress biofilm formation by *E. coli* and *K. pneumoniae* (Van Acker and Coenye 2016). Finally, boeravinone B **66** (Fig. 7), an inhibitor of the *S. aureus* multidrug efflux pump NorA, was recently shown to act synergistically with ciprofloxacin to inhibit biofilm formation by *S. aureus* (Singh et al. 2017).

3 Conclusions

Biofilm-based bacterial infections are inherently unresponsive to the host immune response, and are highly tolerant to conventional antibiotic regimens, rendering them a significant health threat. Multiple mechanisms govern this tolerance, and compounds that overcome or circumvent these mechanisms and eradicate the biofilm have the potential to lead to the development of sorely needed therapeutics for such infections. In addition to the development of compounds that directly kill

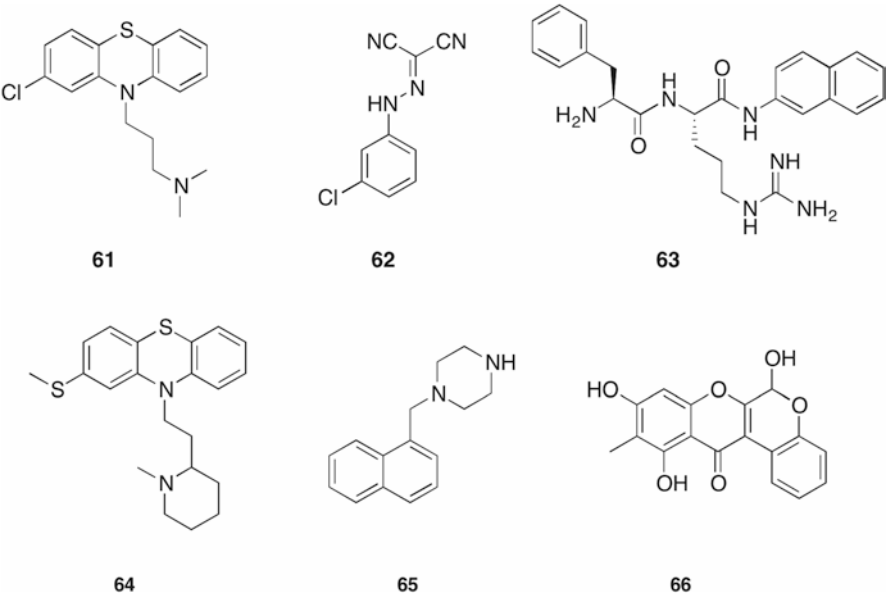

Fig. 7 Efflux pump inhibitors as anti-biofilm compounds

biofilm cells, an adjuvant approach whereby conventional antibiotics are paired with compounds that potentiate their activity against biofilm cells to eradicate the biofilm has received much attention. Compounds that interfere with various aspects of biofilm regulation and antibiotic tolerance have the potential to be used in this manner. These include: compounds that interfere with bacterial signaling and communication pathways such as QS and TCS, compounds that target stress response pathways, compounds that target efflux pumps, and agents that act upon the biofilm matrix. Several promising examples are discussed in this chapter; however, much work is still needed to develop new strategies and therapeutics to tackle the considerable problem of biofilm-based bacterial infections.

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Approaches for Disrupting Tissue-Associated Biofilms

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Abstract

There is no question that new antibiotics are sorely needed as bacteria become more and more resistant. However, another important cause of antibiotic failure is the tolerance bacteria exhibit to treatment when they are present within a biofilm. In contrast to the heritable changes bacteria undergo when acquiring new resistance determinants, tolerance is a reversible phenotype that is dependent upon being in a biofilm. Therefore, approaches that can successfully disrupt biofilms are likely to potentiate the efficacy of antibiotics. Biofilm removal by mechanical means is the conventional and most straightforward approach and includes desloughing and debridement. However, newer experimental approaches that target integral components of the biofilm itself are now showing promise.

Keywords

Biofilm · Wound infection · Dispersal · Debridement · Antibiotic tolerance · Antibiofilm agents

1 Introduction

In their simplest definition, biofilms are communities of microorganisms, adhered to a surface or to each other, and surrounded by an extracellular matrix (ECM). This ECM can be comprised of materials originating from the microbes themselves, or

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from the host or the environment, and serves as an excellent defensive strategy that enables microorganisms to survive in harsh environments. While ECM components vary widely depending on the biofilm environment and the microorganisms present, they are typically very hydrated and composed of polysaccharides, proteins, and extracellular DNA and RNA (Flemming et al. 2000; Dötsch et al. 2012).

Microorganisms make up less than 10% of the dry mass of most biofilms, with the ECM representing the remainder (Flemming and Wingender 2010). Living within the confines of the ECM not only protects the resident microbes from the environment, but also from the host immune system and any antimicrobials that have been administered. In fact, it has been shown that biofilm-dwelling bacteria can be up to 1000 times more tolerant to antibiotic agents than free-living planktonic cells (Mah and O'Toole 2001). Thus, as an alternative approach to traditional therapies, which directly target the causative pathogens, many researchers have turned their attention towards degrading the protective ECM, giving traditional antimicrobials and host defenses a fighting chance against the liberated, more-susceptible pathogens.

The central dogma concerning biofilm development begins with the attachment of a single planktonic cell to a surface, which is usually exposed to an aqueous medium. After attachment, microcolony formation is characterized by the growth of non-motile colonies that begin producing an ECM. Through quorum sensing pathways, other planktonic cells can be attracted to a microcolony in the subpopulation stage. This quickly gives rise to the macrocolony stage with the development of large towers or mushroom-shaped colonies. A macrocolony can become activated by the introduction of stressful environmental factors or even signal molecules produced by other cells that will lead to the final stage of biofilm development known as dispersion (Yang et al. 2011; Yang et al. 2012). However, it should be noted that most of what we know concerning biofilm development is derived from in vitro studies conducted with a few select microorganisms, and it is clearly very different from what occurs in vivo for many different types of infections. For example, to our knowledge, there have been no observations of the characteristic macrocolony shapes (e.g., towers and mushroom-shaped structures) in clinical biofilm samples. And while some biofilms found in the body are surface-associated and quite large (e.g., dental plaque or implant-associated biofilms), most biofilms seen in samples taken from tissue-associated infections, such as lung, wound, and middle ear, tend to be small aggregates that are surrounded by both bacterial and host-derived ECM (DeLeon et al. 2014; Watters et al. 2014; Sonderholm et al. 2017). Despite their size and structural differences, these biofilms are still exceptionally tolerant to antimicrobials and contribute significantly to a chronically infected state.

Biofilm-associated infections are so difficult to treat that once a biofilm forms in vivo, the only real treatment option is to remove it. However, removal of biofilms from an infection site is always problematic. If the biofilm is located on a foreign body, for example, removal could be as simple as taking out a catheter, or as difficult as replacing an artificial joint. While this can involve the expense and risk of a revision surgery, it is still more straightforward than the removal of a tissue-associated biofilm. In these infections, biofilms can be located deep within the tissue, muscle, or bone and often amputation is the only option. In this chapter, we will discuss the current standards of care and experimental strategies for the removal of biofilm

from tissue-associated infections. In most cases, biofilm removal or disruption is not in itself antimicrobial, but is simply a way to reduce bacterial load and/or weaken biofilms so that conventional antimicrobials are more effective.

2 Mechanical Techniques to Remove Biofilms

Despite the enormous effort and resources devoted to combatting biofilms, in some cases, the simplest of means are still the most effective. For example, some studies show that mechanical treatment is the most commonly used and/or effective method of biofilm removal (Sladek et al. 2007; Barbara and Cogan 2015). Mechanical removal of biofilms is traditionally very effective while also being relatively simple, and includes any method that removes biofilm by physical force. This includes everything from surgical debridement of tissue to ablation via non-thermal plasma jet.

While tissue-associated biofilm infections encompass a broad spectrum of infections (Lebeaux et al. 2013), mechanical removal of biofilm is most often associated with wounds. Therefore, most of the focus of this section will be in regard to wound infections, for which the most commonly used methods for removal of biofilm-infected tissue fall under two blanket terms, desloughing and debridement.

2.1 Desloughing

The "slough" present in wounds is composed of devitalized tissue, white blood cells, fibrin, and debris and can present in several forms (Young 2014). It is normally yellow or yellow/brown and can be superficial and wet, thick and slimy, or become dry and encrusted. Removal of slough is an important step for minimizing infection risk because it acts as a safe haven for bacterial colonization and subsequent biofilm formation (Milne 2015, Percival and Suleman 2015). If the slough is hydrated, it can be mechanically scraped, washed, or sucked off a wound. If slough presents itself as a dry crust, then the first step is to hydrate it to allow autolysis to naturally occur. To hydrate the wound, a moist environment can be created by using either an occlusive dressing to trap moisture in the wound, or by applying a hydrating topical treatment, such as, hydrogel, alginate, or hydrofoam (Percival and Suleman 2015). Additionally, hydrating dressings infused with antimicrobials can be used to prevent bacterial colonization of slough.

2.1.1 Wet-to-Dry Gauze

If an individual's wound is not capable of autolytic desloughing, then a series of mechanical desloughing methods can be employed (Young 2014; Milne 2015; Percival and Suleman 2015). A method known as wet-to-dry gauze slough removal has been popular among healthcare providers in the past and, depending on the wound, can also act as a debridement method. Wet-to-dry gauze debridement is implemented through the addition of a saline-soaked gauze bandage to a wound, where it is left to dry. While the bandage dries, it incorporates with outer layers of

necrotic tissue, exudate, and wound scab. After the gauze is given ample time to dry, a healthcare provider will remove the bandage, thereby debriding the wound (Cowan and Stechmiller 2009). While often effective, this method is time-consuming, painful for the patient, and can take some time to show improvement (Milne 2015). New methods, such as monofilament debridement pads, negative pressure wound therapy (NPWT), or slough-trapping wound dressings have gained prevalence in recent years as the beneficial effects of clean, efficient desloughing are being realized (Percival and Suleman 2015).

2.1.2 Monofilament Debridement Pads

Monofilament debridement pads (MDP) are composed of polyester fibers that bind to loose material on the wound surface. They are used to clean wounds in a similar manner to a sponge when cleaning dishes. Laboratory and clinical studies using a commercially available MDP, called Debrisoft®, have shown that it is able to effectively remove wound slough and decrease the bacterial load of both planktonic and biofilm-associated *Pseudomonas aeruginosa* (Wilkinson et al. 2016; Schultz et al. 2018). Additionally, clinical studies have revealed that patient pain is significantly lower when using Debrisoft® in comparison to the wet-to-dry gauze method (Schultz et al. 2018).

2.1.3 Slough-Trapping Dressings

Slough is rich in inflammatory material and can act as a safe-haven for infectious bacteria (Percival and Suleman 2015), so the expedient removal of slough can enhance tissue regranulation and decrease risk of infection (Milne 2015; Percival and Suleman 2015; Young et al. 2016). In addition, molecular methods for detecting bacteria in patient wounds suggests that anaerobic bacteria may play a larger role in chronic and necrotizing soft tissue infections (NSTI) than previously thought (Dowd et al. 2008; Zhao-Fleming et al. 2017). From these findings, we can reasonably surmise that by exposing the underlying tissue to air through slough removal, healthcare providers may be inadvertently impairing infectious anaerobic bacteria. To minimize these risks, healthcare providers must regularly desloughing high slough-producing chronic wounds, and to help automate this process, researchers have developed slough absorbent wound dressings. A 2017 study on KytoCel®, a commercially available slough absorbent dressing, showed that 19 of 27 high slough-producing patients exhibited enhanced healing when treated with the chitosan-composed dressing (Stephen-Haynes et al. 2018). A similar dressing composed of chitosan was imbued with silver nanoparticles to give the slough absorbent dressing antimicrobial properties (Liang et al. 2016). As shown in their study, chitosan alone had no antimicrobial properties, but the inclusion of silver nanoparticles provided significant antibacterial activity against common pathogens, such as *Staphylococcus aureus* and *P. aeruginosa* (Liang et al. 2016).

2.1.4 Negative Pressure Wound Therapy (NWPT)

NPWT is an emerging technique used in the treatment of acute and chronic wounds. Since the early 2000s, NPWT has gained increasing popularity for the treatment of

chronic and burn wounds due to a number of published studies describing increased healing rates and decreases in biofilm formation within the wound (Langer et al. 2015; Young et al. 2016). NPWT works by maintaining constant low vacuum pressure on the wound. This vacuum pressure removes wound slough and bacteria within the slough or on the surface of the wound, and also aids in increasing blood flow to the wound bed. For this reason, NPWT is especially effective in treating wounds, such as diabetic foot ulcers, where peripheral blood flow is low and the risk of chronic infection is high. Alone, NPWT does not usually exhibit a significant reduction in bacterial load within infected wounds (Yang et al. 2017). However, when used in conjunction with topical antimicrobial treatments, NPWT has been shown to aid in a significant reduction of bacterial load (Yang et al. 2017). This is likely due to NPWT's ability to mechanically disrupt biofilm and remove obstacles such as wound slough from the wound bed.

2.2 Surgical Debridement

Wound debridement is the surgical removal of necrotic, infected, or otherwise compromised tissue (Milne 2015), which can be toxic to the surrounding tissue, cause slower wound healing rates, and provide accessible nutrients for bacterial colonization. Surgical removal of compromised tissues is considered invasive when compared to alternative methods of debridement or desloughing, and can also be very painful. For these reasons, surgical debridement is typically only performed on patients with a high risk of sepsis by well-trained personnel. The tools used in surgical debridement have not advanced significantly in recent years, and most healthcare providers still use standard surgical scissors, scalpels, and forceps (Percival and Suleman 2015). Plasma scalpels have been available since the mid-1970s, and while they do offer the benefit of immediate cauterization of the wound and maintenance of sterility, they are cost prohibitive (Guild et al. 2017). Regardless of methodology, early intervention of surgical debridement can prevent significant future morbidity and/or mortality for chronically infected patients (Hsu et al. 2015).

2.2.1 Maggot Wound Therapy (MWT)

As archaic as it sounds, using maggot debridement therapy (MDT) to treat chronic wounds has proven to be an effective method for both desloughing and debridement, resulting in quicker healing rates among a variety of wound types (Sun et al. 2014). Maggots used in MDT are sterile and derived from the *Phaenicia (Lucilia) sericata* fly (Sun et al. 2014). They clean infected wounds by consuming necrotic tissue with a high degree of fidelity and have recently been found to secrete antimicrobials effective at killing a variety of pathogens, most notably methicillin-resistant *Staphylococcus aureus* (MRSA) (Bowling et al. 2007). With this newly realized potential of MDT, researchers have begun experimenting with maggots genetically engineered to secrete new varieties of antimicrobials or wound-healing factors (Linger et al. 2016). Utilizing maggots as both a wound cleaner and producer of healing factors can bring treatment options to the masses that are normally not

affordable, as long-term antimicrobial or growth factor regimens are extremely expensive.

2.2.2 Sound Energy

Sonication is the application of sound energy to an environment. In respect to biofilm disruption, varying intensities of ultrasonication have been used to disrupt a biofilm, or even target and kill the bacteria within a biofilm. Ultrasonication during wound treatment uses frequencies above 20 kHz. Low-intensity sonication is generally nonspecific because it employs non-focused pulses of ultrasound over a broad area of tissue. Results show that low-intensity ultrasound pulses have the ability to disrupt a variety of bacterial biofilms in vitro (Crone et al. 2015). For example, high ultrasound frequency of 580 kHz is optimal for the disintegration of bacterial aggregates without causing cell death, while using lower frequencies of 20 and 40 kHz resulted in colony forming unit (CFU) reduction of *Escherichia coli* and *Klebsiella pneumonia* (Ermolaeva et al. 2011). Alternatively, low-intensity ultrasound can be used to optimize antibiotic treatment of *P. aeruginosa* biofilm infections, resulting in increased CFU reduction over antibiotic or ultrasound treatments alone (Kopel et al. 2011).

High-intensity ultrasound does not actually use higher frequencies of ultrasound than low-intensity ultrasound in most cases, as the name suggests. Instead, high-intensity ultrasound differs from low-intensity in that it is usually composed of multiple ultrasound sources that converge on a single point. This technique allows healthcare providers to focus treatment within a 3D space for the destruction of necrotic tissue by creating a heating effect at the convergence point. High-intensity ultrasound has also been successfully used to destroy *E. coli* biofilms, and the bacteria within them (Bigelow et al. 2009). Treatment using this method raises the effectiveness of ultrasound therapy against biofilm, and even makes targeting infections below the surface of healthy tissue possible. Unfortunately, collateral tissue damage via the heating effect created through converging ultrasound frequencies may limit the number of cases for which this therapy can be considered a viable option.

2.2.3 Cold Plasma

Non-thermal plasma (NTP), or cold plasma, is being investigated as a new method for aiding in the sterilization and debridement of wound infections. While there are many plasma types, NTP denotes plasma operating near normal atmospheric pressure and ambient temperature. The majority of previous investigations into using NTP to fight biofilm contamination has centered on treating delicate surfaces, including heat-sensitive plastics and food items prone to contamination, such as lettuce. A recent study reported that a quick 15-second treatment of *Salmonella* biofilms on food contact surfaces resulted in over a 2-log reduction in CFU (Niemira et al. 2014). Promising results such as these have led to increased interest in applying NTP technology to dentistry and medicine.

2.2.3.1 NTP Treatment of Dental Biofilms

In regard to removing the biofilm (plaque) from teeth, traditional methods include tooth brushing and antimicrobial mouth rinses, such as chlorhexidine (CHX). However, NTP therapy has been adapted for disinfecting dental biofilms, and shows great promise in clinically relevant studies against biofilms grown from the mouth microbiota of healthy volunteers through saliva collection. Researchers often choose to study biofilms grown by isolates collected from saliva because the oral microbiota is very complex and is known to contain a wide variety of pathogenic microorganisms that contribute to severe mouth infection, such as *Pseudomonas*, *Staphylococci*, *Enterococci*, *Candida*, and some species of anaerobic bacteria (Leonhardt et al. 2003; Botero et al. 2005). Proof of concept studies have shown that treating *S. mutans* and mixed-population saliva-derived biofilms grown on Ti disks with NTP resulted in a CFU decrease of over 5 logs for both biofilm types, while CHX decreased *S. mutans* and saliva-derived biofilms by only 3.36 and 1.5 logs, respectively (Koban et al. 2011). Increasingly clinically relevant studies have been performed on surfaces that mimic tooth enamel, and have shown a 96% reduction in *S. mutans* biofilm when a multimodal approach was taken that paired CHX with NTP (Hong et al. 2016).

2.2.3.2 NTP Treatment of Wound Biofilms

With the increasing prevalence of multi-drug-resistant pathogens, the search for new ways to combat biofilm-associated wound infections is becoming a popular endeavor. Naturally, many researchers have begun characterizing NTP efficacy against the most common infection-associated pathogens. Short NTP treatments significantly reduce the bacterial load of all ESKAPE pathogens (Flynn et al. 2015). However, treatment effect is dependent on species and strain (Ermolaeva et al. 2011; Flynn et al. 2015). Moreover, Gram-positive bacteria are more susceptible to NTP than Gram-negative bacteria, but lack of efficacy can be remedied using longer treatment times or higher intensity treatments (Ermolaeva et al. 2011). The consensus is that NTP antimicrobial attributes primarily stem from the production of reactive oxygen species (ROS), which disrupt bacterial membranes (Ermolaeva et al. 2011; Flynn et al. 2015; Xu et al. 2015). In addition to the production of ROS, researchers have noticed that NTP exhibits ablative properties against the outermost surface of the biofilm (Xu et al. 2015; Xu et al. 2017). NTP also effects more than just the bacterial cells themselves, as evidence suggests that NTP may be able to target and significantly disrupt specific quorum sensing-controlled factors such as pyocyanin and elastase production (Ziuzina et al. 2015).

Concerns of biocompatibility issues between NTP and host tissue have also been noted. Reductions in cell epithelial viability have been seen in several cell and NTP types (Haertel et al. 2012, Wende et al. 2014). However, short treatments of approximately 10 seconds resulted in no distinguishable decreases in survival (Wende et al. 2014). Correlating with cell viability, data show that treatment times greater than 10 seconds can result in a significant decrease in DNA synthesis through the

disruption of ssDNA (Wende et al. 2014). These results should provide a cautionary tale to researchers investigating NTP for medical applications, and further investigation into the effects of NTP on host cells is of great importance so that we can carefully optimize therapeutic treatments with the patient's safety in mind.

3 Degrading and Disrupting the ECM

One of the major challenges that researchers are faced with when developing ECM-specific therapeutics is that the composition of the matrix is highly variable, depending on the microorganisms present, the age and maturity of the biofilm, nutrient and substrate availability, environmental forces, and a plethora of other applicable conditions of the host environment (Koo et al. 2017a, b). Considering the variable makeup of the ECM, any realistic approaches to effectively disrupting it should be multifaceted. The most practical and effective strategies, especially for inherently complex, highly polymicrobial biofilms such as those present in chronic wound infections (Wolcott et al. 2015), are those that will target highly conserved ECM components that are produced by a broad spectrum of pathogens, while at the same time avoiding collateral damage to the host. Clinically, such a therapy could be implemented, regardless of the specific microbial makeup of each individual infection and be expected to augment traditional antimicrobial agents.

Targeting specific ECM components as a means of therapeutic intervention is not a novel concept. In 2002, Whitchurch et al. were the first to show that exogenously added DNase I could inhibit the formation of, and degrade established *P. aeruginosa* biofilms in vitro, leading to dispersal (Whitchurch et al. 2002). Similarly, in 2003, Kaplan et al. found that dispersin B, a glycoside hydrolase produced by *A. actinomycetemcomitans*, could degrade the polysaccharide, poly(1,6)-N-acetyl-D-glucosamine (PNAG), by hydrolyzing β(1,6) glycosidic linkages, leading to structural collapse of the biofilm in vitro (Kaplan et al. 2003). However, despite increasing research efforts on medical biofilm dispersal (Fleming and Rumbaugh 2017), clinical application is virtually non-existent, with the exception of Dornase alpha (Pulmozyme®), an FDA-approved therapy for the treatment of lung biofilms presenting in cystic fibrosis patients (Wagener and Kupfer 2012a, b). Thus, despite the promising future of ECM targeting for medical biofilm eradication, there is still quite a bit of work to be done, especially in handling the diverse compositional makeup of biofilms from species to species, and case to case. In this section, we provide an overview of the current state of ECM-targeting strategies, and discuss the logistics of future clinical application.

Active degradation of mature biofilm ECM can be accomplished by various means, and in this section, we discuss three particularly promising approaches: the utilization of ECM-targeted enzymes, antibiofilm peptides, and dispersal molecules. In the interest of broad-spectrum applicability, only the strategies which can be practically effective against the ECM of a range of microbial species will be considered.

3.1 ECM-Targeted Enzymes

Some of the most extensive work in targeting structural ECM components has been the exogenous addition of enzymes that have one or more ECM-integral targets (Fleming and Rumbaugh 2017). Three of the most ubiquitous targets of interest have been proteins, polysaccharides, and extracellular DNA (eDNA).

3.1.1 Exoprotein-Targeting

For many pathogens, proteins constitute a significant percentage of total ECM mass (Lasa and Penades 2006; Jiao et al. 2010; Muthukrishnan et al. 2011; Speziale et al. 2014). Exoproteins are important not only for the role they play in maintaining and modifying the matrix (Zhang and Bishop 2003; Kaplan 2010), but also for surface and ECM scaffolding adhesion and the overall structural stability of the biofilm (Lasa and Penades 2006; Hobley et al. 2015). Therefore, **proteases** are a potential tool of interest for the deconstruction of the ECM matrix. For example, proteinase K is a serine protease that cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids, and has been experimentally effective against the biofilms of multiple Gram-positive and Gram-negative bacterial species (Chaignon et al. 2007; Patterson et al. 2007; Fredheim et al. 2009; Izano et al. 2009; Medina and Kadouri 2009; Kumar Shukla and Rao 2013; Nguyen and Burrows 2014; Cui et al. 2016). Another protease with proven broad-spectrum antibiofilm efficacy is trypsin, which targets peptides at the carboxyl side of the positively charged amino acids, lysine, and arginine (Chaignon et al. 2007; Patterson et al. 2007; Niazi et al. 2014; Banar et al. 2016). It should be noted that, while both proteinase K and trypsin are well-documented antibiofilm agents, potential host toxicity due to the presence of target peptides within eukaryotic tissue must be considered, as is the case with many other proteases.

3.1.2 eDNA-Targeting

Another vital ECM component for the adhesion and stability of biofilms is eDNA, which can play an important role in the initial stages of biofilm development, as well as in protecting mature biofilms from physical stress and antibiotics as both an internal scaffolding and a protective cap (Whitchurch et al. 2002; Das et al. 2013; Jakubovics et al. 2013; Alhede et al. 2014; Okshevsky and Meyer 2015; Sena-Velez et al. 2016). Ribonucleases that can break down eDNA represent an extensively studied and clinically applicable class of antibiofilm agent. In fact, Dornase Alpha, marketed as Pulmozyme®, represents the only widely used ribonuclease currently approved by the FDA (Wagener and Kupfer 2012a, b). Although Dornase alpha is used medically to break up DNA-rich mucus in the lungs of cystic fibrosis patients, it has the potential to treat a wide range of biofilm infections and has been shown effective against multiple pathogens (Hall-Stoodley et al. 2008; Kaplan et al. 2012; Claudius et al. 2015). Similarly, DNAse 1, first shown to break down established biofilms in 2002, has also shown promise as a prospective broad-spectrum antibiofilm agent (Whitchurch et al. 2002; Inoue et al. 2003; Nemoto et al. 2003; Rice et al. 2007; Thomas et al. 2008; Fredheim et al. 2009; Izano et al. 2009; Medina and

Kadouri 2009; Svensson et al. 2009; Tetz et al. 2009; Harmsen et al. 2010; Martins et al. 2010; Tetz and Tetz 2010; Conover et al. 2011; Godeke et al. 2011; Seper et al. 2011; Sahu et al. 2012; Rajendran et al. 2013; Waryah et al. 2016).

3.1.3 Exopolysaccharide-Targeting

The major ECM component for a wide range of biofilm-dwelling microbes are exopolysaccharides (Flemming and Wingender 2010). Their ubiquity is such that "Exopolysaccharide" and "Extracellular Polymeric Substance" are often times used as interchangeable, if not erroneous, descriptors for the "ECM" abbreviation. In addition to being a major biofilm structural component that is vital to overall mechanical stability, polysaccharides play a host of other roles for biofilm initiation and persistence, including but not limited to surface and scaffolding adhesion, microbial aggregation, desiccation tolerance, nutrient sorption and storage, enzyme binding, and physical protection against antimicrobials and host defenses (Wingender et al. 2001; Flemming and Wingender 2010; Bales et al. 2013; Limoli et al. 2015; Watters et al. 2016). Considering their pervasiveness and utility, polysaccharides are a common target for anti-biofilm researchers, and glycoside hydrolases are at the forefront of prospective biofilm dispersal strategies. For example, Dispersin B, a glycoside hydrolase specific for the polysaccharide, PNAG, has been shown to degrade the ECM of both Gram-positive and Gram-negative pathogens that contain the polysaccharide (Kaplan et al. 2004; Itoh et al. 2005; Izano et al. 2007a, b; Yakandawala et al. 2011; Fazekas et al. 2012; Gawande et al. 2014; Waryah et al. 2016) and is currently being pursued by Kane Biotech Inc. as an anti-biofilm agent for use in wound, oral, and lung infections in combination with DNAse 1 (Biotech 2017).

Even with highly conserved targets, however, a single glycoside hydrolase may not be universally efficacious, especially when considering the exceedingly complex, polymicrobial infections that can contain dozens of different microbial species coexisting within one biofilm, as is often the case in chronic wound infections (Wolcott et al. 2016). Thus, combinatorial therapies that contain enzymes with differing targets are of interest. For example, in 2017, researchers showed that α-amylase and cellulase, each targeting a separate, conserved, glycosidic linkage, were able to degrade polymicrobial biofilms and increase antibiotic effectiveness (Fleming et al. 2017). Such multi-glycoside hydrolase combinations, especially when coupled with additional classes of enzymes that target other conserved structural components, such as the previously mentioned exoproteins and eDNA, have the potential to greatly bolster broad-spectrum applicability and clinical practicality. However, it must be considered that the more multifaceted the therapy, the more cautious we must be to avoid collateral damage to the host.

3.1.4 Antibiofilm Peptides

In response to the rapid rise of antibiotic resistance, antimicrobial peptides (AMPs) have received a lot of attention as alternative microbicidal agents, and to date, more than 2600 AMPs have been discovered (Wang et al. 2016). Most of these peptides,

however, have only been tested against planktonic cells, with their efficacy against biofilm-dwelling microbes still understudied. Additionally, due in large part to the fact that they are microbicidal, there is the possibility of resistance arising against AMPs in a similar fashion to antibiotics. Antibiofilm peptides (ABPs), however, possess distinct structure–activity relationships when compared to AMPs and are defined as AMPs that exhibit antibiofilm activity below their minimal inhibitory concentration (MIC) (Pletzer et al. 2016; Pletzer and Hancock 2016). The ability of ABPs to degrade biofilms at sub-microbicidal levels make them promising adjunctive agents to existing antibiotic therapies. An example of an ABP that has shown considerable promise is the human cathelicidin, LL-37, which has demonstrated antibiofilm activity far below MIC. Similarly, innate defense regulator peptide 1018 (IDR-1018), a 12-amino acid synthetic peptide that triggers the degradation of the (p)ppGpp bacterial stringent response signal, is another ABP that has exhibited broad-range efficacy against both Gram-positive and Gram-negative organisms, dispersing the bacteria and augmenting antibiotic efficacy against them (de la Fuente-Nunez et al.2014; Reffuveille et al. 2014; Wang et al. 2015).

3.1.5 Dispersal Molecules

Other molecules that are detrimental to the ECM matrix, but do not exhibit enzymatic activity, and are not derivatives of AMPs, can be classified as dispersal molecules. These are molecules that can directly act on ECM constituents by multiple means, including binding to and destabilizing ECM scaffolding, and acting as a detergent that can disrupt the adhesion of biofilm cells and matrix components. For example, rhamnolipids are a type of biosurfactant that are produced by a number of bacterial species, including *P. aeruginosa* (Boles et al. 2005), that exhibit bimodal activity in biofilms. At lower concentrations, rhamnolipids promote the biofilm mode of life by driving fluid channel maintenance and allowing for robust ECM structure, with rhamnolipid-negative biofilms appearing flat and formless (Davey et al. 2003; Pamp and Tolker-Nielsen 2007; Glick et al. 2010). At higher concentrations, however, rhamnolipids trigger biofilm dispersal for a range of bacterial species by increasing cellular motility, thus inhibiting adherence to the substratum (Boles et al. 2005; Quinn et al. 2013; Bhattacharjee et al. 2016; De Rienzo and Martin 2016). Another example of a potential biofilm dispersal molecule is urea which has been shown effective against the biofilms of both Gram-positive and Gram-negative bacteria, possibly by disrupting structurally important ECM hydrogen bonds (Chen and Stewart 2000, Brindle et al. 2011).

In summary, specifically targeting extracellular ECM components represents a promising approach to degrading biofilms. Additionally, attacking highly conserved biofilm targets with a multifaceted, non-microbicidal therapy may be the most practical way to combat complex, polymicrobial infections with an eclectic, chimeric mix of matrix constituents, while at the same time carrying the decreased risk of the pathogens developing additional resistances. In this way, traditional antimicrobial therapies and host defenses can be augmented, and infections can be more readily cleared.

3.1.6 Dispersal Signal Manipulation

Another avenue of research for the eradication of pathogenic biofilm infections is the manipulation of dispersal signals. Dispersal signals are mediated by certain molecules whose *decreased* or *increased* presence triggers active ECM degradation by biofilm microbes. By harnessing our knowledge of active biofilm dispersal mechanisms, we can theoretically design clinical interventions involving the addition or sequestration of key molecules involved in biofilm dispersal pathways (Fleming and Rumbaugh 2017).

One method of biofilm dispersal signal manipulation is the sequestration or degradation of molecules whose increased concentration is associated with biofilm formation and maintenance. Cyclic di-GMP (c-di-GMP) is a nearly ubiquitous intracellular secondary messenger molecule with broad-spectrum involvement in the biofilm life cycle of both Gram-positive and Gram-negative organisms (Jenal et al. 2017). Universally, increased c-di-GMP levels are almost always associated with the biofilm mode of life, and a plethora of studies over the last decade have focused on the modulation of c-di-GMP as a means of biofilm inhibition and dispersal (Jakobsen et al. 2017). Many of these focus on small-molecule inhibition of diguanylate cyclases, which synthesize c-di-GMP from two molecules of GTP, or by direct degradation by certain phosphodiesterases that deconstruct c-di-GMP into the linear dinucleotide, pGpG (Jakobsen et al. 2017).

An essential nutrient required for the formation and persistence of biofilms in a range of pathogens is iron (Banin et al. 2005; Lin et al. 2012; Oglesby-Sherrouse et al. 2014). Thus, iron chelators and competitive inhibitors (such as gallium) serve as potential agents that can trigger biofilm dispersal (Koo et al. 2017a, b). For example, lactoferrin, a globular, iron-binding glycoprotein vital to the innate immune system, so named because of its high abundance in milk, can sequester iron away from the biofilm microbes and has been shown effective against a variety of pathogens (Ammons and Copie 2013; Reffuveille et al. 2014; Fleming and Rumbaugh 2017).

For other molecules, an increase in concentration can trigger inhibitory changes in ECM-production pathways (i.e., c-di-GMP) or induce the production of matrix degradation enzymes. One example is nitric oxide, which has been shown to regulate the levels of c-di-GMP in a conserved manner across microbial species (Barraud et al. 2009; Barraud et al. 2015; Howlin et al. 2017). While biofilm pathogens produce nitric oxide endogenously in order to trigger dispersal, exogenously added nitric oxide has shown promise as a potential biofilm therapeutic agent, which largely acts by stimulating phosphodiesterase activity (Barraud et al. 2015).

Another molecule whose decreased concentration is associated with dispersal in multiple species is *Cis*-2-decenoic acid (CDA) (Davies and Marques 2009; Rahmani-Badi et al. 2014, 2015; Sepehr et al. 2014). CDA is a fatty acid cross-kingdom signaling molecule, or diffusible signal factor. As an extracellular signaling molecule recognized by both bacteria and fungi, CDA has the potential to trigger the dispersal of complex, polymicrobial biofilms, by inducing the expression of matrix-degrading enzymes by both prokaryotes and eukaryotes (Davies and Marques 2009). It should be noted that, just as with enzymatic therapy, the most applicable therapies are those that would induce multiple dispersal signals, thereby reducing the effect of redundant signaling pathways.

4 Conclusions

The tendency of pathogens to live within the protection of a biofilm affords them greatly increased tolerance to antimicrobials, the host immune system, and environmental stressors, and is a compounding factor in the alarming rise of antibiotic resistance. Therefore, as an alternative to the traditional approach of directly targeting the infecting microbes, many researchers have turned their attention toward anti-biofilm-specific strategies. In this chapter, we highlighted many of the mechanical and chemical approaches to the removal of tissue-associated biofilms, from simple, age-old techniques such as surgical debridement and maggot wound therapy, to cutting-edge technologies like non-thermal plasma, monofilament debridement pads, and small-molecule dispersal signal manipulation. As we better understand the enormous impact that biofilm-associated infections have on medicine, such strategies offer a way forward in their effective management.

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Part IV

Prospects of Nanomaterials: Antibacterials and Drug Delivery Agents

Nanomedicine and Nanoemulsion in Increasing the Availability of Antibiotics

Xinli Liu and Wei Li

Abstract

There is a pressing need for the discovery of new antimicrobials and novel, antimicrobial targeting strategies. Many antimicrobials in preclinical and clinical development have poor aqueous solubility, which limits their clinical development and usage due to the low bioavailability. Enhancement of bioavailability in antimicrobials is, therefore, of great importance for achieving therapeutically effective doses and desired concentrations of drugs for the treatment of infectious diseases. Nanomedicine has enormous potential for the prevention and treatment of infectious diseases. Nanoemulsion is one type of clinically useful formulation that can provide a substantial advantage over the conventional formulation of antibiotics since the antibacterial activity of antibiotics can be significantly enhanced by dispensing them in nanoparticulate dosage form. This sustained delivery system can help to deliver antibiotics with poor solubility and pharmaceutical properties, improve the systemic or local drug bioavailability and enhance the therapeutic efficacy.

Keywords

Nanomedicine · Emulsions · Nanoemulsions · Antibiotics delivery · Antibiotics bioavailability

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1 Introduction of Antibiotics Resistance and Bioavailability

The discovery of penicillin in 1928 and streptomycin in 1943 heralded the age of antibiotics, among the most successful medications in human history. From 1940 to 1960 – the "golden age" of antibiotic discovery – the introduction of more than one-half of the effective antibacterial therapies used today completely revolutionized clinical practices for infectious diseases (Davies 2006). However, due to evolutionary processes, misuse, and overuse of antibiotics, new classes of effective antibiotics will eventually select for survival of the small fraction of bacterial populations that have an intrinsic and/or acquired resistance mechanism. The ability of microorganisms to become resistant to several classes of antimicrobial drugs has become a major concern and poses a global threat to public health (Burnham et al. 2017). There is a pressing need for the discovery of new antimicrobials and novel, antimicrobial targeting strategies. Many antimicrobials in preclinical and clinical development have poor aqueous solubility, which limits their clinical development and usage due to the low bioavailability. Enhancement of bioavailability in antimicrobials is, therefore, of great importance for achieving therapeutically effective doses and desired concentrations of drugs for the treatment of infectious diseases.

2 Introduction of FDA-Approved Nanomedicine

Many antimicrobials showed great promise in preclinical models, but the translation to clinical investigation was hampered by lack of suitable dosage form and poor bioavailability. Nanomedicine may be able to solve these issues and has gained much interest in recent years. Broadly speaking, nanomedicine is a branch of medicine that applies the knowledge and tools of nanotechnology to the prevention and treatment of disease. Nanomedicine involves the use of nanoscale materials, such as biocompatible nanoparticles and nanodevices, for diagnosis, delivery, sensing, or actuation purposes in a living organism (Webster 2006). Specifically speaking, nanomedicine refers to using nanotechnology to incorporate therapeutic drugs into nanoparticles. Nanomedicine has greatly improved delivery of many toxic drugs or drugs with poor water solubility by enhancing solubility and changing the biodistribution of drugs. Appropriately designed nanomedicine has shown great promise as a dosage form for sustained release, attenuated toxicity, and site-specific targeting (Shi et al. 2017). There have been more than 10 nanomedicines approved by the FDA in the past three decades, with the majority of drugs used in oncology and a few in infectious disease treatments. A comprehensive list of these drugs can be found in recent reviews (Shi et al. 2017; Weissig and Guzman-Villanueva 2015; Weissig et al. 2014). Many nanomedicines are currently in early preclinical and clinical development. In the field of infectious diseases, antimicrobials encapsulated in a nanoparticle-based drug delivery system have displayed improved antimicrobial activity and pharmacokinetics, making it possible to reduce the dose and overcome antibiotic resistance (Torchilin 2014). Regulatory approval has been given to some liposome-based or lipid complex

Table 1 Clinically approved nanomedicine for the treatment of infectious diseases

Platforms	Product	API	Excipients	Indication	Approved
Lipid complex	Abelcet®	Amphotericin B	DMPC and DMPG lipids	Invasive fungal infections	1995 (FDA)
Colloidal dispersion	Amphotec®	Amphotericin B	Cholesteryl sulfate complex	Invasive aspergillosis	1996 (FDA)
Liposome	AmBisome®	Amphotericin B	HSPC, DSPG, cholesterol	Fungal infection in febrile, neutropenic patients.	1997 (FDA)
Liposome	Fungisome	Amphotericin B	HSPC and cholesterol	Systemic fungal infection	2003 (India)

API active pharmaceutical ingredient, *DMPC* l-α-dimyristoylphosphatidylcholine, *DMPG* l-α-dimyristoylphosphatidylglycerol, *HSPC* hydrogenated soy phosphatidylcholine, *DSPG* distearoyl phosphatidylglycerol

colloid dispersion nanocarrier systems encapsulating antifungal agent amphotericin B (Tonin et al. 2017); several clinical approved nanomedicine products of amphotericin B on the market are summarized in Table 1.

Nanomedicine has unique physicochemical properties, including ultra-small and controllable size (usually around 100 nm in diameter), large surface area-to-mass ratio, functionalizable surface structure, enhanced drug solubilization, prolonged in vivo circulation time, and potential for organ/tissue/cell targeting. These properties can be applied to facilitate the administration of antimicrobial drugs and enhance the intracellular delivery of drugs, thereby overcoming some of the limitations in traditional antimicrobial therapeutics. Potential advantages of antimicrobial nanomedicine include minimized systemic side effects and the ability to codeliver multiple, synergistic antimicrobial drugs in combination therapy, thus targeting specific organ or tissues of infections (Huh and Kwon 2011).

Nanomedicine has been shown to prevent development of microbial resistance or overcome existing resistance by using multiple mechanisms, including increased cellular penetration and higher cytoplasmic concentration of antibiotics that bypass efflux pumps. Incorporation of antimicrobial drugs into a nanocarrier can improve intracellular delivery and enhance antimicrobial activity, which results in killing the microbe before it can develop mutations that confer resistance (Pelgrift and Friedman 2013).

3 Introduction of Emulsions and Nanoemulsions

There is increasing interest in using emulsion-based, drug nanoparticulate, drug delivery systems to enhance the delivery and bioavailability of antimicrobials (Franklyne et al. 2016). A number of drug-encapsulated emulsion dosage forms have been introduced in the clinic. An example of these clinical emulsion products is summarized in Table 2. Intralipid® is the first safe, metabolizable, sterile,

Table 2 Examples of emulsions used in the clinic

Product	API	Excipients	Indication	Approved
Diprivan®	Propofol	Soybean oil/glycerol/egg lecithin	Sedative, anesthetic	1989 (FDA)
Intralipid®	Fatty acids	Soybean oil/egg yolk phospholipids/glycerin	Parenteral nutrition	1996 (FDA)
Cinvanti™	Aprepitant	Soybean oil/glycerol/egg lecithin/ethanol/sodium oleate/sucrose	Nausea and vomiting	2003 (FDA)
Cleviprex®	Clevidipine	Soybean oil, glycerin, egg yolk phospholipids, oleic acid	Antihypertensive	2008 (FDA)
Smoflipid®	Fatty acids	Soybean oil/ medium-chain triglycerides/olive oil/fish oil/egg phospholipids/glycerin	Parenteral nutrition	2016 (FDA)

nonpyrogenic intravenous fat emulsion that was marketed for intravenous administration as a source of calories and essential fatty acids (MacFie 1999; Wretlind 1999). It was approved by the FDA in 1996, and contains 10% or 20% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerin for tonicity adjustment, and water (Table 2) (Floyd 1999). Intralipid emulsion has a droplet size of approximately 0.5 micron. For intravenous injection, emulsions must be sterile, nontoxic, physically and chemically stable, biocompatible, and nonimmunogenic. The size of the droplet must be small enough without forming aggregates to ensure that the particles do not cause an embolism. Once the emulsions are introduced into the bloodstream, the emulsion particles are rapidly covered by apolipoproteins derived from naturally occurring lipoproteins; thus, natural protein-covered fat emulsion particles are metabolized in the same way as naturally occurring chylomicrons in vivo (Havel et al. 1973).

3.1 Understanding of Emulsions: Types, Composition, and Properties

Emulsion is defined as a thermodynamically unstable dispersion of at least two immiscible liquids, one of which is dispersed in globular form (the dispersed phase) throughout the other liquid phase (the continuous phase) and stabilized by emulsifiers. Emulsifiers are both fat and water soluble, and a single molecular layer of the emulsifier arranges itself and surrounds the lipid droplet (Fig. 1). Phospholipids such as egg lecithin are commonly used emulsifiers. Pharmaceutical emulsions usually consist of water and oil liquid phases. The globular sizes of the dispersed phase range between 100 nm and 25 μm. Simple emulsions are composed oil-in-water (o/w) and water-in-oil (w/o) emulsion types. Only o/w emulsions can be used for intravenous administration, and the amount of oil may vary from 2 to 20% w/w based on the site of administration (Hormann and Zimmer 2016; Rai et al. 2018;

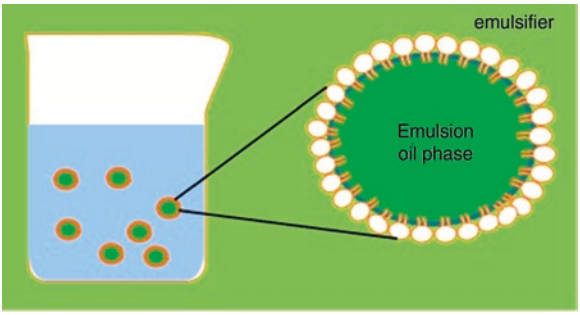

Fig. 1 Schematic representation of emulsion structures

Singh et al. 2017). The oils typically originate from soya beans, sunflower, safflower, olive, coconut, and fish oils, which have different ratios of medium- to long-chain triglycerides.

3.2 Nanoemulsion: Mechanisms and Stabilization

When particulate systems are administered intravenously, if the particle sizes are larger than 7 μm in diameter, they will be trapped in the capillary bed of the lung, as the blood capillary diameter is about 6 μm. If the particle sizes are around 3 and 5 μm, they are taken up by the mononuclear phagocyte system, which includes Kupper cells in the liver and macrophages in the spleen (Kanke et al. 1980). Particles under low nanometers usually circulate longer in the blood. Nanoemulsions have attracted considerable attention due to their ease of preparation and high stability. The nanoemulsions (size 20–200 nm) are fundamentally different from other conventional emulsions (size >500 nm) with respect to thermodynamic stability, preparation methods, and behavior toward dilution and temperature fluctuation. Nanoemulsions are thermodynamically and kinetically stable systems due to smaller particle sizes and lower surface tension. Among the oil and aqueous phase, the Brownian motion of the small droplets overcomes the force of gravity (Rai et al. 2018). Nanoemulsions usually have high colloidal stability with fewer problems of inherent flocculation, coalescence, and creaming, which are common problems associated with conventional emulsions. Nanoemulsions are usually transparent or translucent dispersions, less turbid than conventional emulsions because the diameter of the droplets is often much smaller than the wavelength of light. The nanoemulsions can be administered through oral, intravenous, topical, and nasal routes (Rai et al. 2018). Nanoemulsions have been shown to improve dissolution and bioavailability of poorly water-soluble drugs (Qhattal et al. 2011). Nanoemulsion can help cross biological membranes by reversible alteration of cellular arrangements and by improving interactions with cells after the fusion of a lipid bilayer interface with the cell wall (Baspinar and Borchert 2012). Nanoemulsions are also useful as sustained-release systems by means of deport formation after subcutaneous injection (Prankerd and Stella 1990), and as site-specific drug delivery systems through binding of ligands in various cell surface receptors (Hak et al. 2012). Several poorly

water-soluble drugs were formulated in oral nanoemulsions and approved by the FDA for clinical use, including cyclosporine (Neoral®, Gengraf®), ritonavir (Norvir®), and saquinavir (Fortovase®). Nanoemulsions have many applications as novel drug delivery systems for antimicrobials.

3.3 Methods of Preparation

Emulsions are thermodynamically unstable systems. When two immiscible liquids are placed together in a container, they will form distinct layers with a minimum interfacial area between the liquids, which leads to minimum surface free energy (ΔG). During the emulsification process, external energy needs to be provided by mixing or mechanical agitation to disrupt the bulk liquids and generate fine liquid droplets, thereby increasing the interfacial area between the two liquids and the surface free energy. From a thermodynamic point of view, emulsions have a natural tendency to destabilize to reduce their interfacial energy, so the liquid droplets will spontaneously coalesce (merge and recombine) to form bigger droplets (reduce the interfacial area and minimize ΔG) and eventually, revert back to phase separation. Emulsifying agents (emulsifiers) are added to stabilize the emulsion droplets by reducing the interfacial tension between the oil and water phases and increasing droplet-droplet repulsion through electrostatic and/or steric repulsive forces. Although emulsions are thermodynamically unstable dosage forms, the presence of emulsifiers confers kinetic stability. Most emulsions are stable over a period of time (up to several years), making emulsions useful as a drug delivery system for clinical application.

Nanoemulsions can be prepared by high-energy dispersion techniques using mechanical devices such as homogenization or sonication to increase the water/oil interfacial area or by low-energy spontaneous emulsification methods such as a self-nanoemulsifying drug-delivery system (SNEDDS), which is formed spontaneously by using mild agitation to blend oil, water, surfactant, and cosurfactant in the right proportion (Qhattal et al. 2011). SNEDDS has emerged as a promising approach for the delivery of poorly water-soluble drugs for clinical use.

4 Recent Advances in Nanoemulsion Delivery of Antibiotics

4.1 The Innate Antimicrobial Effect of Blank Nanoemulsions

Antibiotics encapsulated in nanoemulsions with potent antimicrobial activities have been widely reported. However, certain placebo nanoemulsions (devoid of any antibiotic drug) formulated in different oils also have some intrinsic antimicrobial activities. It was initially reported by Hamouda and colleagues in 1999 and 2001 that a surfactant nanoemulsion had broad-spectrum sporicidal activity against *Bacillus* species (Hamouda et al. 1999) and antimicrobial activity against bacteria,

enveloped viruses, and fungi (Hamouda et al. 2001). This surfactant nanoemulsion comprised of soybean oil, tributyl phosphate, and Triton X-100 emulsified in water was shown to have broad-range antimicrobial activity against *Bacillus cereus*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Vibrio cholera*. Additionally, this blank nanoemulsion also demonstrated virucidal activity against *Herpes Simplex virus*, *Influenza A virus*, and *Vaccinia virus* and sporicidal activity against *Bacillus* species. This blank nanoemulsion was safe in animal models. Myc and colleagues reported the fungicidal activity of nanoemulsion X8W60PC against clinically important yeast and filamentous fungi (Myc et al. 2002). The X8W60PC nanoemulsion showed inhibitory activity against *Candida parapsilosis* and clinically important filamentous fungi including *Microsporum spp. Trichophyto mentagrophytes, Trichophyton rubrum, Aspergillus fumigatus, Fusarium oxysporum, and Epidermophyton floccosum* (Myc et al. 2002). The postulated mechanism of the antimicrobial activity of blank nanoemulsions is that when they fuse with the lipid bilayers of outer cell membranes of microorganisms due to electrostatic interaction, the ingredients released destabilize the lipid membrane of the bacteria; killing the microorganism. These features of nanoemulsions make them suitable candidates for wound treatment (Hemmila et al. 2010).

Some essential oils have inherent antimicrobial activities. They have been widely used as functional ingredients in pharmaceuticals, food, and cosmetic applications. Formulating these essential oils as an oily phase into emulsifier-stabilized emulsions improves the aqueous stability of the essential oils and increases their antimicrobial activities (Chang et al. 2013; Liang et al. 2012). Nanoemulsions that incorporate the essential oil of 5% *Cymbopogon flexuosus* (*C. flexuosus*) with emulsifiers including sorbitan monooleate (Span 80) and Tween 80 demonstrate an ability to reduce adhesion to surfaces by pathogenic microorganisms such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, and inhibit the formation of biofilms. The essential oil *C. flexuosus* was shown to be responsible for the observed antimicrobial activity (da Silva Gundel et al. 2018).

4.2 Improved Systemic Bioavailability for Oral and Parental Nanoemulsions of Antibiotics

Nanoemulsions are employed to enhance the stability and bioavailability of lipophilic antibiotics. Capuramycin is a nucleoside antibiotic that inhibits biosynthesis of peptidoglycan. SQ641 is the most potent analogue of capuramycin antibiotics and has a bactericidal effect against *Mycobacterium tuberculosis*. SQ641 is poorly soluble in water and is not absorbed orally. SQ641 has been formulated in phospholipid-based nanoemulsions, which were made of phospholipid Phosal 53 MCT (53% phosphatidyl choline in medium-chain triglycerides) using α-tocopheryl polyethylene glycol 1000 succinate (TPGS) as the emulsifier. The SQ641 nanoemulsions significantly enhanced *Mycobacterium tuberculosis (M. tuberculosis)* intracellular killing activity over a free drug in the *M. tuberculosis*-infected mouse macrophage cell line. SQ641 nanoemulsions are stable up to 1 year at 4 °C,

scalable, and capable of higher drug loading and decreased dosing frequency. Intravenous injected SQ641 nanoemulsions increased the bioavailability and were shown to target lung and spleen in *M. tuberculosis*-infected mice (Nikonenko et al. 2014).

Rifampicin is an antibiotic used to treat several types of bacterial infections, including tuberculosis, leprosy, and Legionnaire's disease. A more bioavailable o/w nanoemulsion was developed by the aqueous phase titration method for intravenous delivery of rifampicin. The formulation contains propylene glycol caprylate (Sefsol 218) and emulsifiers (Tween 80 and Tween 85) and was stable for more than 19 months (Ahmed et al. 2008). Injectable benzathine-penicillin G (PenG) nanoemulsion formulations were prepared by a spontaneous emulsification approach (Santos-Magalhaes et al. 2000). Lipophilic (soya phosphatidylcholine) and hydrophilic (poloxamer) emulsifiers in equal molar ratio promote the stabilization of the nanoemulsion, which release PenG from nanoemulsions within 2 h. PenG nanoemulsion exhibited 100% growth inhibition against *Staphylococcus aureus* in comparison with a standard penicillin solution (Santos-Magalhaes et al. 2000).

4.3 Improved Bioavailability for Local and Regional Delivery of Nanoemulsion Antibiotics

Nanoemulsions can be administered topically to the skin (Rai et al. 2018). Fusidic acid is widely used in the treatment of skin infections caused by *Staphylococcus aureus*. A topical fusidic acid nanoemulsion formulated from phospholipid, Tween 80, and ethanol demonstrated improved wound healing efficacy and better drug permeation in the BALB/c burn wound infection model (Chhibber et al. 2015). Dapsone is a sulfone antibiotic used in the treatment of leprosy and pneumocystis pneumonia. Dapsone was formulated in a nanoemulsion using isopropyl myristate as the oil phase. The topical dapsone-loaded nanoemulsion showed improved epidermal permeability through the skin barrier (Borges et al. 2013).

Pulmonary inhalation of nanoemulsion antibiotics to the lungs is an effective drug delivery approach to enhance the lung bioavailability and improve therapeutic efficacy for lung infections. Pulmonary aerosol delivery in rats of tobramycin antibiotics emulsified within liquid perfluorocarbon demonstrated a 5- to 22 fold greater pulmonary tobramycin concentration at 4-h post-delivery relative to aerosolized aqueous tobramycin. Antibacterial perfluorocarbon ventilation has been proven as an effective means of pulmonary drug delivery, with the potential to significantly improve antibiotic therapy for lung disease patients (Orizondo et al. 2016).

Ocular delivery of nanoemulsion antibiotics is also useful regarding the concentration and retention of antibiotics inside the eye. An ophthalmic o/w nanoemulsion of ofloxacin was prepared by using oleic acid as the oil phase, Tween 80 as the emulsifier and chitosan oligosaccharide lactate as the modifier. In New Zealand rabbits, these nanoemulsions displayed longer preocular residence time and improved efficacy against bacterial keratitis when compared with a commercial ofloxacin solution (Ustundag-Okur et al. 2014).

5 Conclusion

Nanomedicine has enormous potential for the prevention and treatment of infectious diseases. Nanoemulsion is one type of clinically useful formulation that can provide a substantial advantage over the conventional formulation of antibiotics since the antibacterial activity of antibiotics can be significantly enhanced by dispensing them in nanoparticulate dosage form. This sustained delivery system can help to deliver antibiotics with poor solubility and pharmaceutical properties, improve the systemic or local drug bioavailability, enhance the therapeutic efficacy, decrease the therapeutic dose, and shorten the treatment course. Nanoemulsional antibiotics are also more effective at inhibiting biofilm formation and have great potential for overcoming multidrug resistance. Nanoemulsion has been demonstrated as a versatile nanocarrier for systemic, local, topical, rectal, pulmonary, and ocular delivery of many antibiotics. Future efforts can be focused on creating synergistic antibiotic combinations and improving tissue or cell-specific targeting. A holistic understanding of nano–micro interactions, systemic and cellular transport mechanisms of nanoemulsions to bacteria, and targeting nanoemulsions to biofilms will lead to development of more efficacious antibiotic nanomedicine.

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Nanoparticles as New Emerging Antibacterials: Potentials and Limitations

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Abstract

The use and abuse of antimicrobials have led to the emergence of multi-drug resistant (MDR) bacteria and the spread of resistant organisms and is one of the major global threats for healthcare professionals. Alternatives to conventional antibiotics for combating resistant infections are the need of the hour. Nanotechnology-based drugs offer a ray of hope in the fight against MDR bacteria for patients as well as clinicians. Diverse types of nanomaterials have been synthesized from metallic particles with promising antibacterial activity. Efficacy of these nanomaterials depends on their interactions with bacterial cells and their mechanisms of action differ based on their physico-chemical properties. Development of novel and potent nanoantimicrobials requires in-depth knowledge of the physico-chemical properties of nanoparticles and the biological char-

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acteristics of bacteria. However, there is still a long way to go as there are major issues related to the toxicity and stability of nanoparticles. Moreover, the economic feasibility of transferring the technology from bench to bedside needs to be addressed. The present review highlights the antibacterial effects of nanoparticles, their mechanisms of action, factors affecting the activity of NPs and challenges of ongoing and future research.

Keywords
Nanoparticle · Drug resistance · MDR · Biofilm

1 Introduction

The discovery of antibiotics is considered to be one of the most significant advances in the field of medicine (Hwang et al. 2015). However, increasing drug resistance among infection-causing bacteria is slowly but surely leading to the emergence of a post-antibiotic era (Fernandes 2015). The emergence of multi-drug resistant (MDR) microbes has jeopardized the use of current antibiotic therapies (Courvalin 2016). To counter these MDR bacteria, higher doses of antibiotics are prescribed, sometimes causing toxic effects (Poulikakos et al. 2014). The treatment of drug-resistant infections has become difficult, expensive and complicated (Perez et al. 2007; Andrade et al. 2013a, b).

Drug resistance occurs naturally by selective pressure and by the horizontal transfer of resistance genes. This horizontal transfer by conjugation, transformation or transduction further worsens the situation as resistant genes disseminate to species that may be unrelated to the infection and which subsequently persist in natural environments (Doi et al. 2012; Piddock 2016). Another factor playing an important role in the inability of antibiotics to kill bacteria is the formation of biofilms by bacteria. Bacteria residing in biofilms are adhered to a surface and enclosed in a matrix of extra polymeric substances (EPS). EPS acts as a barrier against the entry of the antibiotics, making the bacteria less susceptible to antibacterial drugs. Thus, biofilm formation increases antibiotic tolerance and is a severe health threat (Husain and Ahmad 2013; Baptista et al. 2018; Jamil and Imran 2018).

Many strategies have been introduced to counter the menace of MDR bacteria in recent times and one such approach is the use of nanotechnology-developed novel nanomaterials that have different shapes and sizes and possess broad-spectrum antimicrobial action (Baptista et al. 2018). Nanoparticles (NPs) are promising candidates as they can not only demonstrate bactericidal action but can also act as carriers for conventional antibiotics and antibacterial compounds of natural origin (Wang et al. 2017). A wide range of materials have been investigated from liposomal to polymer-based nano-drug carriers. Metallic vectors, like gold NPs, are attractive core materials as they are essentially inert and non-toxic (Burygin et al. 2009). The most attractive aspect of nanoparticle-based drug delivery is the targeted delivery of various therapeutics to the site of infection effectively and safely. Drugs are delivered to the site of infection either bound to the large surface area of the NPs or

encapsulated within the NP (Gholipourmalekabadi et al. 2017). This review not only summarizes the antibacterial potential of different types of nanoparticles but also discusses various nanostructural factors that contribute to the development of potent nano-antibiotics.

2 Why Nanomaterials as Antibacterial Agents?

Materials that are typically 0.2–100 nm in size, having high surface-to-volume ratio, are termed 'nano'. These nanomaterials differ in their chemical, electrical, mechanical, optical, magnetic and electropotential properties from their bulk materials (Hajipour et al. 2012; Rudramurthy et al. 2016). Among all nanomaterials, most researches have focused on the synthesis and application of NPs. NPs are easy to synthesize, can enhance the solubility and stability of drugs and are biocompatible with target agents, and their modulated release makes them favourable candidates for use as drug vectors. The size of nanoparticles, along with their high surface-to-volume ratios, is responsible for achieving distinct functionality in drug delivery. This gives NPs an advantage over conventional therapies in the treatment of infections caused by drug-resistant bacteria (Rudramurthy et al. 2016; Gholipourmalekabadi et al. 2017). Silver nanoparticles (AgNPs) are the most studied nanomaterials and have been found to be the most effective NPs against pathogenic bacteria. However, other metal and metal oxide NPs synthesized from copper, zinc, titanium, tin, and iron have also demonstrated antibacterial potential (Dakal et al. 2016; Khan et al. 2016; Hemeg 2017; Al-Shabib et al. 2018a, b).

Conventional antibiotics suffer from poor membrane transport and thus have reduced potency (Andrade et al. 2013a, b), while NPs penetrate the membrane of host cells either by endocytosis or through interactions with surface lipids (Huang et al. 2010; Wang et al. 2017). The ability of NPs to confer physical protection against mechanisms of resistance enhances their therapeutic feasibility. Furthermore, since multiple drug combinations can be loaded into NPs, bacteria are less likely to develop resistance due to the complex mechanisms of action of different antimicrobials (Huh and Kwon 2011). However, a report on bacterial resistance against AgNPs has emerged recently (Panáček et al. 2018).

NPs demonstrate broad-spectrum bactericidal activity both against Gram-positive and Gram-negative bacteria and thus, have been employed as carriers for the delivery of antimicrobials (Rai et al. 2016; Wang et al. 2017; Zaidi et al. 2017; Hadiya et al. 2018). NPs are used as carriers because they protect antimicrobial agents from certain enzymes that either destroy or render the drug inactive. Secondly, they have the ability to deliver drugs to the target site and have the capability to carry and deliver multiple drug combinations (Huh and Kwon 2011; Rai et al. 2016; Wang et al. 2017; Zaidi et al. 2017; Hadiya et al. 2018). Moreover, NP vectors thwart pathogenic bacteria by prolonging the retention of drugs at the target site or by conjugation with the active molecules that bind a certain target (Wang et al. 2017). Conjugation of antibacterial drugs with NPs can help to overcome the therapeutic limitations of these drugs. Saha et al. (2007) demonstrated conjugation of the

antibiotics ampicillin, streptomycin and kanamycin to gold NPs, which resulted in lowered minimum inhibitory concentrations (MIC) as compared to their free drugs counterparts against both Gram-positive and Gram-negative pathogens.

3 Antibacterial Action of NPs: Mechanistic Overview

The antibacterial activity of NPs against drug-resistant pathogenic bacteria depends on a number of factors discussed above. These antibacterial effects are attributed to various mechanisms of action such as interaction with the cell wall of bacteria, inhibition of biofilm, triggering host immune response, generation of reactive oxygen species (ROS) and interaction with DNA and proteins (Fig. 1) (Hemeg 2017; Singh et al. 2017; Wang et al. 2017; Zaidi et al. 2017; Siddiqi et al. 2018).

ROS generation that induces oxidative stress is one of the key mechanisms of action of NPs against bacteria (Rudramurthy et al. 2016). Bacteria form ROS via aerobic respiration, and its production is balanced by the antioxidant systems of bacteria, but increases in ROS generation can lead to oxidation of biomolecules and cell components, leading to cell damage (Yang et al. 2012). NPs generate distinctive ROS such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), which can be neutralized by endogenous antioxidants, but singlet oxygen ($^1[O_2]$) and hydroxyl radicals ($^{\cdot}OH$) cause acute death of bacteria (Wang et al. 2017). Metallic NPs demonstrate antibacterial activity as they possess high surface-to-volume ratios, and this increased ratio is usually associated with increased ROS generation, including free radicals. Several investigations on NPs have highlighted the role of ROS-mediated oxidative stress in causing the death of drug-resistant bacteria as shown in Table 1. In one study,

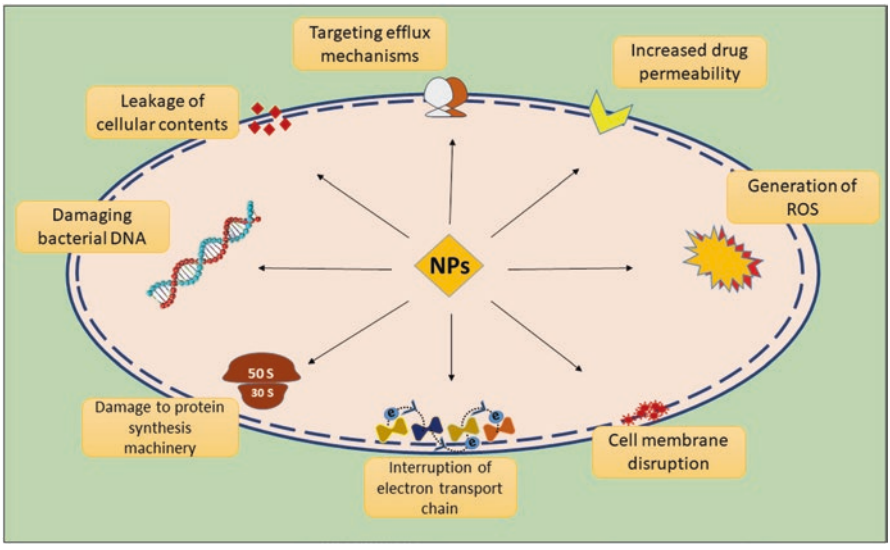

Fig. 1 Modes of action of nanoparticles in bacterial cells

Table 1 Antibacterial activity of metal and metal oxide nanoparticles and their mode of action

Drug-resistant bacterial pathogen		Active nanoparticles	Mode of action	References
Vancomycin resistant	*E. faecalis*	AgNPs	Synergistic action with vancomycin	Esmaeillou et al. (2017)
	S. aureus	AgNPs	Unknown	Saeb et al. (2014)
	S. aureus	AuNPs	Synergistic action with vancomycin	Lai et al. (2015)
	Enterococcus	Ag/Au bimetallic	Photoinactivation	Zhou et al. (2018)
Methicillin resistant	*S. aureus*	AgNPs	Synergistic action with antibiotics	Esmaeillou et al. (2017)
	S. aureus	AuNPs	ROS generation and photoinactivation	Ocsoy et al. (2017)
	S. aureus	ZnONPs	Enzyme inhibition	Cha et al. (2015)
	S. aureus	CuNPs	DNA destabilization	Kruk et al. (2015)
	S. aureus	Al_2O_3NPs	ROS-mediated cell wall disruption	Ansari et al. (2013)
	S. aureus	TiO_2NPs	Protein deactivation	Roy et al. (2010)
	S. aureus	Cu/Zn bimetallic	DNA and protein inhibition	Ashfaq et al. (2016)
	S. aureus	Graphene Oxide NPs	Generation of ROS and heat	Pan et al. (2016a, b)
	S. aureus	SiNPs	Photoinactivation	Zhou et al. (2018)
Ampicillin resistant	*E. coli*, *P. aeruginosa*	AgNPs	Synergistic action with ampicillin	Lara et al. (2010)
	E. coli, *P. aeruginosa, S. aureus*, *E. aerogenes*	AuNPs	Synergistic action with ampicillin	Brown et al. (2012)
	K. pneumoniae	ZnONPs	ROS-mediated cell wall disruption	Reddy et al., (2014a, b)
Erythromycin resistant	*E. coli*, *P. aeruginosa, S. aureus*, *E. faecalis, S. typhimurium, B. subtilis*	AgNPs	Damage to components of cell wall	Otari et al. (2013)
Teicoplanin resistant	*S. pneumoniae*	AgNPs	ROS-mediated cell death	Thapa et al. (2017)
Tetracycline resistant	*E. coli*, *S. aureus*	AgNPs	Synergistic action with tetracycline	Djafari et al. (2016)
Ofloxacin resistant	*P. aeruginosa*	AgNPs	Inhibition of efflux pump	Ding et al. (2018)

(continued)

Table 1 (continued)

Drug-resistant bacterial pathogen		Active nanoparticles	Mode of action	References
Cefotaxime resistant	*E. coli*, *K. pneumoniae*	AuNPs	Cell wall and DNA damage	Shaikh et al. 2017
Kanamycin resistant	*S. bovis, S. epidermidis, E. aerogenes*	AuNPs	Cell wall disruption	Payne et al. (2016)
Carbapenem resistant	*P. mirabilis*, *A. baumannii*	AuNPs	Osmotic imbalance, Cell wall disruption	Shaker and Shabaan (2017)
Multidrug resistant	*E. coli*	AgNPs	ROS-mediated cell death	Zhang et al. (2013a, b)
	S. aureus, Enterococcus spp., P. aeruginosa, A. baumannii	AgNPs	Physico-chemical modification of cellular components	Cavassin et al. (2015)
	P. aeruginosa, S. aureus	AgNPs	Cell wall damage	Acharya et al. (2018)
	A. baumannii	AgNPs	Affecting the permeability of cell membrane	Chang et al. (2017)
	S. aureus, *E. coli*	AgNPs	Upregulation of ATP pumps	Nagy et al. 2011
	S. aureus	AuNPs	Photo inactivation	Galanzha et al. (2012)
	E. coli	AuNPs	ROS-mediated cell death	Zhang et al. (2013a, b)
	E. coli	AuNPs	Inhibition of protein synthesis	Cui et al. (2012)
	E. coli, K. pneumoniae, E. cloacae	AuNPs	Photo inactivation	Khan et al. (2017)
	S. aureus, *E. coli*	AuNPs	Interaction with cellular biomolecules	Kim et al. (2017)
	S. aureus, *E. coli*	AuNPs	Synergism with antibiotics	Pradeepa et al. (2016)
	E. coli	ZnONPs	ROS-mediated cell death	Chakraborti et al. (2014)
	S. aureus, *E. coli*	ZnONPs	Synergism with antibiotics	Ehsan and Sajjad (2017)
	S. aureus, *E. coli*	CuONPs	ROS-mediated cell death	Singh R. et al. (2014)
	Paracoccus denitrificans	CuONPs	Modulation of nitrogen metabolism	Su et al. (2015a, b)
	P. aeruginosa	CuNPs	Hydrosol-mediated cell death	Zhang et al. (2015)

(continued)

Table 1 (continued)

Drug-resistant bacterial pathogen		Active nanoparticles	Mode of action	References
	E. coli	Fe_3O_4NPs	Membrane dysfunction	Chaurasia et al. (2016)
	S. aureus, *E. coli*, *P. aeruginosa*	Fe_3O_4NPs	Modulation of electron transfer system	El-Zowalaty et al. (2015)
	E. coli	Al_2O_3NPs	Accumulation inside the cell wall	Ansari et al. (2014)
	E. coli	TiO_2NPs	ROS generation and cell wall disruption	Li et al. (2012)
	E. coli	TiO_2NPs	Oxidation and decomposition of membrane fatty acids	Joost et al. (2015)
	E. coli	Au/Pt bimetallic	Increasing intracellular ATP levels	Zhao et al. (2014)
	P. aeruginosa	Au/ Fe_3O_4 bimetallic	Cell wall disruption	Niemirowicz et al. (2014)
	S. aureus, *E. coli*, *S. mutans*	Cu/NI bimetallic	Cell wall modulation	Argueta-Figueroa et al. (2014)
	E. coli, E. faecalis	Graphene oxide NPs	ROS-mediated cell death	Govindaraju et al. (2016)
	E. coli, P. aeruginosa, K. pneumoniae, S. aureus	Graphene oxide NPs	Multiple toxicity	Jankauskaite et al. (2016)
	S. aureus, *E. coli*	SeNPs	Cell wall disruption	Huang et al. (2017)

titanium oxide NPs (TIONPs) were shown to elicit ROS generation, causing damage to cellular components and subsequent cell death (Foster et al. 2011). In another investigation on the antibacterial action of NPs, AgNPs inhibited bacterial growth by generating superoxide and hydroxyl radicals, while the bactericidal effect of gold, nickel and silicon was attributed to the production of singlet oxygen (Zhang et al. 2013a, b). NPs made of zinc, graphene and copper have also been shown to trigger oxidative stress, leading to antibacterial effects against drug-resistant pathogens (Reddy et al. 2014a, b; Pan et al. 2016a, b; Ulloa-Ogaz et al. 2017).

Metal oxides demonstrate antibiotic action by releasing metal ions that enter the bacterial cell where they interact with the functional groups of proteins and nucleic acids. This interaction alters the structure of the cell, hinders enzymatic activity and interferes with the physiology of the bacterial cell (Wang et al. 2017). Gold functionalized superparamagnetic iron oxide nanoparticles interact with the disulphide bonds of bacterial proteins, which disrupts their metabolism and redox systems (Niemirowicz et al. 2014). In another study, Copper oxide NPs were shown to cause alteration of the expression of key proteins leading to inhibition of denitrification

(Su et al. 2015a, b). Synergy between several antibacterial mechanisms was described as the possible reason for the bactericidal effect demonstrated by the graphene oxide/Cu/Ag nano-derivatives against drug resistant Gram-negative and Gram-positive pathogens (Jankauskaitė et al. 2016).

The cell wall and membrane protect bacteria from harsh environmental conditions and foreign agents. Thus non-oxidative NP antibacterial mechanisms affect the bacterial cell wall. Structural differences in the cell wall of Gram-negative and Gram-positive bacteria make the latter more vulnerable to the action of NPs (Wang et al. 2017). In a study conducted by Ansari et al., accumulation of NPs in the cell wall resulted in the formation of pits, holes and altered metabolism, leading to cell death (Ansari et al. 2014). TiONPs were shown to inhibit growth of *E. coli* by causing membrane leakage due to increased cell volume (Joost et al. 2015). In another study carried out to investigate the efficacy of gold nanoparticles (AuNPs) against *Corynebacterium pseudotuberculosis*, Transmission electron microscopy images showed that the thick cell wall of *C. pseudotuberculosis* was penetrated by AuNPs and accumulated as agglomerates inside the cell (Mohamed et al. 2017).

4 Nano-inhibitors of Quorum Sensing and Biofilm Formation

Biofilms are aggregations of bacteria adhered on a surface, or to each other, and encapsulated in a self-made matrix of extra polymeric substances (EPS). The EPS protects the cells from the entry of antibiotics and helps to confer tolerance to bacteria (Costerton et al. 1999; Bjarnsholt 2013). NPs disrupt biofilm formation by disturbing the EPS and by interfering with bacterial quorum sensing (QS). QS is a density-dependent communication system in some bacteria that coordinates the expression of various genes including those needed for biofilm production (Rutherford et al. 2014). Since QS systems promote the formation of drug-tolerant biofilms, it is not surprising that the interference of bacterial QS and, consequently, biofilm inhibition forms the basis for the development of new age antipathogenic drugs (LaSarre and Federle 2013; Jakobsen et al. 2017; Reen et al. 2018).

Numerous reports have emerged that have demonstrated the biofilm and QS inhibitory properties of NPs of which some are listed in Table 1. Silver nanowires (SNWs) demonstrated inhibition of QS-mediated biofilm production in *Pseudomonas aeruginosa* and violacein production in *Chromobacterium violaceum* ATCC 12472 (Wagh et al. 2013). AgCl-TiO_2 nanoparticles inhibited QS-regulated production of violacein and synthesis of the QS signalling compounds, acylated homoserine lactones (AHL), in *C. violaceum* (Naik and Kowshik 2014). Lee et al. reported that ZnO nanoparticles considerably inhibited the QS-regulated production of virulence factors and biofilm formation in *P. aeruginosa* without affecting viability (Lee et al. 2014).

Vinoj et al. described the synthesis of an AHL lactonase-coated Gold nanoparticles. These nanoparticles reduced exopolysaccharide (EPS) production and consequently disturbed the biofilm architecture of the pathogen *Proteus*. This reduction in EPS and inhibition of biofilm was attributed to the increased degradation of QS

signals (Vinoj et al. 2015). Green synthesized zinc nanostructures from an extract of *Nigella sativa* seed and demonstrated broad-spectrum QS inhibition against human and food pathogens (Al-Shabib et al. 2016). In another study, ZnONPs synthesized from the leaves of *Ochradenus baccutus* reduced biofilm formation in food-associated bacteria. These NPs were found to possess no serious toxic effects even at high doses. Moreover, they were found to have excellent antioxidant properties (Al-Shabib et al. 2018c). Monophasic tin dioxide nanoflowers (TONFs) assembled by rod-like nanostructures demonstrated inhibition of QS-regulated virulence in pathogens such as *C. violaceum*, *P. aeruginosa* and *Serratia marcescens*. Significant reduction in biofilm formation in all test pathogens was also observed, which was further validated by Confocal laser scanning microscopy images illustrating disturbed biofilm architecture (Al-Shabib et al. 2018a). Recently, phytofabricated AgNPs were shown to exhibit inhibition of QS-controlled functions and biofilm formation in drug-resistant pathogenic bacteria (Hussain et al. 2019).

ROS-mediated inhibition of biofilm has also been reported for NPs. AgNPs synthesized from the leaves of *Mangifera indica* demonstrated altered biofilm architecture in *E. coli* and *S. mutans*. Interaction of AgNPs with bacterial cell walls resulted in membrane damage, ROS production and biofilm inhibition (Qayyum et al. 2017). In another study, superparamagnetic iron oxide (Fe_3O_4) nanoparticles were tested against *S. marcescens, E. coli*, *P. aeruginosa* and *Listeria monocytogenes*. Iron oxide (Fe_3O_4) nanoparticles at sub-MIC levels demonstrated broad-spectrum inhibition of biofilm in all tested bacteria. These NPs also inhibited pre-formed mature biofilms. The proposed mechanism for these effects was the interaction of NPs with bacterial cells, which generated ROS and contributed to reduced biofilm formation (Al-Shabib et al. 2018b).

5 Factors Contributing to Antibacterial Activity of Nanomaterials

Many factors govern the antibacterial efficacy of nanomaterials. These aspects need to be kept in mind when designing and synthesizing nano-antibacterials to combat drug-resistant pathogens. Some of the key factors are discussed below:

5.1 Composition and Type of Nanomaterials

Different nanomaterials display different properties against diverse classes of bacteria. Considering the case of gold nanoparticles, they demonstrate poor antibacterial properties on their own but can potentiate the efficacy of some antibiotics when they are combined. Shaikh et al. reported the synthesis of cefotaxime-conjugated gold nanoparticles that demonstrated antibacterial activity against CTX-M producing drug-resistant strains of *E. coli* and *K. pneumoniae* (Shaikh et al. 2017). Similarly, liposomes conjugated with antibiotics have high efficiency against bacteria (Drulis-Kawa and Dorotkiewicz-Jach 2010). Thus, composition of

nanoantimicrobials is an important consideration because the fundamental make-up of the material defines its surface chemistry and only the surface corona interacts with bacteria. Therefore, it is imperative to consider the composition of the nanomaterials for a specific target organism (Xie et al. 2014).

5.2 Surface Functionalization

Surface modification is performed to facilitate the passage of nanomaterials across biological barriers, but this should be done with caution as it might alter the properties of the original material. Positively charged NPs interact well with negatively charged prokaryotic surfaces, albeit with some toxicity (Thorley and Tetley 2013; Jamil and Imran 2018). Hydrophobic NPs are converted to hydrophilic ones by conjugation with polymers like polyethylene glycol (PEG) and chitosan on the surface of NPs. This process not only makes the NPs hydrophilic but also masks the host immune response and increases blood circulation time of PEGylated NPs. Hydrophobic NPs are captured by the host immune system and are not able to act against infection causing bacteria. Thus, this surface modification helps in the bioavailability of NPs (Kumari et al. 2010). Delivery of drugs encapsulated in nanocarriers to a specific site is termed targeted drug delivery. With targeted drug delivery, the site of infection receives the maximum concentration of the drug, and other organs are protected from side effects. Thus, the action of the drug will be maximized, localized and prolonged (Mahon et al. 2012; Mandal et al. 2013).

5.3 Size of Nanomaterials

Particle size is an important factor in nanotechnology, as the stability of the material depends on its size (Huh and Kwon 2011). Nanosized particles behave differently as compared to their bulk material and display size-dependent effects. As increased surface area contributes to increased activity, nanosize means more surface area is exposed and therapeutic efficacy is enhanced (Yang and Mai 2014). Guo et al. demonstrated that the activity of AgNPs increased with greater surface area, because increased surface area causes the release of more Ag^{+} leading to enhanced antibacterial activity (Guo et al. 2013).

6 From Bench to Bed Side: Clinical Application of Nano-antibacterials

Currently, few NP-based strategies are undergoing clinical trials to treat bacterial infections, probably because the high cost associated with the application of nanomaterials has limited their use as compared to conventional antibacterials. However, in case of specific clinical conditions, or high-risk patients nanobiotics may be particularly useful (Caster et al. 2017). For example, AgTive (NCT00337714) is a

silver-based catheter with improved bactericidal properties. These catheters are manufactured from polyurethanes impregnated with AgNPs. AgTive releases considerably high amounts of Ag upon interaction with body fluids and intravenous solutions to reduce blood-stream infections (Antonelli et al. 2012). Another formulation comprised of AgNPs, chitosan and fluoride was developed and demonstrated antimicrobial properties. During clinical trials, nano silver fluoride was found to be very effective as an antibacterial against *S. mutans* and *Lactobacilli* and is used to prevent dental caries in children (Dos Santos et al. 2014).

Bio-kil® (Cargico, Taiwan) is a patented nanocomposite comprised of an inorganic metal part and organic quaternary ammonium part. This is a high affinity structure and possesses a strong electric field. Bio-kil® acts by damaging the membrane proteins of bacteria through its strong electric charge. Recently, it has demonstrated efficacy against MDR and environmental bacteria in ICUs (Lee et al. 2017). Another nanoformulation called Acticoat has been used for topically against bacteria. It is nanocrystalline silver that acts by releasing Ag into wounds and is reported to inhibit biofilm formation by *P. aeruginosa* and *Acinetobacter baumannii* in vitro (Potgieter and Meidany 2018). Similarly, the efficacy of SilvaSorb (NCT00659204) is being studied at Madigan Army Medical Center and is currently in Phase III clinical trials. SilvaSorb (AcryMed, Inc., Portland) is a gel made from AgNPs and is being investigated to compare the antimicrobial efficacy of a one-time application against standard antibacterial hand gel, in reducing bacterial counts from the hands of 40 patients seeded with *S. marcescens*.

7 Challenges of Ongoing and Future Research

Research carried out across the globe has shown the great potential that nanomaterials possess for the prevention and treatment of drug-resistant infection, but transferring the technology from the lab to the clinics remains a huge challenge. Assessment of interactions between nanoantimicrobials and cells, tissue and organs, dose calibrations and administration route are some of the big hurdles that are being encountered (Sandhiya et al. 2009). Biocompatibility is another issue as most results are based on in vitro studies. In vivo studies are required to investigate the toxicity, degradability and metabolism of NPs (Beyth et al. 2015). Studies have demonstrated the accumulation of NPs injected intravenously into organs like the colon, lungs, spleen, liver and lymphatics. Inhalation of NPs can lead to cytotoxicity at various respiratory organs by systemic circulation (Hagens et al. 2007; Poma and Giorgio 2008). NPs have demonstrated hepatotoxicity and nephrotoxicity due to the free radical-mediated oxidative stress generated via interaction of NPs with bacterial cell (Jong 2008).

Although NPs hold great potential as antibiotics for the future, there are still several questions related to their acute and long-term exposure to humans that need to be addressed. Several routes of exposure (oral and gastrointestinal tract, skin, organs of the respiratory system, blood stream) need very careful consideration when examining NP exposure (Matteis 2017). Physico-chemical properties of NPs

affect their interaction with biological systems and their overall biological activity. This is well documented as various researchers have explored the anti-infection potential of metal and metal oxide NPs both in vitro and in vivo, but questions remain regarding the safety of these NPs. Compounding the problem is the vast number of different shapes, sizes, surface modifications that NPs possess; therefore, different methods are needed to evaluate their safety. Further, most studies report acute exposure rather than long-term exposure (Matteis 2017; Baptista et al. 2018; Warheit 2018). On the contrary, as research has also provided leads as to the specific mechanisms by which NPs exert toxic effects, careful surface modifications can be done to make them safe, stable and less toxic (Matteis 2017). The European Commission sponsored programmes FP7 and H2020 have helped to address concerns related to the safety of the nanoparticles (Warheit 2018). Furthermore, replacement, reduction and refinement policies related to in vivo studies have prompted regulatory agencies to realize the need for standard in vitro methodologies to establish toxicology profiles of NPs and direct labs to construct authentic and reliable databases of NPs based on their toxicological profiles. These steps will surely help in generating information on finding the right dosage at which a NP is safe for use as a therapeutic agent.

8 Conclusion

The emergence of drug resistance among pathogenic bacteria has made clinicians and researchers seek new novel drugs. In the last 10 years' nanotechnology-derived therapeutics have taken centre stage as alternatives to conventional antibiotics. Vast reports on the antibacterial activity of NPs have made it imperative to study and understand the mechanism of their action. Now there is a need for serious consideration of the critical factors related to nanostructures for successful and safe implementation as nanobiotics. Further, detailed study is required to ensure the safety of these NPs before their clinical usage. Finally, we need to understand and address the financial feasibility of transferring these nanomaterials from the lab to the clinics.

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Nanomaterials as a Novel Class of Anti-infective Agents that Attenuate Bacterial Quorum Sensing

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Abstract

Excessive and unabated use of antibiotics has led to the emergence of multi-drug resistant (MDR) bacteria. The ineffectiveness of current antibiotic therapy and the slow development of new drugs with novel modes of action have made the task of combating MDR infections even more difficult. The problem of multi-

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drug resistance among pathogens has prompted the scientific community to look for alternative strategies. One such approach, termed antipathogenic/antivirulence therapy, is considered to be a viable alternative. This strategy is focused on rendering the pathogen ineffective by inhibiting its virulence traits rather than killing it. Since antipathogenic/antivirulence compounds target bacterial virulence, the likelihood of developing resistance is also reduced considerably. The areas of major interest in the antivirulence approach include the inhibition of quorum sensing and biofilm formation. The pioneering discovery of halogenated furanones as quorum sensing inhibitors (QSIs) has prompted the scientific community to search for novel QSIs of both natural and synthetic origin. However, QSIs like furanones are not recommended for human use due to issues related to their toxicity and stability. Recently, researchers across the globe have turned their attention toward nanomaterials as potential anti-infective drugs, targeting QS and biofilm formation. Although there are numerous studies regarding the antibacterial effect of nanoparticles, current reports on the antiquorum sensing and biofilm inhibition properties are still scarce. Therefore, in this article we have made an honest attempt to summarize the reports on the anti-QS properties of nanoparticles and their future as novel anti-infective drugs.

Keywords
Anti-infective · Quorum sensing · Biofilm · Nanoparticles · Nanocarriers · Nano-QSIs

1 Drug Resistance and New Antibacterial Drug Discovery

Globally, a substantial threat to public health is the emergence and spread of multidrug resistance (MDR) in pathogenic bacteria. The development and application of antibiotics in the medical field over the last 70 years is now seriously threatened by a substantial increase in patient mortality and morbidity due to drug-resistant bacterial infections (Payne 2008). The problem of antibiotic resistance is expected to increase in the future due to the excessive and indiscriminate use of antibacterial drugs both in medical and nonmedical settings. In fact, the rise of MDR bacteria was recently ranked as the third greatest threat to human health by the World Health Organization (Bassetti et al. 2011). Resistance in bacteria develops either through mutations, or by acquisition via horizontal gene transfer (transduction and conjugation), and the major reservoir of antibiotic resistance genes may be nonpathogenic strains and the antibiotic producers themselves (D'Costa et al. 2006; Martinez 2008). An example of one of the many complex strategies employed by pathogens to achieve antimicrobial resistance is the novel mechanism of genetic and cellular exchange by nanotube formation between two related or unrelated bacteria (Dubey and Yahuda 2011; Wagner et al. 2017).

The problem of bacterial resistance against the presently approved antibiotics is further exacerbated by the lack of investments by the pharmaceutical industry in the discovery of new antibiotics, given the inherently low rate of return for antibiotics

in comparison to drugs targeted at chronic diseases (Spellberg et al. 2007). Thus, one of the strategies for the treatment of MDR bacterial infections is the development of new antibiotics. However, from 2008 till 2012, only two new antibiotics have been permitted for human use by the US FDA, and neither of them has shown significantly potent activity against Gram-negative bacteria (Spellberg 2012). Additionally, a classical antimicrobial introduced to bacteria invariably will see resistance developed against it, and this solely depends on its bacteriostatic/bactericidal mechanism. In general, resistance appears in a span of a few months to years following the application of a new antibiotic (Walsh 2000). For example, in 2003 the clinical application Daptomycin (an antibiotic) was endorsed for patients infected with Enterococcus faecium and methicillin-resistant Staphylococcus aureus (MRSA), but resistant strains were identified in less than a year after its implementation (Dolgin 2010). Thus, alternative approaches for controlling bacterial infections are desperately needed.

2 Need for New Drug Targets and Nanomaterials

Considering the current scenario of the lack of novel drug development by companies, and the increasing trends of MDR in hospitals, community, and environment-associated bacteria, there is an urgent need to develop new strategies to face the problem. Various possible solutions have been described, including (1) "the magic bullet" concept, i.e., discovery of a powerful new antibacterial, (2) combinational approach, and (3) alternative strategies, such as antipathogenic/ antivirulence methods (Aqil et al. 2006; Ahmad et al. 2009). In the past decade, antipathogenic drug targets have been identified and validated, and antipathogenic drugs are an alternative to killing bacteria or stopping their growth, disarming them instead. Thus, many researchers have begun to develop drugs that inhibit bacterial virulence, rather than affect their viability (Cegelski et al. 2008; Escaich 2010; Zucca et al. 2011; Silva et al. 2016).

Targeting virulence offers several potential advantages, including:

(i) An increased repertoire of pharmacological targets
(ii) Generating antimicrobials with new mechanisms of action
(iii) Decreasing resistance development, owing to reduced selective pressure
(iv) Potentially maintaining gut microbiota

Furthermore, the development of antivirulence therapies offers its own specific challenges. For the development of antivirulence therapies, the use of conventional drug screening systems for the killing or inhibiting of bacterial growth, and the measurement of minimal inhibitory concentrations, is not applicable (Heras et al. 2014). Therefore, for the screening of new compounds that specifically inhibit virulence, novel in vitro and in vivo assays are necessary. However, there is a very narrow spectrum of antivirulence drugs, as the virulence mechanisms vary from one bacterium to another. Real-time diagnostic development will recognize/detect the causative organism and make therapies tailored toward particular infectious agents, thus leading to potentially new drugs for clinical use (Heras et al. 2014).

In the recent years, various targets, such as biofilm development and other virulence factors, as well as quorum sensing (QS) systems, which regulate the expression of many virulence factors in a cell density-dependent manner, have been identified as novel anti-infective drug targets (Roman et al. 2013; Kamal et al. 2017; Soukarieh et al. 2018).

One avenue for the development of new antimicrobial agents involves utilizing novel and/or modulated, conventional antimicrobial activities in the development of nanomaterial as pharmaceuticals. The variable physicochemical and functionalizational (due to tagging of different ligand motifs) properties of nanoparticles (NPs) have given a new dimension to antimicrobial drug development (Ngoy et al. 2011; Gilbertson et al. 2014; Parise et al. 2014). The diverse physicochemical properties of NPs have made them potential candidates for remarkable applications in biomedicine, particularly antimicrobial and drug delivery (Gilbertson et al. 2014; Venkatesan et al. 2014; Qi et al. 2015). Some examples of these biomedical NPs include silver nanoparticles (AgNPs) (Adeli et al. 2013), carbon nanotubes (CNTs) (Subbiah et al., 2011), gold NPs (AuNPs) (Addae et al. 2014), zinc oxide NPs (ZnO-NPs) (Khan et al. 2016), and iron oxide NPs (FeO-NPs) (Sunitha et al. 2013).

The antimicrobial actions of NPs include bactericidal destruction of cell membranes, blockage of enzyme pathways, alterations of the microbial cell wall, and inhibition of nucleic acid synthesis (Galdiero et al. 2011; Hoseinzadeh et al. 2017). However, the full array of antimicrobial mechanisms is yet to be fully elucidated, since some of the NPs are still in their infancy. Regardless, the high potency of NP antiviral, antibacterial, antifungal, and antiprotozoal activities have revolutionized and brought about a turning point to pharmacological therapy. However, although there are numerous studies regarding the antibacterial effect of nanoparticles, reports on the anti-QS and biofilm inhibition activities are still scarce. Therefore, in this chapter, we have summarized the current state of research on the anti-QS and antibiofilm properties of NPs and their future as novel anti-infective drugs.

3 Quorum Sensing

Bacterial cell-to-cell communication is achieved through QS mechanisms that are density- or population-dependent and are governed by the synthesis, exchange, and perception of small signal molecules known as autoinducers. QS signals typically activate specific receptors that function as transcriptional regulators. As the autoinducer signals reach a threshold limit, the transcriptional regulators detect their presence and induce the expression of gene sets responsible for processes such as bioluminescence, pigment production, other virulence factors (Pearson et al. 1994; Ji et al. 1997; Whiteley et al. 1999; Michael et al. 2001; Smith and Iglewski 2003; Chan et al. 2004; Wright et al. 2005; Janssens et al. 2007), biofilm formation (Davies et al. 1998; Singh et al. 2000; Cvitkovitch et al. 2003; Rickard et al. 2006), and antibiotic resistance (Ahmed et al. 2007). Various QS modes have been reported, and some bacteria employ multiple modes for environment monitoring.

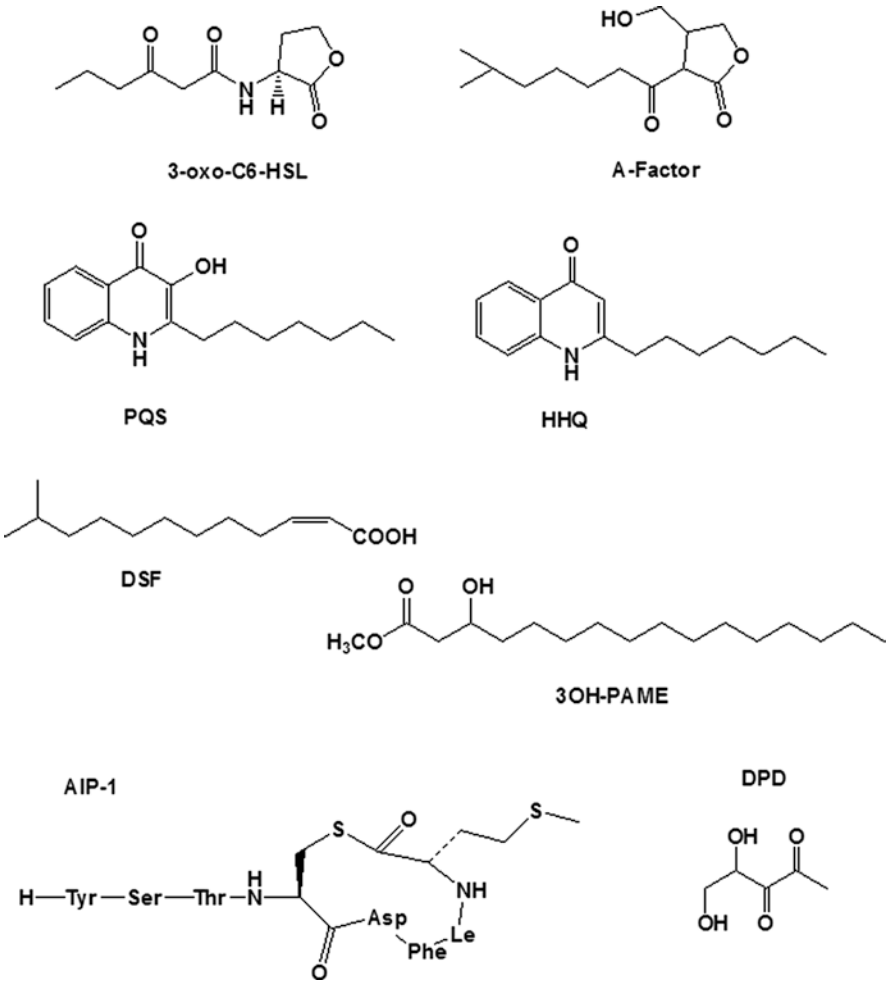

Fig. 1 QS signal molecules. 3-oxo- C6-HSL, N-(3-oxohexanoyl)-L-homoserine lactone; A-Factor, 2-isocapryloyl-3-hydroxymethyl- c-butyrolactone; PQS, *Pseudomonas* quinolone signal, 2-heptyl-3-hydroxy-4(1H)- quinolone; HHQ, 2-heptyl-4(1H)-quinolone; DSF, 'diffusible factor," cis-11-methyl-2-dodecenoic acid; 3OH-PAME, hydroxyl-palmitic acid methyl ester; AIP-1, staphylococcal autoinducing peptide 1; DPD, the AI-2 precursor, 4,5 dihydroxy-2,3-pentanedione

A remarkable array of signaling molecules function as sensors for cell-to-cell communication, as shown in Fig. 1. The most common type of signaling molecule used for intraspecies communication are peptides in Gram-positive bacteria (Shaw et al. 1993; Hiramatsu et al. 1997; Rasheed et al. 1997; Gonzales et al. 2001), and acyl homoserine lactones (AHL; also called AI-1) (Pearson et al. 1997; Waters and Bassler 2005; Geske et al. 2008) in Gram-negative bacteria. Various other

molecules, such as such as quinolones (Tally and DeBruin, 2000), hydroxy ketones (Barbachyn and Ford 2003), and g-butyrolactones (Jones et al. 2002), are also employed as intraspecies communication molecules in Pseudomonas aeruginosa (Pesci et al. 1999), *Vibrio cholerae* (Cámara et al. 2002; Higgins et al. 2007), and *Streptomyces* (Horinouchi et al. 2001), respectively.

The N-AHL molecules vary in N-acyl chain length (4–18 carbon atoms), degree of saturation of the N-acyl chain, and the number of oxygen substitutions, however the L-isomeric form of the homoserine Lactone ring is universal to all AHLs. A particular AHL is synthesized by a specific AHL synthase, which may additionally produce a different AHL in smaller amounts. After synthesis, AHLs majorly diffuse freely across cell envelopes, or actively transported across the cell membrane in certain strains (Evans et al. 1998; Pearson et al. 1994; Chan et al. 2007). The receptor for AHLs is mediated by the *luxR* gene, which translates to form LuxR receptor protein molecules. AHLs, upon reaching the threshold critical concentration in their interaction with LuxR receptor proteins, trigger the formation of a complex which acts at the promoter sites for QS-responsive operons in the bacterial genome. *Chrombacterium violaceum* exhibits a typical example of LuxI/R signaling, as shown in Fig. 2a.

Alternatively, membrane-bound sensor kinases are also found to detect AHLs, such as LuxN of *Vibrio Harveyi*, which initiate phosphorelay signaling cascades upon binding with AHL (Bassler et al. 1993). The N-acyl chain variabilities in length, degree of saturation, and oxidation states governs the binding specificity with the partner receptor protein molecule. Mostly, bacterial species carry a specific cognate synthase/receptor pair that produces and operates with specific AHL

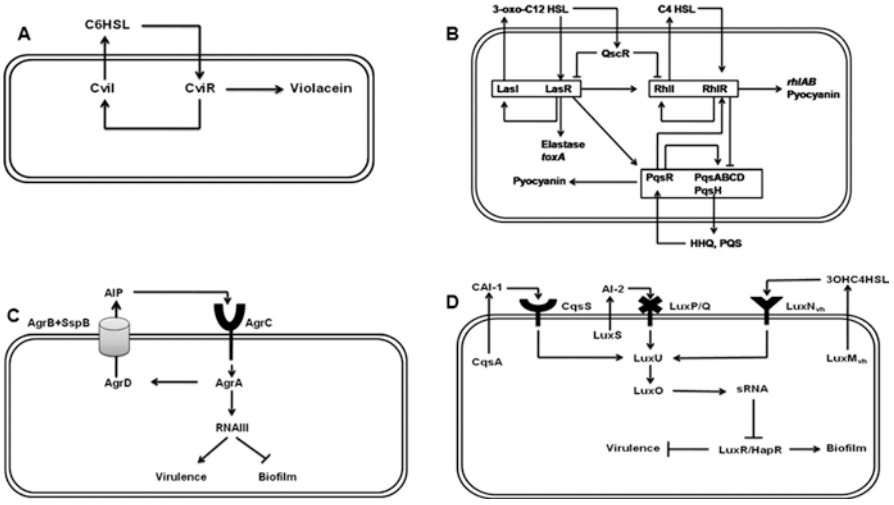

Fig. 2 Simplified schemes of four quorum-sensing networks. Arrows imply information flow. (**a**). *Chromobacterium violaceum*; (**b**). *Pseudomonas aeruginosa*; (**c**). *Staphylococcus aureus*; and (**d**). *Vibrio harveyi*

molecules. P. aeruginosa exhibits multiple synthase/receptor pairs for different AHL molecules, while some other bacteria harbor only a single LuxR homologue without having a cognate LuxI-type synthase for AHL production (e.g., SdiA of Escherichia coli and QscR of P. aeruginosa) (Subramoni and Venturi 2009; Jimenez et al. 2012). P. aeruginosa has complex QS circuitry, harboring three specific signal/receptor pairs. Two *LuxIR* homologous pairs, *LasIR* and *RhlIR*, which mediate 3OC12HSL and C4HSL, respectively, and a non-*LuxIR*-type system, called the *Pseudomonas* quinolone signal (PQS) system. The three QS systems are interregulatory, and control several virulence factor-encoding genes, among which some are coregulated by other QS systems, as depicted in Fig. 2b (Dubern and Diggle 2008; Jimenez et al. 2012).

The autoinducer Type 2 (AI-2) system is the most ubiquitous signaling system expressed by both Gram-negative and Gram-positive bacteria (Fig. 2d) (Xavier et al. 2003; Vendeville et al. 2005). It is mediated by the *luxS* gene locus and related homologues. Structurally, it is derived from 4,5-dihydroxyl-2,3-pentadione (DPD) (Chen et al. 2002; Miller et al. 2004). The AI-2 pathway uses a more complex, two-component, receptor kinase network for signaling amongst bacteria. The receptor for the AI-2 apparatus shows much variability; the LuxP receptor in *Vibrio* species and LsrB in E. coli/*Salmonella* bind to different forms of AI-2. LsrR binds to the phosphorylated, cell-internalized AI-2 molecule and plays the role of a transcriptional activator (Federle and Bassler 2003; Xue et al. 2009). Numerous variations of the AI-2 system occur in bacteria. The existence of a solo AI-2 receptor complex has been reported, where many bacteria do not express the *LuxS* gene. These strains sense and use AI-2 signals generated by other bacteria to regulate their own coordinated transcriptional responses. The role of AI-2 as a mediator of virulence factors has been implicated in *Vibrio vulnificus* (Kim et al. 2003; Shin et al. 2004), *Serratia marcescens* (Coulthurst et al. 2004) and Clostridium perfringens (Ohtani et al. 2002). LuxS, which is the synthase for AI-2, might have an additional role in the regulation of metabolic pathways (Winzer et al. 2002; Vendeville et al. 2005). Interestingly, AI-2 formation from ribulose-5-phosphate, without the involvement of LuxS synthase, has also been proposed (Hauck et al. 2003; Li et al. 2006; Tavender et al. 2008), and the significant role of alternative AI-2 synthesis in QS needs to be further explored.

The autoinducer type 3 (AI-3)-system is far more complicated than the other two. Akin to AI-2, it also uses a two-component, receptor kinase, intracellular signaling complex to activate the genes of the virulome. The characteristic feature of the AI-3 system is that it can use the human stress hormones, epinephrine, or norepinephrine, to signal the system (Walters et al. 2006; Hughes and Sperandio 2008). The periplasmic receptor for the AI-3 system is already characterized and it is known as the QseBC complex, where QseC acts as the sensor kinase, and QseB plays the role of the phosphorylated response regulator that alters the transcription of virulence genes. The AI-3 system is utilized in the pathogenesis of enterohemorrhagic *E. coli* infections and shigellosis (Hughes and Sperandio 2008).

4 Interference Strategies in Quorum Sensing

Being a population-dependent phenomenon and not essentially required for overall survival of bacteria, quorum sensing network could be considered as strategical targets for controlling bacterial pathogenicity. Targeting the quorum sensing network could effectively be used for controlling virulence factor secretion, biofilm formation, and drug resistance. Therefore, antipathogenic drug development via targeting quorum sensing is emerging as an attractive alternative tool for controlling bacterial infections (Hentzer and Givkov, 2003). Based on the overall architecture of the QS circuit, three major targets for QS inhibition have been described as (a) the signal generation assembly, (b) the signaling molecule, and (c) the signal receptor assembly as described in Fig. 3 (Hentzer and Givskov 2003; Rasmussen and Givskov 2006; Ni et al. 2009; Kalia et al. 2013; Defoirdt 2017).

4.1 Targeting Signal Generation

Quorum sensing machinery in the majority of the Gram-negative bacteria employed acyl homoserines lactones of different side chains as a signaling molecule. The AHL synthase complex involves the production of AHL molecules from a corresponding acyl chain and S-adenosylmethionine (SAM) (Schaefer et al. 1996). Thus, QS inhibitors can be designed for targeting AHL synthase, acyl chain biosynthesis, and SAM biosynthetic pathways. Various analogues of SAM such as S-adenosylcysteine, S-adenosylhomocysteine, and sinefungin have been demonstrated as potent inhibitors of AHL synthesis catalyzed by the *P. aeruginosa* RhlI protein (Hoang and Schweizer 1999). Moreover, S-adenosylcysteine specifically inhibited *P. aeruginosa* LuxI homolog RhlI up to 97% (Parsek et al. 1999). After synthesis, all the long-chain AHL molecules are transported out of the bacterial

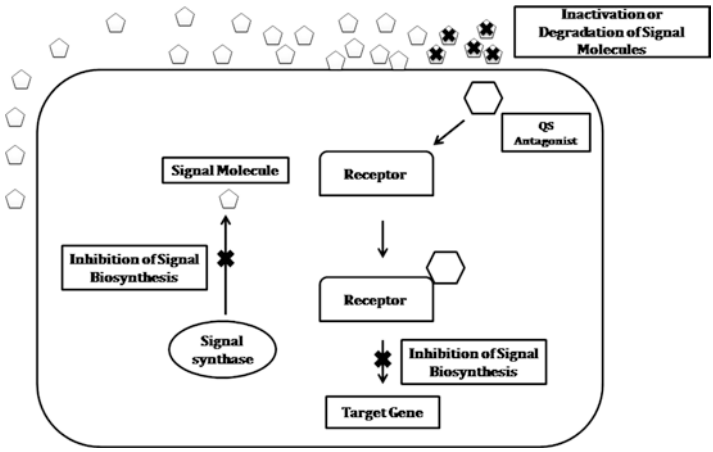

Fig. 3 Schematic view of different QS inhibition strategies

cells, and the process is mediated through efflux pump (Horinouchi et al., 2001). Thus, molecules deactivating other efflux pumps could also be developed as effective AHL-specific efflux pumps inhibitors, ultimately barring QS signal transduction (Varga et al. 2012).

4.2 Degradation of the Signaling Molecules

AHL-mediated QS communication could also be dissipated by direct degradation or inactivation of the signaling molecules. Complete degradation of signaling molecules is mediated either through enzymatic degradation or by chemical-induced inactivation. Different enzymes such as AHL-acylase, AHL-lactonase, oxidoreductase, and paraoxonase have been described to possess AHL degrading activity. Such enzyme-mediated degradation of quorum sensing signaling molecule is termed as quorum quenching (QQ). First report of the AHL-degrading enzyme was the isolation of the *Bacillus* species capable of producing lactonase and cleave the AHL molecule into the lactone ring and acyl chain (Dong et al. 2000). Following the report of lactonase-producing *Bacillus*, acylase-producing *Variovorax* species and *Ralstonia* sp. were isolated (Leadbetter et al. 2000; Lin et al., 2003). Since then, numerous microbes, and eukaryotes as well, displayed efficient QQ activity via the production of degrading enzymes (Chen et al. 2013). These enzymes can be broadly classified into two classes: (i) first group includes the enzymes which contain AHL-lactonase, AHL-acylase, and paraoxonase, while (ii) class second encompasses those AHL-degrading enzymes that reduce carbonyl to hydroxyl (oxidoreductase) (Chen et al. 2013). Moreover, the AHL degrading ability of bacteria in a community is also considered as a means of gaining a selective advantage. Through AHL degradation, some bacteria blocks QS network of competing bacteria while others can use AHLs as the source of energy source (Huang et al. 2003; Bhardwaj et al. 2013).

4.3 Interference of the Signal Reception

Signal reception followed by expression of various QS-linked traits can be inhibited by an antagonistic molecule mimicking the AHL signal molecule structure. The receptor protein is inhibited by the antagonist molecule either by competing or interfering with the corresponding AHL molecule. Numerous synthetic AHL analogues have been designed by the modifications in the AHL structure (Geske et al. 2007; Guo et al. 2015). Halogenated furanones, previously characterized as highly active QS-inhibiting molecules, have also been modified and proven to be potential QS inhibitors (Wu et al. 2004) Also, several natural antagonistic molecules have been isolated and characterized (Koh et al. 2013; Silva et al. 2016; Paczkowski et al. 2017; Khan et al. 2018).

Recently, large number of natural and synthetic molecule library have been screened using in silico tools, and numerous potential signal interfering compound have been identified (Tan et al. 2013; Byeon et al. 2017; Vinothkannan et al. 2018; Ding et al. 2019).

In general, different components of QS systems offer a potential target for the design and development of wide range of QS inhibitors. For instance, inhibitors targeting QseC, the receptor assembly characterized in AI-3, have the potential to affect a wide range of bacteria (Rasko et al. 2008; Ni et al. 2009).

4.4 Criteria for Selecting Quorum Sensing Inhibitors

Hentzer and Givskov (2003), Rasmussen and Givskov (2006), and Vattem et al. (2007) outlined the following criteria for selecting an effective QS inhibitor. An effective QS inhibitor should be (1) a small molecule (2) should be highly specific with no antagonistic effect(s) on the bacteria or the host, (3) should be stable and should not be degraded by the host metabolic system, (4) should be bigger than the native AHL, (5) should not be toxic to the hosts, and (6) should not interfere with the protein synthesis, DNA metabolism, or cell wall formation, as these are targets that commonly lead to the acquisition of drug resistance.

5 Nano-Quorum Sensing Inhibitors: An Emerging Class of Anti-infective Agents

A wide variety of nanostructures have been identified as promising anti-infective agents for modulating the QS circuits of bacteria, rather than their growth (Table 1). The synthesis of nanoparticles is complex, and several physical and chemical methods have used traditionally. Chemical reduction is the most frequently applied method; however, studies have shown that the use of a chemical reducing agent results in more consumption of energy and generation of larger particles with less stability and more agglomeration. Moreover, side products that are formed as result of synthesis are not ecologically friendly (Mukherjee et al. 2001). Therefore, biological methods/green synthesis methods have also been employed, as this route of synthesis is eco-friendly. Moreover, this approach is cost-effective, rapid, less laborious, easily scalable, and more efficient than the chemical methods used previously. In general, green nanoparticles are much more compatible for various biomedical and pharmaceutical applications, as toxic chemicals are not used during synthesis (Bhainsa and D'Souza 2006).

6 Chemically Synthesized Nano-Quorum Sensing Inhibitors

Chemically synthesized metal nanoparticles are considered to be effective antipathogenic drugs. Silver nanowires (SNWs), synthesized by using ethylene glycol (reducing agent) and capping agent, polyvinylpyrrolidone (PVP), demonstrated inhibition of quorum sensing-mediated biofilm production in *P. aeruginosa* NCIM 2948, and violacein synthesis in *Chromobacterium violaceum* ATCC 12472 (Wagh

Table 1 Nano-quorum sensing inhibitors

Nanomaterials	Target bacteria	QS regulated function	References
Silver (Ag) nanowires	*P.* aeruginosa	Biofilm	Wagh et al. (2013)
	C. violaceum	Violacein	
Silver (Ag) nanoparticles synthesized from leaves of *Cymbopogon* citratus	*S. aureus*	Biofilm	Masurkar et al. (2012)
Silver (Ag) nanoparticles synthesized from Sargassum polyphyllum	*C. violaceum* CVO26	Violacein	Arunkumar et al. (2014)
	P. aeruginosa	Swarming motility	
Ag	*P. aeruginosa*	Biofilm	Martinez-Gutierrez et al. (2013)
Ag nanoparticles stabilized by casein	*E. coli*	Biofilm	Radzig et al. (2013)
	P. aeruginosa		
	Serratia proteamaculans		
Ag nanoparticles precipitated from *Bacillus licheniformis*	*S.* epidermidis	Biofilm	Kalishwaralal et al. (2010)
	P. aeruginosa		
Silver chloride-titania (Ag-Cl-TiO_2) nanocomposite	*C. violaceum*	Violacein, acyl homoserine lactone (AHL) production	Naik and Kowshik (2014)
Ag nanoparticles immobilized on titanium	*S. epidermidis*	Biofilm	Qin et al. (2014)
Silver-decorated lithium vanadium oxide (LiV_2O_5/Ag) nanocomposites	*B subtilis*	Biofilm	Gambino et al. (2015)
	E coli		
Ag nanoparticles from *Rhizopus arrhizus (A* fungus of the family Mucoraceae)	*P. aeruginosa*	LasA protease, LasB elastrase, pyocyanin, pyoverdin, pyochelin, rhamnolipid, alginate, biofilm and AHL production	Singh et al. (2016)
Phytofabrication of Silver (Ag) nanoparticles using Piper betle leaf	*P. aeruginosa* *E. coli* *Serratia marcescens* Proteus mirabilis	Prodigiosin, protease, biofilm formation, exopolysaccharides and hydrophobicity productions	Srinivasan et al. (2018)
Ag–TiO2, TiO2–Ag, Ag–Cu and Cu–Ag nanocomposites	*P. aeruginosa* *E. coli* *S. aureus*	Pyocyanin production, swarming motility and biofilm formation	Alavi and Karimi (2018)
Toluidine blue O-silver nanoparticle conjugate	*S. mutans*	Biofilm	Misba et al. (2016)
Gold (Au) based herbo-metallic Nanoformulation	*S. mutans*	QS signalling	Singh et al. (2015)

(continued)

Table 1 (continued)

Nanomaterials	Target bacteria	QS regulated function	References
Gold (Au) nanoparticles coated with AHL lactonase	*Proteus*	EPS production, biofilm	Vinoj et al. (2015)
Gold (Au) nanoparticles synthesis intracellularly using *Laccaria fraternal* mycelia	*P. aeruginosa*	Pyocyanin production and biofilm formation	Samanta et al. (2017)
Zinc oxide (ZnO) nanoparticles	*P. aeruginosa*	Pyocyanin, Pseudomonas quinolone signal (PQS), pyochelin, hemolysis	Lee et al. (2014)
Zinc oxide (ZnO) nanoparticles	*P. aeruginosa*	Elastase, pyocyanin, biofilm	Garcıa-Lara et al. (2015)
ZnO nanostructures synthesized from seed extract of Nigella sativa *(herbal);*	*C. violaceum*	Violacein, exopolysaccharide (EPS), swarming motility, biofilm	Al-Shabib et al. (2016)
	P. aeruginosa	Elastase, protease, pyocyanin, alginate production, EPS, swarming motility, biofilm	
	E. coli	EPS, swarming motility, biofilm	
	L monocytogenes	EPS, swarming motility, biofilm	
ZnO nanoparticles synthesized from leaves *Ochradenus baccatus*	*P. aeruginosa* *C violaceum* *S. marcescens* L. monocytogenes, *E. coli*	alginate production, EPS, swarming motility, biofilm Violacein, EPS biofilm Prodigiosin, EPS, swarming motility, Biofilm EPS, swarming motility, Biofilm	Al-Shabib et al. (2018)
Zno-polyvinyl chloride nanocomposite	*S. aureus*	Biofilm	Seil and Webster (2011)
Iron oxide nanoparticle	*S. aureus*	Biofilm	Thukkaram et al. (2014)
	P. aeruginosa		
Biological nanofactories	*Salmonella typhimurium*	QS modulation	Fernandes et al. (2010)
	E. coli		
Cinnamaldehyde encapsulated chitosan nanoparticles	*P. aeruginosa*	QS modulation and biofilm formation	Subhaswaraj et al. (2018)
Polyethyleneglycol coated CAI-I nanocarriers	*V. chlorae*	Biofilm	Lu et al. (2015)

(continued)

Table 1 (continued)

Nanomaterials	Target bacteria	QS regulated function	References
Silicon-dioxide nanoparticles surface functionalized with –cyclodextrin	*Vibrio fischeri*	AHL reduction	Miller et al. (2015)
Pegylated Ag-coated single-walled carbon nanotubes	*S typhimurium*	Down regulation of *ybeF* and *sdiA* genes involved in biofilm formation and quorum sensing	Chaudhari et al. (2015)
Calcium fluoride nanoparticles	*S. mutans*	Biofilm	Kulshrestha et al. (2016)

et al. 2013). Similarly, AgCl-TiO_2 (AT) nanoparticles inhibited QS-regulated pigment (violacein) production and AHL synthesis in *C. violaceum* (Naik and Kowshik 2014). Lee et al. (2014) found that ZnO nanoparticles considerably inhibited the QS-regulated production of pyocyanin, *Pseudomonas* quinolone signal (PQS), pyochelin, hemolytic activity, and biofilm formation of *P. aeruginosa* without affecting the viability. Observed phenotypic changes were attributed to the induction of the zinc cation efflux pump *czc* operon and several important transcriptional regulators (porin gene *opdT* and type III repressor *ptrA*), and repression of the pyocyanin-related *phz* operon by ZnO nanoparticles. In addition, biofilm inhibition was due to the increased cellular hydrophilicity of *P. aeruginosa* cells after treatment with ZnO nanoparticles. In another study, Lara-Garcia et al. (2015) tested ZnO nanoparticles against six clinical strains from cystic fibrosis patients: a furanone C-30 resistant clinical strain from urine, two PA14 gallium-resistant mutants, a PA14 C-30 resistant mutant, and four environmental isolates of *P. aeruginosa*. They found that ZnO nanoparticles demonstrated broad-spectrum anti-QS activity and significantly reduced the production of virulence factors, such as elastase, pyocyanin, and biofilm formation for most of the strains.

Many other examples of the efficacy of chemically synthesized nano-QS inhibitors can be found in the literature. For instance, Lu et al. (2015) used flash nanoprecipitation for the synthesis of polyethylene glycol (PEG)-coated, water-dispersible CAI-I autoinducer nanocarriers that reduced biofilm forming capabilities of the pathogen *Vibrio cholera.* Also, surface functionalization of silicon-dioxide nanoparticles with β-cyclodextrin (β-CD) reduced the acylhomoserinelactone (AHL) based cell-to-cell communication/quorum sensing (QS) in *Vibrio fischeri* (Miller et al. 2015). Additionally, Chaudhari et al. (2015) synthesized PEGylated silver-coated single-walled carbon nanotubes (pAgC) that interfered with the QS mechanisms of *Salmonella typhimurium*. Further, pAgC nanotube-treated *S. typhimurium* cells showed downregulated expression of *ybeF* (a transcriptional regulator of the *lysR* family that plays and important role in biofilm formation and virulence) and *sdiA* (a receptor for N-acyl-L-homoserine lactones involved in QS). Last, a study was undertaken to examine the mechanism of action of silver nanoparticles in inhibiting QS-regulated biofilm formation by *P. aeruginosa* through the LuxI/LuxR system of

signal transduction. LasR, QscR, RhlR, and Vfr QS systems were included in the study, and molecular docking results suggested that the biofilm formation in Gram-negative bacteria can be inhibited by silver nanoparticles at the level of signal transduction (Vyshnava et al. 2016).

7 Biogenic Nano-QS Inhibitors

Owing to the toxicity and stability issues with chemically synthesized nanoparticles, researchers have turned their attention toward eco-friendlier and cost-effective biological, or "green" synthesis of nanomaterials. For example, Fernandes et al. (2010) synthesized engineered biological nanofactories comprised of antibodies (for targeting) and a fusion protein to modulate the QS response in targeted bacteria, such as *Salmonella typhimurium* and *E. coli*. Green silver nanoparticles synthesized from the leaves of *Cymbopogon citratus* (lemongrass) exhibited significant reduction in the biofilm-forming capabilities of *S. aureus*, and increased quorum quenching activity (Masurkar et al. 2012). Similarly, silver nanoparticles fabricated from alga *Sargassum polyphyllum* (brown seaweed) demonstrated reduced violacein production in a mutant strain of *C. violaceum* CVO26. These biogenic silver nanoparticles also reduced swarming migration of *P. aeruginosa*, indicating broad-spectrum QSI (Arunkumar et al. 2014). Ultrasmall solid lipid (us-SL) nanoparticles were examined for their antivirulence properties in *P. aeruginosa* by quantifying QS-dependent pyocyanin production. The synthesized nanoparticles decreased pyocyanin production by sevenfold in comparison to free compounds (Nafees et al. 2014).

Vinoj et al. (2015) used AHL lactonase (AiiA) proteins, known to inhibit QS signalling molecules, for biological synthesis of AHL lactonase-coated Gold nanoparticles. These nanoparticles reduced exopolysaccharide (EPS) production and consequently disturbed the biofilm architecture of a pathogenic *Proteus* species. This reduction in EPS and inhibition of biofilm was attributed to the increased degradation of QS signals. In another study, herbo-metallic colloidal nanoformulation was developed using gold nanoparticles and an extract of the polyphenol-rich medicinal lichen, Usnea longissima, to assess the anti-QS properties against *Streptococcus mutans* (Singh et al. 2015). These 28 nm crystalline nanoparticles inhibited violacein production in *C. violaceum* 12,472 at sublethal concentrations (5, 10 and 15%). Further, treatment with the synthesized herbo-metallic nanoformulation demonstrated a reduction in the production of an array of virulence factors, including acid production, ATPase, enolase, lactate dehydrogenase, protease, total exopolysaccharide content, and glucosidase. A concentration-dependent decrease in biofilm formation was also recorded. Synergistic interactions of AgNPs and curcumin nanoparticles (Cur-NPs) against *P. aeruginosa* and *S. aureus* microorganisms were studied by Loo et al. (2016). A combination of AgNPs and Cur-NPs at 100 μg/mL disrupted 50% of established bacterial biofilms, as compared to 500 μg/mL of Cur-NP alone. Microscopic analysis also demonstrated that the

combination of silver and curcumin nanoparticles was more potent in eradicating preformed biofilms in comparison to the single drugs.

Metabolites of the fungus *Rhizopus arrhizus* were exploited for the biofabrication of silver nanoparticles in order to assess their effect on QS systems of *P. aeruginosa*. Sublethal concentrations of mycofabricated silver nanoparticles interfered with the production of QS-regulated virulence factors, such as LasA protease, LasB elastase, pyocyanin, pyoverdin, pyochelin, rhamnolipid, and alginate. Biofilm formation was reduced significantly in a dose-dependent manner, and the production of C4 and C12 AHLs was also impaired. Transcriptional studies demonstrated that these nanoparticles reduced the levels of LasIR-RhlIR. Downregulation of various genes encoding the secretion of QS-controlled virulence factors was also observed (Singh et al. 2016).

Green zinc nanostructures synthesized from *Nigella sativa* seed extract have been identified as broad-spectrum QS inhibitors against human and food pathogens (Al-Shabib et al. 2016). Synthesized nanostructures significantly inhibited the QS-regulated functions of *C. violaceum* CVO26 (violacein), as well as elastase, protease, pyocyanin, and alginate production in PAO1. Additionally, biofilm formation capability of four pathogens (*L. monocytogenes, E. coli*, *C. violaceum*, and *P. aeruginosa* PAO1) was impaired significantly in comparison to untreated controls. Key QS-regulated factors, such as EPS production and swarming motility, which help in the formation of biofilms were also reduced considerably in all four pathogens without the inhibition of growth.

8 In Vivo Studies

Nano-QS inhibitors are continually being synthesized from diverse metals, but it is essential to examine potential QS inhibitors in vivo. In this context, various pharmaceutical lipids were used to prepare ultrasmall solid lipid nanoparticles (us-SLNs), and their anti-QS efficacy was investigated by pyocyanin production. Nano encapsulated QS inhibitors ensured prolonged release of the payload, maintained the viability of epithelial cells, increased mucus penetration, and increased the *antivirulence* activity sevenfold (Nafees et al. 2014). Silver nanoparticles immobilized on titanium reduced biofilm formation by Staphylococcus epidermidis, both in vitro and in vivo (Qin et al. 2014), with the immobilized nanoparticle inhibiting biofilm at the adhesion stage in vitro. An in vivo study was carried out on 60 Sprague Dawley male rats, and the results, based on radiographical, microbiological, and histopathological studies, demonstrated the ability of the nanoparticles to fight bacterial infection and reduce the risk of implant-associated periprosthetic infection (PPI).

Biofilm formation on the surface of teeth is the root cause of dental caries and periodontal diseases. Therefore, the effect of calcium fluoride nanoparticles (CaF_2-NPs) on dental carries development in a rat model was investigated, and a significant reduction in the development of caries in treated rat groups, in comparison to the untreated groups, was observed. Moreover, the synthesized calcium fluoride

nanoparticles were found to be nontoxic to a normal human cell line (HEK-293), indicating its potential use as an antibiofilm agent against dental infections (Kulshrestha et al., 2016).

9 Conclusion

In the fight against drug-resistant bacteria, QSIs have attracted a lot of interest among the scientific community as a novel drug class. The discovery of nanoparticles as quorum quenchers, along with the development of nanocarriers as target drug delivery vehicles, has given hope. Despite this, there still exists an urgent need to unearth the molecular mechanism of action of these nanomaterials. However, the promise shown by QSIs in vitro has not been replicated in vivo, and various QSIs were found to be unstable and toxic. That is, although the use of nanomaterials as QS inhibitors in vitro has gained pace in the last few years, very scarce data is available on in vivo efficacy, toxicity testing, and clinical applicability. Therefore, extensive animal model and clinical studies are still needed. Further, a one-drug-fits-all strategy will probably fail in the near future, and it is envisaged that a more combinatorial approach must be adopted that consists of nanomaterials and known drugs that will function to inhibit QS-regulated virulence and biofilm formation, and reduce resistance pressure. This era of nanobiotechnology has the potential to usher in the development of new drugs with novel modes of action, and to aid in combating persistent drug resistant infections.

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Nanoparticle-Based Drug Delivery Systems: Promising Approaches Against Bacterial Infections

Akhilesh Rai, Michela Comune, and Lino Ferreira

Abstract

Despite the arrays of antibiotics available on the market, bacterial infections, notably those produced by multi-drug-resistant (MDR) bacteria and nosocomial pathogens, have become global concerns and are leading factors of morbidity and mortality, especially for immunocompromised and hospitalized patients. The choice of antibiotics is largely empirical and sometimes requires administration of multiple drugs. Recently, the emergence of MDR bacteria has also put pressure on researchers and healthcare experts to discover alternative antimicrobial agents. Additionally, there is growing concern related to biofilm-associated infections that generally inhibit the penetration of antimicrobial agents inside biofilms, leaving almost no therapeutic options. Hence, there is a dire need to develop effective antimicrobial agents. Nanotechnology offers promising new weapons in treating bacterial infections and overcoming resistance, given that it is believed that numerous mechanisms of action, such as multiple gene mutations within same bacterial cell, are required to develop resistance against nanoparticles (NPs). The past decade has seen a surge in the application of innovative nanotechnology-based antimicrobial drugs in fighting bacterial infections. Diverse compositions of NPs and nanocarriers containing antimicrobial drugs have been developed for the efficient treatment of bacterial infections, including those of MDR pathogens in in vitro and in vivo models. This chapter encompasses the emerging efforts in combatting bacterial infections using diverse nanoformulations, such as polymer, liposomal, solid lipid, nanoemulsion, and metal NPs carrying antibiotics, antimicrobial peptides, and other antimicrobial drugs.

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1 Introduction

Bacterial infections are major causes of morbidity, chronic infections, and death (Fauci and Morens 2012). In addition to acute illness in patients, bacterial infections can lead to chronic diseases, where bacteria can produce a biofilm, a three-dimensional microbial community that slows healing (Hall-Stoodley et al. 2004). After the discovery of penicillin in 1928, several classes of antibiotics, including the new generation of aminoglycosides and fluoroquinolones, have been designed and synthesized for the treatment of microbial infections (Appelbaum and Hunter 2000; Poulikakos and Falagas 2013). Antibiotics are the most favored drugs in the healthcare sectors for the treatment of bacterial infections owing to their cost-effectiveness and broad-spectrum antimicrobial activity. In recent years, infections caused by multi-drug-resistant (MDR) bacteria have become growing concerns for human health, imposing a huge burden on public healthcare costs. In Europe, 4 million patients are infected with MDR bacteria annually, causing cost over 1.5 billion euros and killing 25 thousand people per year, according to the European Centre for Disease Prevention and Control, and the European Medicine Agency in 2009 (European Centre for Disease Prevention and Control/European Medicines Agency n.d.). Therefore, despite the availability of antibiotics and other antimicrobial agents, bacterial infections continue to be a major challenge. Additionally, there has been a sharp decrease in the number of approved antibiotics, especially those effective against MDR Gram-negative bacteria, highlighting the immediate need for effective and long-term antimicrobial and biofilm preventing drugs in medicine.

Combining antibiotics with nonantibiotic drugs has shown promise as an alternative strategy to overcome bacterial resistance (Ejim et al. 2011; Farha and Brown 2013), and recently, outdated antimicrobial drugs in combination with current antibiotics have been proposed to combat bacterial resistance (Bush et al. 2011; Imperi et al. 2013). However, in addition to combinatorial therapies utilizing established drugs, it is vital that novel strategies for the effective prevention of deadly bacterial infections are developed, and nanotechnology-based approaches offer an array of promising new weapons.

Over the last few decades, the application of nanoparticles (NPs) for drug delivery has generated significant interest in medicine (Farokhzad and Langer 2009). Various drug delivery platforms, such as liposomes, dendrimers, polymers, and inorganic NPs, have received tremendous attention (Fig. 1). Moreover, NPs are emerging as potent antimicrobial agents, due to their unique physical and chemical

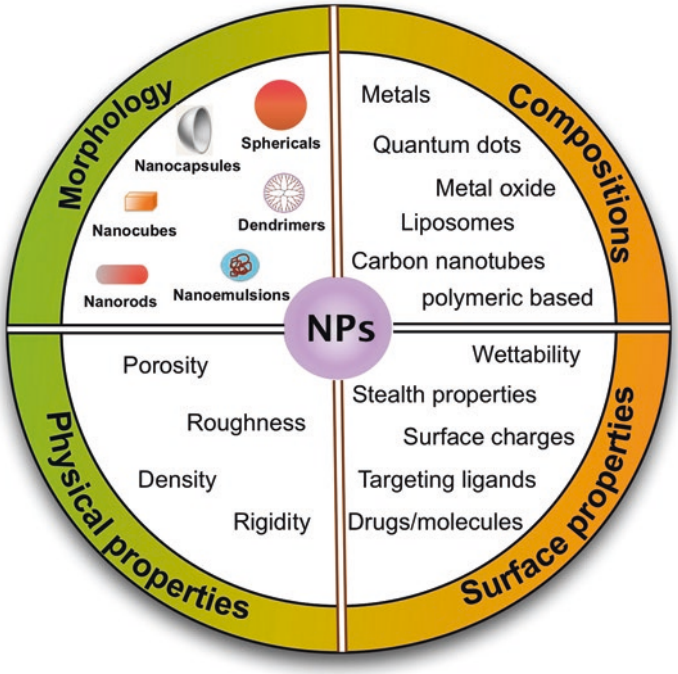

Fig. 1 Schematic representation of a number of NPs used to encapsulate/entrap or conjugate drugs. NPs show unique physicochemical properties, and their surfaces can be functionalized with appropriate molecules for antimicrobial applications

properties, and are gaining recognition as potential agents to overcome the challenges of clinical antibiotic treatments (Wang et al. 2017). For instance, the size of NPs is commensurate with drugs/biomolecules, thus NP-bacterial interactions can be fine-tuned through suitable surface functionalization of NPs with promising synergy resulting from multivalent interactions (Daniel and Astruc 2004). Importantly, NPs have superior activities, gained by the greater loads of therapeutics on their surfaces when compared to their counterparts, leading to smaller required doses of drug-loaded NPs. The combined actions of drugs and NPs make them potent antimicrobial agents against several strains of bacteria. In addition to delivering antimicrobial drugs, NPs employ multiple mechanisms, such as oxidative stress, metal ion release, inhibition of enzyme activity, damage of DNA/RNA, and photocatalysis, to kill bacteria depending on the compositions of NPs. However, the antimicrobial action of NPs is mainly through direct contact with the bacterial membrane, leading to membrane damage and leakage of bacterial content (Fig. 2) (Huh and Kwon 2011; Rai et al. 2010). Moreover, in the last few years, several NP formulations have been developed to improve the antimicrobial efficacy of antibiotics and other drugs (Miller et al. 2015; Zhao and Jiang 2013).

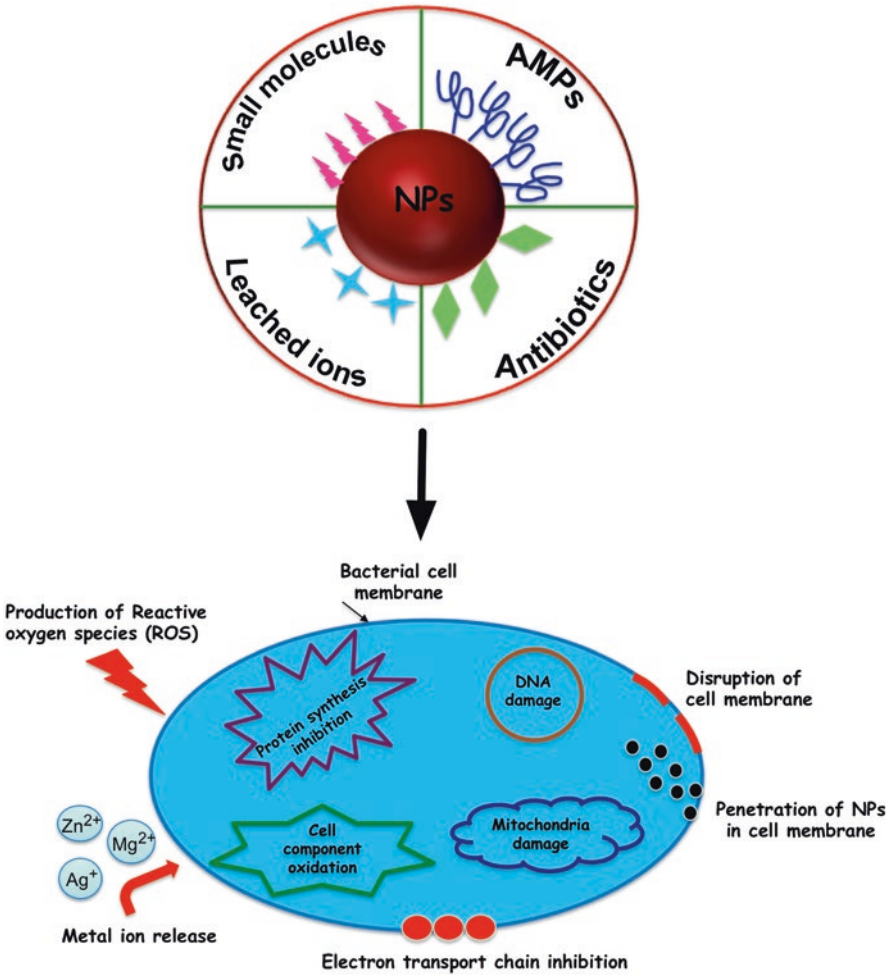

Fig. 2 Representative schematic diagram showing antimicrobial mechanisms of NPs functionalized with different antimicrobial agents

2 Development of Nanoparticles as Drug Vehicles

During the 1950s and 60s, Peter Speiser's group developed polyacrylic beads (Khanna et al. 1969), microcapsules (Merkle and Speiser 1973), and nanocapsules (Birrenbach and Speiser 1976) to achieve prolonged drug release in the blood. Following this discovery, several NP-based drug delivery platforms have been developed for pharmaceutical and biomedical applications. Importantly, NPs above 200 nm in size are not deeply pursued, and nanomedicines often refer to NPs below 200 nm. The advantage of NP-based systems is that they can use lower concentrations of highly toxic drugs in order to reduce side effect, improve drug solubility, prolong systemic circulation, and employ targeted delivery and concurrent delivery of multiple drugs (Singh and Lillard

2009). Drugs of interest loaded into, or onto, NP systems through physical encapsulation/entrapment, adsorption, or chemical conjugation, exhibit improved pharmacokinetic indexes as compared to their free drug counterparts (Singh and Lillard 2009). Additionally, after finding that polyethylene glycol (PEG)-conjugated NPs had prolonged circulation life span and reduced liver uptake (Illum et al. 1987), several studies have been performed on the ability of NPs to cross the blood-brain barrier for the targeting of deep brain tumors or infections (Borchard et al. 1994; Ramge et al. 2000). As the ability to precisely engineer multifunctional NPs continually advances, innovative approaches have emerged to improve the therapeutic efficacy of NPs for treating various diseases. A number of antimicrobial NPs has been approved for clinical use to treat infectious diseases, and several others are currently in the various stages of preclinical and clinical trials (Zhang et al. 2010).

3 Role of Nanotechnology in the Treatment of Bacterial Infections

Due to growing concerns related to bacterial resistance against antibiotics, researchers have turned their attention to nanotechnology-based approaches to produce potent antimicrobial materials to which bacteria may be less likely to develop resistance. Nanotechnology-based approaches have been widely used in a variety of biomedical applications, such as drug-conjugated NP delivery systems for the long-term inhibition of bacterial growth in medical device-related infections, and in the general control of infectious diseases (Huh and Kwon 2011; Gao et al. 2014; Muzammil et al. 2018). Synthesis of metal, metal oxide, and polymeric NPs with different physiochemical properties, such as size, shape, porosity, and surface functionalities, have led to continued evaluation of novel antimicrobial systems (Miller et al. 2015). Various NPs are available to efficiently deliver antibiotics and other antimicrobial drugs to the targeted sites, thus improving their pharmacokinetics and bioaccumulation, while minimizing side effects (Miller et al. 2015). Importantly, local release methods allow the reduction in systemic toxicity and antibiotic dosage. The ability to control the release rate of antibiotics and antimicrobial agents offers consistent therapeutic dosing, and enables the safe delivery of antibiotics over a much longer time span. Importantly, antimicrobial NPs employ multiple pathways to attack microbes, and therefore several mutations at the same time would have to occur in microbes to acquire the resistance against them (Fig. 2).

4 Challenges in Treating Bacterial Infections Using Nanotechnology Approaches

Bacterial resistance has become a serious public healthcare issue, due to the rampant usage of antibiotics, inappropriate drug selection, and the frequent switching between antimicrobial treatments. The emergence of vancomycin-resistant *Enterococcus* (VRE), for example, is a serious clinical threat. VRE has intrinsic resistance to several antibiotics and the capability of acquiring resistance to all

commercially available antibiotics (Gold and Moellering 1996). Likewise, treating vancomycin-resistant *Staphylococcus aureus* (VRSA) infections is one of the most challenging tasks of the twenty-first century, given that vancomycin is a last-resort antibiotic that is assumed to be highly effective in treating *S. aureus* infection (Chakraborty et al. 2010; Perichon and Courvalin 2009).

An attractive alternative to antibiotics is NPs that can cause physical damage to the membranes of the resistant bacteria (Pelgrift and Friedman 2013). However, it is reported that antimicrobial NPs can also induce bacterial resistance in certain cases (Qiu et al. 2012). Biofilms, having unique compositions and structures, provide protection to the resident microorganisms, helping them to escape from antibiotics and antimicrobial NPs, and are a breeding ground for frequent resistance mutations (Khameneh et al. 2016). Moreover, the highly complex structure of biofilms hinders diffusion of NPs to reach the resident microorganisms. Physiological and genetic complexities of biofilms for instance the hydrophobicity of bacterial cell walls control diffusion of NPs inside biofilms (Habimana et al. 2011). A key criterion in the preparation of NPs intended to diffuse inside biofilms is their size and surface charge. Recent studies have shown that the size of NPs governs their diffusion ability inside biofilms, owing to small pore sizes, electrostatic interactions and the uniformity of charges across the biofilms (Peulen and Wilkinson 2011; Stewart 1998; Takenaka et al. 2009). Therefore, understanding the interactions between NPs and biofilm matrices is a fundamental issue in developing effective nanotherapeutics for biofilm treatment.

The search for advanced and effective strategies to combat MDR bacterial infections and biofilms is a top priority in healthcare sectors, and there have been considerable efforts in discovery and synthesis of antimicrobial drugs and nanomaterials with improved efficacy. Challenges to the development of NP-based drug delivery systems include the need to scale-up processes to produce a large quantity of therapeutic materials and multifunctional NPs to meet biological and pharmaceutical requirements.

5 Internalization of Nanoparticles and Delivery of Drugs in Infected Cells

Therapeutic success of NPs or antibiotic/drug-loaded NPs against intracellular microbes is related to their ability to transverse the cellular membrane. Each class of antibiotics, depending on their polarities, has a different propensity to penetrate and be retained by mammalian cells (Briones et al. 2008). Despite rapid intracellular internalization of antibiotics, some of them are cleared from cells via the activation of efflux pumps (Webber and Piddock 2003). On the other hand, the internalization of NPs by cells is governed by various mechanisms, and can be controlled by fine-tuning the physiochemical properties of NPs (Chou et al. 2011). For instance, NPs are opsonized by blood plasma and internalized by the reticuloendothelial system (RES) of cells when administered through the intravenous route. However, the modification of NP's surface governs the interaction with the cell membrane through receptor-mediated, nonspecific, electrostatic or hydrophobic interactions (Chou et al. 2011). Importantly, cells can internalize NPs through several mechanisms at the

same time. Once internalized, NPs are mostly accumulated in endosomes or phagosomes, which further undergo maturation processes involving a number of fusion and fission events. After complex trafficking processes, NPs can translocate to other intracellular compartments or exocytose to the extracellular environment. In most cases, NPs become trapped in endosomes, which mature into lysosomes, where an acidic environment can lead to the degradation of NPs, triggering drug release. This is a challenge to developing suitable NPs that can escape endosomes. NPs that escape the endosomes via endosomal membrane fusion or permeabilization could result in the delivery of drugs to bacteria located in cytoplasm (Peetla et al. 2014). In another approach, NPs can be functionalized in such a way that a caveolin-mediated process internalizes them, thereby bypassing the lysosomes entirely.

6 Antimicrobial Peptides as Promising Therapeutics

Due to the increasing development of antibiotic resistance in bacteria (French 2005), antimicrobial peptides (AMPs) have been attracting considerable attention as potential therapeutics in recent years (Elsbach 2003; Hancock and Sahl 2006). AMPs isolated from bacteria, insects, plants, and vertebrates can kill microorganisms or inhibit their growth (Koczulla and Bals 2003). AMPs are considered as small molecules containing less than 50 amino acid residues. AMPs are mostly positively charged, hydrophobic, and amphipathic in nature (Kang et al. 2017). However, a few AMPs are negatively charged and highly active, but they are less frequent and their mechanisms of action remain elusive (Falcao et al. 2014). AMPs are classified into four major groups based on their composition and secondary structure: (1) amphipathic, α-helical linear peptides, such as cecropin, histatins, magainins, and human ubiquicidin; (2) β-sheet peptides such as β-defensin; (3) peptides with the predominance of one amino acid, such as the indolicidin rich in tryptophan and PR39 peptide rich in proline-arginine residues; and (4) loop structures containing peptides, such as gramicidin (Koczulla and Bals 2003; Peters et al. 2010; Salditt et al. 2006). Cathelicidins and defensins produced by immune cells and histatins produced by salivary glands are the most prominent AMPs in humans (Peters et al. 2010). These AMPs are normally produced at sites of potential pathogen entry in the skin, providing a chemical barrier to keep human skin healthy.

AMPs have potent activities against Gram-positive and Gram-negative bacteria, fungi, and viruses, and thus represent essential players of innate immunity system (Shai 2002). Interestingly, many natural AMPs have been synthetically modified to improve upon their bioactivity, purity, and yield (Gomes et al. 2018; Zasloff 2002). To date, small biotech companies, in association with larger pharmaceutical companies, have studied the biological activities of different AMPs in animals and humans to evaluate their potential as useful drugs for several infectious diseases. Currently, there are numerous AMPs under clinical development for the treatment of infected diabetic foot ulcers, venous leg ulcers, oral mucositis, and skin infections, among other conditions (Mahlapuu et al. 2016). So far, no AMPs have reached the drug market, though many pharmaceutical companies remain enthusiastic about the

prospect of understanding the AMP molecular processing, their mechanisms of action, and their regulation, in the hope for the future use of these novel agents as a new generation of medications, especially for skin therapy.

7 Nanoparticle-Based Antimicrobial Drug Delivery Systems

NPs-based therapeutic approaches have been adopted to combat infections particularly in wounds and other bacterial infections. Antimicrobial NPs can lead to improved outcomes for bacterial infections. Unlike many antibiotics being used in clinics, antimicrobial NPs generally do not cause any acute adverse effects. Antimicrobial NPs can be broadly classified into inorganic and organic NPs that act as carriers to deliver antimicrobial agents.

7.1 Biodegradable Nanoparticles

7.1.1 Polymeric Nanoparticles

Polymeric NPs can be prepared via self-assembly of chitosan (CS), curcumin, poly lactic-co-glycolic acid (PLGA) etc. of different shapes, including nanomicelles (NM), dendrimers, and hydrogels (Kalashnikova et al. 2015). Polymeric NPs have been extensively used in healthcare sectors for the enhanced drug delivery and reduced clearance by the RES of cells (Soppimath et al. 2001). Polymeric NPs containing antimicrobial drugs offer several advantages, including (1) tunable properties (size, shape, surface charge, and controlled drug release) by manipulating polymer lengths, functional groups, and solvents, (2) versatile surface functionalization for conjugating antibiotics and drugs, and (3) structural stability during preparation and storage.

CS NPs (150–300 nm) made of pristine, soluble CS, can be used as prophylactic agents that inhibit infections and promote a significant acceleration of wound healing (Dai et al. 2009; Shrestha et al. 2012, 2014). CS is a positively charged polymer, and has an inherent antimicrobial property. CS has been frequently used as an antimicrobial agent in wound dressings to prevent bacterial infections (Burkatovskaya et al. 2006; Dai et al. 2011). It has been shown that CS NPs, in combination with alginate, can be employed to deliver silver sulfadiazine (SSD) cream to treat infections in open wounds (Huang et al. 2011). Curcumin, a natural product isolated from the root of *Curcuma longa*, has been used as a traditional medicine for centuries. Curcumin-loaded CS NPs have strong antimicrobial activities against *S. aureus* and *Pseudomonas aeruginosa* in murine skin infections (Mirnejad et al. 2014). Also, biomolecules such as lectin-conjugated gliadin-NPs selectively adhered to the carbohydrate receptors of *Helicobacter pylori*, and released antimicrobial agents inside bacterial cells (Umamaheshwari and Jain 2003). For the treatment of lung infection, an antimycobacterial drug, *E-N*2-3,7-dimethyl-2-*E*,6-octadienylidenyl isonicotinic acid hydrazide (JVA), loaded on 180 nm PLGA NPs, exhibited high activity against extracellular

and intracellular mycobacteria (de Faria et al. 2012). Additionally, nebulization of PLGA NPs, loaded with three different antitubercular drugs (i.e., rifampicin, isoniazid, or pyrazinamide), showed superior activity, reduced dosing frequency, and greater drug bioavailability to treat *Mycobacterium tuberculosis* when compared to either oral or intravenous administrations of the parent drugs (Pandey et al. 2003). In another example, ampicillin encapsulated in poly(isohexyl cyanoacrylate) (PIHCA) had greater activity in treating *Salmonella typhimurium* (Fattal et al. 1989) and *Listeria monocytogenes* (Forestier et al. 1992) infection in mice. In another case, penicillin (Turos et al. 2007a) and N-thiolated-β-lactam antibiotics (Turos et al. 2007b) entrapped in polyacrylate (PAA) NPs retained potent antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) in the presence of high concentrations of β-lactamase. Similarly, the potential application of gentamycin entrapped in PLA/PLGA NPs was demonstrated for the treatment of *Brucella* infections, due to the suitable sizes of NPs for phagocytosis (Prior et al. 2000).

In the last few years, advanced techniques using near-infrared (NIR) light has been explored for the spatial-temporal release of cargo molecules from light-triggerable NPs. In the same line, using an antimicrobial photodynamic therapy (PDT) approach, chlorin e6, a photosensitizer, loaded on charge-conversion polymeric NPs, were used to kill pathogens in weakly acidic urinary tract infections under laser irradiation (Liu et al. 2015) (Fig. 3). In recent approach, vancomycin encapsulated in surface charge-switchable NPs (poly(D, L-lactic-co-glycolic acid)-*b*-poly(L-histidine)-*b*-poly(ethylene glycol) (PLGA-PLH-PEG)) demonstrated enhanced antimicrobial activity at a mild acidic condition (pH 6) when compared to physiological condition (pH 7.4) due to their strong binding affinity of positively charged PLGA-PLH-PEGNPs with negatively charged bacterial membranes via electrostatic interactions, and release of vancomycin (Radovic-Moreno et al. 2012).

Recently, AMPs have been considered to be alternatives to antibiotics, given that AMPs mostly perturb the integrity of the bacterial membrane. Temporin B AMPs, derived from frog skin, encapsulated in CS NPs, exhibited enhanced and long-lasting antimicrobial activity against clinically relevant *S. epidermidis*, and a reduced cytotoxicity profile compared to free peptides (Piras et al. 2015). However, in vivo/ex vivo studies are needed to fully evaluate the ability of these AMP-conjugated CS NPs to prevent/treat infections occurring at mucosal/skin surfaces, and in increasing the translational potential of promising AMPs. For example, in ex vivo study, plectasin AMPs, classified as defensins, were encapsulated in PLGA NPs for the treatment of airway *S. aureus* infections with high encapsulation efficiency (71–90%) and continuous release of AMPs over 24 h in infected bronchial epithelial cells (Water et al. 2015). In another work, LL37-capped PLGA NPs were explored to promote wound healing and fight infections (Chereddy et al. 2014). Results showed that the administration of LL37-capped PLGA NPs promoted rapid wound closure due to the sustained release of both LL37 and lactate when compared to PLGA or LL37 administered alone in a full-thickness excisional wound model. In vitro, LL37-capped PLGA NPs induced cell migration and displayed antimicrobial

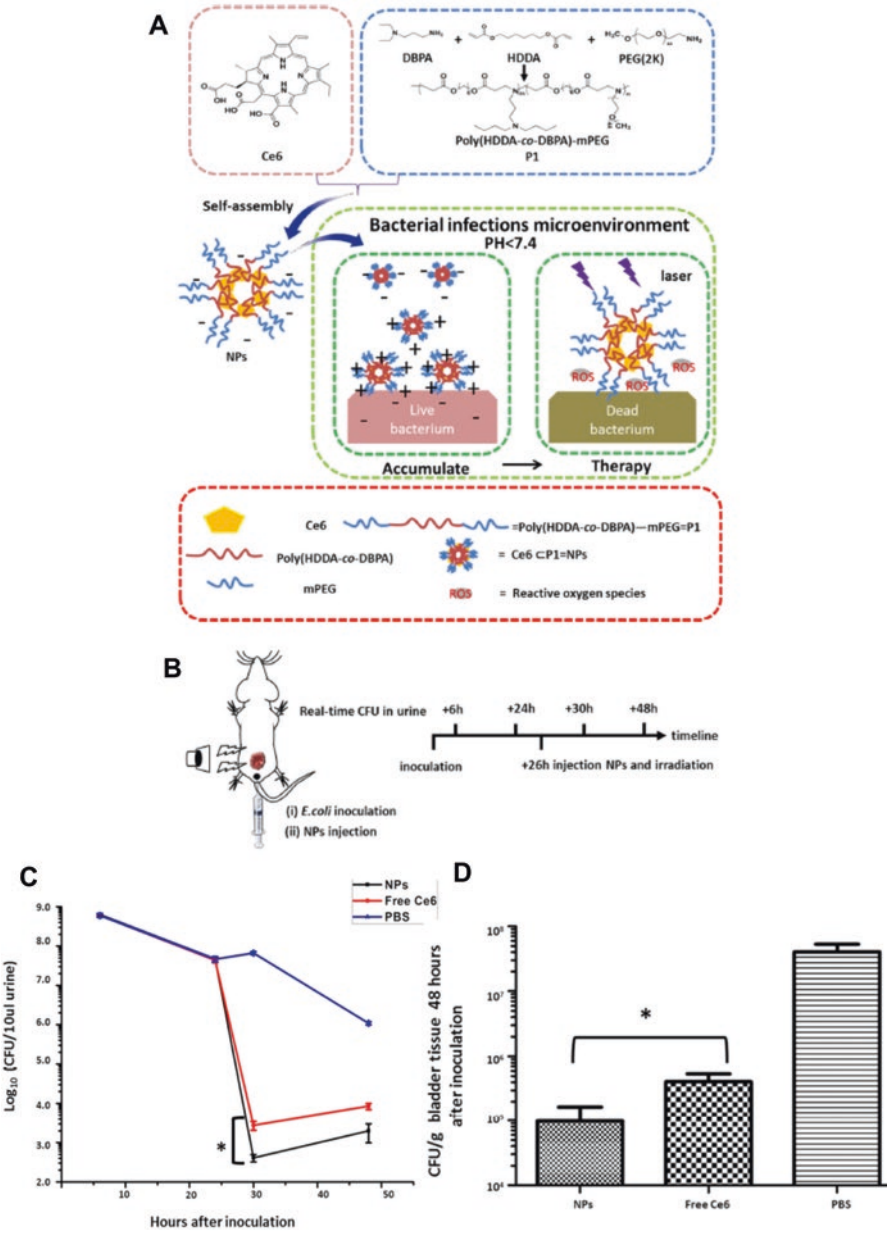

Fig. 3 (**a**) A schematic representation of surface charge-conversion NPs for the recognition and killing of bacteria in the infected site. (**b**) Time-dependent protocol for bladder infection induction and photodynamic therapy. (**c**) Time-dependent reduction of bladder infected *E. coli* in urine after treatment. (**d**) Estimation of *E. coli* in the bladder after treatment. (Reproduced from Turos et al. (2007b) with permission)

activity against *E. coli* (Chereddy et al. 2014). Similarly, nisin immobilized on phytoglycogen (PG)-NPs through electrostatic and hydrophobic interactions showed prolonged antimicrobial efficacy against *L. monocytogenes*, along with minimum loss of peptide activity during storage (Bi et al. 2011).

7.1.2 Dendrimers

Dendrimers are hyperbranched polymers in the nano-scale size range (1–10 nm) with precise nanoarchitecture and monodisperse structures. They are synthesized via a layer-by-layer approach around a core unit, allowing the control of surface functionalization and branching points for conjugation of either hydrophilic or hydrophobic drugs (Svenson 2009). Dendrimers have shown promise as antimicrobial agents in multiple studies. One example is that of dendrimers functionalized with high densities of quaternary ammonium molecules on their surfaces, which displayed greater antimicrobial activity than free antibiotics, due to their ability to destroy bacterial membranes or disrupt multivalent interactions between bacteria and host cells (Chen and Cooper 2002). Additionally, water-insoluble antibiotics, such as nadifloxacin and prolifloxacin, loaded on polyamidoamine-based dendrimers showed enhanced antimicrobial properties (Kalomiraki et al. 2016). In another approach, silver-loaded dendrimers were found to have synergetic anti-inflammatory and antimicrobial properties, allowing them to kill microorganisms and accelerate wound healing (Liu et al. 2014).

7.1.3 Liposomal Nanoparticles

Liposomal NPs are spherical vesicles consisting of phospholipid bilayers with hydrophilic core. Liposomes have been extensively used as clinically acceptable carriers of antimicrobial agents and antibiotics for treating several diseases, given that their lipid bilayer structure mimics cell membranes, allowing them to rapidly fuse with infectious microorganisms (Nisini et al. 2018). Delivery of antimicrobial drugs using liposomal NPs such as liposomal amphotericin-B (Abelcet®, Amphotec®, AmBisome®), approved by the Food and Drug Administration (FDA), have been used in the treatment of aspergillosis, cryptococcal meningitis, and visceral leishmaniasis (Torchilin 2005). For instance, AmBisome® showed improved bioactivities over free drug, including sustained circulation half-life, decreased renal clearance, and enhanced therapeutic efficacy. Another successful example is Polymyxin B-loaded lysosomal NPs, which showed excellent antimicrobial activity against *P. aeruginosa* in a rat lung infection model (Omri et al. 2002). Importantly, free Polymyxin B has limited success due to its toxic side effects, such as nephrotoxicity, ototoxicity, and neuromuscular blockade, and liposomal formulation diminishes the incidence of these side effects (Mugabe et al. 2006). It was observed that lipid molecules of liposomes reorganized in *P. aeruginosa* membranes, leading to bacterial membrane deformation and delivery of high-dose drugs, thus overwhelming the efflux pumps and suppressing the possibility of antimicrobial resistance (Mugabe et al. 2006).

Several antimicrobial drug delivery system based on liposomal NPs have been developed for the treatment of bacterial infections. Ampicillin-loaded liposomes against *Salmonella typhimurium*, bezyl penicillin-loaded liposomes against *S. aureus*, ciprofloxacin-loaded liposomes against *Salmonella dublin*, and gentamicin-loaded liposomes against *Brucella* infections have been developed, and have greater stability and antimicrobial properties than free antibiotics (Gao et al. 2014; Ladaviere and Gref 2015). For example, ciprofloxacin-loaded liposomal NPs accumulate in the spleen and liver and persist in these organs for 48 h after the final administration, suggesting that these liposomal formulations can be an effective therapy for *Salmonella* infection (Magallanes et al. 1993). Recently, co-encapsulation of two or more active drugs within liposomes has become an attractive choice for treating recalcitrant bacterial infections. For example, the synergistic therapeutic efficacy of co-encapsulated gentamicin and ceftazidine show prolonged circulation time and enhanced accumulation at the infection site (Bakker-Woudenberg et al. 1995). Likewise, co-encapsulation of daptomycin and clarithromycin resulted in significant elimination of MRSA infections and increased survival of mice (Li et al. 2015). Also, the combination of levofloxacin and serratiopeptidase loaded on liposomes had improved antimicrobial and antibiofilm efficacy in treating an *S. aureus*-infected lungs in a rat infection model, along with reduced levels of inflammatory cytokines (Gupta et al. 2017). Interestingly, in another study, lipase-sensitive, singlet oxygen-producible and erythromycin-loaded liposomes (LSSPL) were developed for antibacterial therapy in skin disorders by coating erythromycin-loaded liposomes with pullulan-pheophorbide A (PU-Pheo A) in order to produce reactive oxygen species under laser exposure. *Propionbacterium acnes* infections cause skin inflammation and secrete extracellular lipases that promote the degradation of LSSPL, thereby releasing erythromycin and PU-Pheo A. The combined effect of antibiotics and singlet oxygen produced from PU-Pheo A under laser irradiation inhibited *P. acnes* infection in nude mice dorsal skin (Jeong et al. 2017).

Table 1 summarizes antimicrobial liposomal NPs encapsulated with various antibiotics.

7.1.4 Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLNPs) are made of solid lipids and used as drug carriers with the combined properties of liposomes and NPs (Table 2) (Mofazzal Jahromi et al. 2018; Sala et al. 2018). SLNPs are effective against skin infections, as they tend to adhere to the skin surface and continuously release drugs over long periods of time in the stratum corneum. Ciprofloxacin-loaded SLNPs exhibit the prolonged release of drugs, especially in skin and oral infections (Jain and Banerjee 2008). Likewise, Nitrofurazone encapsulated SLNPs was used for topical delivery of drugs to improve their antimicrobial activity in treating infections in burn eschar patients (Shariff et al. 2010). SLNPs have also been tested against eye infections. Tobramicin (Cavalli et al. 2002; Chetoni et al. 2016) and levofloxacin (Baig et al. 2016) -encapsulated SLNPs provided significantly superior bioaccumulation in the aqueous humor, and can be used for treating Pseudomonal keratitis and conjunctivitis, respectively.

Table 1 Liposomal nanoparticles for the delivery of antibiotics

NP composition	Encapsulated molecules	Target bacteria	Therapeutic activity
DPPC and chol liposome	Polymyxin B	*P. aeruginosa*	Decreased bacterial load in the lungs and increased bioavailability (Omri et al. 2002)
PHEPC, chol, and PEG-DSPE liposome	Gentamicin	*Klebsiella pneumoniae*	Increased survival rate of animals infected with bacteria (Schiffelers et al. 2001)
SPC and chol liposome	Ampicillin	*Salmonella typhimurium* and *Micrococcus luteus*	Enhanced stability of encapsulated antibiotics and activity against extracellular bacteria (Schumacher and Margalit 1997)
EPC, DCP, and chol liposome	Vancomycin and Teicoplanin	MRSA	Enhanced intracellular antimicrobial effects due to increased drug uptake by macrophages (Onyeji et al. 1994)
DPPC and chol liposome	Ciprofloxacin	*Salmonella dublin*	Bioaccumulation of NPs to all organs and decreased mortality of infected animals (Magallanes et al. 1993)
PG, PC, and chol liposome	Streptomycin	*Mycobacterium avium*	Targeted delivery of antibiotics to the infected site (Fielding et al. 1998)

Abbreviations: *Chol* cholesterol, *DPPC* 1,2-dipalmitoyl-phosphatidylcholine, *PHEPC* partially hydrogenated egg phosphatidyl choline, *PEG-DSPE* 1,2-disteroyl-sn-glycero-3-phosphoethanol-amine-(N)-(polyethylene glycol-2000), *SPC* soybean phosphatidyl choline, *ECP* egg PC, *DCP* diacetylphosphate, *PG* phosphatidylglycerol, *PC* phosphatidyl choline

Table 2 Lipid-based nanocarriers to deliver antimicrobial drugs

NP composition	Encapsulated molecules	Target bacteria	Therapeutic activity
SA, STC, and SPC	Ciprofloxacin	Gram-negative and Gram-positive bacteria	Drug release over long time (Jain and Banerjee 2008)
SA, STC, and SPC	Tobramycin	*P. aeruginosa*	Increased antibiotic availability (Cavalli et al. 2002)
SA	Rifampicin, isoniazid, pyrazinamide	*M. tuberculosis*	Increased bioavailability of antibiotics in infected organs (Pandey and Khuller 2005)
PC, glycerol monostearate	LL37	*S. aureus and E. coli*	Synergetic effect to kill bacteria and promote wound healing (Fumakia and Ho 2016)

Abbreviations: *SA* stearic acid, *STC* sodium taurocholate

Another advantage of SLNPs is that they can be administered using a nebulizer, and are mostly phagocytosed by alveolar macrophages in the lungs. No tuberculosis bacteria were found in the spleens and lungs when isoniazid, rifampicin, and pyrazinamide-loaded SLNPs were administered via the nasal route to infected guinea pigs every 7 days (Pandey and Khuller 2005). Conversely, daily administration of the free drugs was needed to achieve the equivalent therapeutic efficiency of SLNP-loaded drugs, indicating that antimicrobial-loaded SLNPs are patient-friendly and cost-effective for tuberculosis treatment.

AMPs can also be loaded in SNLPs. Recently, LL-37, an AMP involved in the modulation of wound healing, and serpin A1, an elastase inhibitor, co-encapsulated in SLNPs, were developed for the sustained release of both molecules at specific ratios in order to promote wound closure by inducing the migration of fibroblast and keratinocyte cells, as well enhancing antimicrobial activity against *S. aureus* and *E. coli* (Fumakia and Ho 2016).

Table 2 summarizes various compositions of lipid NPs as carriers for antimicrobial agents.

7.1.5 Nanoemulsions and Nanogels

Nanoemulsions (NEs) are spherical colloidal particulates that have amphiphilic molecules. NEs have generally hydrophobic cores and hydrophilic shells. The hydrophobic core is used to load water-insoluble drugs, and the hydrophilic shell promotes NEs to be soluble in water. NEs made of NB-201, a novel antimicrobial compound (Cao et al. 2017), and chlorhexidine acetate-encapsulated NEs (Song et al. 2016), showed effective and rapid activity against MRSA-infected wounds in a skin burn wound model and hindered the formation of biofilms due to increased leakage of proteins, Mg^{2+}, K^{+}, DNA, and alkaline phosphate from the bacterial cells. NB-201 NEs reduced epidermal and deep dermal inflammation by inhibiting the secretion of pro-inflammatory cytokines in the infected wounds. In another study, NEs containing eucalyptus oil impregnated in CS films exhibited significant antimicrobial activity against *S. aureus*, likely through bacterial membrane damage (Sugumar et al. 2015). Intriguingly, self-assembled cationic peptide micelle NPs containing TAT peptide (a membrane translocation peptide), conjugated with 6 arginine residues and cholesterol, crossed the blood-brain barrier and suppressed *S. aureus* and *Cryptococcus neoformans* infection in a meningitis-infected rabbit model (Fig. 4). These nanoparticles have a broad spectrum of potent antimicrobial activities; much stronger than soluble peptides (Liu et al. 2009; Wang et al. 2010).

Nanogels are three-dimensional nano-sized hydrogels formed by cross-linked, swellable polymer or biopolymer networks. Hyaluronic acid nanogels encapsulated with the LL37 analog, LLKKK18, demonstrated superior killing efficiency against mycobacteria, when compared to the peptide alone, in both

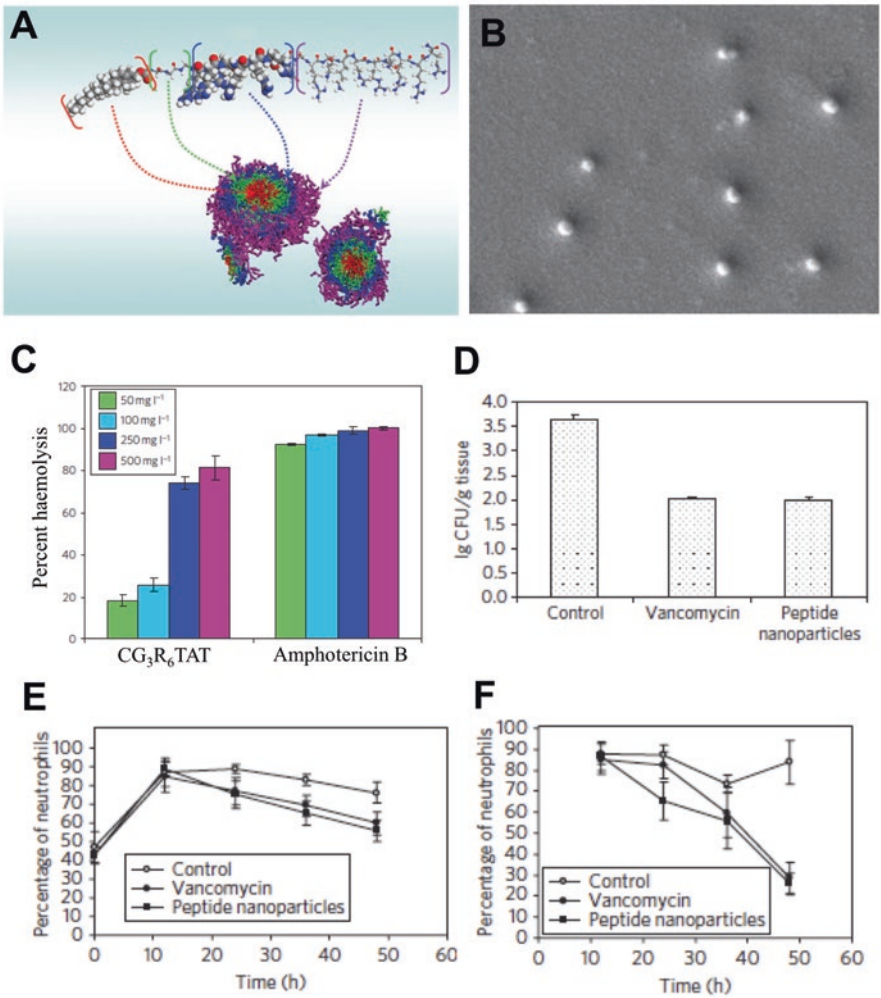

Fig. 4 (**a**) Formulation of micelles simulated through molecular modeling using Materials Studio software. (**b**) Scanning electron microscopy (SEM) image of peptide NPs. (**c**) Dose-dependent hemolytic activity of peptide NPs compared with that of amphotericin-B. (**d**) CFU of *S. aureus* in brain tissues. Percentage of neutrophils in blood (**e**) and CSF (**f**). (Reproduced from Song et al. (2016) with permission)

in vitro and in vivo models, demonstrating their potential to treat tuberculosis. It was found that the hyaluronic acid nanogels were able to protect the peptide from proteolytic degradation and reduce host toxicity (Silva et al. 2016).

7.2 Nonbiodegradable Nanoparticles

7.2.1 Nonantibiotics and Antibiotic-Conjugated Gold Nanoparticles

Typically, gold is considered biologically inert, but gold nanoparticles (Au NPs) exhibit unique physiochemical and biological properties that have triggered tremendous interest for fundamental research and the development of industrial products. For example, Verigene® composed of Au NPs were approved by the FDA in 2012 (Nanosphere) for an in vitro blood infection test for Gram-positive bacteria. Several nonantibiotic molecules conjugated to Au NPs have been explored as potent antimicrobial agents with low induction of bacterial resistance. Jiang's groups have developed a library of amino-substituted mercaptopyrimidine compounds, which have been conjugated to Au NPs (3 nm) in order to test their antimicrobial potency against MDR clinical isolates (Zhao et al. 2010). Out of several compounds, 4,6-Diamino-2-pyrimidinethiol-capped Au NPs (Au-DAPT) showed the best activity when compared with the other two pyrimidine-capped Au NPs. Importantly, Au-DAPT showed very slow induction of resistance compared with gentamicin, with resistance increasing the MIC by 1.3-fold after 50 passages for NPs and tenfold after only 10 passages for gentamicin (Zhao et al. 2010). Recently, Au-DAPT covered in bacterial cellulose (BC) showed excellent physiochemical characteristics, such as water uptake capability, mechanical strain, and biocompatibility, along with inhibition of bacterial growth (*E. coli* and *P. aeruginosa*) in wounds, while promoting wound repair (Li et al. 2017). Similarly, Au NPs capped with a mixture of small molecules can exhibit an antibacterial effect against MDR strains without inducing resistance. For example, 2 nm Au NPs capped with p-mercaptobenzoic acid (pMBA-Au), 3-mercaptopropylsulfonate (MPS-Au), and 2-mercaptoethylamine (MEA-Au) completely inhibited the growth of *E. coli* with an MIC of 0.5 μM, indicating that these NPs could be useful in the treatment of infected wounds (Bresee et al. 2014). 3-mercaptopropylsulfonate and p-mercaptobenzoic acid are inactive against bacteria, while 2-mercaptoethylamine is antimicrobial at high concentrations (2 mM) (Bresee et al. 2014).

Au NPs incorporated in polymeric matrix can accelerate wound closure and aid in fighting infections. In one work, intermediary antibiotic components, such as 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA), and 7-aminodesacetoxycephalosporanic acid (7-ADCA)-coated Au NPs were electrospun with poly(ε-caprolactone) (PCL) and gelatin to produce biocompatible wound dressings to fight MDR infection (Yang et al. 2017). Wettability and tensile strength of Au NP-embedded fiber was similar to PCL/gelatin fiber. Importantly, antibiotic intermediate Au NP-coated fiber significantly decreased the MDR bacterial load in wounds with enhanced wound healing when compared to PCL/gelatin fibers or Ag mat (Fig. 5) (Yang et al. 2017). Similarly, Au NP-chitosan nanocomposites showed improved

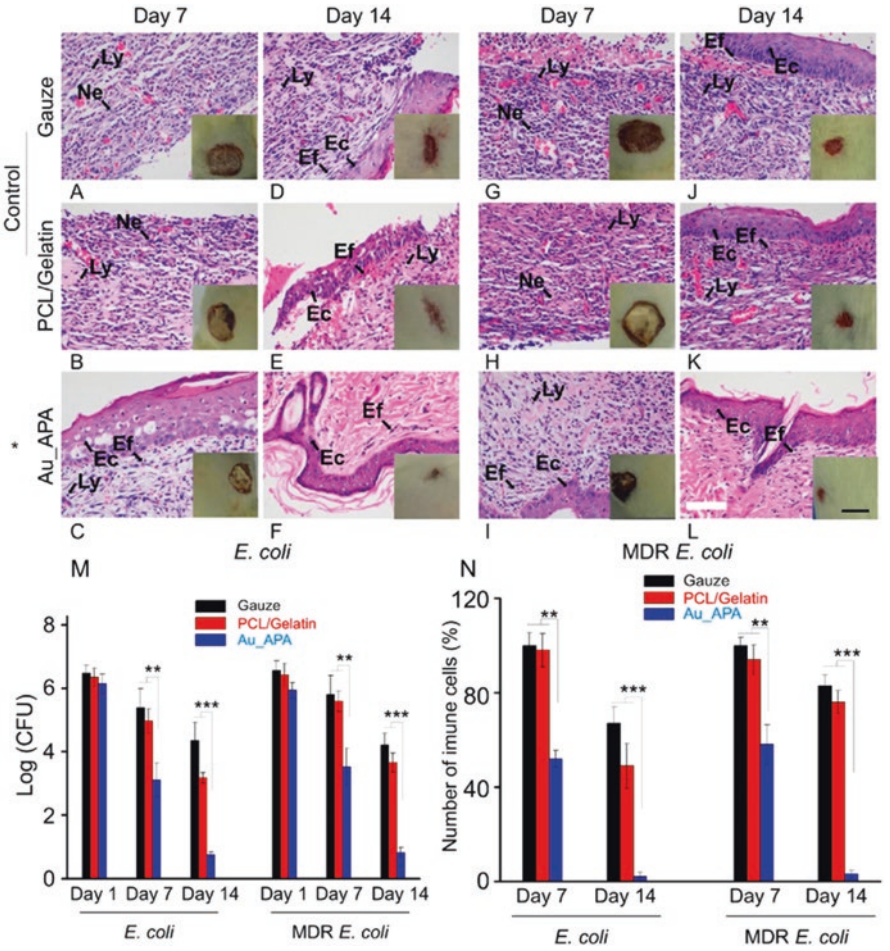

Fig. 5 Animal models of wounds infected with *E. coli* or MDR *E. coli*. Histological images of skin tissues stained by H&E dissected on postoperative day 7 (**a**–**c**, **g**–**i**) and day 14 (**d**–**f**, **j**–**l**). (**m**) Local bacteria count in the wound area and (**n**) relative number of immune cells compared with control group (gauze). Inserted photos show the macroscopic morphologies of wounds. The control groups were treated with wound dressings of gauze (**a**, **d**, **g**, **j**), and PCL/gelatin nanofibers (**b**, **e**, **h**, **k**), and the experimental group was treated with Au-APA electrospun nanofibers as wound dressings (**c**, **f**, **i**, **l**). The presence of Ly and Ne indicates an inflammatory response. Ec and Ef signal reepithelization, and are beneficial for the formation of matured fibrous granulation tissue; ** and *** represent two levels of significant statistical differences (**$P < 0.05$, ***$P < 0.01$). White and black scale bars are 100 μm and 1 cm, respectively (*Ly* lymphocyte, *Ne* neutrophil, *Ec* epithelial cells, *Ef* elongated fibroblasts). (Reproduced from Li et al. (2017) with permission)

wound healing in comparison with Tegaderm (a commercially available wound dressing containing chlorhexidine gluconate) by promoting wound epithelialization and hemostasis (Hsu et al. 2011). In another study, Au NP-embedded collagen scaffolds (AuNPs-SCs) were designed as a skin substitute with good biocompatibility, high

mechanical strength, reduced hydrolytic activity, and stability against enzymatic degradation. AuNP-SCs improved granulation tissue generation, inhibited inflammation, and induced angiogenesis in order to rapidly heal wounds (Akturk et al. 2016).

Several works based on antibiotic-loaded Au NPs have been developed as potent antimicrobial agents (Zhao and Jiang 2013). However, these works showed only in vitro antimicrobial efficacy against bacteria, with none of them having yet been tested in vivo infection models for clinical efficacy. In one report, it was showed that when gentamicin-loaded Au NPs were administered intravenously in mice having intramuscular infections of *S. aureus* in the thigh, most of the NPs were accumulated in the kidneys and blood, with a small amount in the infected thigh but more than in a normal thigh within 60 min postinjection. However, the antimicrobial efficacy and the selectivity of the Au NPs between normal and infected thighs were not studied (Ahangari et al. 2013).

7.2.2 Antimicrobial Peptide-Conjugated Gold Nanoparticles

Several formulations of AMPs conjugated to inorganic NPs have been recently proposed for wound-healing and infection treatments. We have developed a one-step methodology to generate small, homogenous Cecropin-Melittin (CM)-conjugated Au NPs with high loading of the peptide. CM-SH-conjugated Au NPs demonstrated potent antimicrobial activity against both Gram-positive and Gram-negative bacteria in human serum, and in the presence of high concentrations of proteolytic enzymes, than soluble CM-SH, as well as low cytotoxicity to human endothelial and fibroblast cells (Rai et al. 2016). Moreover, CM-SH-conjugated Au NPs demonstrated high antimicrobial activity in chronic wound and systemic infection models. Using the same strategy, Comune et al. immobilized LL37 on Au NPs and demonstrated higher wound-healing properties in comparison to soluble LL37 in both in vitro and in vivo models (Fig. 6) (Comune et al. 2017). The LL37-Au NPs showed antimicrobial activity against *E. coli* and high promigratory activity and low cytotoxicity toward keratinocytes. The LL37-Au NP formulation developed in this work may have great potential to treat chronic wounds, which are often infected by bacteria (Comune et al. 2017). Importantly, the developed nanoformulation is effective in treating wound healing at multiple stages, such as fighting bacterial infection and inducing the migration of keratinocytes to facilitate re-epithelialization in wound with superior efficacy and lower cytotoxicity than LL37 peptide alone.

In another study, the frog skin AMP, esculentin-1a (Esc), chemically conjugated to Au NPs via a PEG linker showed remarkably improved antibacterial activity when compared to the activity exhibited by the same concentration of the free peptide (Casciaro et al. 2017). The developed nanoformulation showed potent anti-Pseudomonal activity of the membrane-active Esc (1-21) approximately 15-fold without keratinocyte toxicity and increased the peptide's re-epithelialization activity on the keratinocyte monolayer (Table 3). These findings make NP attractive candidates for the topical treatment of skin infections. Additionally, Chen et al. prepared gold nanodots (Au-NDs) conjugated with surfactin (SFT; an AMP) and 1-dodecanethiol (Chen et al. 2015). SFT-conjugated

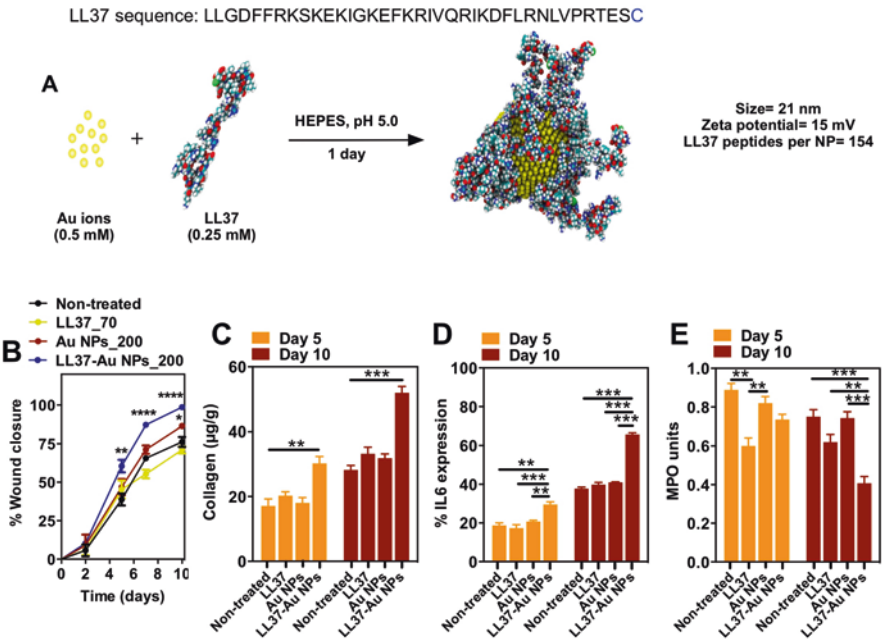

Fig. 6 (**a**) Schematic representation of the synthesis process of LL37-conjugated Au NPs. (**b**) Wound closure in wounds treated with vehicle (0.9% NaCl), LL37 peptide (70 µg per wound), Au NPs (200 µg per wound), or LL37-Au NPs (200 µg per wound). The formulations were administered intradermally at several sites around the wound. Ten animals (therefore 20 wounds) were used per each group. Wound areas were quantified by a high-definition camera. Results are average ± SEM, $n = 20$. (**c**) Quantification of collagen at days 5 and 10 by a sircol assay. Results are average ± SEM, $n = 10$. (**d**) Quantification of IL6 by qRT-PCR at days 5 and 10. Results are average ± SEM, $n = 10$. (**e**) Quantification of myeloperoxidase (MPO) activity at days 5 and 10. Results are average ± SEM, $n = 10$. (Reproduced from Rai et al. (2016) with permission)

Au-NDs showed more pronounced antimicrobial activity against MDR bacteria, and lower cytotoxicity and hemolysis when compared to SFT alone. In vivo MRSA infection studies showed faster healing, improved re-epithelialization, and the efficient collagen fiber production for the SFT-loaded Au-NDs incorporated in a dressing material (Chen et al. 2015).

7.2.3 Silver Nanoparticles

Silver (Ag) has been considered as an antimicrobial material for preventing bacterial infections since ancient times. Clinicians have used Ag, SSD and silver nitrate ($AgNO_3$) for the treatment of the burn wound, dental caries, and bacterial infections. In the last few decades, silver in form of NPs (Ag NPs) has been reported to be effective against several microorganisms such as bacteria, fungi, and yeast. The potent antimicrobial activity of Ag NPs is believed to be due to the release of Ag ions in acidic conditions. It is believed that Ag ions interact electrostatically with the microbial membrane, leading to accumulation on the membrane, loss of intracellular potassium ions, collapse of

Table 3 Antimicrobial NPs

NPs composition	Encapsulated molecules	Target bacteria	Therapeutic activity
CS	Temporin B	*S. epidermidis*	Long-lasting antimicrobial activity, reduced cytotoxicity (Piras et al. 2015)
PLGA	Plectasin	*S. aureus*	High encapsulation efficiency, prolonged release of AMPs over 24 h (Water et al. 2015)
PLGA	LL37	*E. coli*	Synergetic effect to of antimicrobial activity and wound closure (Chereddy et al. 2014)
PG	Nisin	*L. monocytogenes*	Prolonged antimicrobial efficacy, long-term stability (Bi et al. 2011)
Au	CM	Gram-negative and Gram-positive bacteria	High antimicrobial activity and stability in a systemic infection model (Rai et al. 2016)
Au	LL37	*E. coli*	Antimicrobial activity, prolonged cell migration, and in vivo rapid wound closure (Comune et al. 2017)
Au	Esculentin-1a	*P. aeruginosa*	In vitro higher antimicrobial activity and wound healing (Casciaro et al. 2017)
Ag	LL37	*P. aeruginosa*	Antimicrobial and antibiofilm activity (Vignoni et al. 2014)

Abbreviations: *CS* chitosan, *PLGA* poly lactic-co-glycolic acid, *PG* phosphatidylglycerol, *Au* gold, *CM* Cecropin–Melittin, *Ag* silver

the proton motive force, and a decrease in intracellular ATP levels, resulting in bacterial death (Lok et al. 2006). There are several research papers and commercial products (Acticoat® from Smith & Nephew; Aquacel® from Convatec and 3 M Ag mesh from Tegaderm® dressings) available based on wound dressings containing Ag NPs for the treatment of infections in normal and burn wounds (Mofazzal Jahromi et al. 2018; Parani et al. 2016). Recently, alkylated ε-polylysine-capped Ag NPs were synthesized, and used to target bacteria by enhanced multivalent/polyvalent interactions between polylysine and lipopolysaccharide moieties, but showed no cytotoxicity against fibroblasts. Ag nanocomposite-treated wounds had an abundance of pro-inflammatory cells, such as macrophages and $CD3^+$ T lymphocytes, thus helping to control infections and fuel immune responses in order to promote wound healing (Dai et al. 2016). In another approach, Ag NPs synthesized using deoxidizer egg white were mixed with konjac/glucomannan to prepare a composite sponge by freeze-drying. The composite Ag sponge exhibited an excellent antimicrobial activity due to the presence of Ag NPs. The sponge effectively enhanced wound-healing performance within 14 days, owing to its water adsorption properties, which helps to maintain a moist wound environment, and its suitable mechanical strength, which promotes the migration of cells in the wounds (Chen et al. 2018). Beside gold, LL37 peptides have been conjugated to Ag NPs to provide synergetic antimicrobial against *P. aeruginosa*, and have antibiofilm formation activity (Table 3) (Vignoni et al. 2014). Ag NPs functionalized with Polymyxin B peptide showed potent antibacterial activity against MDR *Vibrio fluvialis* and nosocomial

P. aeruginosa. The results of antibacterial assays and live-dead staining showed the peptide-conjugated Ag NPs to display ~threefold higher antimicrobial effects than citrate-capped NPs, due to damage of bacterial membranes. Furthermore, the peptide-conjugated Ag NPs inhibited the biofilm formation and efficiently removed endotoxin (Lambadi et al. 2015).

Ag-related products could cause permanent pigmentation in the skin (argyria) and may induce toxic effects in the kidney and liver, as well as irritation in eyes, skin, and respiratory tract (Drake and Hazelwood 2005). However, Ag NPs are considered to be less toxic than Ag ions, though some reports have shown adverse effects on the mitochondrial activity of cells, and induction of Ag resistance in bacteria, and therefore aggregative clinical use of Ag-based products must be done with caution (Parani et al. 2016; Hussain et al. 2005).

Table 3 summarizes various biodegradable and nondegradable NPs conjugated with AMPs.

8 Future Prospective and Conclusion

Microbial infections, coupled with the emergence of antimicrobial resistance, pose highly complex problems to healthcare systems. The high susceptibility to microbial infections during illness, in addition to the need to achieve rapid and satisfactory treatments, demands the discovery of innovative biomedical technologies. Although topical and intravenous administrations of antimicrobial agents are the most common clinical practice, alternative approaches, including targeted NPs, AMPs, antimicrobial phototherapy, therapeutic microorganisms, and immune-based antimicrobial molecules have also been explored. A range of sophisticated NPs has been developed to control infections and simultaneously promote healing. However, in the future, it is expected that multicomponent and multifunctional NPs will be developed that can deliver drugs on demand. Another challenging task in this area is to target bacteria residing in non-phagocyte cells localized outside the RES using smart NPs loaded with antimicrobial agents. For example, *Salmonella, S. aureus, B. besnlae, chlamydia, L. monocytogenes*, and others mostly accumulate in non-phagocytic cells, such as fibroblast, endothelial, hepatocyte, and enterocyte cells. One possible approach to improve the internalization of antimicrobial NPs within these non-phagocytic cells is to increase the circulation lifetime of NPs by decreasing RES uptake in order to achieve maximum accumulation at infection foci by enhanced retention effects and permeation. Modification of the surface of antimicrobial NPs with specific ligands can also improve the active targeting of infection sites. The most critical issues with NPs are stability during storage, and during their transport from the administration site to the infection site, as well as drug inactivation and premature release. To address these issues, covalent conjugation of drugs and targeted ligands within the same NPs should be explored to reach the infection loci. To tackle notorious MDR infections in wounds, innovative NPs must be developed that not only penetrate and damage the biofilms, but also deliver antimicrobial agents and drugs to

effectively kill MDR bacteria and heal wounds respectively. In the future, smart nanomaterials combined with biotechnologically related advances, such as gene-editing techniques, including CRISPR-Cas systems, will be key players in treating several infectious diseases by suppressing MDR pathogens.

Given the immense research efforts that are currently being invested in developing nanotechnology-based therapies for the treatment of bacterial infections, future progresses in multifunctional and smart antimicrobial nanomaterials are expected to be achieved in the next few years. Importantly, scientific knowledge on the long-term toxicity profile of antimicrobial NPs is desired in order to ensure their successful clinical applications.

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Green Synthesis of Metal Nanoparticles: Characterization and their Antibacterial Efficacy

Faizan Abul Qais, Samreen, and Iqbal Ahmad

Abstract

The global emergence and spread of multi-drug resistance in bacterial pathogens has led the researchers to focus on the development of alternative measures or therapeutic agents to combat microbial infections caused by drug-resistant bacteria. Recent advances in nanotechnology have given a new hope for the development of novel nano-based formulations to address the problem. Green synthesis of metal nanoparticles has advantages over other chemical and physical methods such as, reduced toxicity, one-step process, cost-effectiveness, eco-friendliness, and does not require additional capping or stabilizing agents. The techniques employed for the characterization of nanoparticles are UV–Vis spectroscopy, XRD, FTIR, SEM, EDX, TEM, AFM, DLS, zeta potential, and TGA etc. The antibacterial nanoparticles studied most widely are metal nanoparticles. Metal nanoparticles have demonstrated potent antibacterial activity in vitro with promising therapeutic potential in wound dressings, medical implant coatings, drug delivery, tumour detection, and photoimaging. Many bioagents such as bacteria, fungi, plant extracts, etc. have been exploited for green synthesis of nanoparticles. Medicinal plants as bio-templates for the synthesis of metal nanoparticles might show an immense impact in biomedicine. However, toxicity of nanoparticles to the host is a major concern that needs to be scrutinized properly to monitor its long-term impact. Still, recently developed nano-based formulations, including metal nanoparticles, are expected to become the next generation therapeutic agents against bacterial pathogens, especially against MDR bacteria. In this chapter, we have reviewed the role of medicinal plants in metal nanoparticle synthesis, characterization techniques, and their efficacy against bacterial pathogens.

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Nanoparticles · Antibacterial agents · Toxicity · Nanoparticles synthesis · Characterization

Abbreviations

AFM	Atomic Force Microscopy
DLS	Dynamic light Scattering
FTIR	Fourier Transform Infrared Spectroscopy
SEM	Scanning Electron Microscopy
TEM	Transmission Electron Microscopy
TGA	Thermogravimetric Analysis
UV Vis	UV Visible
XRD	X-ray diffraction

1 Introduction

Over the last few decades, a tremendous increase in the emergence of multi-drug-resistant (MDR) bacterial pathogens has been reported globally. The most common groups of bacteria that are considered problematic includes *S. aureus* (MRSA), *P. aeruginosa*, *Mycobacterium* (MDR), and the extended-spectrum beta-lactamases (ESβL) and carbapenemase-producing *Enterobacteriaceae.* Such MDR bacteria have developed one or more mechanisms by which their susceptibly to antibiotics has been drastically reduced. Therefore, in the era of the emergence of MDR, the efficacy of conventional antibiotics can no longer be trusted for prolonged administration. In the search of novel therapeutic agents to combat MDR bacteria, nanoparticles so far have proven to be a good alternative based on in vitro studies. The recent advances in nanotechnology such as the green route of nanoparticle synthesis has given a new hope for the development of novel nano-based formulations to address the problem. There are numerous bioagents used for the synthesis of nanoparticles. However, the use of plant extracts has clear advantages over other agents (Singh et al. 2016a).

Nanobiotechnology is a fast-developing discipline of science that mainly focuses on synthesis, manipulation, and application of materials at nanometre scale in multiple fields of biology (Shah et al. 2015). Nanoparticles have become of great interest due to their novel physicochemical, optoelectronic, and magnetic properties that are primarily governed by their shape, size, and size distribution (Bogunia-Kubik and Sugisaka 2002; Zharov et al. 2005). Being so small in size and having large surface area-to-volume ratios, they exhibit significant differences in catalytic activity, biological properties, mechanical properties, electrical conductivity etc. in

comparison to the same material in its bulk form or at larger scales (Iravani 2011). The emerging trend of plant-mediated synthesis of metal nanoparticles such as silver, gold etc. has become a focus of attention, leading to the development of multiple methods for their synthesis (Rauwel and Rauwel 2017).

Several efforts have been made to develop environmentally friendly green nanotechnologies and to produce nanoparticles using non-toxic products (Joerger et al. 2000; Chauhan et al. 2012). The synthesis of nanoparticles, using biological entities or green technology, is a one-step procedure resulting in the production of nanoparticles with greater stability and more precise dimensions, which eliminates undesirable processing conditions at a negligible cost (Ingale 2013). Recent advancements in this discipline have been explored for their application in agricultural, biomedical, environmental, and physicochemical areas (Pereira et al. 2015; Rao and Gan 2015; Rai et al. 2016). Silver nanoparticles are the most extensively studied and been found to be potent antibacterial and anti-inflammatory agents that promote faster wound healing. As a result of their therapeutic value, silver nanoparticles are ingredients of pharmaceutical preparations, commercially available wound dressings, and medical implant coatings (Huang et al. 2007b; Pollini et al. 2011; Cox et al. 2011). Similarly, gold nanoparticles have been used in the delivery of certain drugs including paclitaxel, methotrexate, and doxorubicin (Rai et al. 2016). Gold nanoparticles have been also reported for their application in angiogenesis, tumour detection, photoimaging, genetic disorder diagnosis, and photothermal therapy (Singh et al. 2016a). Moreover, being non-toxic, biocompatible, self-cleansing, skin-compatible, antimicrobial, and dermatological in nature, titanium and zinc nanoparticles have been used in cosmetic, biomedical, as UV-blocking agents, and in many other cutting-edge processing technologies (Ambika and Sundrarajan 2015; Zahir et al. 2015). Green synthesis of metal nanoparticles also includes other biological sources such as microbes (bacteria, fungi, algae etc.). In this chapter, we have reviewed plant-mediated synthesis of metal nanoparticles, with a focus on their efficacy against pathogenic bacteria with respect to drug-resistant pathogens. While some excellent review articles are available related to green synthesis of nanoparticles and their antibacterial activity, none of them focussed on MDR bacteria (Shah et al. 2015; Ahmed et al. 2016; Kuppusamy et al. 2016; Singh et al. 2016a).

2 Synthesis of Metal Nanoparticles Using Plants

In last two decades, there has been considerable emphasis on synthesis of nanoparticles using biological agents such as plants, microbes, etc. as these methods are considered less expensive, safe, and eco-friendly alternative to chemical synthesis (Makarov et al. 2014; Gowramma et al. 2015). The use of plants for the synthesis of nanoparticles is even more advantageous than using microorganisms as microbes require culturing, which makes them relatively expensive. In addition, the use of pathogenic microbes or toxic microbial products is often required, adding additional burden (Ahmed et al. 2016). On the other hand, plant metabolites (primary or secondary) such as amino acids, proteins, enzymes, polysaccharides, tannins,

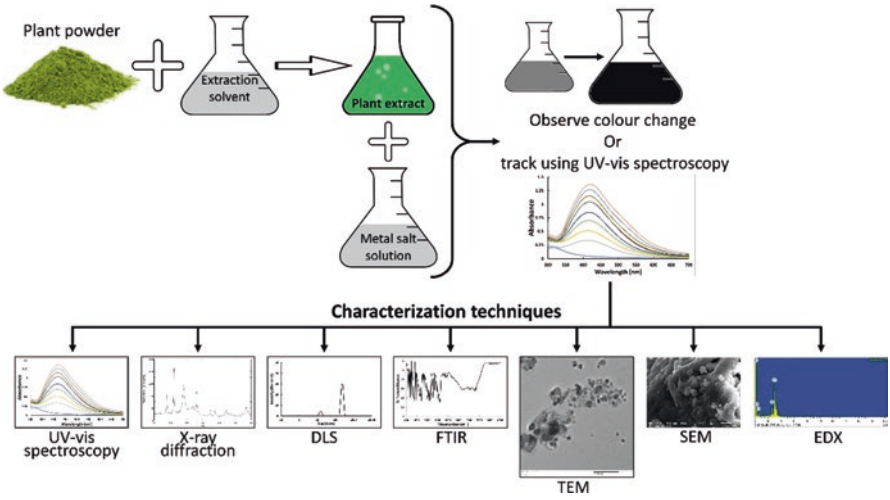

Fig. 1 A representative illustration of metal nanoparticle synthesis and the commonly used techniques for their characterization

alkaloids, phenolics, terpinoids, saponins, and vitamins are used for the reduction of metal ions to make nanoparticles and are environmentally safe (Kulkarni and Muddapur 2014; Kuppusamy et al. 2016). In general, the methods of synthesis of metal nanoparticles have some common stages; (i) collection and washing (distilled water preferably) of the plant's part of interest to remove any debris or dust if present; (ii) shade drying of the sample and then grinding it using a blender to make fine powder; (iii) preparation of plant extract in desirable solvents by dissolving and filtering; and (iv) addition of plant extracts to salt solutions of metals in different combinations for standardization of synthesis (Ahmed et al. 2016). A representative illustration of metal nanoparticle synthesis and the commonly used techniques for their characterization is presented in Fig. 1.

2.1 Silver Nanoparticles

Synthesis of silver nanoparticles using plant material has been commonly reported. To date, a large number of plants have been successfully employed to synthesize silver nanoparticles as mentioned in Table 1, which summarizes the literature from 2009 to 2018 on plant-mediated synthesis, techniques used for their characterization, and organisms against which the nanoparticles were tested, using PubMed and other sources. Some of the recent reports are further elaborated below. Silver nanoparticles have been synthesized using aqueous extract of *Alternanthera dentate* by Kumar et al. (2014a). The nanoparticles were spherical in shape and 50–100 nm in size. It took 10 min to reduce silver ions to silver nanoparticles, validating the technique to be a simple, quick, and economical process. These green synthesized

Table 1 List of plant extract-mediated synthesis of silver nanoparticles, characterization techniques, and the bacteria tested for antimicrobial activity

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
1	*Acacia leucophloea*	Stem bark	Aqueous	17–29 nm	UV–Vis, XRD, TEM, and FTIR	*S. aureus*, *B. cereus*, *L. monocytogenes*, and *S. flexneri*	Murugan et al. (2014)
2	*Acalypha indica*	Leaf	Aqueous	20–30 nm	UV–vis, SEM, HRTEM, XRD, EDS, and SEM	*E. coli* and *V. cholerae*	Krishnaraj et al. (2010)
3	*Actaea racemosa*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *K. rhizophila*, and *B. thuringiensis*	Okafor et al. (2013)
4	*Allium sativum*	Bulb	Aqueous	7.3 ± 4.4 nm	UV–Vis, XRD, TEM, and FTIR	*P. aeruginosa* and *S. aureus*	Rastogi and Arunachalam (2011)
5	*Allophylus cobbe*	Leaf	Aqueous	2–10 nm	UV–Vis, XRD, DLS, TEM, and FTIR	*P. aeruginosa*, *S. flexneri*, *S. aureus,* and *S. pneumonia*	Gurunathan et al. (2014)
6	*Aloe vera*	Leaf	Ethanol	35–55 nm	UV–Vis, SEM, EDX, and FTIR	*B. subtilis*, *K. pneumoniae,* and *S. typhi*	Dinesh et al. (2015)
7	*Alternanthera dentata*	Leaf	Aqueous	10–80 nm	UV–Vis, XRD TEM, and FTIR.	*E. coli*, *P. aeruginosa*, *K. pneumonia,* and *E. faecalis*	Kumar et al. (2014a)
8	*Alternanthera sessilis*	Leaf	Aqueous	20–30 nm	UV–Vis, FTIR, SEM, TGA, and zeta sizer	*S. aureus* and *E. coli*	Niraimathi et al. (2013)
9	*Ananas comosus*	Leaf	Aqueous	12.4 nm	UV–Vis, XRD, TEM, and FTIR	*S. aureus*, *S. pneumoniae*, *P. mirabilis,* and *E. coli*	Emeka et al. (2014)
10	*Anogeissus latifolia*	Gum	Aqueous	5.7 ± 0.2 nm	UV–Vis, XRD, TEM, and FTIR	*S. aureus*, *E. coli*, and *P. aeruginosa*	Kora et al. (2012)
11	*Argemone maxicana*	Leaf	Aqueous	10–50 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli* and *P. syringae*	Singh et al. (2010)

(continued)

Table 1 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
12	*Artemisia nilagirica*	Leaf	Aqueous	70–90 nm	SEM and EDX	*S. aureus*, *E. coli*, *B. subtilis,* and *P. mirabilis*	Vijayakumar et al. (2013)
13	*Artocarpus heterophyllus*	Seeds	Aqueous	10.78 nm	UV–Vis, TEM, SEM, EDX, and FTIR	*B. cereus*, *B. Subtilis*, *S. aureus,* and *P. aeruginosa*	Jagtap and Bapat (2013)
14	*Atrocarpus altilis*	Leaf	Aqueous	25–43 nm	UV–Vis, XRD, SEM, EDX, DLS, and FTIR	*E. coli*, *P. aeruginosa,* and *S. aureus*	Ravichandran et al. (2016)
15	*Azadirachta indica*	Bark	Aqueous	90.13 nm	UV–Vis, XRD, SEM, AFM, DLS, and FTIR	*E. coli*, *P. aeruginosa,* and *B. subtilis,* and *V. cholerae*	Nayak et al. (2016)
16	*Azhadirachta indica*	Leaf	Aqueous	21 nm	UV–Vis, DLS, and FTIR	*K. pneumonia* and *S. typhi*	Lalitha et al. (2013)
17	*Beta vulgaris*	Roots	Aqueous	10–15 nm	UV–Vis, XRD, TEM, EDS, and FTIR	*E. coli*, *P. aeruginosa,* and *S. aureus*	Bindhu and Umadevi (2015)
18	*Boerhaavia diffusa*	Whole plant	Aqueous	25 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*A. hydrophila*, *P. fluorescens,* and *F. branchiophilum*	Kumar et al. (2014b)
19	*Caesalpinia coriaria*	Leaf	Aqueous	40–52 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *S. aureus*	Jeeva et al. (2014)
20	*Cajanus cajan*	Leaf	Aqueous	5–60 nm	UV–Vis, SEM, TEM, and FTIR	*M. luteus*, *S. aureus,* and *E. coli*	Nagati et al. (2012)
21	*Calotropis gigantea*	Leaf	Aqueous	6–12 nm	UV–Vis and AFM	*V. Alginolyticus*	Baskaralingam et al. (2012)
22	*Carica papaya*	Fruit	Aqueous	10–50 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli* and *P. aeruginosa*	Jain et al. (2009)

23	*Centella asiatica*	Leaf	Ethanol	42 nm	UV–Vis, XRD, AFR, and FTIR	*S. aureus*, *P. aeruginosa*, *K. pneumoniae,* and *E. coli*	Logeswari et al. (2013)
24	*Centella asiatica*	Leaf	Aqueous	24 nm	UV–Vis, XRD, SEM, and AFM	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2015)
25	*Ceratonia siliqua*	Leaf	Aqueous	5–40 nm	UV–Vis, XRD, SEM, AAS, and FTIR	*E. coli*	Awwad et al. (2013)
26	*Ceropegia thwaitesii*	Leaf	Aqueous	110.6 nm	UV–Vis, XRD, SEM, EDX, and FTIR	*E. coli*, *V. cholerae*, *S. aureus*, *Corynebacterium*, *P. aeruginosa*, *S. epidermidis*, *Mycobacterium*, *S. typhi*, *S. flexneri*, *K. pneumonia*, *B. subtilis*, *M. luteus,* and *P. mirabilis*	Muthukrishnan et al. (2015)
27	*Chenopodium murale*	Leaf	Aqueous	30–50 nm	UV–Vis and TEM	*S. aureus*	Abdel-Aziz et al. (2014)
28	*Chrysanthemum indicum*	Flower	Aqueous	37–72 nm	UV–Vis, XRD, TEM, and EDX	*B. subtilis*, *S. aureus*, *S. epidermidis*, *E. coli*, *K. pneumoniae,* and *P. aeruginosa*	Arokiyaraj et al. (2014)
29	*Cinnamon zeylanicum*	Bark	Aqueous	31–40 nm	UV–Vis, XRD, TEM, and zeta potential	*E. coli*	Sathishkumar et al. (2009b)
30	*Citrus sinensis*	Peel	Ethanol	41 nm	UV–Vis, XRD, AFR, and FTIR	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2013)
31	*Citrus sinensis*	Peel	Aqueous	59 nm	UV–Vis, XRD, SEM, and AFM	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2015)
32	*Clitoria ternatea*	Leaf	Aqueous	20 nm	UV–Vis, XRD, SEM, and FTIR.	*B. subtilis*, *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa,* and *K. aerogenes*	Krithiga et al. (2015)

(continued)

Table 1 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
33	*Cocos nucifera*	Inflorescences	Ethyl acetate and methanol (40:60)	22 nm	UV–Vis, TEM, and FTIR	*V. alginolyticus*, *P. shigelloides*, *K. pneumoniae*, *S. paratyphi*, *P. aeruginosa*, *V. harveyi*, *B. subtilis*, *E. coli*, *V. mimicus*, and *S. aureus*	Mariselvam et al. (2014)
34	*Coffea arabica*	Seed	Water and ethanol (1:1)	20–30 nm	UV–Vis, XRD, SEM, EDAX, TEM, and FTIR	*E. coli* and *S. aureus*	Dhand et al. (2016)
35	*Coleus aromaticus*	Leaf	Aqueous	40–50 nm	UV–Vis, XRD, SEM, EDAX, and FTIR.	*B. subtilis* and *K. planticola*	Vanaja and Annadurai (2013)
36	*Coriandrum sativum*	Seed	Aqueous	13.09 nm	UV–Vis, XRD, SEM, DLS, and FTIR	*B. subtilis*	Nazeruddin et al. (2014)
37	*Crataegus douglasii*	Fruit	Aqueous		UV–Vis and SEM	*E. coli* and *S. aureus*	Ghaffari-Moghaddam and Hadi-Dabanlou (2014)
38	*Croton sparsiflorus morong*	Leaf	Aqueous	22–52 nm	UV–Vis, XRD, SEM, EDX, and FTIR	*S. aureus*, *E. coli,* and *B. subtilis*	Kathiravan et al. (2015)
39	*Cymbopogon citratus*	Leaf	Aqueous	32 nm	UV–Vis, NTA, TEM, and EDAX	*E. coli*, *S. aureus*, *P. mirabilis*, *S. typhi,* and *K. pneumoniae*	Masurkar et al. (2011)
40	*Dalbergia spinosa*	Leaf	Aqueous	18 ± 4 nm	UV–Vis, TEM, EDS, and FTIR.	*B. subtilis*, *P. aeruginosa*, *S. aureus,* and *E. coli*	Muniyappan and Nagarajan (2014a)
41	*Delphinium denudatum*	Root	Aqueous	≤85 nm	UV–Vis, XRD SEM, and FTIR	*S. aureus*, *B. cereus*, *E. coli,* and *P. aeruginosa*	Suresh et al. (2014)

42	*Dioscorea batatas*	Rhizome	Aqueous	N.A.	UV–Vis, XRD, SEM, EDX, and FTIR	*B. subtilis*, *S. aureus,* and *E. coli*	Nagajyothi and Lee (2011)
43	*Dioscorea bulbifera*	Tuber	Aqueous	8–20 nm	UV–Vis, XRD, TEM, DLS, and FTIR	*A. baumannii*, *E. cloacae*, *E. coli*, *H. influenzae*, *K. pneumoniae*, *N. mucosa,* and *P. mirabilis*	Ghosh et al. (2012)
44	*Diospyros paniculata*	Root	Methanol	14–28 nm	UV–Vis, XRD, SEM, and TEM	*B. subtilis*, *B. pumilis*, *S. pyogenes*, *S. aureus*, *E. coli*, *K. pneumoniae*, *P. vulgaris,* and *P. aeruginosa*	Rao et al. (2016)
45	*Dodonaea viscosa*	Leaf	Aqueous	16 nm	UV–Vis, XRD, AFM, TEM, and FTIR	*E. coli*, *K. pneumonia*, *P. fluorescens*, *B. subtilis,* and *S. aureus*	Kiruba Daniel et al. (2013)
46	*Elettaria cardamomom*	Seeds	Aqueous	40–70 nm	UV–Vis, XRD, SEM, EDAX, and FTIR	*B. subtilis* and *K. planticola*	Gnanajobitha et al. (2012)
47	*Emblica officinalis*	Fruit	Aqueous	10–70 nm	UV–Vis, XRD, SEM, and FTIR	*S. aureus*, *B. subtilis*, *E. coli,* and *K. pneumonia*	Ramesh et al. (2015)
48	*Erythrina indica*	Root	Aqueous	20–118 nm	UV–Vis, XRD, DLS, TEM, EDX, and FTIR	*S. aureus*, *M. luteus*, *E. coli*, *B. subtilis*, *S. typhi,* and *S. paratyphi*	Rathi Sre et al. (2015)
49	*Eucalyptus angophoroides*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*E. coli*, *S. typhimurium*, *S. aureus*, *P. aeruginosa*, *K. rhizophila,* and *B. thuringiensis*	Okafor et al. (2013)
50	*Eucalyptus chapmaniana*	Leaf	Methanol	60 nm	UV–Vis and XRD	*S. aureus*, *S. pneumoniae*, *E. coli*, *P. vulgaris*, *P. aeruginosa,* and *K. pneumoniae*	Sulaiman et al. (2013)

(continued)

Table 1 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
51	*Eucalyptus globulus*	Leaf	Aqueous	5–25 nm	UV–Vis, XRD, SEM, EDX, TEM, and FTIR	*E. coli*, *P. aeruginosa,* and *S. aureus*	Ali et al. (2015)
52	*Euphorbia hirta*	Leaf	Aqueous	40–50 nm	UV–Vis and SEM	*S. aureus*, *E. coli*, *K. pneumoniae*, *B. cereus,* and *P. aeruginosa*	Elumalai et al. (2010)
53	*Euphorbia milii*	Latex	–	105	UV–Vis, particle sizer, and TEM	*P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermis,* and *M. luteus*	Patil et al. (2012)
54	*Euphorbia nivulia*	Latex from stem	Aqueous	5–10 nm	UV–Vis, TEM, and FTIR	*E. coli* and *S. aureus*	Valodkar et al. (2011)
55	*Ficus benghalensis*	Leaf	Aqueous	16 nm	UV–Vis, XRD, TEM, and EDX	*E. coli*	Saxena et al. (2012)
56	*Ficus benghalensis*	Bark	Aqueous	85.95 nm	UV–Vis, XRD, SEM, AFM, DLS, and FTIR	*E. coli*, *P. aeruginosa,* and *B. subtilis* and *V. cholerae*	Nayak et al. (2016)
57	*Garcinia mangostana*	Leaf	Aqueous	35 nm	UV–Vis, TEM, and FTIR	*E. coli* and *S. aureus*	Veerasamy et al. (2011)
58	*Hibiscus cannabinus*	Leaf	Aqueous	8–14 nm	UV–Vis, XRD TEM, EDX, and FTIR	*E. coli*, *P. mirabilis,* and *S. flexneri*	Bindhu and Umadevi (2013)
59	*Impatiens balsamina*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*E. coli*, *S. typhimurium*, *S. aureus*, *P. aeruginosa*, *K. rhizophila,* and *B. thuringiensis*	Okafor et al. (2013)
60	*Iresine herbstii*	Leaf	Aqueous	44–64 nm	UV–Vis, XD, SEM, EDX, and FTIR	*S. aureus*, *E. faecalis*, *E. coli*, *K. pneumoniae,* and *P. aeruginosa*	Dipankar and Murugan (2012)
61	*Jatropha gossypifolia*	Latex	–	62	UV–Vis, particle sizer, and TEM	*P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermis,* and *M. luteus*	Patil et al. (2012)

62	*Lactuca sativa*	Leaf	Aqueous	40–70 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*B. subtilis*, *E. coli*, *K. pneumoniae,* and *P. aeruginosa*	Kanchana et al. (2011)
63	*Lantana camara*	Fruit	Aqueous	12–13 nm	UV–Vis, TEM, and FTIR.	*M. luteus*, *B. subtilis*, *S. aureus*, *V. cholerae*, *K. pneumoniae,* and *S. typhi*	Sivakumar et al. (2012)
64	*Lycopersicon esculentum*	Fruit	Aqueous	10–40 nm	UV–Vis and TEM	*E. coli*	Maiti et al. (2014)
65	*Magnolia grandiflora*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*S. typhimurium*, *P. aeruginosa*, *S. aureus*, *K. rhizophila*, *E. coli,* and *B. thuringiensis*	Okafor et al. (2013)
66	*Mangifera indica*	Peel	Aqueous	7–27 nm	UV–Vis, XRD, TEM, SEM, and FTIR.	*E. coli*, *S. aureus,* and *B. subtilis*	Yang and Li (2013)
67	*Mentha piperita*	Leaf	Aqueous	90 nm	UV–Vis, SEM, EDS, and FTIR	*E. coli* and *S. aureus*	MubarakAli et al. (2011)
68	*Mimusops elengi*	Leaf	Aqueous	55–83 nm	UV–Vis, XRD, SEM, and FTIR.	*K. pneumoniae*, *M. luteus,* and *S. aureus*	Prakash et al. (2013)
69	*Moringa oleifera*	Leaf	Aqueous	57 nm	UV–Vis and TEM	*S. aureus*, *E. coli*, *K. pneumoniae,* and *B. cereus*	Prasad and Elumalai (2011)
70	*Murraya koenigii*	Leaf	Aqueous	40–80 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli*, *S. aureus,* and *P. aeruginosa*	Bonde et al. (2012)
71	*Musa balbisiana*	Leaf	Aqueous	<200 nm	UV–Vis, SEM, TEM, EDS, and FTIR.	*E. coli* and *Bacillus* spp.	Banerjee et al. (2014)
72	*Musa paradisiaca*	Peels	Aqueous	23.7 nm	UV–Vis, XRD, SEM, EDX, TEM, and FTIR.	*B. subtilis*, *S. aureus*, *E. coli,* and *P. aeruginosa*	Ibrahim (2015)
73	*Nyctanthes arbor-tristis*	Flower	Ethanol	5–20 nm	UV–Vis, XRD, TGA, TEM, and FTIR	*E. coli*	Gogoi et al. (2015)

(continued)

Table 1 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
74	*Ocimum sanctum*	Leaf	Methanol	40–50 nm	UV–Vis and SEM	*S. aureus*, *S. saprophyticus*, *E. coli*, *K. pneumoniae*, *E. faecalis*, *E. cloacae,* and *P. vulgaris*	Rout et al. (2012)
75	*Ocimum tenuiflorum*	Leaf	Aqueous	26 nm	UV–Vis, XRD, SEM, and AFM	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2015)
76	*Olea europaea*	Leaf	Aqueous	20–25 nm	UV–Vis, XRD, TEM, and FTIR	*S. aureus*, *P. aeruginosa,* and *E. coli*	Khalil et al. (2014)
77	*Opuntia ficus-indica*	Leaf	Aqueous	12 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli* and *S. aureus*	Gade et al. (2010)
78	*Origanum heracleoticum*	Leaf	Aqueous	30–40 nm	UV–Vis, XRD, SEM, EDX, and FTIR	*E. coli*, *P. aeruginosa*, *S. aureus*, *S. pneumoniae,* and *K. pneumoniae*	Rajendran et al. (2015)
79	*Origanum vulgare*	Leaf	Aqueous	136 ± 10.09 nm	UV–Vis, FTIR, SEM, XRD, and zeta sizer	*A. hydrophilla*, *Bacillus* spp., *E. coli*, *Klebsiella* spp., *Salmonella* spp., *S. paratyphi*, *S. dysenteriae,* and *S. sonnei*	Sankar et al. (2013)
80	*Pedalium murex*	Leaf	Aqueous	20–50 nm	UV–Vis, XRD, SEM, EDAX, TEM, DLS, and FTIR	*E. coli*, *K. pneumoniae*, *M. flavus*, *P. aeruginosa*, *B. subtilis*, *B. pumilus,* and *S. aureus*	Anandalakshmi et al. (2016)
81	*Pedilanthus tithymaloides*	Latex	–	123	UV–Vis, particle sizer, and TEM	*P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermis,* and *M. luteus*	Patil et al. (2012)
82	*Pelargonium graveolens*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*E. coli*, *S. typhimurium*, *S. aureus*, *P. aeruginosa*, *K. rhizophila,* and *B. thuringiensis*	Okafor et al. (2013)
83	*Petroselinum crispum*	Leaf	Aqueous	3–50 nm	UV–Vis, XRD, TEM, DLS, and FTIR	*K. pneumoniae*, *E. coli,* and *S. aureus*	Roy et al. (2015)

84	*Piper longum*	Fruit	Aqueous	15–200 nm	UV–Vis, SEM, DLS, and FTIR	*S. aureus*, *B. cereus*, *P. aeruginosa,* and *B. subtilis*	Reddy et al. (2014)
85	*Piper nigrum*	Leaf	Aqueous	5–50 nm	UV–Vis, XRD, TEM, and FTIR	*E. coli* and *S. aureus*	Augustine et al. (2014)
86	*Pistacia atlantica*	Leaf	Aqueous	10–50 nm	UV–Vis, XRD, SEM, TEM, EDAX, and FTIR.	*S. aureus*	Sadeghi et al. (2015)
87	*Plectranthus amboinicus*	Leaf	Aqueous	20 nm	UV–Vis, XRD SEM, EDS, TEM, and FTIR.	*E. coli*, *Staphylococcus* spp., *Pseudomonas* spp., and *Bacillus* spp.,	Ajitha et al. (2014b)
88	*Polyalthia longifolia*	Leaf	Aqueous	15–50 nm	UV–Vis, TEM, and FTIR	*E. coli*, *P. aeruginosa,* and *S. aureus*	Kaviya et al. (2011b)
89	*Prosopis farcta*	Leaf	Aqueous	8–11 nm	UV–Vis, XRD, and TEM.	*S. aureus*, *B. subtilis*, *E. coli,* and *P. aeruginosa*	Miri et al. (2015)
90	*Rhinacanthus nasutus*	Leaf	Aqueous	<22 nm	UV–Vis, XRD, TEM, DLS, and FTIR	*S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *K. pneumonia*	Pasupuleti et al. (2013)
91	*Rhizophora mucronata*	Leaf buds	Aqueous	4–26 nm	UV–Vis, XRD, TEM, and FTIR	*Proteus* spp., *P. florescence,* and *Flavobacterium* spp.	Umashankari et al. (2012)
92	*Rosmarinus officinalis*	Leaf	Aqueous	10–33 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*S. aureus*, *B. subtilis*, *E. coli,* and *P. aeruginosa*	Ghaedi et al. (2015)
93	*Sansevieria trifasciata*	Leaf	Aqueous	3–15 nm	UV–Vis, TEM, and AFM	*E. coli*, *S. typhimurium*, *P. aeruginosa*, *S. aureus*, *K. rhizophila,* and *B. thuringiensis*	Okafor et al. (2013)
94	*Sargassum wightii*	Whole plant	Aqueous	5–22 nm	UV–Vis, XRD, TEM, AFM, and FTIR	*P. aeruginosa*, *V. cholerae*, *K. pneumoniae*, *S. aureus*, *E. coli*, *S. pneumoniae,* and *S. typhi*	Shanmugam et al. (2014)
95	*Securinega leucopyrus*	Leaf	Aqueous	11–20 nm	UV–Vis, SEM, EDX, TEM, and FTIR	*P. aeruginosa*, *B. subtilis*, *K. pneumonia*, *S. aureus*, *P. putida,* and *E. coli*	Donda et al. (2013)

(continued)

Table 1 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
96	*Sesbania grandiflora*	Leaf	Aqueous	10–25 nm	UV–Vis, XRD SEM, EDX, TEM, AFM, and FTIR	*S. enterica* and *S. aureus*	Das et al. (2013)
97	*Skimmia laureola*	Leaf	Aqueous	38 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. vulgaris,* and *S. aureus*	Ahmed et al. (2015)
98	*Solanum nigrum*	Leaf	Aqueous	28 nm	UV–Vis, XRD, SEM, and FTIR.	*B. subtilis*, *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa,* and *K. aerogenes*	Krithiga et al. (2015)
99	*Solanum tricobatum*	Leaf	Ethanol	52 nm	UV–Vis, XRD, AFR, and FTIR.	*S. aureus*, *P. aeruginosa*, *K. pneumoniae,* and *E. coli*	Logeswari et al. (2013)
100	*Solanum tricobatum*	Leaf	Aqueous	20 nm	UV–Vis, XRD, SEM, and AFM	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2015)
101	*Solanum xanthocarpum*	Berry	Methanol	4–18 nm	UV–Vis, XRD, and TEM	*H. pylori*	Amin et al. (2012)
102	*Spinacia oleracea*	Leaf	Aqueous	40–70 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*B. subtilis*, *E. coli*, *K. pneumoniae,* and *P. aeruginosa*	Kanchana et al. (2011)
103	*Syzygium cumini*	Leaf	Ethanol	53 nm	UV–Vis, XRD, AFR, and FTIR	*S. aureus*, *P. aeruginosa*, *E. coli,* and *K. pneumoniae*	Logeswari et al. (2013)
104	*Tagetes erecta*	Flower	Aqueous	10–90 nm	UV–Vis, XRD, TEM, EDAX, and FTIR	*S. aureus*, *B. cereus*, *E. coli,* and *P. aeruginosa*	Padalia et al. (2015)
105	*Tephrosia purpurea*	Leaf	Aqueous	5–35 nm	UV–Vis, XRD SEM, EDX, TEM, and FTIR	*E. coli*, *Bacillus* spp., *Pseudomonas* spp., and *Staphylococcus* spp.	Ajitha et al. (2014a)

106	*Terminalia arjuna*	Leaf	Aqueous	3–50 nm	UV–Vis, DLS, TEM, and FTIR	*E. coli* and *S. aureus*	Ahmed and Ikram (2015)
107	*Terminalia chebula*	Fruits	Aqueous	<100 nm	UV–Vis, XRD, FTIR, TEM, EDAX, and AFM	*E. coli* and *S. aureus*	Mohan Kumar et al. (2012)
108	*Trianthema decandra*	Roots	Aqueous	10–50 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli* and *P. aeruginosa*	Geethalakshmi and Sarada (2010)
109	*Tribulus terrestris*	Fruit	Aqueous	16–28 nm	UV–Vis, XRD, TEM, AFM, and FTIR	*S. pyogenes*, *E. coli*, *P. aeruginosa*, *B. subtilis,* and *S. aureus*	Gopinath et al. (2012)
110	*Vitex negundo*	Leaf	Methanol	10–30 nm	UV–Vis, XRD, and TEM	*S. aureus* and *E. coli*	Zargar et al. (2011)
111	*Vitis vinifera*	Fruit	Aqueous	30–40 nm	UV–Vis, XRD, SEM, EDX, and FTIR	*B. subtilis* and *K. planticola*	Gnanajobitha et al. (2013)
112	*Withania somnifera*	Root	Aqueous	40–60 nm	UV–Vis, XRD, SEM, EDX, TEM, DLS, and FTIR	*S. aureus*, *S. mutans*, *P. aeruginosa,* and *S. typhimurium*	Qais et al. (2018)
113	*Zingiber officinale*	Root	Aqueous	10–20 nm	UV–Vis, SEM, EDS, TEM, and FTIR	*Staphylococcus* spp., *Listeria* spp., and *Bacillus* spp.	Velmurugan et al. (2014a)

silver nanoparticles exhibited antibacterial activity against *P. aeruginosa*, *K. pneumonia*, *E. coli*, and *E. faecalis* (Kumar et al. 2014a).

Similarly, silver nanoparticles were also synthesized from aqueous extracts of *Withania somnifera* with an average size of 52.19 nm. The silver nanoparticles exhibited broad-spectrum antibacterial and antibiofilm activities against Gram-positive and Gram-negative pathogens. The antibacterial mechanism described was the disruption of cellular membrane and production of intracellular reactive oxygen species in test bacteria (Qais et al. 2018). Rathi Sre et al. (2015) synthesized silver nanoparticles using aqueous root extract of *Erythrina indica*. The 20–118 nm sized silver nanoparticles were characterized using UV–Vis, XRD, DLS, TEM, EDX, and FTIR and exhibited a wide range of antimicrobial activity against *S. aureus*, *M. luteus*, *E. coli*, *B. subtilis*, *S. typhi*, and *S. paratyphi* (Rathi Sre et al. 2015).

Rhizome extracts of *Acorous calamus*, an Indian medicinal plant used in traditional medicine, was used for silver nanoparticle synthesis and showed multifaceted biological activities like antibacterial, antioxidant, and anticancer effects (Nakkala et al. 2014). Extracts from green and black tea (*C. sinensis*) leaves have been used for green synthesis of silver nanoparticles that showed antibacterial activity against Gram-positive bacterial species, i.e. methicillin- and vancomycin-resistant *S. aureus* (Asghar et al. 2018). *Boerhaavia diffusa* extract, a medicinal plant, was used as a reducing agent for the synthesis of silver nanoparticles. Characterization using TEM and XRD revealed the average particle size to be 25 nm and having face-centred cubic (fcc) structure. The nanoparticles exhibited antibacterial activity against fish bacterial pathogens viz. *P. fluorescens*, *A. hydrophila*, and *F, branchiophilum* (Kumar et al. 2014b). Banana peels (*Musa paradisiaca*) have also been shown to reduce $AgNO_3$ to produce silver nanoparticles. The nanoparticles were characterized using UV–Vis, XRD, SEM, EDX, TEM, and FTIR with an average size of 23.7 nm and exhibited antibacterial activity against *B. subtilis*, *S. aureus*, *P. aeruginosa*, and *E. coli* (Ibrahim 2015). Likewise, leaf extracts of *Ceratonia siliqua*, *Musa balbisiana*, *Azadirachta indica*, *Ocimum tenuiflorum*, *Ocimum sanctum*, *Argemone maxicana* have all been used for synthesizing silver nanoparticles with antibacterial activity against numerous pathogens (Singh et al. 2010; Rout et al. 2012; Awwad et al. 2013; Banerjee et al. 2014).

2.2 Gold Nanoparticles

After silver nanoparticles, gold nanoparticles are the most routinely synthesized nanoparticles using plant extracts. Various plants used in the synthesis of gold nanoparticles are listed in Table 2, which gives the details of the plants used, characterization techniques, and the test organisms against which the gold nanoparticles were active. A brief description of reports is discussed here.

Root extract of *Panax ginseng* has been documented to reduce auric acid to produce gold nanoparticles within 5 min at 80 °C without using any additional capping agent. The gold nanoparticles exhibited antibacterial activity against *B. anthracis*, *B. cereus*, *E. coli*, *S. aureus*, and *V. parahaemolyticus* (Singh et al. 2016b). Gold

Table 2 List of plant extract-mediated synthesis of gold nanoparticles, characterization techniques, and the bacteria tested for antimicrobial activity

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
1	*Abelmoschus esculentus*	Pulp	Aqueous	14 nm	UV–Vis, XRD, SEM, EDX, DLS, and FTIR	*B. subtilis*, *B. cereus*, *P. aeruginosa*, *M. luteus,* and *E. coli*	Rahaman Mollick et al. (2014)
2	*Anacardium occidentale*	Nuts shell	Aqueous	5–20 nm	UV–Vis, XRD, SEM, EDS, TEM, and FTIR	*A. hydrophila*, *A. bestiarum*, *P. fluorescens,* and *E. tarda*	Velmurugan et al. (2014b)
3	*Ananas comosus*	Fruit	Aqueous	16 nm	UV–Vis, XRD, SEM, and EDX	*S. aureus* and *P. aeruginosa*	Bindhu and Umadevi (2014)
4	*Areca catechu*	Nuts	Aqueous	13.7 nm	UV–Vis, XRD, TEM, and FTIR	*E. coli*, *K. pneumonia*, *P. aeruginosa*, *Enterobacter* spp., and *S. aureus*	Rajan et al. (2015)
5	*Carica papaya*	Leaf	Aqueous	15–28 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*B. subtilis*, *S. aureus*, *P. vulgaris,* and *E. coli*	Muthukumar et al. (2016)
6	*Catharanthus roseus*	Leaf	Aqueous	15–28 nm	UV–Vis, XRD, SEM, TEM, and FTIR	*B. subtilis*, *S. aureus*, *P. vulgaris,* and *E. coli*	Muthukumar et al. (2016)
7	*Citrullus lanatus*	Rind	Aqueous	20–140 nm	UV–Vis, XRD, SEM, EDX, TGA, and FTIR	*B. cereus*, *L. monocytogenes*, *S. aureus*, *E. coli,* and *S. typhimurium*	Patra and Baek (2015)
8	*Coleus aromaticus*	Essential oil	–	28 nm	UV–Vis, XRD, TEM, EDS, and FTIR	*E. coli,* and *S. aureus*	Vilas et al. (2016)
9	*Curcuma pseudomontana*	Essential oil	–	20 nm	UV–Vis, SEM, TEM, and FTIR	*B. subtilis*, *P. aeruginosa*, *S. aureus,* and *E. coli*	Muniyappan and Nagarajan (2014b)
10	*Dracocephalum kotschyi*	Leaf	Aqueous	8–22 nm	UV–Vis, XRD, TEM, EDAX, and FTIR	*S. aureus*, *B. subtilis*, *B. cereus*, *E. coli*, *P. aeruginosa*, and *P. vulgaris*	Dorosti and Jamshidi (2016)
11	*Euphorbia hirta*	Leaf	Aqueous	6–71 nm	UV–Vis, XRD, TEM, EDS, AFM, DLS, and FTIR	*E. coli*, *P. aeruginosa,* and *K. pneumonia*	Annamalai et al. (2013)

(continued)

Table 2 (continued)

S. No.	Plant	Part used	Extraction solvent	Average size/ range of NPs	Characterization techniques	Test bacteria	References
12	*Gloriosa superba*	Leaf	Aqueous	20–50 nm	UV–Vis, XRD, TEM, EDX, AFM, and FTIR	*S. aureus*, *S. pneumoniae*, *E. coli,* and *K. pneumoniae*	Gopinath et al. (2016)
13	Grapes	Fruit	Aqueous	N.A.	UV–Vis, TEM, TGA, and FTIR	*S. aureus*, *C. koseri*, *B. cereus*, *E. coli,* and *P. aeruginosa*	Lokina and Narayanan (2013)
14	*Hovenia dulcis*	Fruit	Aqueous	15–20 nm	UV–Vis, XRD, TEM, EDX, TGA, and FTIR	*E. coli* and *S. aureus*	Basavegowda et al. (2014)
15	*Jasminum sambac*	Leaf	Aqueous	20–50 nm	UV–Vis, XRD, SEM, EDX, TGA, and FTIR	*S. aureus*, *E. coli*, *S. typhi,* and *P. aeruginosa*	Yallappa et al. (2015)
16	*Mentha piperita*	Leaf	Aqueous	150 nm	UV–Vis, SEM, EDX, and FTIR	*E. coli* and *S. aureus*	MubarakAli et al. (2011)
17	*Musa paradisiaca*	Peels	Aqueous	N.A.	UV–Vis, XRD, SEM, EDX, and FTIR	*Shigella* sp., *E. aerogenes*, *Klebsiella* spp., and *P. aeruginosa*	Bankar et al. (2010)
18	*Nepenthes khasiana*	Leaf	Aqueous	50–80 nm	UV–Vis, XRD, SEM, and FTIR	*E. coli* and *Bacillus* spp.	Bhau et al. (2015)
19	Nigella sativa essential oil	Essential oil	–	15–28 nm	UV–Vis, XRD, TEM, and FTIR	*S. aureus* and *V. harveyi*	Manju et al. (2016)
20	*Panax ginseng*	Root	Aqueous		UV–Vis, XRD, SEM, EDX, and TEM	*B. anthracis*, *V. parahaemolyticus*, *S. aureus*, *E. coli,* and *B. cereus*	Singh et al. (2016b)
21	*Panax ginseng*	Root	Aqueous	10–30 nm	UV–Vis, SEM, EDX, and TEM	*V. parahaemolyticus*, *S. aureus,* and *B. cereus*	Singh et al. (2016c)
22	*Pistacia integerrima*	Gall	Methanol	20–200 nm	UV–Vis, SEM, and FTIR	*K. pneumonia*, *B. subtilis,* and *S. aureus*	Islam et al. (2015a)
23	*Plumbago zeylanica*	Root	Aqueous	20–30 nm	UV–Vis, XRD, TEM, EDX, DLS, and FTIR	*A. baumannii*, *E. coli,* and *S. aureus*	Salunke et al. (2014)
24	*Plumeria alba*	Flower	Aqueous	15.6 ± 3.4 nm	UV–Vis, XRD, TEM, EDX, TGA, and FTIR	*E. coli*	Mata et al. (2016)

25	*Punica granatum*	Fruit	Aqueous	5–20 nm	UV–Vis, XRD, TEM, TGA, and FTIR	*S. aureus*, *S. typhi,* and *V. cholerae*	Lokina et al. (2014)
27	*Rivea hypocrateriformis*	Aerial part	Aqueous	10–50 nm	UV–Vis, XRD, SEM, EDX, TGA, TEM, and FTIR	*K. pneumonia*, *S. aureus*, *B. subtilis*, *P. aeruginosa,* and *E. coli*	Godipurge et al. (2016)
28	*Salicornia brachiata*	Aerial part	Aqueous	22–35 nm	UV–Vis, XRD, TEM, and FTIR	*P. aeruginosa*, *S. typhi*, *E. coli,* and *S. aureus*	Ayaz Ahmed et al. (2014)
29	*Salix alba*	Leaf	Aqueous	50–80 nm	UV–Vis, SEM, AFM, and FTIR	*K. pneumonia*, *B. subtilis,* and *S. aureus*	Islam et al. (2015b)
30	*Solanum nigrum*	Leaf	Aqueous	32 ± 6 nm	UV–Vis, XRD, TEM,DLS and FTIR	*S. saprophyticus*, *B. subtilis*, *E. coli,* and *P. aeruginosa*	Muthuvel et al. (2014)
31	*Solanum torvum*	Fruit	Aqueous	5–50 nm	UV–Vis, SEM, EDX, and FTIR	*B. subtilis*, *P. aeruginosa,* and *E. coli*	Ramamurthy et al. (2013)
32	*Trianthema decandra*	Leaf	Aqueous	38–80 nm	UV–Vis, SEM, EDX, and FTIR	*E. faecalis*, *S. aureus, S. faecalis*, *B. subtilis*, *Y. enterocolitica*, *P. vulgaris*, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*	Geethalakshmi and Sarada (2013)
33	*Trianthema decandra*	Root	Aqueous	33–65 nm	UV–Vis, SEM, EDX, and FTIR	*S. aureus*, *S. faecalis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *B. subtilis,* and *Y. enterocolitica*	Geethalakshmi and Sarada (2012)
34	*Turbinaria conoides*	Whole plant	Aqueous	2–19 nm,	UV–Vis, XRD, SEM, EDX, TEM, and FTIR	*Salmonella* spp., *E. coli*, *A. hydrophila,* and *S. liquefaciens*	Vijayan et al. (2014)
35	*Zizyphus mauritiana*	Leaf	Aqueous	20–40 nm	UV–Vis, XRD, SEM, EDS, TEM, and FTIR	*S. aureus*	Sadeghi (2015)

nanoparticles (8–22 nm) have also been synthesized using leaf extracts of *Dracocephalum kotschyi*. The nanoparticles were found to be active against *S. aureus*, *B. subtilis*, *B. cereus*, *E. coli*, *P. aeruginosa*, and *P. vulgaris* (Dorosti and Jamshidi 2016). Aqueous extracts of grapefruit have been documented for their ability to synthesize gold nanoparticles and were reported to have dual activities, i.e. antibacterial and anticancer activities (Lokina and Narayanan 2013). In another finding, *Trianthema decandra* root extract-mediated synthesis of gold nanoparticles were reported to be active against Gram-positive and Gram-negative bacteria such as *S. aureus*, *S. faecalis*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *P. vulgaris*, *B. subtilis*, and *Y. enterocolitica* (Geethalakshmi and Sarada 2012). Gold nanoparticles synthesized from *Indigofera tinctoria* leaf extracts were found to exhibit antimicrobial, anticancer, antioxidant, and catalytic properties (Vijayan et al. 2018). Similarly, leaf extracts of *Ziziphus zizyphus* reduced Au^{3+} ions to Au^{0} to form gold nanoparticles. The 51.8 ± 0.8 nm sized nanoparticles were characterized using (TEM), scanning electron microscope (SEM), and UV–Vis spectroscopy, AFM, XRD, EDX, and TGA (Aljabali et al. 2018).

2.3 Other Metal Nanoparticles

Apart from silver and gold nanoparticles, many other metal nanoparticles have also been synthesized using different parts of various plants. Zinc oxide nanoparticles synthesized from the leaf extracts of *Tabernaemontana divaricata* have been reported. Antimicrobial studies revealed that the zinc oxide nanoparticles exhibited higher antibacterial activity against *S. aureus* and *E. coli* compared to *S. paratyphi* (Raja et al. 2018). Similarly, zinc oxide nanoparticles were also synthesized from the leaf extracts of *Glycosmis pentaphylla*. XRD revealed the crystalline nature of these nanoparticles and EDAX confirmed the presence of highly pure zinc oxide metal (20.70%). The nanoparticles inhibited the growth of *B. cereus*, *S. aureus*, *S. dysenteriae*, and *S. paratyphi* in agar well diffusion assays (Vijayakumar et al. 2018a). Vijayakumar et al. also reported the synthesis of zinc oxide nanoparticles using leaf extracts of *Atalantia monophylla*. Their study mentioned better bacterial and fungal destruction by zinc oxide nanoparticles than plant extracts and standard drugs (Vijayakumar et al. 2018b). In another report, zinc oxide nanoparticles were synthesized from leaf extracts of *Azadirachta indica*. The MIC values of these synthesized nanoparticles were found to be in 12.5–50 μg/ml range against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *P. mirabilis*, and *E. coli* (Elumalai and Velmurugan 2015).

Copper oxide (CuO) nanoparticles have been synthesized using leaf extracts of *Gloriosa superba*. Characterization revealed the nanoparticles to be in 8–17 nm range. The nanoparticles exhibited antibacterial activity against *K. aerogenes*, *E. coli*, *S. aureus*, and *P. desmolyticum* (Naika et al. 2015). Similarly, the juice of *Citrus medica* has been documented for its ability to produce copper nanoparticles. The nanoparticles were 10–60 nm in size and showed antibacterial activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. acne*, and *S. typhi* (Shende et al. 2015). Apart from plant extracts, copper oxide nanoparticles were synthesized using gum

karaya as a biotemplate. The nanoparticles exhibited antibacterial activity against *E. coli* and *S. aureus* with MIC values of 103 ± 4.7 and 120 ± 8.1 μg/ml respectively (Černík and Thekkae Padil 2013).

Titanium dioxide nanoparticles have been reported to be synthesized using ethanolic leaf extracts of *Nyctanthes arbor-tristis* with particle sizes of 150 nm (Sundrarajan and Gowri 2011). Likewise, titanium dioxide nanoparticles were also synthesized using aqueous leaf extracts of *Psidium guajava*. The synthesized nanoparticles exhibited antibacterial activity against *A. hydrophila*, *P. mirabilis*, *E. coli*, *S. aureus*, and *P. aeruginosa* (Santhoshkumar et al. 2014). Many other metal nanoparticles such as nickel, aluminium oxide, magnesium oxide, calcium oxide, vanadium, chromium oxide, manganese oxide, iron, and cobalt have also been synthesized using the green route and tested for their antibacterial efficacy (Ramesh et al. 2012; Ansari et al. 2015; Pandian et al. 2016; Aliyu et al. 2017; Varaprasad et al. 2017; Haneefa 2017; Sharma et al. 2017; Ijaz et al. 2017; Devatha et al. 2018).

3 Factors Affecting Green Synthesis of Metal Nanoparticles

A number of factors are known to influence the synthesis of metal nanoparticles via the green route that are responsible for nucleation and stabilization of nanoparticles. The major controlling factors are concentration of reactants, pH, temperature, and reaction time (Shah et al. 2015). These are discussed below.

3.1 Role of Reactant Concentration

The concentration and nature of phytocompounds present in plant extracts has a remarkable effect on the synthesis and stabilization of metal nanoparticles. The increasing concentration of leaf extract *Cinnamomum camphora* (camphor) in the presence of a fixed amount of precursor (chloroauric acid) resulted in a change of shape of the nanoparticles from triangular to spherical (Huang et al. 2007a). Similarly, Kora et al. (2010) studied the efficacy of silver nanoparticle synthesis at different silver nitrate concentrations and reaction times. A reaction mixture containing 0.1% gum kondagogu and 1 mM $AgNO_3$ resulted in 55.0 and 18.9 nm sized nanoparticles at 30 and 60 min of reaction time, respectively, with polydispersed morphology. When a reaction mixture of 0.5% gum kondagogu and 1 mM $AgNO_3$ were tested, the nanoparticles were mostly spherical in shape with an average particle size of 11.2 and 4.5 nm at 30 and 60 min reaction time respectively (Kora et al. 2010). Similarly, it was found that the concentration of *Aloe vera* leaf extract had an influence on the ratio of gold triangular plates to spherical nanoparticles. It was also determined that the carbonyl compounds in the extract assisted in shaping particle growth and the concentration of extract had a modulatory effect on particle size (Chandran et al. 2006). Likewise, silver nanoparticles with hexagonal, decahedral, triangular, and spherical morphology were synthesized by altering *Plectranthus*

amboinicus leaf extract concentration in the reaction mixture (Narayanan and Sakthivel 2010). Hence, the concentration of reactant in reaction mixture affects both the shape and size of the synthesized nanoparticles.

3.2 Role of pH

The pH of the reaction medium also plays a crucial role in the synthesis of nanoparticles (Shah et al. 2015). It has been found that changes in the pH of the reaction medium result in variability in the size and shape of the synthesized nanoparticles. Broadly, nanoparticles synthesized at higher pH are usually smaller compared to the ones produced at lower pH (Dubey et al. 2010; Sathishkumar et al. 2010). For instance, the size of silver nanoparticles synthesized from bark extracts of *Cinnamon zeylanicum* were large and highly dispersed when synthesized at lower pH. However, there was a reduction in particle size when they were synthesized at higher pH. It was speculated that lower pH tends to cause the aggregation of nanoparticles resulting in the formation of larger particles. It has been suggested that the availability of a large number of functional groups at higher pH produces more silver nanoparticles with smaller diameters (Sathishkumar et al. 2009b). Similarly, gold nanoparticles synthesized using *Avena sativa* at pH 2 were larger in size (25–85 nm) compared to the particles synthesized between pH 3 and 4 (5–20 nm). It was suggested that at a lower pH there are fewer functional groups available, resulting in the aggregation of particles to form larger nanoparticles whereas, at pH 3 and 4, there are more accessible functional groups available for particle nucleation (Armendariz et al. 2004). In another finding, gold nanoparticles synthesized using *Elaeis guineensis* extracts at lower pH (4.5) ranged from 4.49 nm to 17.56 nm (average size 9.61 nm). The same nanoparticles, when synthesized at pH 7.5, ranged in size from 4.32 nm to 16.12 nm with an average diameter of 8.51 nm (Irfan et al. 2017). On the contrary, palladium nanoparticles synthesized from bark extracts of *Cinnamon zeylanicum* at lower pH were smaller in size compared to those synthesized at higher pH. The palladium nanoparticles ranged in size from 15–20 nm when synthesized at pH <5 and from 20 to 25 nm at pH >5 (Sathishkumar et al. 2009a).

3.3 Role of Temperature

In the green synthesis of metal nanoparticles, temperature also plays a crucial role. It has been reported that reaction temperature not only determines the size of the nanoparticles, but also in their shape and yield (Song et al. 2009; Sathishkumar et al. 2010). For example, the average size of gold nanoparticles synthesized at 25 °C using peel extracts of *Citrus sinensis* was around 35 nm. However, upon increasing the reaction temperature, the average size of gold nanoparticles became 10 nm (Kaviya et al. 2011a). Similarly, leaf extracts of *Diospyros kaki* were used to synthesize silver nanoparticles at different temperatures (25–95 °C). It was shown that the size and shape of the nanoparticles could be controlled by changing leaf

broth concentration and the reaction temperature (Song et al. 2009). Moreover, variation in thermal conditions for *Avena sativa*-mediated synthesis of gold nanoparticles of varied shape and size (Armendariz et al. 2004). It was revealed by Gericke and Pinches (2006) that higher temperatures promote the formation rate of synthesis of gold nanoparticles. They found that at lower temperatures, spherical-shaped nanoparticles were predominant while plate-like nanoparticles were more common at higher temperatures (Gericke and Pinches 2006). At higher temperatures there is a faster particle formation rate; however, there is also a decrease in average particle size with increasing temperature (Shah et al. 2015).

3.4 Role of Reaction Time

Reaction time also contributes to the green synthesis of metal nanoparticles by influencing the shape, size, and stability of nanoparticles. A study on the green synthesis of silver nanoparticles using *Ananas comosus* (Pineapple) extract showed rapid colour change within 2 min due to the reduction of $AgNO_3$ to form nanoparticles. When the reaction time was increased to more than 5 min, a slight change in the colour of the reaction mixture was observed (Ahmad and Sharma 2012). Similarly, the green synthesis of silver nanoparticles from silver nitrate using leaf extracts of *Azadirachta indica* exhibited variation in particle size upon changing the reaction time. When reaction time was varied from 30 min to 4 h, the particle size also changed from 10 to 35 nm. Additionally, the zeta potential of nanoparticles was −47 mV at 3.5 h reaction time and increased to −40 mV at 4 h reaction time (Prathna et al. 2011). The *Withania somnifera* (aqueous root extract) synthesized silver nanoparticles also showed a similar trend. The absorption band increased up to 5 h and then became almost constant (Qais et al. 2018). In another similar finding, there was increase in absorbance of UV–Vis spectra and peak sharpening was observed with increasing reaction time. In the synthesis of gold and silver nanoparticles, the absorption band increased up to 2 h and then only slight variations were observed (Dwivedi and Gopal 2010).

4 Advantage of Nanoparticle Synthesis by the Green Route

The green route of metal nanoparticle synthesis has several advantages over chemical or physical synthesis. One of the most advantageous characteristics of biosynthesized metal nanoparticles is their biological compatibility (Singh et al. 2016a). For biomedical applications, nanoparticles with negligible or reduced toxicity is required. Biogenic metal nanoparticles are usually free from the toxic contamination of by-products created during the physical or chemical synthesis of nanoparticles and become attached to particles, consequently limiting their biomedical utility (Baker et al. 2013). Other advantages of biological synthesis of metal nanoparticles include rapid synthesis, eco-friendly nature, and their cost-effective production

(Singh et al. 2016a). In the chemical synthesis of metal nanoparticles, stabilizing agents are required; however, phytoconstituents available in the reaction mixture itself act as capping and stabilizing agents (Makarov et al. 2014). Furthermore, metal nanoparticles contain many functional groups due to the presence of phytocompounds. These groups progressively interact and adsorb biomolecules when they contact biological fluids, resulting in the formation of a corona, providing added efficacy over bare biological nanoparticles (Monopoli et al. 2012).

Another advantage to biological synthesis of metal nanoparticles is that there are fewer steps required, such as in the attachment of some desired functional groups to the surface to make them biologically active (Baker et al. 2013). Therefore, the nanoparticles produced from biological sources become more therapeutically effective due to the attachment of bioactive phytoconstituents of medicinal importance. For instance, the attachment of metabolites with pharmacological activity from medicinal plants to synthesize nanoparticles can provide an additional benefit with enhanced efficacies (Sintubin et al. 2012; Mukherjee et al. 2012; Makarov et al. 2014).

5 Characterization of Nanoparticles

Recently, nanoparticles are being tailored more precisely as per their application. Therefore, the synthesized nanoparticles must be verified for the presence of desired characteristics before their application especially for biomedical usage. The common attributes for which nanoparticles are usually characterized include shape, particle size or size distribution, surface area, zeta potential, hydrated surface analysis, solubility, adsorption tendency, porosity and pore size, presence of functional groups, stability, etc. (Ingale 2013). Some routinely used techniques for characterization of nanoparticles are UV–Vis spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), atomic force microscopy (AFM), X-ray diffraction (XRD), dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TGA), nuclear magnetic resonance (NMR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), etc. (Khomutov and Gubin 2002; Choi et al. 2007; Gupta et al. 2013; Patra and Baek 2014). Some of these techniques are briefly described below.

5.1 UV–Vis Spectroscopy

UV–Visible spectroscopy is primarily used to track and confirm the formation of various types of metal nanoparticles by measuring the Plasmon resonance and evaluating the collective oscillations of conduction band electrons (Ingale 2013). The technique provides preliminary information about structure, size, stability, and aggregation of the nanoparticles being synthesized (Daniel and Astruc 2004). Metal nanoparticles exhibit specific bands in absorption spectra when the incident light enters into resonance with the conduction band electrons on their surface (Patra and

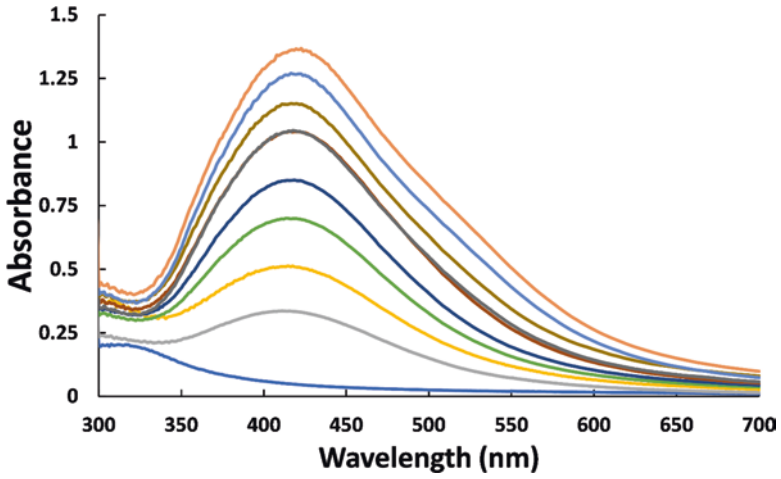

Fig. 2 UV–Visible spectra of silver nanoparticle synthesis using root extract of *Withania somnifera*

Baek 2014). For instance, silver nanoparticles exhibit a specific absorbance band in 400–450 nm range, while gold nanoparticles produce an absorbance maximum from 500 to 550 nm, due to surface plasmon resonance that may vary depending on the size and other related characteristics of the metal nanoparticles (Asharani et al. 2008; Rao and Savithramma 2011; Devi et al. 2012). A representative UV–Visible spectra of silver nanoparticle synthesis using *Withania somnifera* is shown in Fig. 2.

5.2 X-Ray Diffraction

X-ray diffraction (XRD) is a commonly used technique to assess the crystallinity of nanoparticles including metal nanoparticles (Chauhan et al. 2012).The technique is employed for identification and quantification of various crystalline forms or elemental composition materials such as nanoparticles (Tiede et al. 2008). With this technique incident light is diffracted from a powder specimen and then the diffraction pattern is analysed by measuring the angle of diffraction. The width of the particles can be determined by measuring the diffraction peaks and using Scherrer formula (Gupta et al. 2013). A representative X-ray diffraction pattern of silver nanoparticle is shown in Fig. 3.

5.3 Fourier Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) spectroscopy is employed to identify the various functional groups present on nanoparticles. The transmission spectra of nanoparticles are obtained by forming a thin and transparent layer of potassium

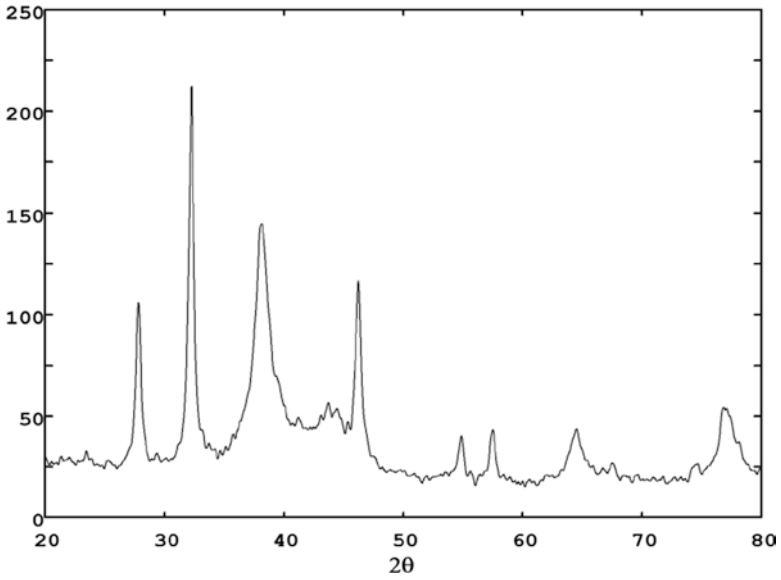

Fig. 3 A characteristic X-ray diffraction pattern of silver nanoparticles

bromide (KBr) pellets along with nanoparticles. The KBr mixtures are then placed in a vacuum line for sufficient time for formation of pellet before use. The transmission spectra are recorded after purging in dry air and the background is corrected using a reference blank sample. i.e. KBr only (Gupta et al. 2013). Using advanced computational software tools, quantitative analysis of nanoparticles can be performed in very short duration (Priya et al. 2011; Kumar et al. 2011).The FTIR spectrum is the representation of fingerprint of absorption or transmission peaks that corresponds to the frequencies of vibrations and rotations between the bonds of atoms present in/on nanoparticles. The functional groups present in the nanoparticles can be identified using FTIR spectroscopy as each bond and functional groups contains a unique combination of atoms (Faraji et al. 2010). The number of functional groups present in nanoparticles can be determined by the size of the peaks in the spectrum (Faraji et al. 2010; Chauhan et al. 2012). A representative FTIR spectrum is shown in Fig. 4.

5.4 Microscopic Techniques

Particle size distribution and morphology of nanoparticles are important traits that can be characterized using microscopic techniques such as SEM, TEM, and AFM (Pal et al. 2011). Particle size or size distribution is a vital attribute of nanoparticles that plays an important role in determining drug release and targeting, toxicity, in vivo distribution, and biological fate (Patra and Baek 2014). It has been reported that nanoparticles are more biologically effective than microparticles owing to their

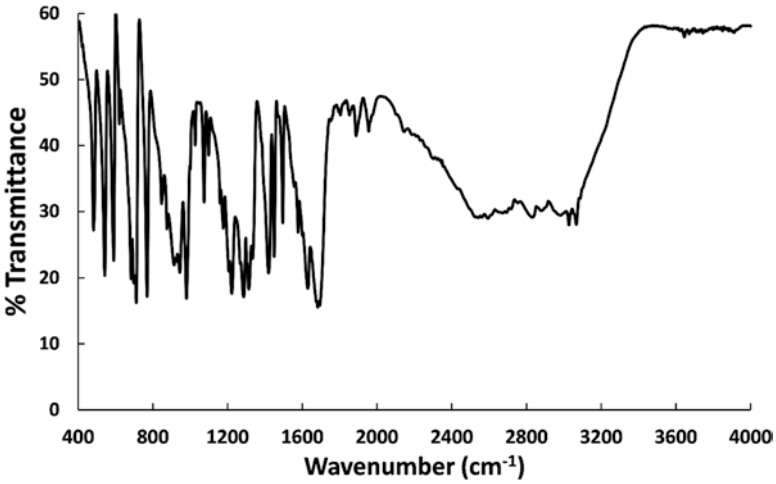

Fig. 4 Representative FTIR spectra

larger size (Kreuter 1991; Panyam et al. 2003). Some common microscopic tools used in size determination of nanoparticle are discussed below:

5.4.1 Scanning Electron Microscopy

SEM characterizes the morphology and size distribution of nanoparticles through direct visualization. This electron microscopic technique has advantages for size and morphological analysis. However, one weakness of this tool is that it provides limited information about true population and size distribution (Pal et al. 2011). Another disadvantage is that only the specimens that can withstand high vacuum pressure and adverse effects of the electron beam can be analysed. With SEM, the particles are dried if they are present in solution and mounted on a sample holder. The sample is then coated with conductive metal such as gold, gold/palladium alloy, osmium, platinum, iridium, chromium, or tungsten (Suzuki 2002). For conducting metal nanoparticles, no coating is required. A beam of high-energy electrons is directed to the sample, which generates a variety of signals on the surface of the specimens (Jores et al. 2004). The signals received from the sample are recorded by a detector that deciphers useful information about the sample such as crystalline structure, external morphology, and chemical composition (Rao and Savithramma 2011; Devi et al. 2012). A two-dimensional image with spatial variations is generated representing the surface of the nanomaterial (Prashanth et al. 2011; Priya et al. 2011). A representative scanning electron micrograph of silver nanoparticles synthesized using *W. somnifera* is shown in Fig. 5.

5.4.2 Transmission Electron Microscopy

TEM is another commonly used method for evaluation of size, shape, and morphology of nanoparticles (Zargar et al. 2011; Chauhan et al. 2012). Sample preparation in TEM is relatively time-consuming and complex as samples must be ultrathin

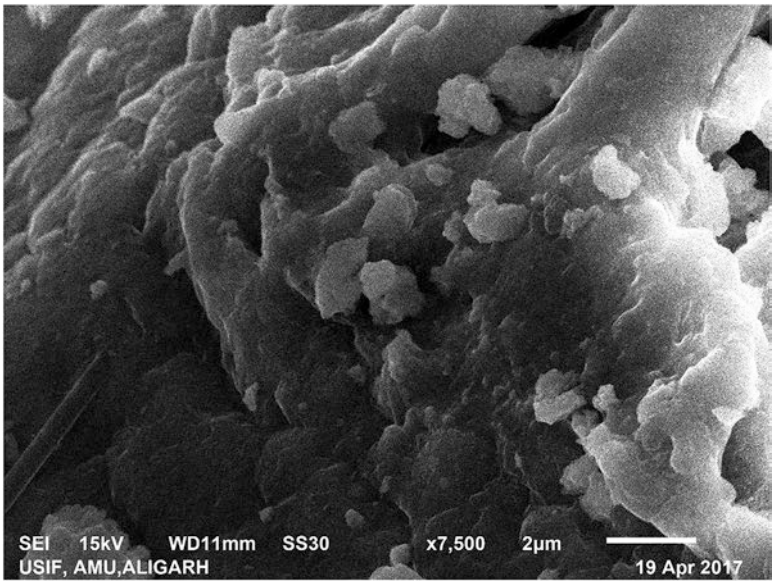

Fig. 5 Representative scanning electron micrograph of silver nanoparticle synthesis using root extract of *Withania somnifera*

such that electrons can be transmitted through them. A thin film containing sample is prepared on copper grids by placing a very small amount of sample solution onto the grid and then removing the excess solution with blotting paper. The sample, including nanoparticles, are fixed with a negative stain followed by embedding in plastic or exposing to liquid nitrogen (Pal et al. 2011). The sample is then allowed to dry under a mercury lamp. In TEM, the sample is exposed to a monochromatic beam of electrons that penetrates through the sample and the beam is projected onto a screen to generate an image (Vahabi et al. 2011; Kumar et al. 2011). Even 0.1 nm sized particles can be visualized, and their crystallographic structure can be obtained at atomic scale (Pal et al. 2011). Using high-resolution transmission electron microscopy (HR-TEM), even the arrangement of atoms such as lattice fringe, lattice vacancies, glide plane, and the their atomic arrangement can be analysed (Brice-Profeta et al. 2005). A representative transmission electron micrograph of silver nanoparticle synthesized using *W. somnifera* is shown in Fig. 6.

5.4.3 Atomic Force Microscopy

AFM provides three-dimensional images in which the height and volume of nanoparticles can be evaluated (Vesenka et al. 1993; Mucalo et al. 2002). Using a probe tip, physical scanning of the sample is performed to produce an ultra-high-resolution image of nanoparticles (Mühlen et al. 1996). Processing of images with the help of software yields quantitative information regarding nanoparticles such as size (in all three dimensions), morphology, and texture of the surface (Chauhan et al. 2012). An additional advantage of this technique is that it can be performed either in gas or

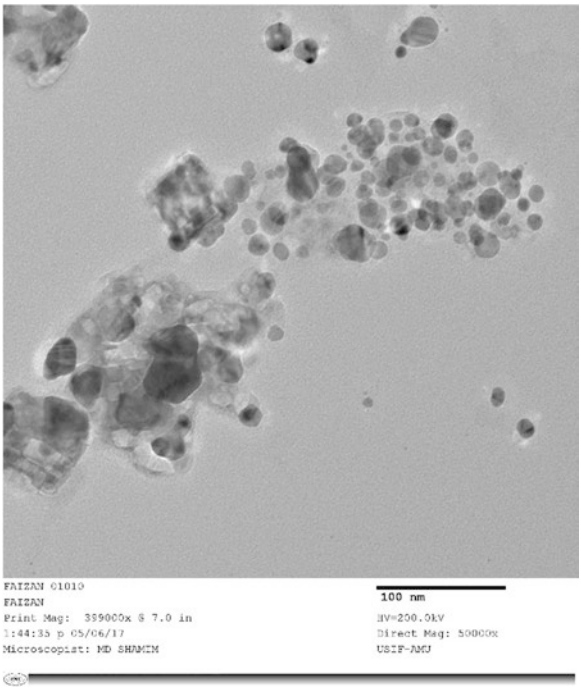

Fig. 6 Representative transmission electron micrograph of silver nanoparticle synthesis using root extract of *Withania somnifera*

liquid medium. Samples such as nanoparticles or biomolecules are spread on a glass cover slip mounted on the AFM stub and then dried with nitrogen gas. For better interpretation of data, multiples images (6–10) from a single sample are taken. AFM produces a topographical map of the sample by scanning in contact mode which is based on forces between the tip and surface of sample. In non-contact mode, a probe hovers over the conducting surface. Another advantage of this technique is that it is capable of imaging non-conducting samples without any specific treatment such as in case of delicate biological or polymeric nanostructures (Shi et al. 2003).

5.5 Dynamic Light Scattering

DLS is one of the fastest methods used for the determination of particle size and distribution. The technique is also known as photon-correlation spectroscopy and is one of the most popular methods for imaging nanoparticles. DLS is routinely used for the measurement of size of Brownian nanoparticles in colloidal suspensions (De Jaeger et al. 1991; Chauhan et al. 2012). In DLS, a monochromatic beam of light (laser) hits the nanoparticles present in a solution causing a Doppler shift by movement of the particles. Changes in the wavelength of the incident beam of light is related to the size of the particle. DLS computes the size, motion, and distribution of nanoparticles in the medium by measuring the diffusion coefficient of nanoparticles (Saxena et al. 2010).

6 Antibacterial Activity of Metal Nanoparticles

In last two decades, a large number of green synthesized metal nanoparticles have been tested for their antimicrobial efficacy; although, silver and gold nanoparticles are most commonly studied for their antimicrobial activity. Other nanoparticles such as nickel, aluminium oxide, magnesium oxide, calcium oxide, vanadium, chromium oxide, manganese oxide, iron, and cobalt nanoparticles have also proved to possess antibacterial tendency (Ramesh et al. 2012; Ansari et al. 2015; Pandian et al. 2016; Aliyu et al. 2017; Varaprasad et al. 2017; Haneefa 2017; Sharma et al. 2017; Ijaz et al. 2017; Devatha et al. 2018). A list of plant-mediated synthesis of silver nanoparticles and their antibacterial activity is given in Table 1 and some of the reports on drug-resistant bacteria are discussed here briefly.

Due to excellent antimicrobial properties, silver nanoparticles are the most commonly used in food storage, health industry, textile coatings, and many other environmental applications (Ahmed et al. 2016). This has led to the approval of silver nanoparticles for a wide range of uses by numerous accredited bodies such as US EPA, US FDA, Korea's Testing, SIAA of Japan, FITI Testing and Research Institute and Research Institute for Chemical Industry (Veeraputhiran 2013). The medicinal properties of silver (including its antimicrobial properties) have been appreciated for more than 2000 years (Prabhu and Poulose 2012). Silver has been used for many medical treatments, such as preventing microbial growth in wounded soldiers during World War I (Ankanna et al. 2010). The exact mechanism of action of silver nanoparticles is still under investigation and a debatable topic. According to a widely accepted theory, the antimicrobial action of silver ions is due to their positive charge. When in solution or coming in contact with moisture, the inert form of silver releases silver ions (Klueh et al. 2000). It has been documented that there is an electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles (Cao et al. 2001). This interaction makes silver nanoparticles a suitable candidate for bactericidal action (Eby et al. 2009).

Silver ions are capable of forming complexes with nucleic acids preferentially by interacting with the nucleosides. All forms of silver with observed antimicrobial properties are the sources of silver ions in one way or another (Sondi and Salopek-Sondi 2004). The nanoparticles penetrate into cells and subsequently accumulate inside the cellular membrane, causing damage to cell membranes and/or cell walls. It is believed that silver ions bind to thiol groups of enzymes with thiol containing compounds, causing the deactivation of enzymes present in cell membranes that are involved in trans-membrane energy generation via the electron transport chain (Ahmed et al. 2016). It has been also proposed that silver ions intercalate between purine and pyrimidine base pairs resulting in disruption of hydrogen bonding between the two strands and ultimately leading to denaturation of DNA. Lysis of bacterial cells might also be a possible reason for the antimicrobial activity of silver nanoparticles. Susceptibility of Gram-positive bacteria are comparatively less than Gram-negative bacteria due to the fact that the cell wall of Gram-positive bacteria is composed of peptidoglycan. More silver ions get stuck in the negatively charged peptidoglycan is thicker in cell wall of Gram-positive bacteria than Gram-negative

bacteria. Other proposed mechanisms of the antibacterial activity of silver nanoparticles include free radical production and interaction between silver and biological macromolecules (enzymes and DNA) through an electron-release mechanism (Sharma et al. 2009; Ankanna et al. 2010). The inhibition of protein and cell wall synthesis and ATP leaking by silver nanoparticles has also been suggested in the literature (Park et al. 2011).

A study conducted by Jeeva et al. (2014) on the synthesis of metallic silver nanoparticles using *C. coriaria* leaf extract-mediated biosynthesis found that they had antimicrobial activity against multi-drug-resistant clinical isolates including *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *S. aureus* (Jeeva et al. 2014). Similarly, *T. terrestris* fruit extract-mediated synthesis of silver nanoparticles exhibited antibacterial activity against clinically isolated MDR bacteria such as *S. pyogens*, *P. aeruginosa*, *E. coli*, *B. subtilis*, and *S. aureus* (Gopinath et al. 2012). It has been reported by Prakash and co-workers that silver nanoparticles of 55–83 nm were synthesized using leaf extracts of *Mimusops elengi*. The nanoparticles showed enhanced antibacterial activity against MDR clinical isolates such as *K. pneumoniae*, *M. luteus*, and *S. aureus* (Prakash et al. 2013). Veerasamy et al. (2011) reported that silver nanoparticles synthesized using leaf extracts of *Garcinia mangostana* that were found to be highly effective against MDR human pathogens (Veerasamy et al. 2011). In another study, *Sesbania grandiflora* leaf extract-mediated synthesis of silver nanoparticles exhibited potent antibacterial activity against MDR bacteria including *S. enterica* and *S. aureus* (Das et al. 2013). Green synthesis of silver nanoparticles using *Ananas comosus* leaf extracts showed good antimicrobial activity against *S. aureus*, *S. pneumoniae*, and *E. coli* (Emeka et al. 2014). *Pedalium murex* leaf extract-mediated synthesis of silver nanoparticles has also been reported. The nanoparticles were characterized using UV–Vis spectroscopy, XRD, FTIR, FESEM, EDAX, DLS, and TEM. The size distribution was found to be 10–150 nm and exhibited antibacterial activity against *E. coli*, *K. pneumoniae*, *M. flavus*, *P. aeruginosa*, *B. subtilis*, *B. pumilus*, and *S. aureus* (Anandalakshmi et al. 2016). Similarly, silver nanoparticles were synthesized using *Zingiber officinale* root extracts and proved to be both bacteriostatic and bactericidal (Velmurugan et al. 2014a).

Likewise, other metal nanoparticles have also proved to be good antibacterial agents. A list of green synthesized gold nanoparticles exhibiting antibacterial activity is presented in Table 2. Gold nanoparticles synthesized using *Euphorbia hirta* leaf extracts exhibited antibacterial activity against many bacterial pathogens. The growth of *E. coli*, *P. aeruginosa* and *K. pneumonia* were found to be inhibited at 1.25–200 μg/ml concentration range (Annamalai et al. 2013). Biogenic gold nanoparticles synthesized using *Coleus aromaticus* essential oil have also been reported. The nanoparticles demonstrated antibacterial activity against *E. coli* and *S. aureus* (Vilas et al. 2016). Another study reported antibacterial activity of green synthesized silver/gold bimetallic nanoparticles using *Gloriosa superba* leaf extracts (Gopinath et al. 2016). *Mentha piperita* leaf-mediated synthesized gold nanoparticles also exhibited antibacterial activity against *E. coli* and *S. aureus* (MubarakAli et al. 2011).

An eco-friendly synthesis of zinc oxide nanoparticles using the leaves of *Passiflora caerulea* found that amines and alkanes induced the synthesis of particles and exhibited antibacterial activity against urinary tract infection pathogens (Santhoshkumar et al. 2017). A report on *Citrus medica* juice-mediated synthesis of copper nanoparticles found that the nanoparticles were antibacterial against *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *P. acnes* and *S. typhi* (Shende et al. 2015). Biogenic aluminium oxide synthesized nanoparticles exerted bactericidal effects against clinical isolates of multi-drug-resistant *P. aeruginosa* (Ansari et al. 2015). Nickel nanoparticles synthesized using leaf extracts of *Ocimum sanctum* inhibited the growth of *E. coli*, *K. pneumoniae*, *S. typhi*, *B. subtilis*, and *S. epidermidis*. The enhanced antimicrobial activity of nanoparticles was attributed to the formation of ROS that led to the loss of cellular proteins and LDH resulting in cell death (Pandian et al. 2016). Manganese nanoparticles synthesized using lemon fruits were found to inhibit the growth of *S. aureus*, and *B. subtilis*, *E. coli*, and *S. bacillus* (Jayandran et al. 2015). A large number of other metal nanoparticles synthesized using various plants has been reported with potent antibacterial activity.

7 Conclusion and Future Prospects

With advancements in nanobiotechnology, a new hope has arisen regarding the eco-friendly synthesis of metal nanoparticles using plants. Such biogenic nanoparticles are an important aspect of nanotechnology with multiple biomedical applications. The plant-mediated synthesis of metal nanoparticles is a single step and quick way to produce nanoparticles compared to other biological entities such as microbes, which require time-consuming methods to maintain and culture them. Therefore, plants as bio-templates for the synthesis of nanoparticles may show an immense impact in the near future by identifying and standardizing the phytocompounds responsible for this process. Toxicity is the major concern that needs to be properly addressed in suitable animal models to monitor possible adverse effect on long-term administration prior to their therapeutic application. So, it is expected that such nano-based formulations including metal nanoparticles may become the next-generation therapeutic agents against bacterial pathogens with special reference to drug-resistant bacteria. Moreover, large-scale production of nanoparticles using the green route will need to be scaled up to make them commercially available.

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