Chapter 10
Microbial Options Against Antibiotic-Resistant Bacteria

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Abstract Antibiotics are routinely used to treat human and animal infectious diseases since the time of invention. However, due to continuous and long-term usage of antibiotics, infectious organisms naturally developed resistance over time through genetic changes. Further indiscriminate use of antibiotics in public health care is accelerating the emergence of drug-resistant bacteria. The spread of antimicrobial resistant bacteria among people, animals, food, and environment is of growing concern that requires urgent attention to control the widespread occurrence of antibiotic-resistant bacteria. Transition from antibiotics to nontraditional treatments is one option to overcome this global challenge. Small peptides like bacteriocin, synthesized by certain bacteria, showed good antimicrobial activity against pathogenic bacteria. Use of microbial cell-free probiotic along with regular antibiotics has significantly increased the antibacterial activity against multidrug-resistant bacteria. The application of phage therapy and quorum sensing inhibitors are also well-known options against antibiotic-resistant bacteria. Recent developments in genome editing showed successful cleavage of specific target gene, coding for pathogenesis or re-sensitizing pathogenic organisms for antibiotics, this strategy proves their ability to kill specific pathogenic bacteria based on their sequence rather than targeting group of bacteria. Similarly, nanotechnology has attracted worldwide interest due to its promising results in drug delivery system, and the versatile characteristics

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like potent antimicrobial activity of nanoparticles make it extremely outstanding candidate for the management of infectious diseases.

Keywords Antibiotics · Antimicrobial resistance · Penicillin · Bacteriocins · Phage therapy · Quorum sensing

10.1 Introduction

Antibiotics are a class of compounds naturally produced by certain types of microorganisms for inhibition of other competitive microorganisms in their habitat. These compounds can be isolated or synthesized by various mechanisms which can be used as medications for the treatment of infectious disease caused by pathogenic bacteria/etiology. The invention of antibiotics has made a tremendous contribution in medical field; many diseases that once known to cause huge damage on human health have been successfully treated after the invention of antibiotics (CDC report 2013). However, over a period of time, some bacteria naturally developed resistance against antibiotics due to prolonged usage of these antibiotics (Kaliwal et al. 2011). Antibiotic resistance is the ability of bacteria to develop some mechanism to neutralize the action of antimicrobials against them. As a result, use of such antimicrobials against these bacteria becomes ineffective. Recently, the World Health Organization has announced the alarming level of resistance among various species of bacteria (Tacconelli et al. 2018). The development of resistance among bacteria goes beyond the available antibiotics and also exceeds the rate of antibiotic discovery at present situation. Studies suggest that around 444 million people could suffer infectious disease by 2050 (Gould and Bal 2013). High prevalence of antibiotic resistant bacteria has become one of the major public health problems and especially control of mortality due to nosocomial infections poses a biggest challenge to the health care professionals.

The evolution of multidrug-resistant bacteria in hospital environment is the result of prolonged exposure of bacteria to various antibiotics and also transfer of resistant bacteria between individuals; another important factor for evolution of resistant bacteria is transmission of resistance genes from resistant bacteria to susceptible bacteria (Guillemot et al. 2001). In the pre-antibiotic era, *S. aureus* infection resulted in 80% mortality (Smith and Vickers 1960). With the inventions of antibiotics, the organism was reported to be successfully controlled by the earliest antibiotics like penicillin. However, during the 1950s, the extensive applications of these antibiotics against infectious disease resulted in the emergence of beta-lactamase-producing bacterial strains (Fisher and Knowles 1978). Further beta-lactamase-resistant penicillins were developed to overcome the emergence of beta-lactamase-producing strains, but resistant bacteria developed against this new antibiotic were first reported in the 1960s from Europe and in the 1970s from the USA (Peacock et al. 1980). Furthermore, in the 1980s, *S. aureus* strains were also frequently reported to have developed resistance against potent antibiotics like methicillin in hospital

environment (Hughes 1987). By the 1990s, studies reported the emergence of bacteria resistance to semi-synthetic penicillins like nafcillin and oxacillin. Moreover, several bacteria also developed resistance against antibiotics like macrolides, tetracyclines, and aminoglycosides, compromising the use of these drugs for empiric therapy for infectious diseases in a number of regions. This has led to the introduction of glycopeptide antibiotic known as vancomycin for the management of methicillin-resistant *S. aureus* (MRSA) infections (Hiramatsu et al. 2001). However, Hiramatsu and his coworker (1997) reported the first vancomycin-resistant *Staphylococcus aureus* from Japan. Similarly, other countries like the USA (Smith et al. 1999), Belgium (Denis et al. 2002), and India (Assadullah et al. 2003) also reported reduced susceptibility of *S. aureus* to vancomycin. Hence, it is necessary to understand the genetics and defense pathway mechanism of the resistant bacteria at individual level in order to develop effective therapy against infectious disease. Therefore, comprehensive efforts are required to discover novel molecules or new technologies for the control of multidrug-resistant bacteria. In recent times, several progressive approaches have been made with unique properties, which include use of probiotic, phage therapy, quorum sensing inhibition, genome editing technology, and nanoparticle therapeutics, and they prove their potential ability to control resistant bacteria. Though the alternative strategy for combating bacterial infection is in its infancy, its potential to re-sensitize or eliminate resistant bacteria cannot be underestimated. Therefore, in this chapter, we summarized novel strategies for handling the crisis of bacterial infections, and overview of new strategies for the management of multidrug-resistant bacteria is represented in Fig. 10.1.

10.2 Small Peptide as a Novel Antimicrobial Agent

As the prevalence of resistant bacteria increases, it is necessary to search for a new molecule that plays an important role in controlling the widespread occurrence of resistant bacteria. Bacteriocins are ribosomally produced small peptide molecules known for their potential antimicrobial agent. Bacteriocins are broadly classified into two classes based on the mode of their production. Class I bacteriocins are produced after modification during post-translation process. This class of bacteriocins is also identified by the presence of unusual amino acids where threonine and serine residues are dehydrated to dehydrobutyrine and dehydroalanine, respectively, during post-translation modification (Cotter et al. 2013). Class II bacteriocins are unmodified with cyclic structure and further divided into class IIa to class IIe (Cotter et al. 2013). Several class I bacteriocins are isolated, among which the most commonly studied bacteriocins include nisin, lacticin, staphylococcin, mersacidin, etc. (Brotz et al. 1995; McAuliffe et al. 1998; Navaratna et al. 1998; Xie et al. 2004; Field et al. 2008). Similarly, class II bacteriocins are also extensively studied which revealed their strong affinity with mannose phosphotransferase receptor suggesting their specificity in antimicrobial activity against pathogens (Oppegard et al. 2007). Bacteriocin usually recognizes either a general or specific receptor molecule on a

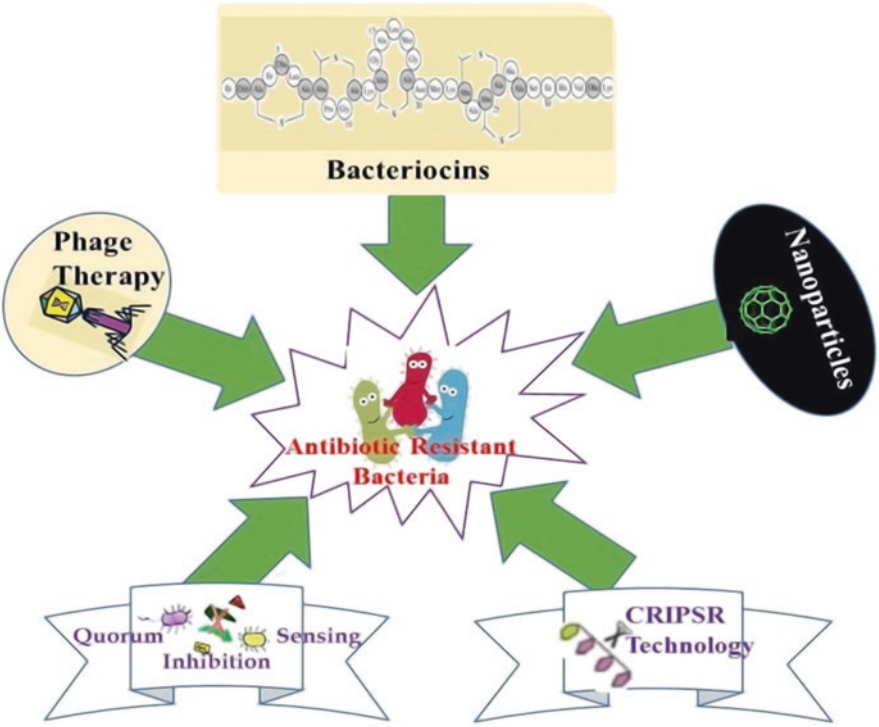

Fig. 10.1 Overview of new strategies for the management of antibiotic-resistant bacteria

target cell to which it binds and disrupts membrane structure by pore formation (Brogden 2005). Bacteriocins produced by different types of bacteria vary in their structural and functional characteristics. Extensive studies on the mode of action of bacteriocins showed that bacteriocins produced by Gram-positive bacteria have broad-spectrum antimicrobial activity (Sang and Blecha 2008). Nisin is a common bacteriocin produced by *Lactobacillus* and *Lactococcus* species which is well known for its antimicrobial activity against foodborne pathogens (Dimitrieva-Moats and Unlu 2012). *Staphylococcus* species also produce different types of bacteriocins such as lysostaphin, aureocin, and nukacin. Lysostaphin is produced by coagulase-negative staphylococci and is proven to be an outstanding candidate for the control of human and animal infection caused by multidrug-resistant *S. aureus* (Bastos et al. 2010). Aureocin is produced by *S. aureus*; successful isolation and application of aureocin were demonstrated to control the udder infection in domestic cattle (2007). Similarly, nukacin is another bacteriocin produced by *S. simulans* also used in the therapeutic applications of animal disease (Ceotto et al. 2010). *Carnobacterium* spp. also produce bacteriocins such as carnobacteriocin X and carnocyclin A which have potential to kill *Listeria* spp. (Martin-Visscher et al. 2008; Tulini et al. 2014). Likewise, *Enterococcus* spp. produce different types of bacteriocin such as enterocin, enterocin X, and enterocin A. All these bacteriocins showed their efficacy

against foodborne and also clinical pathogens (Gálvez et al. 2007; Hu et al. 2010; De la Fuente-Salcido et al. 2015).

Different types of bacteriocins are also produced by Gram-negative bacteria. It is important to note that the first reported bacteriocin in 1952 was isolated from Gram-negative bacteria. Among Gram-negative bacteria, *Escherichia coli* is the predominant producer of bacteriocins known as colicins, which are reported to have the ability to kill the target organism by the action of pore formation or nuclease activity (Bakkal et al. 2010). Similarly, Klebcins are proteinase bacteriocins produced by *Klebsiella pneumoniae* (Gillor et al. 2004). Pyocin is another small antimicrobial peptide produced by *Pseudomonas aeruginosa* (Gulluce et al. 2013). Further, bacteriocins with their potential antimicrobial ability are shown to inhibit the growth of multidrug-resistant bacteria such as vancomycin- and methicillin-resistant bacteria. This suggests that cell wall peptidoglycan acts as a receptor for bacteriocins.

10.3 Phage Therapy as a Complimentary Strategy for the Control of Antibiotic-Resistant Bacteria

In recent years, increased prevalence of antibiotic-resistant bacteria has emerged as a major threat to the global population. Presently available antibiotics fail to control the spread of infectious diseases caused by resistant bacteria. Therefore, rather than the old antibiotic therapy, a new strategy such as phage therapy is required to control the adverse effect of resistant bacteria on human health. Phages are considered as natural predators of bacteria; bacteria feeding viruses are generally known as bacteriophages. Bacteriophages are very specific in their host. Hence, they are effectively used for the biotyping of bacterial strains, since they are specific to their target bacteriophages. They have been considered as a promising strategy against bacterial infections. Several studies have shown the use of phage therapy in treating bacterial infections, but still, it has not gained much interest all around the world. Phage therapy has several advantages such as target specificity and does not harm the other normal microflora and replication takes place inside the infected cell. The mode of action of phage therapy involves adsorption of phages to their target bacteria and killing the host bacteria after making several copies of itself with the host DNA replication process. The newly formed phages, lysis the host bacterial cell and released into surrounding environment, further infect the nearby target bacteria. This process continues until the target bacteria get eliminated from the surrounding environment. Once all the bacteria are killed from the surrounding, bacteriophages are eliminated through natural cleansing process without affecting the human tissue. To date, clinical applications of phages in treating infectious disease have been extensively studied (Morello et al. 2011; Vieira et al. 2012; Waters et al. 2017). Around 137 different phages have been characterized for targeting *Pseudomonas* genus (Pires et al. 2015). Several institutes in Europe have carried out extensive research on application of phages on human trials to treat common bacterial

infections caused by *E. coli*, *S. aureus*, *Streptococcus* spp., *Proteus* spp., *P. aeruginosa*, *S. dysenteriae*, *Salmonella* spp., and *Enterococcus* spp. (Kutateladze and Adamia 2008). Similarly, specific phages were successfully used to treat diabetic foot ulcers caused by multidrug-resistant *S. aureus* (Fish et al. 2016). In another study, patients administered with phage cocktail consisting of various phages targeting different types of bacteria responsible for dysentery such as *Salmonella typhi*, *Shigella*, *E. coli*, *Salmonella paratyphi*, *Proteus* spp., *P. aeruginosa*, *Shigella flexneri*, and *Staphylococcus* spp., *Streptococcus* spp., and *Enterococcus* spp. were found to recover from the symptoms within 24 h of phage cocktail treatment (Chanishvili and Sharp 2008). Recently, Forti et al. (2018) demonstrated the successful use of six different phages in treating *P. aeruginosa* infection in mice and *Galleria mellonella* models. It is observed that some phages also disrupt the *P. aeruginosa* biofilms. This ability of phages is one of the important contributions over traditional antibiotic treatment (Waters et al. 2017; Fong et al. 2017). Recent studies showed promising results in controlling *E. coli* infection in mice (Vahedi et al. 2018). Kumari and coworkers confirm that topical application of phage on burn wounds of mouse showed significant reduction in mortality of mice (Kumari et al. 2011). Apart from clinical application, phages have potential to control the growth of food pathogen and are considered to be safe; several commercial phages were used for biocontrol of bacterial pathogens, viz., *Pseudomonas syringae*, *Listeria monocytogenes*, *Salmonella* spp., *E. coli*, and *Campylobacter* spp. (El-Shibiny and El-Sahhar 2017). Another progress in phage therapy research is that phages and purified phage lytic proteins can be genetically engineered, thereby increasing the efficacy of treatment. A variety of phages are also used as a vehicle in drug delivery process. M13 phages were successful in delivering the coding sequence to the target cell resulting in the death of the target cell (Westwater et al. 2003). Correspondingly, a variety of bioengineered phages were constructed to control *E. coli* infection by destroying biofilms and interrupting DNA replication and delivery of RNA-guided virulence nucleases. Further, some phages also function as an effective adjuvant that increases the efficacy of antibiotics against bacterial infections (Citorik et al. 2014; Lu and Collins 2007, 2009).

10.4 Quorum Sensing Inhibition as a Novel Approach to Diminish Bacterial Resistance

For many years, the search for an effective treatment to fight against infectious diseases has been one of the biggest challenges to the scientific community. Use of plant-based bioactive molecules to control infectious agent was one of the well-known methods practiced before the invention of antibiotic. However, the use of such molecules to treat infectious disease was substituted by chemotherapy due to its broad spectrum activity and low toxicity. Prolonged usage of antibiotics has led to the development of resistant strains, and in recent years, emerging antibiotic-resistant strains have become a serious threat worldwide. Currently, revival of bioactive molecules that block quorum sensing in bacteria is necessary to mitigate

infectious diseases. Quorum sensing is a unique mechanism of bacteria that regulates the expression of genes; most pathogenic bacteria acclimatize to their habitat by expressing virulence genes via quorum sensing system (Heilmann et al. 2015). For instance, in *Pseudomonas aeruginosa*, Las and Rhl are two different quorum sensing transcription factors responsible for production of biofilm and expression of multiple virulence genes (Rutherford and Bassler 2012). Similarly, in *Staphylococcus aureus*, quorum sensing system controls the expression of accessory gene regulator (AGR) genes that are responsible for the production of several toxins and exoenzymes (Martin et al. 2013). The survival of *S. aureus* in the host environment against the immunity of the host is attributed to the self-defense mechanism expressed by *S. aureus* (Kurjogi et al. 2010). Hence, quorum sensing system plays an important role in regulation of various virulence genes and biofilm formation leading the resistant bacteria. Therefore, quorum sensing inhibition approach is considered to be a promising strategy for the management of antimicrobial resistant bacteria. Available reports show that use of anti-quorum sensing agents can obstruct the quorum sensing signals among the bacteria; several bacterial enzymes like lactonase, acylase, oxidoreductases, and 3-hydroxy-2-methyl-4(1H)-quinolone 2, 4-dioxygenase have been reported to be potential quorum sensing inhibitors (Jiang et al. 2019). Similarly, bioengineered *E. coli* was successful to disrupt the proteolytic activity and pyocyanin production of *Pseudomonas aeruginosa* (Dong et al. 2018). Liu et al. reported that fishes supplemented with lactonase were found to be resistant to *Aeromonas hydrophila* infection (Liu et al. 2016). In addition, studies suggest that lactonase can also disrupt the biofilm formation by *Vibrio parahaemolyticus* in shrimps (Torres et al. 2018). Acylase is another bacterial enzyme known as quorum sensing inhibitor found to be successfully used for the control of *Pseudomonas aeruginosa* in healthcare sectors (Grover et al. 2016). Further it is also noted that oxidoreductases by bacteria can abolish the biofilm formation and inhibit the growth of *Klebsiella oxytoca* and *K. pneumoniae* (Wildschut et al. 2006; Zhang et al. 2018). Overall, the quorum sensing inhibitors are the promising alternative strategy to tradition antibiotic therapy. Use of anti-quorum sensing agent in health sectors not only kills the pathogenic bacteria but also controls the spread of antibiotic-resistant bacteria. However, further studies are needed to ensure the stability of quorum sensing inhibitors to convey the potential ability of quorum sensing therapy for management of infectious diseases.

10.5 Gene Editing Technique for Management of Infectious Diseases

Invention of antibiotics is one of the greatest discoveries that revolutionized the medical field. Antibiotics have saved millions of lives since their discovery. However, increased use of antibiotics to treat common infectious disease has led to the development of resistant strains. Therefore, in recent years, most pharmaceutical companies have stopped the production of several antibiotics due to declined use of such antibiotics. On the other hand, use of genetic engineering to

edit the bacterial gene to re-sensitize the bacteria to antibiotic is providing a novel approach for management of infectious diseases.

CRISPR-Cas system not only protects bacteria against invaders but also controls endogenous transcription and the pathogenicity of bacteria. For example, *Francisella novicida*, which is known as intracellular parasite, can successfully replicate by surpassing the host immune system. This bacterium has several mechanisms to mitigate the defense mechanisms of the host. On macrophage engulfment, *F. novicida* enters the phagosome, where numerous antimicrobials and immune recognition receptors are present (Jones et al. 2012). Toll-like receptor 2 (TLR2) is one of those receptors that can detect bacterial lipoproteins (BLPs). TLR2 activation initiates a pro-inflammatory response and triggers immune cells, thereby eliminating pathogen. However, *F. novicida* uses cas9, sacRNA, and tracrRNA to inhibit BLP expression (Sampson and Weiss 2014). Therefore, by preventing TLR2 activation, this pathogen can survive within the host. *F. novicida* induces inflammatory response in the absence of these regulators, as it was stated that cas9, sacRNA, and tracrRNA deletion mutants induce stronger inflammatory immune response compared to wild type. In contrast, deletion mutants of sacRNA, cas9, and tracrRNA are not capable of causing lethal infection in mice, further emphasizing the importance of CRISPR-cas system as an *F. novicida* virulence regulator. In addition, cas9 is required for invasion and attachment of *Campylobacter jejuni* (Louwen et al. 2013). Nevertheless, *C. jejuni* attachment to host cells protects this bacterium from the inherent complementary mechanism of the host. A study recently confirmed that *C. jejuni* has a role to play in controlling CRISPR-cas9 associated virulence genes (Shabbir et al. 2018).

Resistance may evolve by inactivating CRISPR-Cas loci through mutations or deletions in target cleavage cas genes or by deleting targeting spacers (Bikard et al. 2012; Jiang et al. 2013). At present, more than 20 distinct acr gene families have been identified, both type I and II CRISPR-Cas systems (Pawluk et al. 2018; Borges et al. 2017). Many of the Acr protein families targeting type I CRISPR-Cas systems have been associated with *Pseudomonas aeruginosa* as well as other Proteobacteria species. While most of these Acr proteins tend to target only one CRISPR-Cas subtype, one Acr targeting both the type I-E and I-F CRISPR-Cas subtypes has been published. More recently, Acr proteins have been established as target type II systems—including the CRISPR-Cas9 systems used for gene editing—one of which is particularly wide in its target range (Pawluk et al. 2016). The massive sequence diversity and high specificity of Acrs indicate that they are likely ubiquitous and possibly carried by MGEs such as phages and plasmids to circumvent targeting by CRISPR-Cas (Harrington et al. 2017). The implications of CRISPR-Cas targeting resistant genes and their effect on other population need to be studied in detail, especially using clinical pathogens to understand the ecological and evolutionary risks.

Until CRISPR-Cas can be used to target antibacterial resistance, several hurdles remain to be overcome. Future research is needed to identify the effective method to explore CRISPR-Cas technology. However, the social and legislative challenges are to draft guidelines for regulation of CRISPR technology and to encourage the proper use of this technology to ensure its responsible and safe use (Makarova et al. 2015).

10.5.1 Green Nanotechnology to Combat Against Antibiotic-Resistant Bacteria

For many years, the search for an effective treatment to fight against infectious diseases has been one of the biggest challenges to the scientific community. The concern of drug-resistant clinical pathogens is not only limited to humans but also reported in domestic animals. Several studies show that bacteria have developed resistance to many commonly used veterinary antibiotics (Kaliwal et al. 2011; Kurjogi and Kaliwal 2011). It is essential to explore novel antimicrobial agents with potent antimicrobial activity as an alternative to traditional antibiotics. In this context, nanotechnology has attracted worldwide interest due to its promising results in drug delivery system, and it is not surprising to see the antimicrobial activity of nanoparticles against clinical pathogens. The versatile characteristics of nanoparticles make them extremely outstanding candidate in several research fields like clinical, agricultural, and physical sciences (Chaudhuri and Paria 2012; Tran et al. 2013; Rauwel et al. 2015). Nanoparticles can be synthesized in different ways. Till date, several chemical and physical approaches have been made for the synthesis of nanoparticles. However, nanoparticles synthesized by chemical and physical processes are not suitable for clinical application since the reducing or stabilizing agent used in chemical or physical process is not biocompatible and hazardous to environment. Therefore, recently environmentally benign biological methods like green synthesis of nanoparticles have gathered global scientific attention (Lee et al. 2016; Cerda et al. 2017; Prasad 2014, 2016, 2019). Nanoparticles synthesized by biological approach are more advantageous in terms of safety, efficiency, and biocompatibility (Kumar et al. 2015; Quester et al. 2016; Prasad et al. 2017). Several microbes are considered as a novel source for green synthesis of nanoparticles since microbes are rich source of enzymes and other metabolites that act as a reducing or stabilizing agent in the process of nanoparticle synthesis (Prasad et al. 2016, 2018). Another advantage of using microbes is they can be easily cultured in a controlled condition. Several studies have proved the antimicrobial efficacy of microbe-based nanoparticles in different way. Nanoparticles can be used as adjuvant with available antibiotics that increases the efficiency of antibiotics against the target pathogen (Hassan and Hemeg 2017). On the other hand, several metal nanoparticles like silver, copper, gold, zinc, etc. are known to be used directly as an antimicrobial agent against pathogenic bacteria (Aziz et al. 2014, 2015, 2016). Recently, studies reported that silver nanoparticles synthesized by *Ganoderma applanatum* demonstrated in vitro antibacterial activity against clinical pathogens (Jogaiah et al. 2017). Similarly, silver nanoparticles were synthesized by edible mushrooms such as *Pleurotus pulmonarius* and *Pleurotus djamor* which showed high bactericidal activity against clinically important *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Shivashankar et al. 2013). In the same way, silver nanoparticles synthesized using *Trichoderma viride* also showed good bactericidal activity against Gram-positive and Gram-negative bacteria (Chitra and Annadurai 2013). Pandey and colleagues prepared nano-capsulation for oral dosage of streptomycin and other antibiotics that

are non-injectable (Pandey et al. 2003). Elechiguerra et al. (2005) demonstrated the inhibition of HIV from binding to host cells when treated with silver nanoparticles. Nanoparticles produced by *R. stolonifer* successfully inhibited the growth of antibiotic-resistant *P. aeruginosa* isolated from burn patients (Afreen and Ranganath 2011). Studies also revealed that *P. glomerata*-based nanoparticles performed synergistic antibacterial activity against *E. coli*, *P. aeruginosa*, and *S. aureus* when combined with standard antibiotics (Birla et al. 2009). The authors also used saprophytic fungi like *Nigrospora oryzae* for nanoparticle production and successfully demonstrated their efficacy against several clinical pathogens like *E. coli*, *B. cereus*, *Proteus vulgaris*, *P. aeruginosa*, and *Micrococcus luteus* (Saha et al. 2011). The mechanism involved in antimicrobial activity of nanoparticles is attributed to their size and shape. Further, surface charge of the nanoparticles is also an important factor of antibacterial activity where bacterial cell wall electrostatically attracts the oppositely charged nanoparticles, causing damage to the cell membrane leading to the death of the bacterial cell (Aziz et al. 2014, 2015, 2016, 2019).

10.6 Conclusions

Current studies on alternative strategies specifically against multidrug-resistant bacterial infections suggest that these novel therapies have all the potential ability to control antibiotic-resistant bacteria. Further research has to be carried out to show the application of these therapies in large population through clinical trials. However, ever remaining challenges in these therapies are purification of bacteriocins, development of phage bank that includes collection of various identified phages against the resistant bacteria, identification of quorum sensing inhibitors, implementation of CRISPR technology, and controlled synthesis of microbe-based nanoparticles.

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