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Emerging Modalities in Mitigation of Antimicrobial Resistance

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About This Book

Antimicrobial resistance (AMR) is one of the deadliest threats to global public health. This book focuses on dynamics in the landscape of AMR while informing about the latest technologies and strategies to mitigate it. The menace of AMR in different niches, routes of penetration across various domains, socio-economic impact, and the need for a "One Health" approach in mitigating AMR have been emphasized. Factors involved in AMR, underlying mechanisms, and pharmacometrics in developing antimicrobials are highlighted. Emphasis is given to emerging technologies that are sustainable, scalable, and applicable to the global community, such as big data analytics, bioactive agents, phage therapy, and nanotechnology. The book also explores current and alternative treatment strategies to combat AMR, emphasizing the use of nanoparticles to target pathogens and as a viable alternative to antibiotics.

Contents

About the Editors

Nadeem Akhtar, PhD currently works at the University of Guelph, Canada, as a Research Associate. His current research focuses on biotechnology in animal nutrition, where the main objective is to improve animals' health for a better and disease-free lifestyle. Before the Research Associate role, he has worked as a Postdoctoral Researcher in the Department of Chemical Engineering, University of Waterloo, Canada (2019–2021), and in the Department of Animal Biosciences, University of Guelph, Canada (2015–2019). Dr. Akhtar was awarded a Ph.D. from the Department of Biotechnology, Thapar University, India. He has qualified National Eligibility Test (NET-2010) and Graduate Aptitude Test (GATE) in Life Sciences (2010) and Biotechnology (2011). His scientific contributions include more than 23 articles in peer-reviewed international journals, 10 book chapters, and a patent. He reviewed several research articles for reputed journals, including *RSC Advances*, *Journal of Applied Microbiology*, *Scientific Reports*, *Poultry Science*, and many more. Department of Animal Biosciences, University of Guelph, Guelph, Ontario, Canada

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Part I
Current Status and Active Dynamics of AMR

Chapter 1
Antimicrobials in Livestock Production and Its Cross-Domain Dynamics

Bishwo Pokharel and Sandeep Raj Karna

1.1 Introduction

Antimicrobials are natural, seminatural (semisynthetic), or synthetic substances that kill or inhibit the growth of microorganisms (Page and Gautier 2012). Antimicrobials are effective against various classes of microorganisms such as virus, bacteria, protozoa, and fungi; however, the antimicrobials of common interest are those that are effective against bacteria. This is because bacteria can mutate to variants that are resistant to the antimicrobials used against them. These bacteria, after the mutation, can become a public health concern and jeopardize livestock and human health. The coexisting nature of livestock and human and their dependency on each other provide a greater host range to these resistant bacteria. Fear is growing among the scientific community that such resistance could result in another costly pandemic. Therefore, this chapter focuses on the use of antimicrobials in livestock production; dynamics of antimicrobial flow between humans, animals, and the environment; antimicrobial resistance; and potential alternatives to antimicrobials. This chapter also discusses One Health approach to antimicrobial resistance and provides information on antimicrobial stewardship to provide guidelines to stakeholders involved in the use of antimicrobials.

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1.2 Antimicrobial Use in Livestock

1.2.1 History

Tracing the date when antimicrobials started to have a dramatic impact on livestock farming can be daunting. Kirchhelle (2018), in their review, mentioned that antimicrobials started to play a bigger role in food production since the 1930s when synthetic sulfonamides came into existence. Sulfonamides were found to be effective against streptococcal infection providing a therapeutic effect on agricultural animals. Food products coming from livestock became even more important during World War II when there was a need to optimize livestock production to meet the increasing demand for food products. Researchers started to study alternatives in the form of antimicrobials to produce more meat at a cheaper cost; however, the practice started to come under scrutiny with the emergence of antibiotic residues in food products and antibiotic resistance (Table 1.1).

Table 1.1 Summary of major events on antimicrobial use in livestock

1920–1930	Discovery of penicillin (1928)
1930–1940	Development of the first agricultural antibiotic (1935): sulfochrysoidine (prontosil), first-time use of sulfonamides in animals in Britain (1938)
1940–1950	A rapid surge in the production of antibiotics during world war II, first-time use of penicillin/gramicidin against mastitis, antibiotics to control fish infection (1947), sulfaquinoxaline in poultry feed against coccidiosis (1948)
1950–1960	For the first time, concerns started to emerge on the use of antimicrobials in food animals, antimicrobial use boomed in several European countries, and nearly all piglets had access to food with tetracycline in the late 1950s
1960–1970	Agricultural antibiotics became widespread in Japan; the Food and Drug Administration (FDA) started monitoring programs against antibiotic residue in milk and meat; *animal machines*—a revolutionary book published backlashing antimicrobials (1964); nearly 80% of animal feed in Germany had some form of antimicrobials
1970–1980	Antimicrobial use in livestock boomed in the United States, South Africa, and several other countries
1980–1990	Sweden banned antimicrobial use as a growth promoter (1986) and prophylactic medications (1988)
1990–2000	Denmark banned the prophylactic use of antimicrobials (1994), initiation of European lobby to ban antimicrobial growth promoters, ban of several antimicrobials in EU; the World Health Organization (WHO) concluded that the use of antimicrobials as growth promoter should be stopped
2000–2010	Ban of all antibiotic growth promoters by the EU (2006); the WHO, Food and Agriculture Organization (FAO), and Office International des Epizooties (OIE) held a workshop that coined the term “critically important antimicrobials” to tackle antibiotic resistance
2010–2020	Substantial publications on antimicrobial use in livestock and antimicrobial resistance, accelerated discussion on one health approach and antimicrobial stewardship

Sources: Cogliani et al. (2011) and Kirchhele (2016, 2018)

1.2.2 Numbers Behind Antimicrobial Use in Livestock

Globally, more than 27 billion chickens, 1.7 billion cattle and buffaloes, 850 million pigs, and 2.3 billion sheep and goats are farmed (FAOSTAT 2020). Also, other groups of livestock share a significant proportion among the total livestock population. This suggests that a significant portion of the global population relies on some forms of livestock farming directly or indirectly. For those directly dependent on livestock, poor health and productivity of their animals could be devastating with a serious negative impact on their economy for years. Thus, many of them knowingly or unknowingly use substances such as antimicrobials that enhance the productivity of their livestock and prevent/protect their livestock from diseases.

In recent years, the use of antimicrobials is growing at an unprecedented rate in food animals. This is expected to grow similarly for some time as demand for animal protein is growing rapidly (Tilman et al. 2011). An estimated 63 thousand tons of antimicrobials were used in 2010 in livestock, which doubled in 2013 (131,109 tons). This use is further expected to rise up to 67% by 2030 (Van Boeckel et al. 2015). Figures are even more alarming in Asia, where antimicrobial use in chicken and pigs are expected to rise by 129% and 124%, respectively, by 2030 (Van Boeckel et al. 2015). In India, industrial poultry production is expected to grow by 312% by 2030, further increasing the demand for antimicrobials. Developed countries such as Denmark, Sweden, and Norway have been cautious in using antimicrobials; however, the developing countries have not shown any signs of reducing the use of antimicrobials for agricultural purposes (for instance, 8 mg/PCU of antimicrobial use in Norway compared to 318 mg/PCU in China) (Van Boeckel et al. 2017).

In 2010, the top five countries that shared the largest proportion of global antimicrobial use in livestock were China, the United States, Brazil, India, and Germany (Van Boeckel et al. 2015). The more alarming data from BRICS (Brazil, Russia, India, China, and South Africa) suggest that the use of antimicrobials is expected to grow by more than 99% in those countries in the next 10 years, making them future hotspot of global antimicrobial use. The global rise in the use of antibiotics is attributed to the shift toward more intensified livestock farming where a large group of animals is kept in an enclosed environment, increasing antimicrobial pressure to maintain and improve health and productivity. Also, livestock farming has seen significant changes in the last few decades owing mainly to the genetic advancements. Genetic selection has been practiced heavily, and the focus is mostly laid on improving productivity, which has unintentionally given rise to undesirable side effects such as increased frequency of rare recessive alleles. As a result, immune incompetence is more common leading to increased occurrence of pathologies and compromised animal welfare (Rauw et al. 1998; Hocking 2014). Compromised immune system is also one of the reasons that has caused increase in prophylactic and therapeutic use of antimicrobials, possibly giving rise to increased antimicrobial resistance.

Most of the abovementioned data come from poultry, cattle, and pig. There is hardly any accurate data available on antimicrobial use from fish farming. However, fish farming may already be contributing to the major proportion of antimicrobial use globally. The data from South Asia and South America already suggest an extremely high rate of antimicrobial use up to 1400 mg/kg (Van Boeckel et al. 2015) in fish farming. Like any other livestock farming, aquaculture is also shifting toward more efficient and intensive farming with the potential to become a major shareholder of global antimicrobial use.

1.3 Why Are Antimicrobials Used in Livestock?

1.3.1 Antimicrobials as a Growth Promoter

The single most controversial use of antimicrobials in livestock is its use as a growth promoter, which dates to the 1950s in the United States, Australia, and some European countries (Dibner and Richards 2005). Studies have reported improved feed conversion and growth in cattle, pigs, poultry, and other animals (Gallo and Berg 1995; Cromwell 2002; Castanon 2007; Chattopadhyay 2014) with some of these studies reporting productivity improvement of up to 10% after the use of antimicrobial growth promoters. The interaction between gut, microbiota, and antimicrobials is thought to be the reason behind growth-promoting effects of antimicrobials, more specifically the reduction of microbial metabolites that cause growth reduction in animals (Visek 1978; Anderson et al. 1999). Antimicrobials reduce the population of opportunistic pathogens and subclinical infections, limiting competition for food and thereby improving growth (Visek 1978). Antimicrobials also increase nutrient availability and absorption by maintaining gut microflora compositions, thereby thinning the barrier in the small intestine, and assisting in the digestion of high-energy diets (Peng et al. 2014).

Although antimicrobials are being used as a growth promoter for decades, there is a lack of reliable recent data on the effect of antimicrobials as growth promoters. Most of the studies on antimicrobials as growth promoter were conducted in the decades of the 1980s and 2000s (Teillant 2015). With the readily available antimicrobials to be used for growth promotion, it is often ignored by farmers that similar results could be achieved by selecting high growing lines, good hygiene, nutrition, and health management. Focusing on these things rather than just relying on antimicrobials for growth-promoting effects could dramatically reduce the use of antimicrobials in livestock.

Additionally, it is important to understand the economic aspects of using antimicrobials as a growth promoter and the potential economic effect of banning antimicrobials as growth promoters. There is limited knowledge on these; however, studies from the countries such as Denmark and Sweden, where antimicrobials have already been banned as a growth promoter, suggest that there is minimal impact on

economy (Graham et al. 2007; Sneeringer et al. 2015). The most likely cost after banning antimicrobials as a growth promoter will be to improve hygiene and management, which is significant, but with a long-term positive effect both on animals and humans. Developing countries are a major concern where the production is less controlled, and the impact of the ban is likely higher compared to that of developed countries. The ban could become counterproductive if not handled properly as it could lead to more therapeutic use of antimicrobials to keep animals healthier and more productive. Additionally, to meet the demand of increasing world population, more animals need to be raised if growth-promoting antimicrobials are prohibited, which may subsequently lead to negative impacts on environment and other areas (Hao et al. 2014). Therefore, this is an extraordinarily complex issue requiring intervention from each country to make a common alliance with common goal.

Compared to very few positive effects (such as improved growth and improved feed efficiency) of antimicrobials used as growth promoters, there are numerous negative effects (Edqvist and Pedersen 2001; Hao et al. 2014). They are summarized below:

- Increases the pool of antimicrobial-resistant genes.
- Camouflages bad feed, subsequently discouraging improvement in feed development and its alternatives.
- Helps to hide the subclinical diseases and associated stress.
- Promotes intensive farming that is less animal-friendly.
- Disrupts disease treatment by increasing antimicrobial resistance.
- Provides the best possible environment to bacteria that are mutating to become antimicrobial-resistant.
- Indirectly impacts human health due to the transfer of antimicrobial resistance.

Based on the above, it is of utmost importance to identify alternatives of growth-promoting antimicrobials and implement those alternatives as soon as possible. Some of the alternatives to antimicrobials are discussed later in this chapter.

1.3.2 Prophylactic Use of Antimicrobials in Livestock

Farmers do not have any other choices but to use antimicrobials when animals are sick. The use depends on the animal species, stage of production, and disease risk. Similarly, when only a few individuals are sick, farmers choose to use antimicrobials to prevent the spread of disease to other animals. Usually, such antimicrobials are administered at critical points during the livestock production cycle to prevent diseases.

When antimicrobials are used as a prophylactic agent against certain diseases, they are generally used for a short duration and administered via feed or water to a group of animals. For example, most feedlot cattle in the United States (~83%) are administered with at least one antimicrobial in feed and water to control different disease outbreaks such as diarrhea and pneumonia (Animal and Plant Health

Inspection Service 1999). Similarly, broilers are usually administered with bacitracin and sulfonamides via feed to prevent necrotic enteritis and coccidiosis, respectively (McEwen and Fedorka-Cray 2002). In pigs, several antimicrobials such as tiamulin, sulfonamides, tetracyclines, and ceftiofur are used to prevent pneumonia. Additionally, most pigs receive antimicrobials during weaning to prevent them from infectious disease as weaning is one of the most stressful periods in a pig's life (McEwen and Fedorka-Cray 2002).

1.3.3 Therapeutic Use of Antimicrobials in Livestock

Antimicrobial use as a therapeutic agent is a common practice throughout the world and is the least controversial among the three uses of antimicrobials. Usually, antimicrobials are administered to a targeted individual(s) via feed and water or through direct injection. During disease outbreaks, especially in large pig and poultry farms, antimicrobials are administered through the water as a disease can depress feed intake in animals and it is usually believed that animals continue to drink water despite reducing the feed intake during sickness.

Gentamicin, apramycin, and neomycin are used to treat bacterial diarrhea in pigs caused by *E. coli* and *C. perfringens* (McEwen and Fedorka-Cray 2002). Similarly, nearly all weaned piglets have access to some form of antimicrobials to control disease outbreaks because of stress during weaning (Dewey et al. 1999). Fluoroquinolones are used to treat *E. coli* infections in poultry, and it is a common practice to use ionophores and sulfonamides to control coccidiosis. Hatchery use of antimicrobials is also common to control omphalitis in day-old chicks (Ouckema and Phillipe 2009). In dairy cattle, antimicrobials such as penicillins, cephalosporins, and erythromycins are used to treat mastitis (Erskine 2000). Such drugs are a routine practice in cattle, which are usually administered to the entire herd during nonlactating periods (Erskine 2000).

1.4 Antimicrobial Resistance

Bacteria are referred to as resistant to antimicrobials when they become nonsusceptible to one or more antimicrobials. When they become resistant to three or more antimicrobials, they become multidrug-resistant bacteria and then called pan drug-resistant if they are immune to any antimicrobials (Magiorakos et al. 2011).

Many antimicrobials (especially antibiotics) that are used in livestock are also essential for human use. When antimicrobials are used in livestock to prevent disease, it suppresses and eliminates bacteria that are susceptible to the antimicrobials. However, such antimicrobials cannot eliminate those bacteria that are resistant to them. Bacteria have an extraordinary potential to be adaptive to the new environment including the environment with antimicrobials. Those bacterial that are

tolerant to antibiotics can multiply within the host and likely become the dominant bacterial population. Such bacteria are also able to transfer the resistant genes to other bacteria. When humans consume food products coming from animals, such bacteria can enter human being and subsequently colonize in the intestine. Once these tolerant bacteria are widespread within the human population and the antibiotics stop working against those bacteria, the treatment strategies can fail and lead to the devastating outcome (Hall et al. 2011; Marshall and Levy 2011).

1.5 How Antimicrobials and Antimicrobial Resistance Flow Between Humans, Livestock, and the Environment?

The dynamics behind the movement of antimicrobials and antimicrobial resistance from food animals to humans and vice versa is a complex phenomenon. The emergence of antimicrobial resistance and dissemination across and within different species has been summarized below:

1.5.1 Agricultural Production Method

Housing is one of the major drivers increasing the rate of emergence and dissemination of antimicrobials and antimicrobial resistance. In modern housing systems, a large group of animals is confined within a building or closed space (e.g., battery housing, feedlot cattle, pig barns, etc.). Hundreds of animals share food, water, air, and bedding for a long period. Animals are exposed to their own and other wastes containing antimicrobials and resistant bacteria (Gormaz et al. 2014). Additionally, workers get exposed to a large group of animals and resistant pathogens, who further transmit these pathogens to communities through contaminated clothing, shoes, and surfaces (Fey et al. 2000; Rinsky et al. 2013). Humans are not only exposed to these pathogens in farms but can also get these pathogens from a slaughterhouse. In slaughterhouses, workers are in close contact with animal bodies and equipment used in slaughtering, handling, cutting, processing, and storage of carcasses (Madden 1994; Sammarco et al. 1997). Besides, cross-contamination of pathogens is also linked to the trucks and other vehicles, when such vehicles are not thoroughly cleaned and decontaminated after their use in transporting other animals and food products (Hennessy et al. 1996; Pell 1997). Overcrowding, lack of appropriate sanitary measures, and cross-contamination during handling, transport, and slaughterhouse operations can amplify the dissemination of resistant pathogens, further worsening the situation.

Housing (especially intensive) is the major stressors to animals compromising their immune function. A compromised immune system leads to increased shedding of different kinds of pathogens. Animals are exposed to a series of stressors

throughout their life from housing, handling, transport, and lairage at a slaughterhouse. Studies have suggested that stress can result in an increased prevalence of infections (Hayes et al. 2004; Verbrugghe et al. 2012), leading to an increase in the demand for antimicrobials.

1.5.2 Livestock Waste

Livestock farming results in a large volume of waste products often bigger than the carrying capacity of the environment. Livestock waste may contain resistant pathogens and genes, feed wastes, and spilled antimicrobials (from the feed, water, and excreta). In many countries, these waste products are largely unregulated, which means they are not treated before going into solid and water. This can lead to the release of a large number of antimicrobials and antimicrobial-resistant pathogens to the environment. From the environment, other animals and humans can get exposed to it, which might create an uncontrolled and widespread transfer of resistant pathogens across different species.

1.5.3 Exposure to Other Animals and Insects

Often livestock buildings are intruded by rodents, birds, insects, and other animals, mostly due to poor biosecurity. Nazni et al. (2005) reported similar pathogens to that found in poultry houses in the flies found in the poultry barn. Rodents in poultry and swine barn have been found to carry antimicrobial-resistant pathogens and disseminate them to the environment (Backhans and Fellstrom 2012). There is a potential transfer of such pathogens and antimicrobials from domesticated animals to wild animals.

1.5.4 Movement of Animals and Food

There is extensive movement of live animals across the different parts of the world, for example, the movement of poultry breeding stock from Europe to Asia and within Europe and live sheep export from Australia to the Middle East. If the use of antimicrobials is permitted in exporting countries but not in the importing countries, there is a likelihood of antimicrobial-resistant pathogen transfer from the exporting country to the importing one.

In addition to live animals, there is an extensive export and import of food products throughout the world. Major producers of pork, poultry, fish, and beef extensively export these products to other countries (Silbergeld and Dailey 2017). This extensive trading makes it impossible for countries to assess the flow of pathogens

through food products between the countries. Food can be contaminated with resistant bacteria through several routes, i.e., from bacteria present in animals, from bacteria added during culture, and from bacterial cross-contamination during the processing of foods (Verraes et al. 2013).

Especially in developing countries, antimicrobials are misused due to poor regulations in the supply chain. Moreover, a large population in such countries is in close contact with the animals. It hence burgeons the chances of transmissions of resistant microorganisms from animals to humans from handling of the animals.

1.5.5 Environment

The environment is not only a significant reservoir of many pathogens but also facilitates their dissemination by forming a cycle of pathogen contamination. In addition to getting pathogens from livestock wastes, antimicrobials used as crop pesticides also lead to soil and water contamination (Bhandari et al. 2019), subsequently leading to the emergence of resistant bacteria. Moreover, globalization and urbanization have led to environmental pollution, further compromising livestock and human health and increasing the demand for antimicrobials (Balakrishnan et al. 2019). Antimicrobials used in agriculture, human, and veterinary medicine are partially metabolized by animals and humans and end up being released into the environment through sewage systems. Antimicrobials used in aquaculture are directly added into the water, leading to a high antimicrobial concentration in water and the sediments. Studies in various countries have detected a low concentration of antimicrobials in different environmental compartments such as municipal wastewater, sewage plant effluent, and even groundwater (Kümmerer 2004; Kolpin et al. 2002; Sacher et al. 2001). Most of the commonly used antimicrobials are not biodegradable and persist in the aquatic ecosystem (Kümmerer 2003). These antibiotics may have direct effects upon the resident microbial community of sediments in the ecosystem (Nygaard et al. 1992). The presence of active antibiotic compounds in the environment exerts a selective pressure which might create the occurrence of antibiotic-resistant phenotypes that may spread in the environment through the microbial species (Thanner et al. 2016). In addition to the release of antibiotics leading to the development of resistant bacteria, bacteria themselves are also excreted by humans and animals which end up in the ecosystems.

Humans and animals are a part of a complex environmental phenomenon. Several human activities such as traveling, contact with livestock, and contact with wild animals lead to the dissemination of antimicrobials and pathogens that are resistant to antimicrobials. The environment in which both human and animal live completes the cycle of this dissemination. Therefore, we must reduce the release of antimicrobials to the environment to disrupt this cycle and to slow down the zoonotic transmission of antimicrobial resistance (Fig. 1.1).

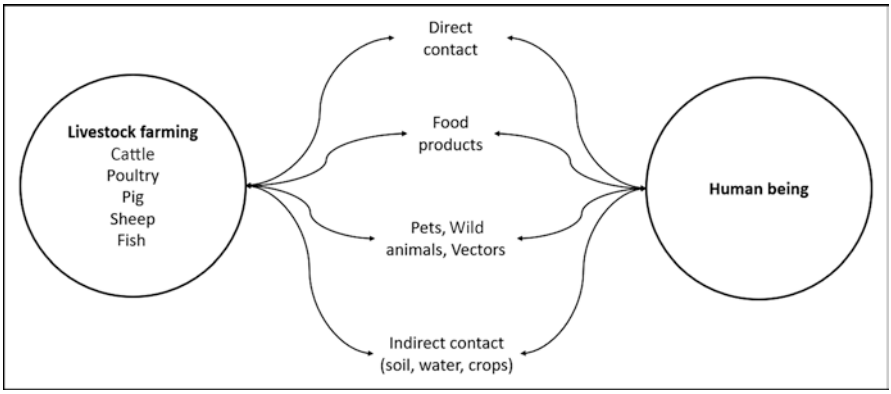

Fig. 1.1 Potential route of exchange of antimicrobials and antimicrobial-resistant pathogens between animals, humans, and the environment

1.6 Zoonosis of Antimicrobial Resistance

It is estimated that more than 50% of pathogens that can infect human beings can also infect other animals (Taylor et al. 2001). Therefore, there is a huge potential for transfer of antimicrobial resistance from animals to human beings and vice versa. The earliest documented evidence of animal-human transmission of antimicrobial resistance was in the 1970s during the *Salmonella* epidemic in a human hospital that was traced back to the calves infected by *Salmonella* (Labro and Bryskier 2014). Since then, documentation of antimicrobial resistance in livestock and humans and the spread of resistant bacteria between animals and humans is large and readily available (Woolhouse and Ward 2013).

In the United States alone, more than 2.8 million cases of illnesses are due to some form of antimicrobial-resistant infections leading to more than 35,000 deaths per year (Centers for disease control and prevention (CDC) 2019). Antimicrobial resistance contributes to 700,000 deaths annually with estimated 214,000 neonatal deaths attributed to resistant sepsis infections (Pokharel et al. 2020). The data on livestock deaths due to antimicrobial resistance is scarce; however, studies have reported antimicrobial resistance to antibiotics in *E. coli*, *Salmonella* spp., *Campylobacter* spp., and *Enterococcus* spp. that are responsible for most infections in livestock (McEwen and Fedorka-Cray 2002; WHO 2003; Aarestrup et al. 2008).

Increased resistance to antimicrobials usually coincides with the use of such antimicrobials in livestock that are used for food production. For example, in poultry, fluoroquinolones are heavily used to treat respiratory diseases, and it is no surprise that increased resistance to fluoroquinolones has been heavily documented in humans, mostly linked to poultry consumption (Endtz et al. 1991; Nelson et al. 2007). In a more recent study, approximately 90% of isolates from poultry showed some form of resistance to antimicrobials such as sulfonamides, tetracyclines, fluoroquinolones, and third-generation cephalosporins (Kaesbohrer et al. 2012).

Similarly, methicillin-resistant *Staphylococcus aureus* (MRSA) is a growing concern among people that are in contact with animals, both livestock and pets (Labro and Bryskier 2014). *Enterococcus* is another commensal bacteria found in both human and animal guts, which are intrinsically resistant to cephalosporins and can also acquire resistance to quinolones, macrolides, and tetracyclines (Murray 1990).

As a global public health threat affecting both humans and animals, antimicrobial resistance has warranted several national and international communities to work together on implementing policies to preserve the efficacy of medically important antimicrobials. The concept of critically important antimicrobials was developed in a second workshop held between the WHO, FAO, and OIE in 2004. The WHO classified antimicrobials into five groups based on their importance to human medicine and released a guideline in 2018, which recommended that the highest priority critically important antimicrobials (HPCIA) should not be used in food-producing animals (WHO 2017). The HPCIA includes five classes of antimicrobials: quinolones; third-, fourth-, and fifth-generation cephalosporins; macrolides and ketolides; glycopeptides; and polymyxins. Study in some European countries has shown that it is possible to maintain health and productivity with no use of cephalosporins and fluoroquinolones in livestock; however, total exclusion of macrolides is difficult as they are critically important in managing respiratory disease in pigs, poultry, cattle, and other animals. This makes it more complex as respiratory diseases in livestock are associated with significant economic losses in most countries (Lhermie et al. 2020).

1.7 One Health and Antimicrobial Resistance

Antimicrobial resistance is a multifaceted global issue (Pokharel et al. 2020). Both human and veterinary medicine are the major contributor to the emergence of antimicrobial resistance. The issue is not going to affect one single species in the world; it could well become the most widespread pandemic in the future, affecting the largest number of species throughout the world. Therefore, there is a need for a multidisciplinary approach involving humans, animals, and the environment, which is referred to as One Health.

The WHO defines One Health as a "concept and approach to designing and implementing programs, policies, legislation and research in which multiple sectors communicate and work together to achieve better public health outcomes" (WHO 2017). The origin of One Health is centuries old and recognizes both human and animal health. More recently, this concept recognizes environmental health too. In summary, there are three domains in this approach: human health, animal health, and environmental health.

Among the three domains, human health takes a major emphasis. Antimicrobial resistance genes have been reported to be highly prevalent in some common pathogens in humans such as *E. coli*, *K. pneumonia*, and *S. aureus* (Robinson et al. 2016). Livestock has played a major role in the transmission of antimicrobial resistance,

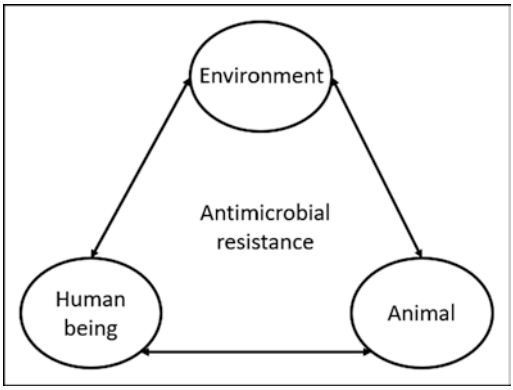

Fig. 1.2 Relationship between human being, animal, and environment in a One Health concept

which was already discussed previously in this chapter. Livestock and associated products will continue to play a significant role in the dissemination of antimicrobial residue and antimicrobial resistance in the future. At present, there is a lack of knowledge transfer between human and veterinary medicine, causing inconsistencies in the use of antimicrobials in humans and animals. The collaborative approach between human and veterinary medicine can mitigate this and provide sustainable solutions (Fig. 1.2).

The third domain, environment, is getting considerable recognition in recent years. As discussed earlier, the environment is a significant transmission reservoir for most of the pathogens in humans and animals, without which the disease cycle cannot be mostly completed (Pornsukarom and Thakur 2017). Soil and water contamination of antimicrobials can lead to the emergence of antimicrobial-resistant bacteria that are already in soil and water (Grenni et al. 2018). Similarly, other aspects of the environment such as air pollution have led to increased infections in humans and animals, subsequently increasing the demand for antimicrobials that further aids in the emergence of antimicrobial resistance.

1.8 Third-Generation Cephalosporins: A One Health Example

Third-generation cephalosporins are widely used in humans and animals. These are classified as critically important antimicrobials by the WHO (2017). Third-generation cephalosporins have a broad-spectrum activity, and some of their uses include controlling bovine respiratory disease in cow, preventing *E. coli* infections in chicks, and treating pneumonia, arthritis, and other conditions in humans (Greko et al. 2009). Resistance to third-generation cephalosporins has been reported in *E. coli* and *K. pneumonia* (Park 2014; de Kraker et al. 2011). Several studies reported that voluntary withdrawal of third-generation cephalosporin use in chicks was followed by the drop in its resistance in *E. coli* (Hiki et al. 2015; Dutil et al. 2010).

Countries such as Denmark, Australia, and Canada have placed a voluntary ban on the use of these drugs recognizing the resulting human health risk with their use in animals (Collignon and McEwen 2019).

1.8.1 Antimicrobial Stewardship

Antimicrobial resistance is a one-world issue. Therefore, it is in everyone's interest to preserve the efficacy of antimicrobials by properly using them, following the guidelines, monitoring their use and resistance, and implementing good stewardship programs. Antimicrobial stewardship is a set of actions that promote the responsible use of antimicrobials and can be summarized with 5Rs: responsibility, review, reduce, refine, and replace (Page et al. 2014). The 5R approach guides livestock farmers, veterinarians, physicians, and other relevant stakeholders who are involved in antimicrobial use to adopt best practice and management of antimicrobial use. With regard to good stewardship, prevention of disease in livestock is more important than the treatment, which means vaccination and good husbandry are critical in putting antimicrobial use in check (Table 1.2).

1.9 Alternatives to Antimicrobial Use in Livestock

As suggested by good antimicrobial stewardship, we can identify and implement the practices that can either replace or reduce antimicrobial use and also reduce the likelihood of infections in animals. Such practices can include early intervention long time before the infections such as vaccinations. There are several vaccines available that can help prevent several infections in livestock (e.g., cattle, *E. coli*, *Salmonella* vaccine; pigs, *E. coli* vaccine, vaccine against bacterial pneumonia; and poultry, vaccine against pasteurellosis, *Salmonella* vaccine). Another important

Table 1.2 The 5R approach to tackle misuse of antimicrobials

Responsibility	Everyone using antimicrobials need to understand that antimicrobial use can be a risk to both human and livestock. Therefore, responsible use of antimicrobials should be practiced to reduce public health risk
Review	Everyone using antimicrobials should review the use regularly and make strategies to reduce the use of antimicrobials
Reduce	Whenever possible, there should be an attempt to look for the ways to reduce antimicrobial use
Refine	Right drugs at the right time and right dose should be used for the right amount of time
Replace	Whenever possible, strategies should be implemented to consider replacing antimicrobials with non-antimicrobial products such as probiotics, herbal medicines, vaccines, and immune modulators

strategy that can reduce the antimicrobial load includes good husbandry practices. Good sanitation in and around the farm can reduce bacterial load around the farm, good air and water quality can prevent horizontal transmission of diseases, and good feed can help protect animals against many conditions such as salmonellosis and mycotoxins. Good air quality and appropriate ventilation in the animal farm can help control high gaseous levels (e.g., ammonia level in poultry houses) subsequently reducing several bacterial infections.

Good husbandry practices also involve farmers following appropriate biosecurity measures. Controlling what goes into the farm can help prevent a lot of diseases in animals. For example, the use of appropriate clothing and foot baths, control of vectors, control of birds and rodents, and use of *Salmonella* free food can be easily practiced on the farm. Another less common practice involves the use of beneficial bacteria in the form of probiotics, which can act as an antibiotic growth promoter in animals (Reid and Friendship 2000); however, more studies are yet to be conducted to understand more about probiotics and their role in the farm as an alternative to antimicrobials. In addition to probiotics, prebiotics and organic acids can also provide health benefits to animals by stimulating growth, metabolism, and composition of beneficial bacteria in the gastrointestinal tract and eliminating the harmful one (Solis-Cruz et al. 2019).

Genetic selection is another avenue that could provide potential solution to the widespread use of antimicrobials. Herds that are resistant to certain diseases can be selected that could possibly eliminate the use of antimicrobials for that disease. Studies are scarce on the use of genetic selection to achieve pathogen-resistant animals but can be food for thought for animal scientists to tackle the issue. More recently, bacteriophages have emerged as a potential alternative to antimicrobials, which works by specifically attacking bacteria; however, lack of regulatory guidance and clinical trials has hindered the possibility of using bacteriophages in large scale (Romero-Calle et al. 2019). Different alternatives to antimicrobials and how they work have been summarized in Table 1.3.

Table 1.3 Summary of alternatives to antimicrobials in livestock

Alternatives to antimicrobials	Mechanism of action
Vaccines	Preparing immune system to recognize and combat pathogens
Good husbandry practices	Reducing microbial load in the farms and thereby lowering exposure to microbes
Prebiotics, probiotics, and organic acids	Promoting growth, selectively stimulating beneficial bacteria, and eliminating harmful ones
Genetic selection	Selecting animals that are resistant to certain diseases
Bacteriophage	Attacking and killing bacteria

1.10 Conclusion

Antimicrobials are the most important discoveries of human and animal health, and ironically, antimicrobial resistance is one of the greatest crises to public health. The use of antimicrobials in the livestock sector in different parts of the globe is indiscriminate and unregulated. There is a lack of data about the scale of their use, and more studies are required to understand the fate of these antimicrobials in the environment and their consequences on human health. Livestock farming should urgently be recognized as a major contributor to the development of antimicrobial resistance, and countries need to develop legislation regulating prophylactic use of antimicrobials in farming. The evidence presented across countries indicates that it is possible to reduce antimicrobial use and gain highly intensive and productive production systems (Cogliani et al. 2011). A coordinated effort between governments, industry, and scientists is required for effective action on antimicrobial resistance. An immediate step to tackle the problem would be to develop strategies for improved antimicrobial stewardship involving both human medicine and livestock industry and develop alternative approaches to combat microbial disease and improve livestock production.

Key Notes

- Antimicrobial is a complex subject.
- Antimicrobial use in livestock is rising at an alarming rate, driven by increasing demand for animal protein globally.
- Data on the antimicrobial use is not sufficient, which warrants more study on the topic.
- Antibiotic resistance is a public health crisis.
- Livestock farming is a major contributor to the development of antimicrobial resistance.
- Keeping animals healthy is important in reducing the use of antimicrobials.
- Antimicrobial resistance is a One Health issue. More than that, it is a one-world issue.
- 5R approach can help become a good antimicrobial steward and help tackle antimicrobial resistance.
- Strategies that can help reduce the use of antimicrobials include good farm management, vaccination, biosecurity, probiotics, and genetic selection.

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Chapter 2
Antibiotics and Resistance in Environment

Rahil Changotra, Atul Chalotra, and Himadri Rajput

2.1 Introduction

In 1928, the penicillin discovery by Alexander Fleming is often considered to be one of the most vital discoveries in the field of medical technology (Levy and Bergman 2003). After that, in between the 1940s and 1980s, the worldwide growth of pharmaceutical industries led to the production of over 160 antibiotics and semi-synthetic pharmaceutical compounds which soon became the foundation for the treatment of several infectious diseases (Davies 2006). Despite great success in the reduction of morbidity and mortality due to infectious diseases, these pharmaceuticals have been regarded as emerging pollutants and invoked increasing concern worldwide in the past few decades (Rivera-Utrilla et al. 2013). Even at subtherapeutic concentrations in the environment, they have adverse effects on human health and ecosystems. However, the risk associated with the exposure of pharmaceuticals is essentially vital in areas that practice indirect water reuse, where pharmaceutical effluent is discharged to rivers and streams that are in turn used as source of drinking water for the nearby human communities. Pharmaceutical manufacturing activity has been classified into "red category" owing to its production of huge volume, complexity, and hazardous waste. The process involved in the pharmaceutical manufacturing industry includes the manufacture, extraction, processing, purification, and packaging of solid and liquid materials to be utilized for the ailments of humans and animals. In the manufacturing unit, the synthesis of bulk drugs involves several reactants with or without fermented natural antibiotics depending on the type of bulk drugs being synthesized. Moreover, the increasing rate of unused medicines

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from hospitals and households and its disposal malpractices have also been considered as a source of pharmaceutical waste (Tong et al. 2011; Vellinga et al. 2014). The effective disposal and management of pharmaceutical waste pose a serious challenge for the scientific research community, particularly due to the occurrence of pharmaceuticals and their metabolites in the outlets of wastewater treatment plants, which may find their ways to enter the aquatic environment. Knowing the existence of pharmaceutical compounds in the aquatic environment, there are growing concerns to explore their fate, transport, effects, and toxicity as well as their resistance in the environment.

2.2 Treatment Systems and Antibiotics in the Environment

The pharmaceuticals comprise of nonsteroidal anti-inflammatory drugs, antibiotics, analgesics, antiepileptics, hormones, steroids, toxic substances, volatile organic compounds (VOCs) and surfactants, etc. Among these pharmaceuticals, antibiotics are the major class of pharmaceuticals with extensive demand and use with the growing attention. These antibiotics despite being intended to treat certain ailments in animals and humans have exhibited adverse impacts on the ecosystem due to their existence in wastewater, surface water, groundwater, and drinking water. The previous years have witnessed significant and wide research on the detection of more than 160 different antibiotics and their metabolites in the various environment samples ranging from few ng L^{-1} to several μg L^{-1} (Kümmerer 2010; Kanakaraju et al. 2017), and as a result, these pharmaceuticals are now categorized as an emerging pollutant in various water bodies. Antibiotics enter into the environment from the pharmaceutical production industries and direct and improper disposal of unused/expired medicines by humans and through human urine or feces and hospital wastes and household sewage which enter into the influent of wastewater treatment plants (WWTPs) in an unmetabolized form or as moderately active metabolites (Mompelat et al. 2009; Changotra et al. 2019). It has been reported that about 10 to 90% of the administrated dose of antibiotic drug is excreted from the human body as a parent compound, whereas the rest is excreted as metabolites and/or in transformed forms (Kümmerer 2009; Changotra et al. 2017). The recent research showed that numerous pharmaceutical manufacturing units were found to be sources of surpassing antibiotic concentrations into the aquatic environment than those caused by the usage of drugs (Kessler 2010). The excreted antibiotics reach the WWTPs and are finally discharged as raw or treated wastewater into the rivers, surface water, groundwater, oceans, and soil (Fig. 2.1).

A major pathway of antibiotic mobilization in the environment is considered to be from WWTPs as they accumulate discharges from pharmaceutical industries, hospitals, households, veterinaries, and pharmacies (Tarpani and Azapagic 2018; Changotra et al. 2018). This is due to the inefficient removal of antibiotics in the conventionally used treatment technologies which are intended to treat biodegradable, nonpolar, and large compounds, as opposed to polar tendency, small

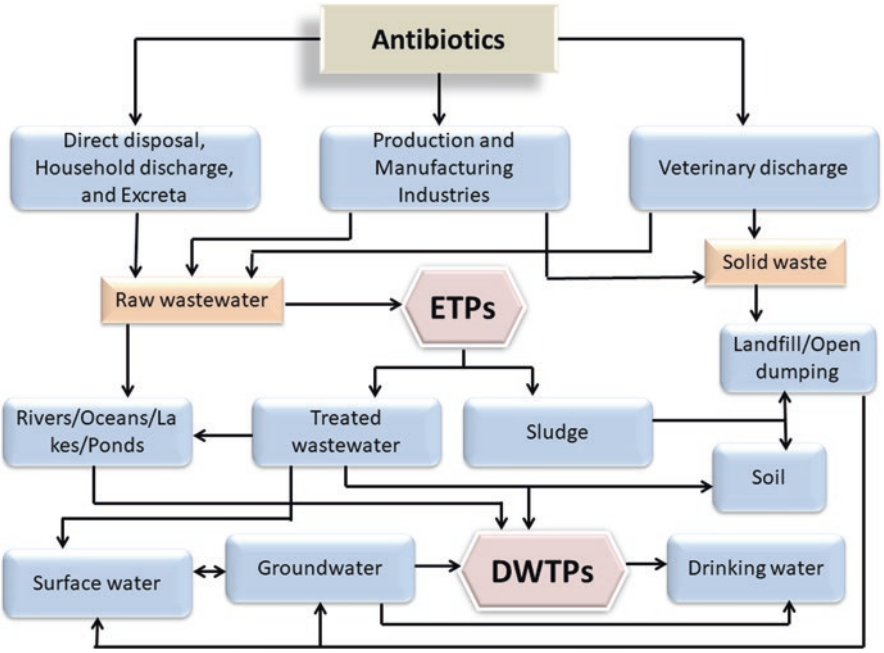

Fig. 2.1 Fate and transport of the antibiotics in the environment. *ETPs* effluent treatment plants, *DWTPs*, drinking water treatment plants

compounds, and nonbiodegradable nature of antibiotic compounds (Ratola et al. 2012; Changotra et al. 2020). It is notable that antibiotics enter in the soil and surface waters and eventually in the ground and drinking water after their excretion (as active metabolites or in unmetabolized form) in urine or feces from humans and animals (Klavarioti et al. 2009). Besides excretion, antibiotics may find their pathways into water bodies due to the disposal of medicines used in industry, agriculture, households, and hospitals. Veterinary pharmaceuticals may directly contaminate soil through manure and surface water and groundwaters by surface runoff (Khetan and Collins 2007). Moreover, numerous transformation products and metabolites are also likely to form in WWTPs as a consequence of numerous chemical reactions in the course of treatment (Zuccato et al. 2000) that could facilitate synergetic effects and become more bioactive when compared to parent compounds. In order to treat the wastewater containing antibiotics, most of the pharmaceutical industry utilized general approaches like recovery of antibiotic drugs which are expected to be present in wash waters and solvents, physicochemical treatments like sedimentation or floatation, conventional treatment technologies like aerobic/anaerobic biological treatment, inactivation of active compounds using UV oxidation, sterilization or inactivation of bioactive substances, and a new hybrid technology specific to the pharmaceutical industry (Gadipelly et al. 2014). Due to the inadequate design, complex operations, lack of technical manpower, and frequent electricity breakdowns,

the treatment plants do not function appropriately (CPCB 2007). As a result, improper disposals of effluents from the treatment plants are considered to be the major pathways for the antibiotic drugs to enter in aquatic environment (Gothwal and Shashidhar 2017).

2.3 Impacts of Antibiotics on Environment

Chemical structure of antibiotics is stable enough to produce therapeutic activity, and due to their constant input, they persist in the environment for a longer duration of time; and their existence at trace, low, and high concentration is dangerous to the environment and ecosystem (Klavarioti et al. 2009). These antibiotics are characterized by low volatility and high polarity and tend to be easily transported and distributed in different water bodies (Zhang et al. 2014). Many antibiotics have the potential to bioaccumulate and are highly persistent in the environment. Antibiotics have been detected in various bodies like surface water and groundwater (WHO 2011; Balakrishna et al. 2017; Lee et al. 2017), drinking water (Zuccato et al. 2000; Jones et al. 2005; Buffle et al. 2006), tap water (Halling-Sørensen et al. 1998; Doll and Frimmel 2003), soil and sediments, ocean water (Halling-Sørensen et al. 1998; Klavarioti et al. 2009), and sewage influents and effluents (Heberer 2002; Arlos et al. 2015). Additionally, few studies confirmed the presence of pharmaceuticals in Indian rivers (Fick et al. 2009; Ramaswamy et al. 2011; Mutiyar and Mittal 2014; Subedi et al. 2015; Archana et al. 2016). Studies on the treatment of wastewater comprising antibiotics revealed that antibiotics are not readily biodegradable and their genotoxicity was not eliminated by biological treatment (Alexy et al. 2001). The most commonly used activated sludge treatment process cannot remove antibiotics efficiently that are persistent to biodegradation (Celiz et al. 2009). In Japan, Matsuo et al. (2011) detected the presence of 100–2000 ng L^{-1} of amoxicillin concentration in an activated sludge-based biological treatment of wastewater. A similar presence of amoxicillin was reported in the influent of WWTP involving activated sludge treatment in Australia by Watkinson et al. (2007), whereas 20 times higher concentration was found in the effluent of WWTP comprising biological treatment in Hong Kong (Minh et al. 2009). The concentrations of amoxicillin, ofloxacin, sulfamethoxazole, ciprofloxacin, and metoprolol were found to be 40 times higher in the treated wastewater of Indian WWTPs when compared to the treated effluents of WWTPs in Europe, Japan, North America, and Australia (Balakrishna et al. 2017). Although biological treatment is considered to be effective in the abundance of industrial wastewater treatment applications, its efficacy decreases in the presence of complex and tough antibiotic compounds in wastewater. Moreover, the complex design, overall cost, and economic return are the major problems associated with the treatment of antibiotic-laden wastewater at an industrial scale.

Antibiotics discharged in the environment may impose serious toxicity at many levels of life, i.e., humans, animals, microorganisms, ecosystems, or the ecosphere. They are resistant to biodegradation processes and frequently escape from the

conventional treatment plants. Moreover, antibiotics may cause irreversible change and lasting effects on the microorganisms, making them resistant to antibiotics, even at trace concentrations (Bredhult et al. 2007; Kristiansson et al. 2011). The antibiotic's presence in the environment has effects such as retardation of nitrite oxidation and methanogenesis, disruption of the human endocrine system, and increased toxicity of chemical combinations and metabolites (Gadipelly et al. 2014). The scientific community is more concerned about the development of antibiotic-resistant bacterial strains and the endocrine-disrupting effects on aquatic organisms exposed to antibiotics. Hence, the presence of antibiotics or their metabolites in the aquatic environment in particular poses a serious environmental problem on a local and a global scale.

2.4 Antibiotic Resistance in Environment

At present, the emergence of pathogenic microbes that are able to resist antibiotic treatments is regarded as one of the most concerning public health issues (CDCP 2013). According to the report by the CDCP, nearly 25,000 people die per year in Europe due to antibiotic-resistant bacterial infections with a worldwide mortality of half a million people (Davies et al. 2013). Antibiotic resistance also poses a significant impact on the world economies, with an approximate investment of 35 billion USD per annum by the United States alone for the treatment of antibiotic-resistant infections (CDCP 2013). To further worsen the situation, the worldwide discoveries of new antibiotics have declined in the past few years due to economic and technical challenges, thereby leading to the "antibiotic crises" (Livermore 2011). The existence of an increased variety of bacteria harboring antibiotic resistance genes in different environmental samples is one of the most prominent consequences of antibiotic pollution. At an early stage, the antibiotics were found to be effective in treating bacterial infections, leading to short and intense research on developing new antibiotics. However, the prolonged and larger-scale use of antibiotics led to the emergence of antibiotic-resistant bacteria. For instance, an example of the remarkable speed of resistance could be observed when 70% of *S. aureus* strains became resistant to erythromycin within 6 months after the beginning of treatment (Finland 1979). Antibiotic resistance can be disseminated through a wide range of mechanisms, such as inactivation of antibiotics (e.g., cleavage of beta-lactams, penicillin, through beta-lactamases), transportation of antibiotics from the bacterial cells through efflux pumps (e.g., tetracyclines pumped out of the cell through *TetA* proteins), or modifications of antibiotic's target cell (e.g., ciprofloxacin binding to cell is prevented by point mutations in *gyrA*) (Gullberg et al. 2011).

Due to the complex and multifaceted ecological role of antibiotics, it is believed that antibiotic resistance is an ancient phenomenon. It has been investigated that antibiotic resistance genes have been found in a range of nonhuman impacted and pristine environments, from permafrost soils to caves, and have enlightened our understanding of the natural occurrence of antibiotic resistance in microbial

Table 2.1 List of antibiotic-resistant "priority pathogens" (WHO 2017)

Priority 1	Critical • *Acinetobacter baumannii*, carbapenem-resistant • *Pseudomonas aeruginosa*, carbapenem-resistant • *Enterobacteriaceae*, carbapenem-resistant, ESBL-producing
Priority 2	High • *Enterococcus faecium*, vancomycin-resistant • *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant • *Helicobacter pylori*, clarithromycin-resistant • *Campylobacter* spp., fluoroquinolone-resistant • *Salmonella*, fluoroquinolone-resistant • *Neisseria gonorrhoeae*, cephalosporin-resistant, fluoroquinolone-resistant
Priority 3	Medium • *Streptococcus pneumoniae*, penicillin-non-susceptible • *Haemophilus influenzae*, ampicillin-resistant • *Shigella* spp., fluoroquinolone-resistant

ecosystems (D'Costa et al. 2011; Perron et al. 2015). For instance, 70% of Gram-positive and 65% of Gram-negative bacterial isolates from a pristine cave carried several functional antibiotic resistance genes (Bhullar et al. 2012). As a human, the important concern is not the presence of resistance genes in the pathogens, but the main concern is the horizontal transfer of the resistance genes in the environmental microbes, thereby limiting our ability to fight against infectious diseases with antibiotics. There are different mechanisms for the transfer of resistance genes into the microbes including phage-supported transmission (transduction) (Xu et al. 2011), uptake of DNA from the environment (transformation) (Springman et al. 2009), and transmission through conjugative plasmids (Palmer et al. 2010).

From the perspective of public health, environmental microbes carrying the resistance genes against the variety of antibiotics, also known as "superbugs," are an important concern. In 2017, the World Health Organization (WHO) released a list of "superbugs" comprising of 13 families of bacteria that pose the greatest threat to the public health (WHO 2017). The list is divided into three categories according to the urgency of need for new antibiotics: critical, high, and medium priority as shown in Table 2.1. The list highlights in particular the threat of Gram-negative bacteria that are resistant to multiple antibiotics. The high consumption and anthropogenic use of antibiotics have triggered the experimental evolution of the antibiotics in each environment. In the subsequent sections, we shall discuss how the resistance genes are selected and flow through different environments and different vectors that contribute to the speed of gene transfer.

2.4.1 Resistance Genes in the Environment

Due to the increasing awareness of the risks posed by the use of antibiotics and the availability of a wider range of detection techniques, there is a substantial increase in the research focusing on the detection of antibiotic resistance in the environment

through qPCR, conventional PCR, culture-dependent techniques, or mutagenomic methods (Graham et al. 2016; Ju et al. 2019). The possibility of the environment affected with antibiotic resistance genes is based on the flow of antibiotic resistance genes between the different environments as outlined based on a schematic model in Fig. 2.2. Extensive investigation has been done to explore the hotspot environments of antibiotic resistance genes, where microbes could grow rapidly due to the availability of nutrients and are able to quickly encounter heavy and repetitive doses of antibiotics (Berendonk et al. 2015). The major hotspot environments of antibiotic resistance genes include animal feeding operations, hospitals, aquaculture operations, and WWTPs.

With the emergence of hospital-acquired infections due to antibiotic-resistant microbes, the hospital facilities and intensive care units are important to explore the evolution and distribution of antibiotic resistance genes. The various members of human-associated microbes existing in hospital water and airflow systems are the major microbial communities in hospitals with diverse groups of antibiotic resistance genes (Kizny Gordon et al. 2017). The extended use of a wide variety of antibiotics in hospitals could drive de novo evolution of resistance, for instance, during the long-term treatment of chronic infectious diseases in patients (Folkesson

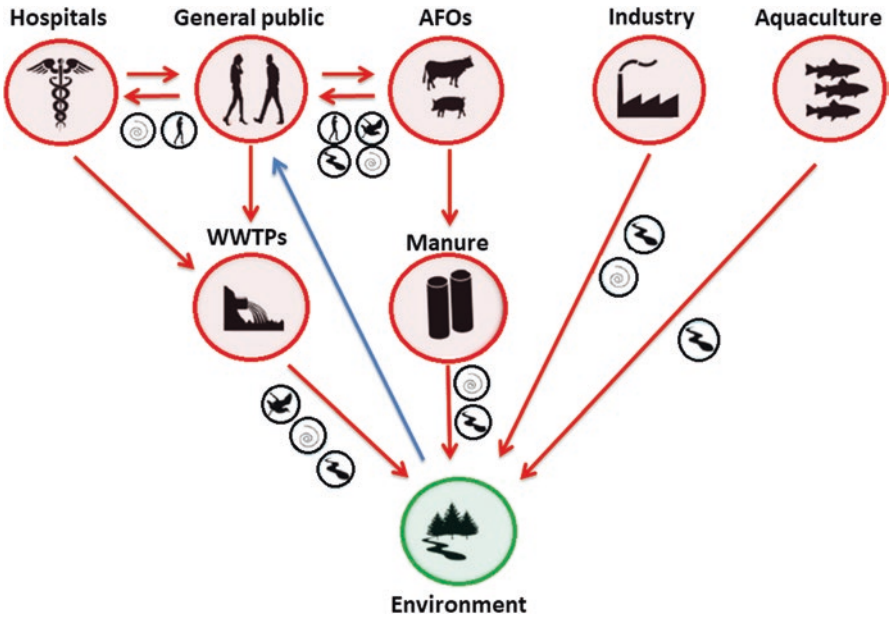

Fig. 2.2 Representation of flow of antibiotic resistance genes and antibiotic-resistant bacteria to the environment (green circle) from hotspots of transmission and evolution (red circles). Red arrows designate the known flows of antibiotic resistance genes and antibiotic-resistant bacteria, blue arrow designates the probable route of transmission from the polluted environments back to the general public. Black circles indicate the possible vectors that might assist in the transmission of infection between environments including waters, air, humans, surface, and animals. (Kraemer et al. 2019)

et al. 2012). In addition, the microbes with newly evolved antibiotic resistance genes could spread epidemically in patients, or the direct gene transmission may occur in different genetic hosts through horizontal gene transfer. Thus, in addition to resistance evolution, the microbes carrying the antibiotic resistance genes could enter the hospital environments through infected humans, where they could enter the new genetic host or could spread epidemically. The antibiotic-resistant microbes that have been found in hospital effluents are vancomycin-resistant enterococci (VRE), *P. aeruginosa*, and extended-spectrum beta-lactamases (ESBL) carrying *E. coli* (Hocquet et al. 2016). Even though the hospital encompasses a variety of antibiotic-resistant microbes, however, the overall role of the hospital as a source of environmental resistance is highly uncertain due to their relative low contribution to the total wastewater in comparison to the total population (≈1%) (Kümmerer 2009). Even though hospitals are under extensive surveillance, they offer controlled environments for the use of antibiotics, and resistance evolution is easily tracked due to the testing of hospital effluents, thus eliminating the problem. Contrary, the inappropriate use of in-home antibiotics by the general population always remains unsupervised. Moreover, even if the antibiotics are correctly utilized, approximately 70% of antibiotics are excreted in the environment unaltered via the human digestive system (Changotra et al. 2019, 2020). In comparison to hospital-derived sewage, the small quantity of hospital independently generated municipal sewage is the major contributor of antibiotic resistance genes from the general public (Kümmerer 2009).

Municipal and hospital sewage are combined and transferred to WWTPs, which employs various physicochemical and biological treatments to treat the waste and diminish the amount of pathogens before their discharge. Sewage comprises of different types of antibiotics, seeded with new antibiotic-resistant bacteria and antibiotic resistance genes, and contains a nutrient-rich environment to support the high quantities of bacteria. Since the wide varieties of antibiotics are present at subminimum inhibitory concentrations (MIC) in the sewage, thus they could easily encourage horizontal gene transfer mechanisms to increase the transmission of antibiotic resistance genes (Szczepanowski et al. 2009; Ju et al. 2019). Due to these reasons, WWTPs and sewage are regarded as the hotspot environments for the evolution, recombination, and transmission of antibiotic resistance (Rizzo et al. 2013). While the abundances of antibiotic-resistant bacteria and antibiotic resistance genes are greatly reduced in the water fraction in sewage passage through WWTPs, the WWTPs are ineffective in the removal of antibiotic resistance genes in biosolids (Chen and Zhang 2013; Yang et al. 2014). The current policies allow the use of biosolids as fertilizers for crops, which were subjected to bury in landfills in the past. Thus, the potential contamination of crops, grazing livestock, and soils with resistant microbes and antibiotic resistance genes needs to be examined. Sewage, hospitals, and WWTPs are enriched with human-associated antibiotic-resistant bacteria and antibiotics. As it is very difficult to control the usage of antibiotics in the general public, WWTPs may provide an essential role in the intervention to curb the transmission and evolution of resistance genes.

Independent of the application of antibiotics on the general public, antibiotics are extensively utilized in animal feeding operations for livestock production and aquacultures. Generally, the amount of antibiotics used in such facilities is much higher than that being utilized by hospitals (Chee-Sanford et al. 2009). In Canada, the amount of antibiotics used by biomass in animals is twice the amount used by the general public, and in the United States, approximately 80% of antibiotics sold are used for veterinary purposes (Ventola 2015; PHAC 2016). Waste manure containing antibiotic-resistant bacteria from animal feeding operations is generally collected and treated in waste lagoons and is independent of WWTPs and municipal wastes (Chee-Sanford et al. 2009). Although manure storage could decrease the concentration of culturable bacteria, still manure and lagoons have been found to contain an extensive amount of antibiotic-resistant bacteria and resistance genes (Pruden et al. 2006; Peak et al. 2007). As a result, manure is being used as a fertilizer for crops and soil, where the surviving antibiotic-resistant bacteria and resistance genes come in contact with the soil environment. Subsequently, antibiotic-resistant bacteria and resistance genes from manure enter the surface water and groundwater via surface runoff. In addition to animal feeding operations, the installations of aquaculture, where fish are farmed, are an example of livestock production in which an extensive amount of antibiotics are frequently used (Cabello et al. 2016). Among the open- and closed-water aquaculture, the closed ones are of superior interests, in which animal or human waste is fed to the fish, thereby promoting the resistance gene transfer between the systems (Watts et al. 2017). In contrast, in open-water aquaculture, antibiotics are directly added to open waters to promote the growth and prevent the fishes from disease, leading to the transmission and evolution of antibiotic resistance genes and antibiotic-resistant bacteria in open waters and sediments (Shah et al. 2014). This method of administration of antibiotics leads to their affecting both diseased and healthy fish (metaphylaxis) in the aquaculture (Sørum 2006). Antibiotics not only are utilized in animals for therapeutic use to treat infectious diseases and prevent postsurgery infections but also can be added in low concentrations to the animal feed as antibiotic growth promoters (AGPs) or as prophylaxis to increase the farm productivity and profitability (Turnidge 2004; Hao et al. 2014). In 2006, AGPs were banned in the many European Union (EU) countries as well as in the United Kingdom, leading to subsequent lower antibiotic consumption especially for colistin and tetracycline (Cogliani et al. 2011). Till date, an amended regulation on the veterinary medication in the EU proposed the limit of antibiotic use in the metaphylaxis and prophylaxis was confirmed by the European Parliament and EU Council in 2018 (Council of the European Union 2018). In 2017, subtherapeutic antibiotics in water and feed to enhance feed efficiency and animal growth became illegal in the United States (U.S. Food and Drug Administration 2018). In the rest of the world (particularly low- or middle-income countries), the usage of antibiotic remains partially regulated provoking significant risks for the environmental management and transfer of resistance genes due to the release of antibiotics to water bodies and soils affected by animal farming operations (Van Boeckel et al. 2019; Moore 2019). Based on a One Health framework, a global action plan was launched by the World Organisation for Animal Health (OIE) in 2015 on antibiotic resistance

genes and antibiotic-resistant bacteria, and over 100 countries have now established their national action plans against antibiotic resistance genes and antibiotic-resistant bacteria in animals (Góchez et al. 2019). Following the One Health framework policy by the OIE, the Chinese government published "The National Action Plan on controlling antibiotic-resistant bacteria on animal origins (2016–2020)" integrated with the One Health concept for food safety and public health. In the end, the OIE has built a global database on antibiotic agents proposed for the use in animals and published the methodology for data collection in 2019 for the global development of monitoring and surveillance in line with OIE international standards (Góchez et al. 2019).

2.4.2 Transmission Vectors

The transport of antibiotic resistance genes and antibiotic-resistant bacteria to the pristine environments from hotspots of evolution resistance takes place through a variety of vectors (as outlined in Fig. 2.2). Vectors can either allow the transportation or occur as reservoirs where antibiotic resistance genes and antibiotic-resistant bacteria could persist, evolve, or multiply. In most cases, the antibiotic resistance genes are transported within the antibiotic-resistant bacteria between environments. Thus, the genetic framework of most of the existing antibiotic resistance genes in the environment is still anonymous.

2.4.2.1 Air and Surface Water

In the past, the dissemination of antibiotic-resistant bacteria through air has received considerable attention. For instance, antibiotic resistance genes against the seven frequently utilized antibiotics have been identified with high relative abundances in particulate matter in the air throughout the major cities in the world (Li et al. 2018). Similarly, the antibiotic resistance genes were found to be enriched in particulate matter from smog (Hu et al. 2018). Most of the antibiotic resistance genes are concomitant with the wide range of bacteria bounded to the particulate matter floating in the air and disperse in the air through fog, wind, and precipitation (Dueker et al. 2017). Some species of *Pseudomonas*, such as *P. syringae* and *P. aeruginosa*, have been identified to contain multiple resistance genes associated with precipitation (Hwang et al. 2005; Christner et al. 2008). Antibiotic resistance genes have also been identified in the seed air originating during the animal feeding operations (Gao et al. 2017) and in close vicinity and downwind of WWTPs (Li et al. 2016), thereby posing the health risk to the workers. At last, the airborne transmissions of antibiotic-resistant bacteria from hospitals have not been extensively explored; however, airborne antibiotic resistance genes have been found there (Gilbert et al. 2010). On the other hand, surface waters receive industrial and WWTP effluents as well as runoff from animal feeding operations and manure-fertilized fields and hence located at a

central hub for the dissemination and transportation of the antibiotic-resistant bacteria (Pei et al. 2006; Graham et al. 2011). Profiles of antibiotic-resistant bacteria and antibiotic resistance genes have been found in rivers through surface runoff and were strongly impacted by the contaminations from animal feeding operations and WWTP effluents.

2.4.2.2 Animal Vectors

In comparison to air and water vectors, animal vectors including humans provide nutrient-rich environments to antibiotic-resistant bacteria to amplify or change their genetic context within the animal vectors (Langelier et al. 2019). Labors and farmers have shown to develop augmented resistance on direct contact with livestock (Hanselman et al. 2006; Price et al. 2007). Antibiotic-resistant bacteria originating from animals or animal feeding operations have to cross the species barrier for their transmission in humans, whereas human-originated antibiotic-resistant bacteria from sewage and hospitals can easily be transmitted to humans. Certainly, hospital workers are more vulnerable to increased transmission risk of antibiotic-resistant bacteria and thus develop increased antibiotic resistance (Lis et al. 2009). Interestingly, the antibiotic-resistant bacteria could easily be transferred to the family members of hospital workers, thereby promoting the possible chain of transmission.

In addition to workers from hospitals and farming, antibiotic-resistant bacteria have been found to spread through international travel. Studies have confirmed that antibiotic-resistant bacteria and resistance genes could persist for a longer period in a traveler's microbiome and thus could remain there even after the return of travelers to their origin country (Bengtsson-Palme et al. 2015; Langelier et al. 2019). Apart from humans, insects such as cockroaches and houseflies have been identified as potential antibiotic-resistant bacteria-carrying vectors for the transmission of pathogens from WWTPs (Doud et al. 2014), animal feeding operations (Zurek and Ghosh 2014), and hospitals (Tilahun et al. 2012). A wide range of vertebrates, specifically small rodents, have been associated to carry and transmit antibiotic-resistant bacteria from animal feeding operations, water feeding birds, and even WWTPs (Allen et al. 2011); however, a direct link between the hotspots of resistance evolution and resistance gene carriage has been rarely established. Recent research has established that birds exposed to wastewater, especially ducks, are characterized by high diversity and abundance of antibiotic resistance genes. As such, the ducks are regarded as a potential vector for the transmission of resistance genes due to their habitats and feeding habits (Marcelino et al. 2018). Another group of birds that is responsible for carrying the antibiotic-resistant bacteria (for instance, pathogenic bacteria such as ESBL carrying *E. coli*) from the contaminated water is seagulls (Poeta et al. 2008; Marcelino et al. 2018).

Although the hotspots (such as WWTPs, animal feeding operations, and hospitals) for the transmission and evolution of antibiotic-resistant bacteria have been extensively evaluated, the risk posed by the transmission vectors between the

environments has not been explored thoroughly. As transmission vectors provide the selective conditions for the recombination of resistance genes and multiplication of resistant bacteria, they are regarded as the central hub for understanding the flow of resistant genes between the environments. Indeed, the risks associated with the combination of resistance genes from animals and humans and the passage of species barrier must be assessed.

2.5 Cytotoxic Effects of Antibiotics in the Environment

Even though the primary role of antibiotics is to prevent and treat microbial infections, research is still underway to know whether antibiotics could harbor inhibitory activities under the natural condition (Davies and Davies 2010). Nevertheless, the resistance genes, for example, β-lactamases, are anticipated to have originated millions of years ago, signifying that antibiotics have been affecting the roles of pathogens long before their use in clinical medicines (Hall and Barlow 2004; Aminov and Mackie 2007). The quantities of antibiotics discharged and accumulated into the environment as a result of human activities have negatively affected the local communities of animals and microorganisms.

2.5.1 Toxic Effects of Antibiotic Pollution on Microbial Communities

To favor the transmission and evolution of resistance genes in environments, the selective pressures build up by antibiotic pollution could adversely affect the dynamics of evolution in microbes in different ways (Martínez 2017). Diverse populations of bacteria present different tolerance levels to antibiotics because of variations in physiological traits or changes in gene expression (El Meouche and Dunlop 2018). Under strong selective pressures, the antibiotics could decrease the microbial population's diversity by favoring the growth of tolerant or resistant microbial lineages. Under weak selective pressures, the lower concentrations of antibiotics could increase the genotypic and phenotypic diversity by selectively favoring the growth of resistant microbial lineages. In addition to the fluctuations in microbial population's diversity, exposure of microbial population to even low concentration of antibiotics could also increase the genetic diversity in microbes via the direct mutagenic effect on the DNA (Andersson and Hughes 2014) and via activation of bacterial SOS response, causing an augmented mutation rate in the bacterial genome (Foster 2007). Antibiotics have been found to enhance the horizontal gene transfer between bacterial populations either by augmenting the competence leading to uptake of extracellular DNA (Slager et al. 2014) or by conjugation (Maiques et al. 2006). At last, antibiotics could also affect the regulation of the gene at the transcription level

(Davies et al. 2006), either via regulatory mechanisms such as quorum sensing (Rémy et al. 2018) and riboswitches (Blount and Breaker 2006) or via direct binding, leading to increase virulence and phenotypic variability (Rémy et al. 2018). As a whole, these mechanisms could increase the existing pond of phenotypic and genetic diversity in microbial populations with the exposure to antibiotics. Consecutively, if selective pressure increases, these mechanisms may promote the evolution of antibiotic resistance according to Fisher's Fundamental Theorem of Evolution by Natural Selection (Lee et al. 2018). Selective pressures related to antibiotic pollution could also act on the composition of the overall microbial community by shifting microbial composition or by decreasing taxa diversity. For instance, exposure to antibiotics tends to favor a decrease in Gram-positive bacteria as opposed to Gram-negative bacteria. The former displayed increased susceptibility to disinfectants and antibiotic exposure due to the lack of outer cell membrane. Antibiotic exposure to microbial populations may also lead to the loss of critical ecological roles. For example, the presence of antibiotics in the aquatic environments tends to reduce the overall diversity of microbial populations, including primary productivity and microbial taxa accountable for carbon cycling (Grenni et al. 2018; Eckert et al. 2019). Likewise, the antibiotic pollution in soil environments could alter the physiology of microbes leading to reduction in microbial activity such as respiration, denitrification, nitrification, loss of biomass, and loss of enzyme activity of bacteria (Thiele-Bruhn and Beck 2005; Cycoń et al. 2019). Lastly, the disruption of microbial communities by antibiotics could also increase the plenty of toxic pathogens and parasites in soil and water environments, for example, *Cyanobacteria* species, triggering eutrophication in freshwater and soil environments and causing health threats to humans (Drury et al. 2013).

2.5.2 Toxic Effects of Antibiotic Pollution on Higher Organisms

2.5.2.1 Physiological Effects

In addition to the adverse effects of antibiotics on microbial communities, these antibiotics have a strong ability to affect higher organisms in aquatic environments where these organisms are exposed to pollutants even at low concentrations. It has been reported that the concentration of antibiotics in environmental samples is quite low and exhibits negligible risks to humans (Mojica and Aga 2011). Nevertheless, the presence of low concentrations of antibiotics in the environment could also accumulate in humans via long-term exposure to food, drinking water, or other consumer goods inducing some unknown health hazards. For instance, quinolones and macrolides have been identified in chlorinated drinking water (Ye et al. 2007). Triclosan, an antimicrobial agent used in consumer goods, for example, clothes and soap, has been detected in human urine and serum which are not using this antimicrobial and was found to pose health hazards from muscle weakness to reproductive

problems (Weatherly and Gosse 2017; Bever et al. 2018). Approximately 70% of the US population has been exposed to various antibiotics and triclosan via consumer goods (Calafat et al. 2008). As a result, triclosan has been detected in rivers and streams across the world (Halden and Paull 2005).

Exposure to low concentrations of antibiotics (such as erythromycin and streptomycin) has been found to negatively impact the behaviors and survival of smaller organisms and micro-invertebrates such as *Artemia* (Migliore et al. 1997) and *Daphnia magna* (Flaherty and Dodson 2005) in laboratory conditions. The presence of subinhibitory concentrations of macrolides in the aquatic environments has also been found to affect the vertebrates such as zebra fish by causing malformations, such as uninflated swim bladder and yolk sac edema, and manipulating movement frequency of the embryo (Wang et al. 2014). Similar malformations have been observed in the case of other fish species on the exposure to antibiotics, including quinolone (Wang et al. 2009), sulfonamide (Lin et al. 2014), and tetracycline (Zhang et al. 2015). Besides, quinolones and their metabolites could persist in the human body for a longer time, thereby contributing toward bioaccumulation risk as well as chronic toxic effects (Liu et al. 2018). The presence of antibiotic tetracycline in aquatic environments has also affected the amphibians, thereby inducing malformations such as pericardial edema and shortened body length in *Xenopus tropicalis* (Liu et al. 2018).

Besides the physiological effects, the exposure of organisms to antibiotics could also lead to interfere with behavior and development changes, possibly resulting from the modifications in gene regulations (Zhang et al. 2016). In 2017, Kim et al. found that exposure to antibiotic tetracycline of zooplankton species *D. magna* caused a gene regulation in the species, impacting the carbohydrate and protein metabolism as well as promoting general stress response. Additionally, these alterations in gene regulation can carry over in subsequent generations of organisms even in the absence of tetracycline, thereby impacting the organism population even after the removal of antibiotic compounds from the environment (Kim et al. 2017).

2.5.2.2 Effect on Host Microbiome

It has been established that antibiotics could adversely affect the higher organisms by disrupting the microbial populations related to the host animals. Host microbiomes in animals accomplish different roles for the host animals, ranging from bone formation to effective nutrient metabolism (Raymann and Moran 2018). Thus, the disruption of host's microbiomes, also known as dysbiosis, could promote health consequences, such as metabolic diseases, allergies, developmental defects, or augmented susceptibility to pathogens (Jie et al. 2017). Recent investigations have shown that the low concentrations of antibiotics in the aquatic environment could also impact other local organisms, such as the fish microbiome, which is highly susceptible to variations in the aquatic environment (Schmidt et al. 2016). In aquaculture, a lower concentration of antibiotics can increase mortality and decrease microbial diversity in the fish gut (Navarrete et al. 2008). Even exposure to the high

concentration of certain chemicals and antibiotics in waters contaminated with WWTP effluent could impact the fishes by increasing mortality. Previous research also demonstrated that the adult fish can recover from the short-term exposure to antibiotics, whereas, in some cases, even the lower concentration of antibiotics (tetracycline and streptomycin) as low as 1 μg mL^{-1} could increase mortality and cause dysbiosis in zebra fish embryos (Pindling et al. 2018). Another negative impact of antibiotic exposure on higher organisms includes reduction in fertility and disruption of the growth cycle of host microbiomes (Yan et al. 2016). Thus, there is an imperative need to investigate the extent of negative impact of antibiotic pollutions on aquatic organisms, as antibiotic pollution is only providing additional threats to aquatic organisms in the world due to anthropogenic activity.

2.6 Gaps in Current Policies to Tackle Antibiotic Pollution

With the aim of tackling the global crisis due to antibiotic resistance and pollution, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) under the global action plan in 2015, hence providing a framework for action plans for individual nations to develop regulations and policies to combat antibiotic resistance and pollution problems (WHO 2019). GLASS is a platform for global data-sharing worldwide that aimed to support countries in establishing national antimicrobial resistance (AMR) surveillance systems as part of national action plans to enable collection, analysis, and sharing of data. Till date, just 82 countries (less than half of the WHO member states) have enrolled in GLASS and 66 have submitted AMR data. Technically, this means that national AMR data is either unavailable in a shareable format or not readily accessible, thus hindering the comparison of local or global trends in AMR. Another surveillance system, namely, the National Antimicrobial Resistance Monitoring System (NARMS), was established by the United States in 1996 (FDA 2020). The NARMS is a collaborative program of state and local public health universities and departments, the Centers for Disease Control and Prevention (CDCP), the FDA, and the US Department of Agriculture (USDA). This surveillance system tracks changes in the antimicrobial susceptibility of enteric (intestinal) bacteria found in retail meats (FDA), ill people (CDCP), and food animals (USDA) in the United States. NARMS data are used by the FDA to make regulatory decisions to preserve the effectiveness of antibiotics for animals and humans. In 2009, the Global Antibiotic Resistance Partnership (GARP) was started to create a platform for developing actionable policy proposals on antibiotic resistance in low-income and middle-income countries including India, South Africa, Kenya, Vietnam, Mozambique, Tanzania, Nepal, Uganda, and Bangladesh (CDDEP, New Delhi n.d.). GARP is a project of the Center for Disease Dynamics, Economics and Policy (CDDEP), funded by the Bill & Melinda Gates Foundation.

According to the report of the European Commission, the increasing extent of antibiotic resistance and pollution crisis leads to approximately 70,000 deaths per year on a global scale, making it a global problem due to economic and social

fallout (European Commission 2017). With the aim to collect comparable and reliable data on antibiotic use in Europe, the European Surveillance of Antimicrobial Consumption Network (ESAC-Net) project was granted by the European Commission in 2001 (ECDC 2020). The international network continues collecting reference data on the consumption of antimicrobials for systemic use in the community and in the hospital sector in EU countries through the European Surveillance System. Another surveillance network in EU, namely, European Antimicrobial Resistance Surveillance Network (EARS-Net), is the largest publicly funded system for AMR surveillance in Europe. EARS-Net is based on routine clinical antimicrobial susceptibility data from local and clinical laboratories reported to the European Centre for Disease Prevention and Control by appointed representatives from the EU Member States. In South Africa, the South African Society for Clinical Microbiology (SASCM) was implemented with the objectives to include the monitoring of antimicrobial resistance patterns in the public and private medicine sector in South Africa including the publication of antibiotic resistance patterns in selected publications (Singh-Moodley et al. 2018). Due to the lack of reliable and accurate information in African countries, there is an urgent need for the implementation of standardized surveillance programs. In view of estimates of AMR burden, the Indian Council of Medical Research (ICMR), in 2013, initiated the Antimicrobial Resistance Surveillance and Research Network (AMRSN) to enable compilation of data on pathogenic groups on antimicrobial resistance from the country (Walia et al. 2019). The ICMR has developed a real-time online AMR data entry system for its network and will have AMR data analysis capacity. It is a user-friendly web-based informatics solution/suite for collection, storage, and analysis of surveillance data.

The major benefit of having a surveillance system is the collection of real-time accurate data on AMR including the mechanisms of resistance and representativeness to community; sustaining the current effort and expanding the current activities to next levels of healthcare settings are the major challenges. As per the UNEP report, present research emphasized the investigation of health threats associated with the accumulation of antibiotics, antibiotic-resistant bacteria, and resistance genes in the environmental reservoirs (UNEP 2017). However, most of the regulations and action plans laid by different nations fail to address this critical issue and do not aim to combat the pollution problems associated with antibiotics and resistance in the natural environments. Similarly, all national action plans do not include any regulations and policies to control the environmental release of antibiotics by the pharmaceutical manufacturing industries. Considering the magnitude of antibiotics released from industries and accumulation in the local environments, the negligence of such issue might facilitate the localized hotspots of evolution and transmission of antibiotic-resistant bacteria and resistance genes that may consequently spread worldwide. Lastly, there are no policies and regulations in any national action plans to address the problems of antibiotic pollution of WWTPs. Currently, the design of WWTPs is not entitled to remove antibiotics, antibiotic-resistant bacteria, and resistance genes; thus, policies need to be amended to establish WWTP technologies to allow an acceptable load of antibiotics and resistance genes in biosolids and WWTP effluents.

2.7 Conclusion

The presence of antibiotics and resulting antibiotic resistance is a critical problem, leading to serious health problems worldwide. The environmental constituents of this issue, such as evolution and transmission of resistant genes between bacteria, and environmental hotspots of resistant bacteria and genes have received much attention in the past years. However, many elements of antibiotic resistance and pollution are still unknown and need further research. Hospitals, veterinary use, and WWTPs are the major sources of antibiotic pollution in the natural environments. Studies need to be focused on linking the abundance and presence of antibiotic resistance genes directly to the general public's risk of becoming infected on exposure to antibiotic-resistant bacteria. Besides the predominance of antibiotic resistance in natural environments, antibiotic pollution has negatively impacted the ecosystem's functions in the animals and subsequently affected the human health directly. The antibiotic's presence in the environment has effects such as retardation of nitrite oxidation and methanogenesis, disruption of the human endocrine system, and increased toxicity of chemical combinations and metabolites. For such reasons, along with the contribution of antibiotic pollution toward antibiotic resistance, it is vital to consider the overall impacts of antibiotic pollutions on natural environments as well as on human health. Current policies and regulations are based on the surveillance data, but more descriptive studies are required to develop efficient action plans and policies to combat the global problems of antibiotic pollution. Antimicrobial resistance surveillance in healthcare-associated facilities, e.g., hospitals, dialysis clinics, long-term care facilities, etc., is vital in order to understand the prevalent healthcare-associated pathogenic organisms, the antimicrobial resistance patterns of critical organisms, and their mechanisms of resistance. National laboratory systems should support the surveillance for antimicrobial resistance and implement standardized practices, manuals, and quality systems. Besides, this information will support the upliftment and development of national treatment guidelines, regulating policies and strategies for antimicrobial infection prevention and control.

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Chapter 3
Antimicrobial Agents in Agriculture and Their Implications in Antimicrobial Resistance

Shweta Singh and Arun Goyal

3.1 Introduction

The spread of antimicrobial resistance genes, among microbial pathogens, is becoming a global threat to environment, human, and animal health (Fig. 3.1). Antibiotics are the chemotherapeutic compounds used to treat bacterial diseases (Amábile-Cuevas 2016). Its development is the greatest achievement in the history of chemotherapy. The first antimicrobial compound discovered by Sir Alexander Fleming was penicillin: a β-lactam antibiotic (Fleming 1929). Antibiotics treat patients with an active infection and permit clinicians to execute medical techniques safely. The use of antibiotics allows organ transplantation, myelosuppressive chemotherapy, basic surgery, and implantation of cardiac devices successfully without infection (Amábile-Cuevas 2016). The misuse and overuse of antibiotics have led to the selection of resistant bacterial strains to every antibiotic which has been introduced in the past 70 years and is a major threat to human health. The center for disease control and prevention in the USA reported that nearly two million illnesses and 23,000 deaths annually are due to antibiotic resistance (Chang et al. 2015).

In agriculture, the use of antibiotics is carried out: (1) to treat bacterial infections in sick animals, (2) for prophylactic use when there is the risk of infection, and (3) in small quantities in water and feed to promote animal growth (Marshall and Levy 2011; Economou and Gousia 2015). The flocks and herds can be given antimicrobial agents in early feeding period when there is a risk of an outbreak of infectious disease, and this mass medication is known as metaphylaxis (Checkley et al. 2010).

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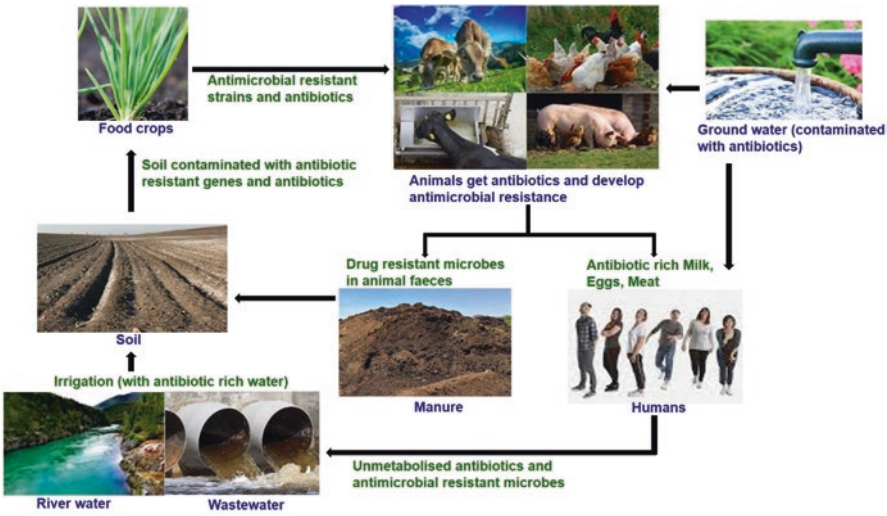

Fig. 3.1 Transfer pathway of antimicrobial resistance gene within microbial community via agriculture, environment, and food chain

Feed prophylaxis and injectable metaphylaxis in cattle improve the feed efficiency and daily weight gain. Some important antibiotics used in the treatment of animals in their food source are amoxicillin, bacitracin, cephalosporins, erythromycin, fluoroquinolones, gentamicin, lincomycin, neomycin, penicillin, streptomycin, tetracycline, and sulfonamides (Shea 2003). Similarly, antifungal drugs used in agriculture served as the environmental drivers for the evolution of resistance in pathogenic fungal strains. Therefore, to decrease the development of resistance in fungal strains for use in agriculture, a mixture of antifungal compounds with different mechanisms of action is supported (Thomson 1982). Antimicrobial agents used for veterinary and human as growth promoters are not fully metabolized and therefore excreted by animals and end up in manure. This manure is used as agricultural fertilizer because of which antimicrobial agents penetrate the soil and enters the groundwater (Thanner et al. 2016). In many countries, the unmetabolized antimicrobial agents are disposed of in the sewage system. If these drugs are not metabolized during sewage treatment, then they seep into the soil or in other environments, and then they can mix with surface water and groundwater (Sanseverino et al. 2018). For example, tetracyclines have been detected in concentrations of up to 0.2 μg/kg in soil (Hamscher et al. 2002). The antibiotics detected in sewage effluent, municipal sewage, and surface water and groundwater include quinolones such as sulfonamides, ciprofloxacin, roxithromycin, erythromycin, and others (Kümmerer 2003). Ciprofloxacin and ampicillin were found in concentrations between 0.7 and 124.5 μg/L and 20–80 μg/L at the hospital effluent which was equal to the minimum inhibitory concentration for susceptible pathogenic bacteria (Kümmerer 2001). Most of the time this untreated sewage water is used for irrigation purposes, and thus it contaminates the soil with antibiotics even above minimum inhibitory concentrations of antimicrobial agents (Kümmerer 2003). In addition to this, the

antibiotic concentration in soil at subminimum inhibitory concentrations also selects the antimicrobial-resistant soil microflora via transfer of antimicrobial resistance genes and associated mobile genetic elements (transposons and plasmids) and brings about the genetic modifications in bacterial genome (Cycoń et al. 2019). Gullberg et al. (2011) reported that 1/4 and 1/10 of minimum inhibitory concentration of streptomycin and ciprofloxacin, respectively, select resistant *Salmonella typhimurium* LT2 strain and *Escherichia coli* strain, respectively. This leads to the transmission of antimicrobial drug resistance genes into the soil microflora and as a result to the plants. Therefore, the use of these plant products can transmit drug resistance to humans and animals. In 2013, the US Food and Drug Administration marked a change in its stance, calling on the industry to eliminate the use of essential medical antibiotics.

Various studies have shown that the treatment of foodborne infections in human caused by multidrug-resistant *Campylobacter*, *Salmonella*, *Vibrio*, *Shigella*, *Helicobacter*, and *E. coli* is difficult and needs last-resort antibiotics (Karunasagar et al. 1994; Mousavi et al. 2014; Giacometti et al. 2021; Zachariah et al. 2021). Young children and infants are at higher risk of developing foodborne gastrointestinal infections (Shea 2003). Bacterial populations acquire resistance against antibiotics through either horizontal transfer, vertical gene transfer, or chemical modification of antibiotics. For example, the β-lactamase enzymes were expressed from plasmids; therefore, the horizontal gene transfer is the important mechanism of drug resistance in β-lactam antibiotics (Barlow and Hall 2002). Li et al. (2019) showed the antimicrobial resistance against sulfonamides in *E. coli* via vertical gene transfer. The resistance against β-lactam and glycopeptide antibiotics via chemical modification of penicillin-binding proteins and structure of peptidoglycan precursor inhibits the binding of the drug to the target in *Staphylococcus aureus* (Reygaert 2018).

The human gastrointestinal tract provides an environment for antibiotic resistance genes to spread in normal gut microflora (Thanner et al. 2016). Therefore, to control the acceleration of antibiotic resistance in the microbial community, an adequate understanding of molecular biology of the mobile genetic elements is needed. The knowledge of interactions between the health system and the food chain will reduce the infections caused by antimicrobial resistance microbes and thus improve the patient health. It also guides policymakers and consumers to minimize the nontherapeutic uses of antimicrobial agents (Van Hoek et al. 2011).

3.2 Antibiotics in Agriculture

3.2.1 Antibiotics

The word antibiotic comes from the word “antibiosis” which means “against life.” Antibiotics produced from microorganisms or synthesized wholly or partly via synthetic means can inhibit bacterial growth (bacteriostatic) or completely able to kill bacteria (bactericidal). They are differentiated as antibacterials, antivirals, and antifungals on the basis of group of microorganisms they act upon. Antibiotics are

classified on the basis of chemical or molecular structures, mode of action, and spectrum of activity (Etebu and Arikekpar 2016).

3.2.1.1 On the Basis of Chemical or Molecular Structures

On the basis of chemical or molecular structures, antibiotics are grouped as β-lactams, tetracyclines, macrolides, quinolones, sulfonamides, aminoglycosides, oxazolidinones, and glycopeptides (van Hoek et al. 2011; Frank and Tacconelli 2012).

3.2.1.1.1 β-Lactams

The member of β-lactam antibiotics contained highly reactive one-nitrogen and three-carbon ring. The β-lactam antibiotics are penicillins, monobactams, cephalosporins, and carbapenems (Etebu and Arikekpar 2016). These antibiotics inhibit the protein essentially required for the synthesis of peptidoglycan layer of bacterial cell wall. Penicillin was the first antibiotic discovered by Alexander Fleming in 1929 from *Penicillium notatum* (McGeer et al. 2001). Penicillin group of antibiotics contained a common nucleus, 6-animopenicillanic acid. This class included several antibiotics such as penicillin G, oxacillin, penicillin V, methicillin, ampicillin, carbenicillin, nafcillin, amoxicillin, piperacillin, ticarcillin, and mezlocillin (Boundless 2016). The monobactam group of antibiotics was produced by bacterium *Chromobacterium violaceum*, and in this group of β-lactam antibiotics, the β-lactam is not fused with other ring (Bonner and Sykes 1984). Aztreonam antibiotic of this group is the only antibiotic which is commercially available. It has narrow range of antimicrobial activity against aerobic Gram-negative bacteria (Sykes et al. 1981). Cephalosporin group of antibiotics are produced by fungus *Cephalosporium acremonium* that contains a common nucleus of 7-aminocephalosporanic acid and a side chain 3,6-dihydro-2 H-1,3-thiazane ring. The cephalosporins are subdivided into five generations depending on their target organism (Abraham 1987). Carbapenem group of β-lactam antibiotics displayed resistance against β-lactamase enzyme (Papp-Wallace et al. 2011). Therefore, these antibiotics are administered to the patients, when they have acquired the infection with resistant microbial strains. The two members of this group are clavulanic acid and thienamycin isolated from *Streptomyces clavuligerus* and *Streptomyces cattleya*, respectively (Brown et al. 1976; Butterworth et al. 1979). They have a broad-range antimicrobial activity against both Gram-positive and Gram-negative bacteria.

3.2.1.1.2 Tetracyclines

Tetracycline produced by *Streptomyces* was discovered by Benjamin Duggar in 1945 (Sánchez et al. 2004). The members of this class of antibiotics have four hydrocarbon rings, and on the basis of the method of synthesis, they are grouped

into three different generations (Fuoco 2012). First-generation tetracyclines are produced from biosynthesis, which includes tetracycline, oxytetracycline, chlortetracycline, and demeclocycline. Second-generation tetracyclines are derivatives of semi-synthesis, which includes doxycycline, meclocycline, lymecycline, rolitetracycline, minocycline, and methacycline. The third-generation tetracyclines are produced from total synthesis which includes tigecycline. The target of tetracyclines is ribosome in bacteria, which inhibits the antimicrobial activity by the disruption of the addition of amino acids to polypeptide chains during protein synthesis. They are used in the treatment of amoebic infections, malaria, rickettsia, and elephantiasis (Sánchez et al. 2004).

3.2.1.1.3 Quinolones

Quinolones were first discovered as nalidixic acid. The members of quinolones have been dived into two major groups, i.e., quinolones and naphthyridones. These include nalidixic acid, cinoxacin, ofloxacin, norfloxacin, temafloxacin, ciproxacin, sparfloxacin, temafloxacin, and sparfloxacin (Domagala 1994). The chemical structure of quinolones consists of two rings, but the recent generations also have an added ring which enables them, the broad-spectrum antimicrobial activity. Quinolones interfere the transcription and DNA replication in bacteria.

3.2.1.1.4 Aminoglycosides

In this class of antibiotics, streptomycin was the first drug isolated in 1943 (Mahajan and Balachandran 2012). The members of this class were produced from soil: actinomycetes. They have a broad-spectrum antimicrobial activity. The aminoglycosides consist of 3-amino sugars which are connected by glycosidic bonds. These include streptomycin, neomycin, gentamicin, amikacin, and tobramycin. The members of this class inhibited the protein synthesis by binding with one of the subunits of ribosome. These drugs have been extensively used against *Mycobacterium tuberculosis* (Etebu and Arikekpar 2016).

3.2.1.1.5 Sulfonamides

Sulfonamides are the first group of antimicrobial agents used as therapeutic medicine (Eyssen et al. 1971). They inhibit Gram-positive and Gram-negative bacteria and some protozoa (Eyssen et al. 1971). The antimicrobial sulfonamides are synthetic and bacteriostatic (Henry 1943). They also demonstrated that at higher concentrations they may become bactericidal.

3.2.1.1.6 Glycopeptides

Glycopeptide antibiotics are obtained as natural products or as semisynthetic derivatives. The glycopeptides are consisted of a cyclic peptide having seven amino acids and two bound sugars (Kang and Park 2015). They bind to its target via formation of five hydrogen bonds from the peptidic backbone of the glycopeptide. In few glycopeptides, an additional sugar or chlorine is attached to the backbone of glycopeptide (oritavancin) at the time of synthesis. Similarly, the lipophilic side chain prolongs the half-life of glycopeptides (Etebu and Arikekpar 2016).

3.2.1.1.7 Oxazolidinones

These antimicrobial agents are a group of synthetic antibiotics. Linezolid is the first member of this group and approved for medical application. Earlier studies showed that these drugs inhibited antimicrobial activity via interfering with protein synthesis. The members of this class have a broad-spectrum activity against Gram-positive bacteria (Shinabarger et al. 1997; Bozdogan and Appelbaum 2004). Oxazolidinones are given in surgical infections as they can easily penetrate in the tissue, i.e., the lung, bone and vegetations (plant-like growth in tissues), and cerebrospinal fluid (Bozdogan and Appelbaum 2004).

3.2.1.1.8 Macrolides

The first macrolide, erythromycin A, was isolated from fungus *Streptomyces erythraeus* (Moore 2015). The members of this class have 14-, 15-, or 16-membered macrocyclic lactose rings along with unusual deoxy sugars D-desosamine and L-cladinose (Moore 2015). These antibiotics have broad-range spectrum for antimicrobial activity. Macrolides can inhibit growth or kill microorganisms by inhibiting the protein synthesis. The members of this class are erythromycin, clarithromycin, and azithromycin. These antibiotics showed broad-range spectrum antimicrobial activity. These antibiotics generally cause inflammation in the body; therefore, clinicians recommended low-dose administration (Hamilton-Miller 1973).

3.2.1.2 On the Basis of Mode of Action

In this mode of classification, antibiotics are divided into groups as follows: (a) inhibits the synthesis of bacterial cell wall, (b) disrupts the function or structure of cell membrane, (c) inhibits the function or structure of nucleic acid, (d) inhibits the protein synthesis, and (e) alters the metabolic pathways.

3.2.1.2.1 Antibiotics Inhibits the Synthesis of Bacterial Cell Wall

The members of this class of antibiotics are cephalosporins, carbapenems, and penicillins (Josephine et al. 2004). Antibiotics of glycopeptide class such as vancomycin also comes in this class (Kahne et al. 2005). These antibiotics inhibit the synthesis of peptidoglycan, a main component of bacterial cell wall. The rigid layer of peptidoglycan protects the bacterial cell by maintaining the high osmotic pressure in the cell (Bugg and Walsh 1992).

3.2.1.2.2 Antibiotics Disrupts the Function or Structure of Cell Membrane

The members of this class of antibiotics are daptomycin (Alborn et al. 1991) and polymyxins (Falagas et al. 2010). On the basis of differences in the type of lipids in cell membrane, the above class of antibiotics are grouped. Daptomycin depolarizes calcium-dependent membrane, whereas polymyxins binds to the lipopolysaccharide, i.e., lipid moiety of the cell membrane, and resulted in the disintegration of bacterial cell membrane (Falagas et al. 2010).

3.2.1.2.3 Antibiotics Inhibits the Synthesis of Nucleic Acid

The members of this class of antibiotics are quinolones (Etebu and Arikekpar 2016). The synthesis of nucleic acid is essential for the survival of bacterial cell. This group of antibiotics interferes the synthesis of nuclei acid by stopping replication or transcription. In DNA replication, helicase enzyme unwinds the double helical structure of DNA (Gale et al. 1981). Antibiotic quinolones disrupt the functionality of the helicase enzyme because of which it no longer unwinds the DNA in bacteria. In bacteria, this group of antibiotics also inhibits the functionality of topoisomerase II and topoisomerase IV because of which they prevent the RNA synthesis (Etebu and Arikekpar 2016).

3.2.1.2.4 Antibiotics Inhibiting the Protein Synthesis

Antibiotics inhibiting the protein synthesis are divided into two subclasses: (a) 30S inhibitors and (b) 50S inhibitors. The members of 30S ribosome inhibitors of antibiotic subclass are spectinomycin, streptomycin, and tetracycline (Hong et al. 2014). These antibiotics inhibit the entry of aminoacyl-tRNAs to the ribosome. The members of 50S ribosome inhibitors of antibiotic subclass are chloramphenicol, clindamycin, erythromycin, lincomycin, and linezolid (Douthwaite 1992; Katz and Ashley 2005). These antibiotics inhibit the initiation or the elongation phase of protein synthesis (Patel et al. 2001).

3.2.1.2.5 Antibiotics Blocking the Key Metabolic Pathway

The members of this class of antibiotics are sulfonamides and trimethoprim. These antibiotics mimic the structure of substrate required in cellular metabolism of bacteria. Due to this, the antibiotic attaches to the bacterial enzyme instead of the original substrate. The sulfonamide antibiotics resembles tetrahydrofolate required in the synthesis of folic acid in bacteria (Talaro and Chess 2008). This folic acid plays a vital role in the nucleic acid and amino acid metabolism. Therefore, these sulfonamide drugs inhibit the nucleic acid synthesis (Talaro and Chess 2008).

3.2.2 Crop Protection

In agriculture, streptomycin was the first antibiotic used against an enterobacterium *Erwinia amylovora* for controlling fire blight of pome fruits (McManus et al. 2002). The annual economic loss due to fire blight and control cost in the USA was estimated to be more than USD 100 million (Norelli et al. 2003). It was estimated to be USD 42 million and USD 68 million in Michigan and Washington, respectively (Aćimović et al. 2015). Therefore, the antibiotic streptomycin was registered in 1959 in the USA for its use in crop protection (McManus et al. 2002). Blasticidin S is the first antibiotic developed selectively for crop protection by Japan. It was isolated from *Streptomyces griseochrogenes* and used against rice blast disease (Kidd and James 1991). The rice blast causes nearly 30% of global rice production loss which was equivalent to the feeding of nearly 60 million people (Nalley et al. 2016). The control on rice blast motivated other researchers to develop antifungal antibiotics such as aureofungin (Nene and Thapliyal 1993), kasugamycin (Tomlin 2006), and validamycin (Liao et al. 2009) for crop protection. Aureofungin is a broad-spectrum fungicide registered in India against gummosis in citrus. The disease gummosis is caused by *Phytophthora* species which causes major economic losses to the citrus industry. The epidemic outbreak of gummosis in California caused 46% decrease in the annual yield of citrus plants which resulted in 12.9 million dollars of annual economic loss to the citrus industry (Savita and Nagpal 2012). Validamycin is a non-systemic fungicidal antibiotic used against soilborne disease caused by *Rhizoctonia solani* in potatoes, rice, and vegetables. In addition, validamycin is also used for curing infection in vegetable seedlings such as sugar beets, cotton, and rice (Thomson 1982). In India, tetracycline and streptomycin are registered against many plant diseases (Vidaver 2002). Earlier report of Vidaver (2002) showed that gentamicin, an aminoglycoside antibiotic, is used to control various bacterial diseases of vegetable crops caused by *Pseudomonas*, *Pectobacterium*, *Ralstonia*, and *Xanthomonas*. Oxolinic acid is a quinolone antibiotic used to treat fiber blight of pear and blight of rice (Maeda et al. 2004). So, to avoid the global economic losses, antibiotics were used in the plant agriculture for controlling plant pathogens. Most of the above antibiotics used in controlling plant pathogens are also

used for treating human diseases. The antibiotic streptomycin is used in the treatment of tuberculosis caused by *Mycobacterium* in humans (Grosset and Singer 2013). It is also used in the treatment of bacterial infections in pig (Luk'aš et al. 1963), poultry (Barbour et al. 1985), and sheep and goat (Radwan et al. 1992) and as growth promoters. The tetracyclines are used in the treatment of cholera, acne, malaria, plague, brucellosis, pneumonia, and syphilis in humans (Chopra and Roberts 2001) and as growth promoter and in bacterial infection treatment in animals (Di Cerbo et al. 2019). The antibiotic gentamicin is used in the treatment of meningitis (Mccracken et al. 1980) and infections in the lungs (Pines et al. 1967), abdomen (Gomez et al. 1999), urinary tract (Chong et al. 2003), and skin (Gemeinder et al. 2021) in humans, and it is a common medicine used in treating bacterial infections in veterinary practices (Jensen et al. 2006).

3.2.3 Animal Husbandry

The antibiotics are used since the late 1940s in veterinary medicine to treat animals from preventing infections in flocks or herds. In the early 1950s, the Food and Drug Administration (FDA) in the USA approved the use of antibiotics in animal feed (Mellon et al. 2001; Amábile-Cuevas 2016). This practice improves animal growth with a better economy to farmers in a shorter period of time (Mellon et al. 2001). The antibiotics used in livestock are classified as therapeutic agents, prophylactic agents, and metaphylactic agents (growth promoters) (Sarkar et al. 2018). Antimicrobials as therapeutic agents are used in relatively high doses for treating infected animals in a shorter period of time. Antimicrobial agents as prophylactic agents are given to animal at sub-therapeutic doses via drinking water or feed to prevent disease, when symptoms of infection are not present but suspected. Antimicrobial agents as growth promoters are given to animals at a very low dose on regular basis over the lifetime through fodder to increase productivity and growth rate. In animal husbandry, the worldwide use of antibiotics was 63,000 tons in 2010, out of which 3%, 3%, 9%, 13%, and 23% were consumed in Germany, India, Brazil, the USA, and China, respectively (Van Boeckel et al. 2015; Amábile-Cuevas 2016). This use of antibiotics is anticipated to increase by two-third to 105,600 tons by 2030. Most of the antibiotics used in animal husbandry are also used for humans. The key classes of in-use antibiotics shared by humans and animals are shown in Table 3.1. The antibiotics from classes, macrolide, penicillin, and tetracycline are mostly used for treating infected animals, each in the annual order of USD 500 million (Laxminarayan et al. 2015). Earlier study of Sarkar et al. (2018) reported that the worldwide average annual consumption of antibiotics for pig, chicken, and cattle is estimated to be 172 mg/kg, 148 mg/kg, and 45 mg/kg, respectively. The World Health Organization (WHO) has given a list of critically important, highly important, and important antibiotics that must be well preserved for human use (Table 3.2) to prevent the spread of antimicrobial resistance (Scott et al. 2019).

Table 3.1 Key classes of antibiotics shared by humans and animals

Antibiotic class	Used in humans	Used in animals	References
β-lactams	Amoxicillin, penicillin G	Amoxicillin	Neu (1974), Sarkar et al. (2018)
Macrolides	Erythromycin, azithromycin	Erythromycin, azithromycin	Noli and Boothe (1999)
Tetracyclines	Doxycycline, chlortetracycline, oxytetracycline	Doxycycline, oxytetracycline	Di Cerbo et al. (2019)
Fluoroquinolones	Lomefloxacin, ciprofloxacin, ofloxacin	Ciprofloxacin, enrofloxacin, sarafloxacin, lomefloxacin, ofloxacin	Ihrke et al. (1999)
Streptogramins	Quinupristin-dalfopristin	Virginiamycin	Schwarz et al. (2016)
Glycopeptide	Vancomycin, teicoplanin, avoparcin	Avoparcin	Pyörälä et al. (2009), Sarkar et al. (2018)
Phenicol	Chloramphenicol, thiamphenicol	Florfenicol	Schwarz et al. (2016)
Polypeptides	Bacitracin, polymyxin B	Enramycin	Dowling (2013a, b)
Pleuromutilins	Retapamulin	Tiamulin, valnemulin	Novak and Shlaes (2010), Schwarz et al. (2016)

3.2.4 Aquaculture

The Food and Agriculture Organization estimated that the annual supply of fish in fisheries is around 110 million tons. However, the pathogenesis in fisheries causes unpredictable mortalities and hinders fish production in aquaculture (Cabello 2006; Defoirdt et al. 2011). The Food and Drug Administration has approved the use of antibiotics such as florfenicol, oxytetracycline, and sulfadimethoxine/ormetoprim in aquaculture to prevent this loss (Romero et al. 2012). In India, antibiotics such as erythromycin, streptomycin, chloramphenicol, and co-trimoxazole have been used in aquaculture (Sarkar et al. 2018). Antibiotics are given to fish after mixing them in the formulated feed. However, the fish subsequently passed the unmetabolized antibiotics into the environment via feces. In India, the Marine Products Export Development Authority has listed the banned pharmacologically active substances and antibiotics for use in aquaculture such as nitrofurans, chloramphenicol, nalidixic acid, neomycin, sulfamethoxazole, chloroform, colchicine, chlorpromazine, dapsone, dimetridazole, sulfonamides, fluoroquinolones, nitroimidazoles, ipronidazole, and ronidazole (Sarkar et al. 2018).

Table 3.2 Class of antibiotics used in the treatment of infection in humans and animals

Antibiotic class	Animal use	Human medicine importance	References
Cephalosporin	Treatment of infection in cattle and swine	Critically important	Doyle et al. (2013), Asai et al. (2011)
Macrolide	Treatment of disease in cattle and swine	Critically important	Doyle et al. (2013), Pyörälä et al. (2014)
Fluoroquinolones	Treatment of infection in cattle, swine, and poultry	Critically important	Huq (2006), Doyle et al. (2013)
Tetracycline	Treatment of infection in cattle and swine	Highly important	Sengeløv et al. (2003), Doyle et al. (2013)
Penicillins	Treatment of infection in cattle, poultry, and swine	Highly important	Doyle et al. (2013), De Briyne et al. (2014)
Lincosamide	Treatment of infection in cattle and swine	Highly important	Doyle et al. (2013), Pyörälä et al. (2014)
Streptogramin	Treatment and prevention of infection in cattle, swine, and poultry	Highly important	Giguère (2013), Doyle et al. (2013)
Phenicol	Treatment of infection in cattle and swine	Not	Dowling (2013a, b)
Sulfonamide	Treatment of infection in swine	Not	Stahl et al. (2016)
Pleuromutilin	Treatment of infection in swine	Not	Alban et al. (2017)
Bambermycin	Treatment in cattle and chickens	Not	Dealy and Moeller (1977), Letellier et al. (2000)
Carbadox	Treatment of infection in swine	Not	Looft et al. (2014)

3.3 Routes of transmission of Antimicrobial-Resistant Bacteria in the Environment

According to the methods of waste management released from information for all program, the United Nations Educational, Scientific and Cultural Organization (UNESCO), the antimicrobial-resistant bacteria are released into the environment via air, water, and soil through disposals from animal houses and feedlots (Islam et al. 2019). The earlier report of the US Department of Agriculture found that food animals nearly produce 335 million tons of manure annually which is 40 times more than the mass of human biosolids (7.6 million) (Silbergeld et al. 2008; Linville et al. 2015). The unmetabolized antimicrobial agents and resistant microbial strains are excreted by the animals into the manure, and its applications in soil are the main cause of spread of antimicrobial resistance in soil microflora. The surface water and groundwater get contaminated by the animal waste and sewage disposal which leads antimicrobial resistance pathogenic strains in drinking water and irrigated soil (Islam et al. 2004). Food crops grown in wastewater-irrigated soil and manure

application soil get contaminated with antibiotic-resistant bacteria and are the major source of transmission of multidrug resistance in humans. Mass gatherings in religious congregations, sports events, beach sports, and water parks are another source of transmission of antibiotic-resistant bacteria and antibiotic-resistant genes in the water bodies and resulted in the spread of waterborne diseases, i.e., endemic cholera (Mutreja et al. 2011; Fouz et al. 2020). Recently, antimicrobial resistance genes corresponding to different classes of antibiotics, including β-lactams, fluoroquinolones, aminoglycosides, macrolide-lincosamide-streptogramins (MLS), sulfonamides, and rifampicin, were identified in samples collected from the Ganges-Yamuna river confluence site (Samson et al. 2019). Hajj is a religious gathering in Saudi Arabia where pilgrims stayed in tents in Mina for 3–5 days. The wastewater from septic tanks of Hajj pilgrim camps showed the antibiotic resistance genes for β-lactam antibiotics and aminoglycoside (Fouz et al. 2020). In addition to this, water sports in water and sea parks cause the spread of antibiotic resistance genes by direct ingestion of water by many people (Leonard et al. 2015; Williams et al. 2016). The wastewater from antibiotic manufacturing plant or pharmaceuticals disposed antimicrobials in aquatic environment which is directly associated with the spread of antibiotic resistance genes in the community. β-Lactams, tetracyclines, aminoglycosides, glycopeptides, fluoroquinolones, macrolides, sulfonamides, and trimethoprim have been detected in wastewater from pharmaceutical industries (Felis et al. 2020). Hospital wastewater is the hotspot of antibiotic resistance genes as it contains the mixture of antibiotics, metabolized antibiotic from the patient excreta, and multidrug-resistant bacteria having resistance to extended-spectrum β-lactams, tetracyclines, carbapenems, and sulfonamides (Zhang et al. 2020).

3.4 Consequences of Antibiotic Use in Agriculture

3.4.1 Consequences of Antimicrobial Resistance in Crop Disease

The antibiotics have been used against crop disease since the 1950s. After the continuous use of antibiotics in agriculture for years, the plant pathogens became resistant to antibiotics (Sarkar et al. 2018). Streptomycin was used against plant pathogen, *Xanthomonas campestris*, for controlling disease in tomato and pepper plants (Minsavage et al. 1990). Nowadays, *Xanthomonas campestris* strain has acquired resistance against antibiotic streptomycin. This is due to the single point mutation in *rpsl* chromosomal gene and thus leads to the inhibition of the binding of streptomycin to ribosome (Sarkar et al. 2018). Similarly, streptomycin-resistant *Erwinia amylovora*, the causative agent of fire blight in apple and pears, and *Pseudomonas syringae*, the causative agent of necrosis in plants, evolved (Sundin and Bender 1993; Russo et al. 2008). *Erwinia amylovora* showed resistance against oxolinic acid (Chen et al. 2019). *Pectobacterium carotovorum*, the causative agent of soft rot

potato tubers and bacterial canker, showed multidrug resistance against penicillin, polymyxin B, erythromycin, and vancomycin (Koh et al. 2012). *Acidovorax avenae*, the causative agent of bacterial leaf blight, showed resistance against kasugamycin (Chen et al. 2019). Tetracycline resistance was reported in plant pathogenic bacteria, *Pseudomonas syringae* and *Agrobacterium tumefaciens* (Chen et al. 2019). The bacterium *Pseudomonas syringae* pandemically affected *Actinidia chinensis* and *Actinidia deliciosa* in China, Italy, New Zealand, Spain, Portugal, France, Japan, and South Korea (Scortichini et al. 2012). Therefore, countries having kiwifruit industries suffered from grievous economic losses. The symptoms of the disease are brown leaf spots along with chlorotic haloes, discoloration of cankers, and bud damage fruits (Cameron and Sarojini 2014). This report showed that the development of antibiotic resistance in *Pseudomonas syringae* is a major threat to plant ecosystem. *Agrobacterium tumefaciens* is the most important plant pathogen known to cause crown gall disease eudicots (De Cleene and De Ley 1976). Antimicrobial resistance in plant pathogenic bacteria is a major threat to pathosystems because it decreases the productivity of the agrosystem and transfers resistance to human pathogenic strains (Williams-Nguyen et al. 2016; Sundin and Wang 2018; Raman et al. 2020).

3.4.2 Medical Consequences of Antibiotic Use in Agriculture

Several studies showed increasing multiple drug resistance among key enteric pathogens such as *Escherichia coli*, *Salmonella* spp., *Vibrio cholera*, *Shigella* spp., *Campylobacter* spp., and *Helicobacter* sp. to nearly all commonly available antibiotics. Therefore, it is imperative that this trend should be reversed.

3.4.2.1 *Escherichia coli*

Escherichia coli is the most predominant facultative anaerobic bacteria in the gastrointestinal tract of animals and humans. It is the most common enteric pathogen that also has harmful strains which causes serious urinary tract infection (Varughese and Beniwal 2015). So, multidrug resistance in *Escherichia coli* is of great concern. Rasheed et al. (2014) found that *Escherichia coli* isolates from raw chicken, raw meat, vegetable salad, unpasteurized milk, and raw egg surface showed multidrug resistance. The isolates from raw chicken possessed multidrug resistance against ampicillin, amoxicillin, aztreonam, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, co-trimoxazole, gentamicin, ofloxacin, streptomycin, and tetracycline (Rasheed et al. 2014). Isolates from unpasteurized milk showed drug resistance against ampicillin, amoxicillin, amoxyclav, cefotaxime, chloramphenicol, ciprofloxacin, ofloxacin, streptomycin, and tetracycline. Food chain is the most common mode of cycling of pathogens among humans and animals (Laxminarayan et al.

2015). Therefore, good hygienic practices are required to avoid contamination from poultry and cattle products in humans (Marshall and Levy 2011; Meng et al. 2017).

3.4.2.2 *Salmonella* spp.

Salmonella enterica causes salmonellosis, the most commonly reported foodborne bacterial disease (Shang et al. 2021). Chicken meat is the second largest source of animal protein for humans. So, the dissemination of antimicrobial resistance *Salmonella* sp. through the food chain, by chicken, has important consequences for the failure of salmonellosis treatment (Shang et al. 2021). A recent report showed multidrug resistance monophasic *Salmonella typhimurium* against streptomycin, ampicillin, and tetracyclines (Giacometti et al. 2021). The *Salmonella* serotype isolates from humans were found to be identical to *Salmonella typhimurium*, *Salmonella enteritidis*, and *Salmonella infantis* isolated from food (Rospotrebnadzor 2018). All *Salmonella* serotypes were resistant to amoxicillin-clavulanic acid, 93.8% to ceftazidime and cefuroxime, 68.8% to tetracycline, 47.9% to gentamicin, 39.6% to ciprofloxacin, 41.7% to nalidixic acid, 31.3% to ofloxacin, 27.1% to cefixime, and 52.1% to multidrug resistance against all these antibiotics (Rospotrebnadzor 2018).

3.4.2.3 *Vibrio* spp.

Vibrio sp. are the causative agent of diarrheal and systemic diseases in humans. They grow at temperatures from 10 to 30 °C in aquatic environment. Several *Vibrio* sp. such as *Vibrio harveyi*, *Vibrio splendidus*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio anguillarum*, *Vibrio alginolyticus*, and *Vibrio parahaemolyticus* induce infections in the aquatic ecosystem. *Vibrio vulnificus* cause wound infections of soft tissues, gastroenteritis, and septicemia (Armstrong et al. 1983). *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus* strains are human pathogens. *Vibrio cholerae* is the most common causative agent of cholera in humans. Earlier report of Pan et al. (2008) showed that *Vibrio cholera* has resistance against various antibiotics, ampicillin, tetracycline, streptomycin, chloramphenicol, gentamicin, and trimethoprim-sulfamethoxazole. Other *Vibrio* sp. showed resistance against antibiotics: ampicillin, cefazolin, ciprofloxacin, cefotaxime, streptomycin, and cefuroxime sodium (Yano et al. 2014).

3.4.2.4 *Shigella* spp.

Shigella are Gram-negative bacteria and cause acute gastrointestinal infection, shigellosis, or bloody diarrhea (Shahin et al. 2019). *Shigella dysenteriae* type 1 was first isolated in Japan in 1896 by Kiyoshi Shiga, as it caused severe dysentery epidemic. The global report on mortalities due to shigellosis was more than 700,000 and 60% of them occurred in children below the age of 5 years (Puzari et al. 2018). Shigella

strains are transmitted through fresh vegetables (Jo et al. 2019), contaminated meat and dairy products (Ahmed and Shimamoto 2014), contaminated food and water, and person-to-person contact in human beings (Niyogi 2005). Shigellosis can occur in the pandemic, epidemic, and sporadic forms. Epidemics have been reported from India, Myanmar, Bhutan, Nepal, Maldives, Sri Lanka, Bangladesh, and Central American countries (Muthuirulandi Sethuvel et al. 2017). In the year 2017, the World Health Organization (WHO) released a report that for treating *Shigella* infections, there is an urgent need for new antibiotics or the alternative approaches to be explored. The National Antimicrobial Resistance Monitoring System reported that in the year 1999, only 57%, 56%, 1.6%, 0.3%, and 1% of *Shigella* isolates were resistant to tetracycline, streptomycin, nalidixic acid, ciprofloxacin, and ceftriaxone, respectively, whereas in recent years these rates increased to 87%, 57%, 98.3%, 23.1%, and 10.8%, respectively (Shahin et al. 2020). Zachariah et al. (2021) reported that *Shigella* spp. showed 91.7%, 83.3%, 82.1%, 73.1%, 66.7%, and 54.2% against erythromycin, doxycycline, ampicillin, co-trimoxazole, minocycline, and cefuroxime, respectively.

3.4.2.5 *Campylobacter* spp.

Campylobacter spp. are microaerophilic curved comma or s-shape Gram-negative bacteria. They are part of the normal intestinal flora of domestic ruminants. Food products from ruminants such as meat and milk predominantly spread this bacterium to humans. *Campylobacter coli* and *Campylobacter jejuni* cause the most commonly detected enteric infections, campylobacteriosis in humans (Samie et al. 2007). Earlier studies of Hong et al. (2007) showed that *Campylobacter* isolates from chicken raw meat showed resistance to doxycycline, ciprofloxacin, nalidixic acid, tetracycline, enrofloxacin, and erythromycin. Kinana et al. (2006) reported the predominance of macrolide- and fluoroquinolone-resistant *Campylobacter jejuni* and *Campylobacter coli* is increasing globally. In tropical developing countries, *Campylobacter* infection among children is hyperendemic. The community-based studies assessed the occurrence rate of *Campylobacter* infection in children below 5 years of age which is between 40,000 and 60,000 notifications per 100,000 population (Coker et al. 2002). According to a recent report of Zachariah et al. (2021), *Campylobacter jejuni* showed 87.5%, 75%, 73.7%, 73.3%, and 68.8% resistance against erythromycin, doxycycline, ampicillin, co-trimoxazole, and minocycline, respectively.

3.4.2.6 *Helicobacter* spp.

Helicobacter pylori is a curved spiral shape, microaerophilic Gram-negative bacterium. It is the causative agent of peptic ulcer disease, gastric adenocarcinoma, type B gastritis, and mucosa-associated lymphoid tissue lymphoma and the fourth common type of cancer (Mousavi et al. 2014). Earlier studies showed that *Helicobacter*

pylori is the natural host in the gut of domestic ruminants, i.e., cow, sheep, buffalo, goat, and camel species without any gastritis (Rahimi and Kheirabadi 2012). It was found that the milk and feces of these domestic species showed the presence of *Helicobacter pylori* (Rahimi and Kheirabadi 2012). *Helicobacter pylori* can survive in water, milk, fresh meat, fresh fruit and vegetables, and some dairy products at temperatures below 30 °C and provide optimal conditions for its transmission to humans (Fan et al. 1998). *Helicobacter pylori* infects about 50% of persons above the age of 60 years and 20% of persons below the age of 40 years (Sosa et al. 2010). Metronidazole and clarithromycin belong to nitroimidazole and macrolide class of antibiotics, respectively, and are used with amoxicillin for the treatment of gastritis and peptic ulcer caused by *Helicobacter pylori*. They are the first-line treatment antibiotics for infections caused by *Helicobacter pylori*. Therefore, resistance in Helicobacter pylori against these antibiotics hampers the treatment (Abadi 2017). Resistance in *Helicobacter pylori* against these antibiotics is low in Asia and Europe (0.8–9.1%) and higher (nearly 18%) in developing countries (Lwai-Lume et al. 2005).

3.4.2.7 Urinary Tract Infection Causing Pathogens

In urinary tract infection, bacteria colonize in the urinary tract. The lower urinary tract infection is known as cystitis (bladder infection) and in the upper urinary tract infection is known as pyelonephritis. The common symptoms of lower urinary tract infection are pain with frequent urination and feeling the need to urinate and are flank pain, fever, and sometimes bloody urine. The urinary tract infections are caused by Gram-positive and Gram-negative bacteria, some fungi, viruses, and protozoa. The most common bacteria causing urinary tract infection are *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococci*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterobacter* spp. (Ziółkowski et al. 2021). Mechal et al. (2021) reported that *Escherichia coli* and *Staphylococcus aureus* acquired high level of resistance against ampicillin and tetracycline. The antimicrobial resistance in urinary tract infection causing microbes causes recurrent infections and fails to antibiotic therapy (Vachvanichsanong et al. 2021).

3.5 Mechanisms of Antibiotic Resistance

Bacteria have different mechanisms to survive antibiotics, which in turn are dependent on the mode of action, biochemical properties, and structures of different antibiotics (Barker 1999). The mechanisms of antimicrobial resistance can be divided into five major groups:

1. Prevention of the antimicrobial agent from reaching its cellular target by decreasing active export or uptake of the antimicrobial agents, e.g., a decrease in the

number of porin channels decreases the antibiotic entry into the cell, and efflux pump decreases the concentration of antibiotics in cell by exporting them outside the cell (Fernández and Hancock 2012),
2. Alterations of the antimicrobial agents, e.g., resistant microbes convert penicillin into penicilloic acid (Cole et al. 1973).
3. Microorganism protects the target site of antimicrobials via phosphorylation, acetylation, or nucleotidylation, e.g., in aminoglycosides (Mingeot-Leclercq et al. 1999).
4. Formation of alternative metabolic pathways, which bypass some steps which are inhibited by antimicrobial agents (Reynolds 1989).
5. Alteration of the target protein through mutation (Barker 1999; Morar and Wright 2010; Nguyen et al. 2014; Munita and Arias 2016).

3.5.1 Transfer of Antibiotic Resistance in Clinical Isolates via Agricultural Sources

Humans are at high risk of exposure to resistant pathogens through direct contact during handling, contact with infected humans, and ingestion of contaminated food and water (Chang et al. 2015). The mechanisms by which antibiotic resistance through agricultural microbes is transferred to human microbes are mainly via horizontal transfer of mobile genetic elements such as broad host range plasmid DNA, integrons and transposable elements, and vertical gene transfer.

3.5.1.1 Horizontal Gene Transfer

Horizontal gene transfer is the process of transmission of the genetic material between unicellular and multicellular organisms from donor to recipient. It is carried out via three main mechanisms such as conjugation, transformation, and transduction (Stearns and Hoekstra 2005). It occurs in the digestive system of humans and animals, food, soil, and water. Most of the antimicrobial resistance genes are carried on plasmids, integrons, or transposons. These can act as vectors for transferring antimicrobial resistance genes to other members of the same species or other genus or species (Keeling and Palmer 2008). The frequency of horizontal gene transfer mainly depends on the characteristics of the donor and recipient populations, properties of mobile genetic elements, and the environment (Verraes et al. 2013). Moreover, the high intrinsic recombination ability in certain microbes such as *Escherichia coli*, *Salmonella enterica*, *Bacillus subtilis*, and *Streptococcus pneumoniae* increases the rate of horizontal gene transfer (Dutta and Pan 2002). *Salmonella typhimurium* transferred ampicillin resistance gene in *Escherichia coli* in milk, and *Enterococcus faecalis* transferred tetracycline and erythromycin resistance in *Leuconostoc monocytogenes* (Von Wintersdorff et al. 2016).

3.5.1.1.1 Conjugation

Conjugation is the process of transfer of genetic material (DNA) between the live bacteria that requires a direct contact between the recipient and the donor cells (Fig. 3.2). The antimicrobial resistance genes are present on the mobile elements, such as transposons, plasmids, insertion sequence, and integrons. The transposons and insertion sequences can translocate the antimicrobial resistance genes only within the bacterial cells (Lyras et al. 2004). An integron is an immobile element that expresses and integrates or releases the gene cassettes. It is divided into two groups: (1) the chromosomal integrons which are associated with the bacterial chromosome and (2) the mobile integrons associated with plasmids and transposons (Cambray et al. 2010). In Gram-negative bacteria, the production of extended-spectrum beta-lactamase (ESBL) enzymes commonly imparts resistance against β-lactam antimicrobial agents. The genes encoding ESBL enzymes are usually acquired or transferred in microbes belonging to *Enterobacteriaceae* by conjugation (Liu et al. 2019). A recent study by Johansson et al. (2021) showed that an isolate of *Salmonella enterica* from animal origin has translocatable mobile resistance elements for tetracycline, sulfonamide, and aminoglycoside.

3.5.1.1.2 Transformation

Transformation is the process of uptake, integration, and expression of foreign genetic material from the environment by a recipient cell as shown in Fig. 3.3 (Stearns and Hoekstra 2005). *Bacillus subtilis*, *Campylobacter* spp., *Helicobacter pylori*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Acinetobacter baylyi*, and *Streptococcus* spp. have natural tendency of competence (Lorenz and Wackernagel 1994; Verraes et al. 2013). Natural transformation is observed in various environments, such as in soil, water, foodstuff, and human fluids, and contributed to the

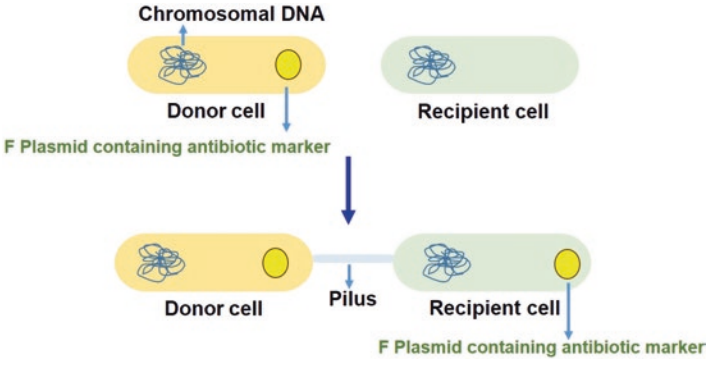

Fig. 3.2 Conjugation by direct contact between two bacterial plasmids by forming a mating bridge between the bacteria which results in DNA transfer for antibiotic resistance genes to a recipient cell

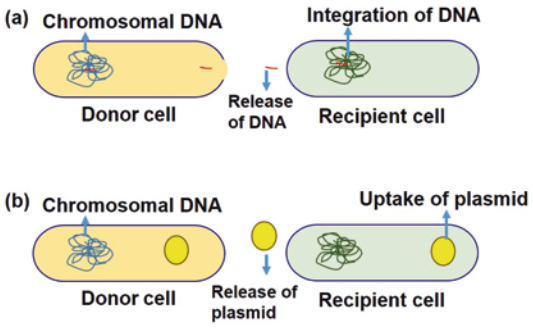

Fig. 3.3 (**a**) Transformation by transfer of antibiotic resistance gene from donor to a recipient cell and its integration into its chromosome. (**b**) Uptake of a plasmid DNA by the recipient cell from a donor cell

genetic variations in human pathogens (Domingues et al. 2012). The report of Domingues et al. (2012) showed that *Pseudomonas aeruginosa* acquired resistance against ampicillin and sulfamethoxazole and *Acinetobacter* sp. acquired resistance against ampicillin, ceftazidime, gentamicin, cefotaxime, kanamycin, and sulfamethoxazole via transformation. A recent report of Jin et al. (2020) showed that the anthropogenic activity, i.e., the chlorination of drinking water by sodium hypochlorite, enhanced the transmission of antibiotic resistance genes encoding tetracycline, ampicillin, kanamycin, and nalidixic acid markers in enteric pathogens *Enterococcus faecalis* and *Escherichia coli*.

3.5.1.1.3 Transduction

Transduction is the process in which bacterial DNA is transferred from one bacterium to another by a bacteriophage. The mobilization of antibiotic resistance genes by bacteriophages has been reported in strains of *Streptococcus pyogenes* for tetracycline, in enterococci for tetracycline and gentamicin, in *Escherichia coli* for β-lactamase, and in *Salmonella* for multidrug resistance (Von Wintersdorff et al. 2016).

3.5.1.2 Vertical Gene Transfer

In vertical gene transfer, the genetic material is inherited by the transmission of genes from parents to offspring (Fig. 3.4). A recent report of Li et al. (2019) showed that transconjugants of *E. coli* have tetracycline, trimethoprim, and amoxicillin resistance genes by vertical gene transfer. Meng et al. (2017) found that amoxicillin resistance gene was transferred through vertical gene transfer in *Phocoenobacter*, *Ignavibacterium*, *Aminobacterium*, *Spirochaeta*, and *Cloacibacillus*, which are present in wastewater. The BLAST analysis of homologous sequences of methicillin-resistant gene showed that it was transferred through vertical gene transfer in *Staphylococcus aureus*, which is a common pathogenic species of skin infection (Ito et al. 2003).

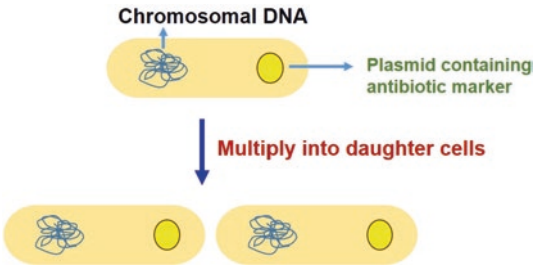

Fig. 3.4 Vertical gene transfer in bacterial species from parent to daughter cells

3.6 Antimicrobial Resistance and Health Risks to Humans

The severity of infections in humans has increased worldwide due to the antimicrobial-resistant bacteria. This is because of the following reasons:

1. *Failure or delay in treatment*: Antibiotic administration to patients, mainly in severe cases, which required intensive care units (ICU) is usually done before the antibiogram results. This results in the increase of the multidrug resistance in microbial strain. This leads to worsening of the patient's condition during relapse time. This condition in patients is avoided by increasing the awareness in people regarding antibiotic use (Khilnani et al. 2019). Further, the switching from broad-spectrum antibiotics to narrow-spectrum antibiotics, i.e., de-escalation, is recommended in the guidelines of antibiotic use to avoid increase in multidrug resistance in microbial strains (Leone et al. 2014). The recent studies showed that the de-escalation reduces the number of mortalities in patients (Guo et al. 2016; Tabah et al. 2016).
2. *Choice of antimicrobial agents is limited*: The antimicrobial agents' categories are limited due to the appearance of antimicrobial-resistant strains (Khilnani et al. 2019).
3. *Selection of antimicrobial-resistant strains*: The antibiotic treatment in patients selects the suppressed resistant pathogenic strains. This is because the prolonged exposure of microbial strains to antimicrobial agents helps them to evolve resistance against antimicrobial agents. Every year in the USA, nearly 35,000 deaths of human were reported due to multidrug-resistant microbial strain (Jordt et al. 2020).

3.7 Antibiotic Stewardship and Public Awareness to Mitigate the Health Risks to Humans

Antimicrobial stewardship is a coordinated program that promotes the appropriate use of antibiotics by selection of optimum dosage and time of antibiotic treatment, and it improves patient outcomes, reduces microbial resistance, and decreases the spread of infections caused by multidrug-resistant organisms (Gerding 2001). In

this the first aim of medical practitioners is to prescribe the correct dose and duration of antimicrobial agents for the patients. The second aim is the prevention of misuse and overuse of antimicrobial agents, and the third aim is the minimization of the development of antimicrobial agent resistance in individual patient and in community level (Doron and Davidson 2011). The increase in antimicrobial resistance in community can be avoided or decreased via public awareness regarding antibiotic use and misuse. This can be done by increasing awareness in school and college students by adding lectures on the subject. The campaign regarding the use of antibiotics should be done to increase the knowledge of rural community and less educated people (Manyi-Loh et al. 2018). The consumer associations and groups can increase the awareness of antibiotic uses in food animals, which increases the demand of antibiotic smart food. They are also guiding farmers to do antibiotic free farming to avoid spread of antimicrobial resistance (Mathew et al. 2019).

3.8 Alternatives to Antimicrobial Agents in Agriculture

One of the most important alternatives for reducing the use of antimicrobial agents is immunization by vaccination (Hoelzer et al. 2018). The use of prebiotics, probiotics, and synbiotics also reduces the usage of antibiotics because they improve the health of human gut microflora (Hume 2011). The health of human digestive tract microbiota generally contributes to the immune system functionality and offers less area for pathogen colonization. In poultry, the major decrease in *Salmonella* colonization was observed after the administration of anaerobic bacterial commensal. Phages are also used as an alternative of antimicrobial agents, and they neutralize the selective pathogen in lytic cycle (Lin et al. 2017). Moreover, phages can also be used with other antimicrobials without any side effects. The oral administration of virus in humans reduces the symptoms of inflammatory bowel disease caused by *Butyricicoccus pullicaecorum*. The use of phages is limited to neutralization of foodborne pathogens in animal husbandry, curing of tropical diseases in humans, and control of phytopathogens.

Other useful agents are antimicrobial peptides which can act as alternative to antibiotics (Baltzer and Brown 2011). Bacteriocins are the antimicrobial peptides which are used as food preservative. The US Food and Drug Administration has approved the daily uptake of nisin A (bacteriocin) to 2.9 mg per person per day (Delves Broughton et al. 1996; Joo et al. 2012). It is used in food products and recognized as safe ferments. Bacteriocins have been used in food industries to reduce the growth of *Listeria*. Predatory bacteria are also used as an alternative to antimicrobial agents (Allende et al. 2007). *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus* have been used as prey against multidrug-resistant pathogens of genus *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Aeromonas*, *Salmonella*, *Shigella*, *Serratia*, *Yersinia*, *Vibrio*, *Enterobacter*, *Proteus*, *Bordetella*, *Burkholderia*, and *Pseudomonas putida* (Dashiff et al. 2011). They act as both probiotics and

antibiotics. *Bdellovibrio bacteriovorus* has been used for treating ocular diseases in rabbits caused by *Shigella flexneri* and in cows caused by *Moraxella bovis* (Shanks et al. 2013; Galvão and Angelos 2010).

3.9 Regulatory Agencies and Their Role in Regulating Antibiotic Use and Global Partnerships in Antimicrobial Resistance

The World Health Organization introduced the Global Action Plan so that every country must develop the national action plans in an attempt to reduce the spread of antibiotic resistance (World Health Organisation 2015). Further, the monitoring and surveillance of antibiotic use and antibiotic resistance are important strategy against spread of antibiotic resistance. Surveillance systems regarding consumption of antimicrobial agents are South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD), Pretoria, South Africa; Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in Wageningen, Netherlands (MARAN); and Danish Integrated Antimicrobial Resistance Monitoring and Research Programme (DANMAP), Kongens Lyngby, Denmark (Schaekel et al. 2017). The antimicrobial stewardship is implemented in agriculture sector as well as in clinical practices in order to maintain the efficiency of available antimicrobial agents and to reduce the spread of antibiotic resistance from animal- and plant-derived food products (Moudgil et al. 2017). The stakeholders involved in reducing the spread of antibiotic resistance must report the antimicrobial stewardship from the stand point of surveillance, regulations, treatment guidelines, research, education, infection control, and awareness (Sharma et al. 2018). The National Farmed Animal Health and Welfare Council stated that the antimicrobial stewardship can be implemented in agriculture with the following perspectives such as clinical microbiology, biosecurity, surveillance on antibiotic use and antimicrobial resistance, alternatives to antibiotics, and animal management (National Farmed Animal Health and Welfare Council 2016). Some European countries banned the use of antimicrobial agents as growth promoters (Cogliani et al. 2011), whereas in New Zealand and Australia the partial ban is implemented. The tripartite alliance among the World Health Organization, Food and Agricultural Organization of the United Nations, and World Organisation for Animal Health was formed in 2003. This alliance helps in the categorization of medicines used in veterinary practices into important drugs for human health, highly important and critically important. Therefore, it is helpful in guiding the use of antimicrobial agents in animal agriculture and thus helps in reducing the spread of antimicrobial resistance (Manyi-Loh et al. 2018).

3.10 Conclusions

The continued increase of antimicrobials in the environment from agriculture and other sources has disrupted the natural balance between the antimicrobial agents and microbes. This has resulted in the dissemination of antimicrobial resistance in the microbial communities via horizontal gene transfer and vertical gene transfer. The multidrug resistance genes from commensal or beneficial microbes of human or plant origin are often transferred to pathogens and ultimately create superbugs. Therefore, to control the overuse and misuse of antimicrobial agents, alternative approaches have been used such as vaccination, prebiotics, probiotics, phages, and predatory bacteria. In addition to this, awareness should be spread against indiscriminate antibiotic usage, and strict regulatory policies should be implemented for nonhuman usage of antibiotics.

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Chapter 4
Modern Diagnostic Tools for Rapid Detection of Multidrug Resistance

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4.1 Introduction

In the past few decades, there has been a consistent rise in cases exposing the prevalence of multiple drug resistance throughout the world. The emergence of antimicrobial-resistant pathogens is the greatest epidemiological menace in this twenty-first century (Prestinaci et al. 2015). It has become a major challenge for calculated antimicrobial therapies to treat various types of infectious diseases and thus presents a serious threat to patient's treatment. Various clinically important bacteria including Gram-positive and Gram-negative such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and members of family Enterobacteriaceae like *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus* sp. rapidly develop and transmit antimicrobial resistance in hospital environment

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(Basak et al. 2016; Lee et al. 2012; Livermore 2012). Further, it results in increased rate of morbidity and mortality with long stay of patients in hospitals.

Keeping in view, the WHO declared "combat drug resistance: no action today, no cure tomorrow" in 2011 (Sharma 2011). In the twenty-first century, MDR represents the largest threat to health globally according to the WHO and needs immediate actions in place. Annually, death of more than 700,000 people results from drug-resistant infections, and this number is speculated to reach ten million by 2050 (UN 2016; Vasala et al. 2020).

Treatment of common infections being a challenge due to an exponential rise in antimicrobial drug resistance worldwide has led to the development of several novel antimicrobials in recent years. However, it also needs to focus on using the currently available antimicrobials. This highlights the importance of detection of susceptibility profile of targeted pathogens toward different drugs for particular infection. The use of appropriate diagnostic methods to determine drug resistance holds great potential to play significant role in initiating the right type of treatment (Frickmann et al. 2014).

Methods used for evaluating the multiple drug resistance and susceptibility profile mainly include phenotypic and genotypic types. Classical AST systems chiefly include the use of culture-based approaches which require more detection time due to long incubation steps usually requiring 48–72 h for completion (van Belkum et al. 2020). This contributes toward the indiscriminate use of antibiotics that further pave the way to drug resistance.

Therefore, various attempts have been made to develop methods offering rapid detection of drug resistance in the past few years. According to most clinical microbiologists, a testing can be defined as rapid if it is feasible during a single working shift, i.e., within 8 h or less (van Belkum et al. 2020).

This chapter presents the origin and transmission of multiple drug resistance with major emphasis on the current progress toward different types of AST systems including microscopy; molecular, i.e., PCR-based, RNA-based, isothermal amplification, and DNA microarrays; flow cytometry; biosensing; immunodiagnostic; mass spectrometry; colorimetric imaging; and optical approaches for rapid detection of MDR. In addition, we discuss the existing challenges and future prospects of new techniques for rapid detection of antimicrobial resistance.

4.2 Origin and Transmission of Multidrug Resistance

The development of AMR is considered as a phenomenon of natural selection that happens usually through genetic manipulations naturally over time due to the selective pressure posed by unchecked applications of drugs. The resistant strains may develop and be found in humans, animals, plants, and environment, but what makes the condition worse is the possibility of transmission of such AMR strains from one carrier to another, and the carrier may be a person, animals, or food of animal origin. Several factors include misuse of drugs as prophylactic, metaphylactic, or growth

promoter (Woolhouse et al. 2015), unhygienic practices, ignorance, improper health-care facilities, unaffordable and inaccessible quality treatments, lack of awareness and knowledge, as well as negligence and/or lack of law enforcement.

The scientific literature has shown an existence of a positive correlation between resistance to single drug and resistance to multiple drugs within a bacterial population. Numerous mechanisms have been proposed to explain the multidrug resistance among different pathogens but still need to be assessed and tested critically. Several unanswered questions still exist like whether mechanisms are taxon-specific, do pathogens share the pathways to accumulation of MDR to some extent, and why do resistance determinants aggregate in certain strains of bacteria. The increasing cases of MDR may be attributed to unexpected high rates of origin, high rates of spread of MDR strains or determinants, or both. Horizontal gene transfer is believed to be a key factor that can disseminate resistance to multiple drugs in a single step. However, it is better to understand the MDR concept in terms of origin and transmission.

Various possible mechanisms explained so far for the emergence of resistant strains include biochemical mechanisms, genetic linkage, highly mutable or recombinogenic bacterial lineages, and multidrug therapy with accelerated treatment failure in resistant infections. The biochemical mechanisms employed by resistant strains are modification of the target molecules, inactivation of the drug, bypassing of the target molecule, or efflux of drugs. Each biochemical mechanism mentioned can confer resistance to more than one drug. Also, the resistant strain may employ one or many different biochemical mechanisms to resist one or multiple drugs (Fig. 4.1).

Other reports have mentioned that the determinants of resistance to multiple drugs are genetically linked. They are arranged in a sequence on bacterial

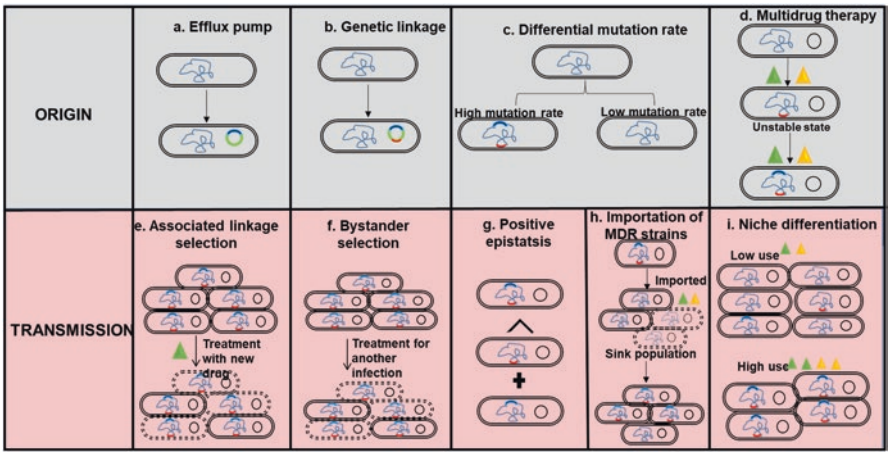

Fig. 4.1 Mechanisms for the origin and transmission of the MDR strains. Green and yellow triangles indicate the treatment with drug A and drug B. Red and blue marks represent drug A and drug B resistance determinants, respectively

chromosome or on the horizontally transmittable elements such as plasmid and conjugative transposons. Hence, the determinants can be co-inherited vertically and potentially co-transformed when shared horizontally. Researchers have believed that once the strain has acquired one resistance phenotype, then it can acquire many. The elements like plasmids, transposons, and integrons that transmit horizontally are highly extensible in nature and hence can incorporate new elements with time (Hall 2012; Roberts and Mullany 2009). Such elements do carry genes encoding for the specific enzymes like transposases and other site-specific recombinases that facilitate genetic incorporations. These elements may be organized on the chromosome or plasmid and may get transferred among interspecies or intraspecies through conjugation, transformation, and/or transduction (Alekshun and Levy 2007). The molecular mechanisms involved in transfers are well known, but how the resistance elements stay together under selective pressure is still unclear.

The rate of mutational changes varies among bacterial lineages which are attributed to variability in DNA proofreading mechanisms such as the mismatch repair system and also their ability to accept and integrate transforming DNA (Croucher et al. 2013). Therefore, the highly recombinogenic lineages possess higher frequencies of acquiring MDR determinants. An exposure to sublethal concentrations of antibiotics is considered as a selective pressure that can induce the mutation in bacteria (Baquero et al. 1998; Kohanski et al. 2010). Under antibiotic stress, the bacteria generate the reactive oxygen species that elevates the rate mutational changes irrespective of their specificity to the AMR (Kohanski et al. 2010). Acclimatization to the environmental conditions with antibiotics may provide second-order selection for high mutation rates (Mao et al. 1997).

Multidrug therapy is commonly recommended to treat infections, which may be involving strains resistant to single drug, with a belief that a combination of multiple drugs may control the resistance strain and minimize the probability of the emergence of resistance. Such belief was based on the assumptions that the host will harbor no MDR bacteria. However, the frequency of bacteria with resistance to multiple drugs increases if the infection carries bacteria already resistant to two or more drugs used in a cocktail. Simply, the single drug-resistant strains are highly unstable and prone to undergo multiple genetic recombinations that facilitate the selection and emergence of MDR (Hingley-Wilson et al. 2013; van Rie et al. 2005). Once the resistance has emerged, then the proliferation depends on the fitness and survivability of the host cells as well as stability and transmittance ability of the responsible genetic elements.

4.3 Methods for Detection of Multidrug Resistance

4.3.1 Microscopy

Microscopy has been used as a rapid means of detecting drug resistance in many bacteria. In March 2010, the WHO approved the microscopic observation drug susceptibility (MODS) assay to test susceptibility of mycobacteria against isoniazid

and rifampicin. The rapid growth of *Mycobacterium* in liquid medium than solid medium forms the basis of this assay. Rope-like growth/cord formation in liquid medium is characteristic of *M. tuberculosis* which is detected by an inverted microscope (Caviedes et al. 2000; Moore et al. 2006; Bwanga et al. 2010). This is an easy, simple, sensitive, rapid, and inexpensive assay for detection and drug resistance testing of mycobacteria. Sanogo et al. (2017) in their study conducted in West Africa evaluated the performance of this technique by comparing the results with other validated methods, i.e., mycobacteria growth indicator tube/antimicrobial susceptibility testing/streptomycin, isoniazid, rifampicin, and ethambutol. Caviedes et al. (2000) also analyzed sputum samples known to be positive for *M. tuberculosis* for susceptibility to isoniazid and rifampin using MODS and microwell alamar blue assay (MABA) in concordance with 89% of cases. MODS showed results rapidly with a median of 9 days and 9.5 days for diagnostic and susceptibility testing, respectively. Thus, the findings of these studies show the great potential of MODS for better management of multidrug-resistant TB cases throughout the world.

Accelerate Diagnostics (USA) has commercialized a multiplexed automated digital microscopy (MADM) based on fluorescence in situ hybridization (FISH) as a rapid online technique for AST (Metzger et al. 2014; Chantell 2015). Accelerate Pheno uses a preliminary step of electrophoresis to separate impurities like blood and urine from clinical samples. Subsequent change of electric field polarity leads to repel the target microorganism again toward the liquid. Here, the sample representing microbial culture growing in Mueller-Hinton medium is used to detect fluorescence signal after every 10 min (Charnot-Katsikas et al. 2017). Till date, this method is the only FDA-approved rapid diagnostic AST known which is based on microbial growth (Doern 2018).

4.3.2 Molecular Approaches

Molecular-based approaches have been increasingly used to trace the antibiotic resistance determinants (ARDs) in clinically relevant pathogens by targeting their specific sequences of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polymerase chain reaction (PCR)- and RNA-based detection, loop-mediated isothermal amplification (LAMP), and DNA microarrays are some of the molecular techniques used for early detection of antibiotic resistance which are discussed below.

4.3.2.1 PCR-Based Approach

The usage of PCR in clinical settings for detection of ARDs was first described to detect β-lactam resistance genes among *Enterobacteriaceae*. Since then, PCR and real-time PCR (qPCR) have proven to be a great diagnostic asset not only for precise identification/quantification of infectious agents but also for profiling their ARDs. Conventional PCR usually takes 12 h to present result and is composed of three steps. Firstly, the genetic material has to be extracted from the specimen

(blood, urine, etc.) followed by amplification of target DNA in a thermocycler. Secondly, thermocycler allows continuous amplification of DNA in around 25–35 cycles of denaturation, annealing, and extension. Lastly, the amplified target is detected through agarose gel electrophoresis, southern blotting, DNA fingerprinting, and other sequencing-based techniques (March Rosselló and Bratos Pérez 2016). To further shorten the turnaround time (TAT) of diagnosis to few hours, real-time PCR (qPCR) allows simultaneous amplification and detection of amplicons via different methods. PCR had also been adopted for detection of many ARDs in Gram-negative bacteria such as carbapenemase and cephalosporinase encoding genes like NDM, VIM, KPC, IMP, SHV, TEM, and OXA (Monteiro et al. 2012; Bogaerts et al. 2012).

qPCR can be used to differentiate antibiotic-susceptible strains from resistant ones by monitoring their growth under different antibiotic concentrations. For example, antibiotic susceptibility of *A. baumannii* against ciprofloxacin, imipenem, and colistin has been concluded within 6 h after culturing for isolated colonies wherein highly conserved region of *ompA* gene was used as bacterial growth indicator (Martín-Peña et al. 2013). Recently, a 2-D multiplex qPCR approach was used to detect four cephalosporin and five carbapenemase genes simultaneously in a single tube using a combination of probe colors and Tm values with high sensitivity of 30–3000 copies per reaction (Li et al. 2020). However, the limitation of qPCR is time-consuming culturing protocols and overestimation of viable cells, i.e., amplification of DNA from nonviable bacteria leading to increased false positives.

As the PCR technology refined in terms of sound DNA extraction protocols, multiplexing, and high-throughput automation, several commercial PCR instruments and/or kits came into force for enhanced antibiotic stewardship. Examples include Cepheid Xpert, Roche SeptiFast, Check-Points, Check-Direct CPE, Molzym's SepsiTest, Seegene MagicPlex, and BD GeneOhm MRSA (Li et al. 2017a). BD GeneOhm MRSA is suitable for antibiotic assessment from less complex samples like nasal swabs. These assays deliver results in the form of sample to answer and have short TAT (1–8 h). The GeneXpert® system (Cepheid) is one such example of fully automated qPCR-based point-of-care diagnostic tool to detect several carbapenemase genes directly from rectal samples without the need for skilled technicians. The Xpert® MRSA/SA BC and MRSA/SA SSTI cartridge could detect methicillin-resistant *S. aureus* (MRSA) in grown blood bottles and skin-soft tissue infections, respectively, in an hour with high sensitivity (93%) and specificity (close to 100%) (March Rosselló and Bratos Pérez 2016). This instrument proved useful in clinical laboratory settings as evident by saving 25.4 h of time for optimal antibiotic treatment (Carver et al. 2008). Similarly, Xpert® MTB/RIF detects *Mycobacterium tuberculosis* and its rifampicin resistance within 2 h with 86.8% sensitivity and 93.1% specificity in sputum samples (March Rosselló and Bratos Pérez 2016). SeptiFast test (Roche) is another rapid culture-independent amplification assay studied extensively for diagnosis of bloodstream infections directly from EDTA whole blood samples. It uses dual FRET probes for identification plus quantification and can detect around 25 bacteria and fungi in about 6 h with limit of detection of 3–30 cfu/mL (Mongell et al. 2015). Although SeptiFast possesses

specificity and sensitivity values in the range of 74–100% and 60–95%, respectively, the technology is costly and labor-intensive and provides no information on antibiotic susceptibility testing (AST) except for MRSA (Jordana-Lluch et al. 2015). FilmArray (bioMérieux) Blood Culture Identification Panel is another closed and fully automated culture-independent system which is approved by the FDA and CE. The instrument could detect 24 etiological agents (5 *Candida* spp. and 11 Gram-positive and eight Gram-negative bacteria) and three resistance genes (*mecA*, *KPC*, and *vanA/B*) with a TAT of 1 h (Salimnia et al. 2016). It can detect contaminant species also and consists of automated steps of cell lysis, DNA purification, multiplex PCR, nested PCR, and lastly melting curve analysis. The instrument demonstrated overall sensitivity and specificity in the range of 50–100% and 77–100%, respectively (Maugeri et al. 2019). The Curetis Unyvero system employs comprehensive resistance panels for diagnosing many ASDs per test in pneumonia, bloodstream infection, implant and tissue infection, and intra-abdominal infection (Gadsby et al. 2019).

Compared to conventional and real-time PCR, digital PCR (dPCR) has the ability to improve sensitivity as well as robustness in detecting low-abundance targets by partitioning the extracted nucleic acids into many individual picoliter droplets containing either one or zero molecules per droplet reaction. This system allows absolute quantification of target DNA or RNA in digital format (1 or 0) without the need for normalization leading to enhanced precision and reproducibility (Zhang et al. 2015). Besides, the partitioning also offers unique characteristics of determining ASTs by evaluating the delay in DNA replication. This property had been adopted to determine the antibiotic susceptibility of clinical UTI isolates after 15 min of exposure to four clinically relevant antibiotics (Schoepp et al. 2016).

However, PCR-based approaches are rapid, specific, and sensitive and could possibly reduce the turnaround times in antibiotic stewardship but suffer from drawbacks such as expensive, poor correlation between phenotypic and genotypic resistance especially for ESBLs and CREs, and their inability to detect novel and uncharacterized genes or the coincidental mutations thereof especially for continuous emerging variants of carbapenemases in gram negative bacteria (Pulido et al. 2013). Finally, their complicated upstream sample processing makes them more difficult to automate than the conventional phenotypic-based culture methods. Recently, a rapid sample processing-free microfluidic diagnostic platform based on single-step blood droplet digital PCR coupled with 3D particle counter system for bacterial identification and ARD determination directly from whole blood had been reported (Abram et al. 2020). This sample-to-answer device can detect CTX-M-9 family ESBLs with high sensitivity of 10 cfu/mL within 1 h assay time.

4.3.2.2 RNA-Based Approach

This approach of measurement of gene transcripts (RNA) instead of gene itself (DNA) could resolve the inherent disadvantages of DNA-based detection techniques, i.e., the lack of differentiation between viable and nonviable bacteria and

between silent and expressed genes (Mancini et al. 2014). NucliSENS EasyQ KPC Test® (bioMérieux) is based on detection of RNA levels of blaKPC gene variants with TAT of 2 h (McEwan et al. 2013). Fluorescence in situ hybridization (FISH) is a non-amplification technique typically utilizing bacterial and fungal rRNA target-specific oligonucleotide probes to capture naturally abundant rRNA genes circumventing the amplification step (Wellinghausen et al. 2006). PNA-FISH is the modified version to detect clinical bacterial isolates using peptide nucleic acid (PNA) probes (Pellestor et al. 2008). In PNA probes, negatively charged phosphate backbone of nucleic acids is replaced by neutral *N*-(2-aminoethyl)-glycine scaffold allowing robust hybridization and less susceptibility to inhibition by impurities.

Furthermore, direct targeting the mRNA could provide both genotypic and phenotypic information via its unique sequence and abundance, respectively. Bhattacharyya et al. (2019) developed a novel assay GoPhAST-R combining the phenotypic and genotypic AST via hybridization-based multiplexed RNA detection for improving molecular epidemiology and identifying emerging resistance mechanisms (Bhattacharyya et al. 2019). The assay presented with 94–99% accuracy in strain classification through machine learning analysis and determined key antibiotic resistance markers based on differential transcriptional responses to antibiotic exposure in <4 h from positive blood cultures compared to 24–36 h by conventional phenotypic ASTs.

4.3.2.3 Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is another rapid technique for real-time evaluation of antibiotic susceptibility testing (ASTs). Based on the strand displacement activity of Bst DNA polymerase, the gene of interest is amplified to detectable levels at isothermal temperature of 60–65 °C, thereby circumventing the need for thermocycler (Li et al. 2017b). LAMP had been evaluated for detection of carbapenem resistance in *Acinetobacter baumannii* through amplification of OXA-type genes (Vergara et al. 2014). The eazyplex system (Amplex Biosystems GmbH) utilizes LAMP reagents in lyophilized form in a tube and amplifies extracted DNA when added to tube within 30 min at constant temperature maintained in Genie II apparatus (OptiGene) followed by detection of amplicons in real time (March Rosselló and Bratos Pérez 2016). Notably, eazyplex SuperBug CRE had been developed to detect genes encoding for ESBL (CTX-M) and carbapenemases (NDM, KPC, VIM, and OXA-48) directly either from urine samples or from isolated colonies. Hicke et al. (2012) utilized helicase-dependent isothermal amplification with DNA array for multiplexing of 64 distinct targets on a single platform. This technology had been evaluated for detection of *C. difficile* on 130 patient samples with sensitivity and specificity of 97% and 100%, respectively. The ultrafast real-time digital LAMP for phenotypic antibiotic susceptibility measurements of *E. coli* from urine samples based on quantification of transcriptional responses upon antibiotic exposure had been developed which completes its entire workflow within 30 min (Schoepp et al. 2017).

4.3.2.4 DNA Microarrays

DNA microarrays are promising technologies for rapid screening of antibiotic susceptibility as these are capable of detecting broad range of resistance markers in a single step. Multiple cDNA probes on nylon membranes are closely attached to a glass or silicon platform, and specific hybridization of labeled probes with target antibiotic gene marker helps in determining the resistance (Khan et al. 2019). DNA microarrays had been used successfully for evaluating rifampin and isoniazid resistance in *M. tuberculosis* (Huang et al. 2014). An automated system VERIGENE (Luminex) for multiple detection of individual targets is composed of two panels. One panel (BC-GN) detects 8 Gram-negative bacterial targets and their six ARDs (*IMP*, *OXA*, *NDM*, *VIM*, *KPC*, and *CTX-M*), while the other (BC-GP) detects 12 Gram-positive targets and their three ARDs (*mecA*, *vanA*, and *vanB*) with turnaround time of around 2.5 h (Minejima and Wong-Beringer 2016). The cartridges of this system composed of glass slide printed with several capture probes for target DNA sequence-specific binding. A mediator probe binds to target DNA region and has polyA tail for further attachment to polyT region of probe labeled with gold nanoparticles. Afterward, silver nanoparticles surround the gold nanoparticles, and their aggregation is detected by light scattering (Ledeboer et al. 2015). BC-GP/BC-GN have sensitivity and specificity of 92.6–100%/97.1% and 95.4–100%/99.5%, respectively, for bacterial identification (Bork et al. 2014; Buchan et al. 2013). The Check assay (Check-Points) is based on ligation-mediated multiplex qPCR and microarray hybridization using different probes specific to genetic variants of β-lactamases and carbapenemases. It can provide results within 2–6 h; however, DNA extraction has to be performed outside of this platform (Lau et al. 2015). Examples include Check-MDR, Check-MD, and Check-CPE kits. After whole-cell DNA extraction, multiplex ligation reaction produces DNA molecules followed by PCR amplification using universal primers labeled with biotin. The amplicons are hybridized with resistance gene-specific probes, and image-acquired results in the form of presence or absence of marker gene are automatically interpreted by software (March Rosselló and Bratos Pérez 2016).

Microarray offers potential of high-throughput screening of resistance genes unlike PCR which can detect only handful of genes at a time, but it suffers from the same limitations as PCR such as poor correlation between phenotypic and genotypic markers, no information about MIC, and failure to detect novel or uncharacterized mechanisms of resistance. Moreover, the technology is also cost-intensive. Lately, a proof of concept for development of low-cost microarray platform for detection of highly clinically relevant antibiotic genes was developed (Wolff et al. 2019). It is based upon cost-effective in-house elongation of biotin-labeled oligonucleotide probes using terminal deoxynucleotidyl transferase reaction and has comparable sensitivity and specificity at one-tenth the cost of commercial-labeled probes.

4.3.3 Flow Cytometry

Flow cytometry (FC) has also been studied for AST by monitoring the multiple characteristics (cell membrane abnormality, cell size, enzyme activity, and microbial concentration) of microbial populations exposed to different concentrations of antibiotics over time in the presence of relevant fluorescent stains (Huang et al. 2018). After extensive sample preparation and labeling with fluorescent dyes, FC conducts automated data interpretation for over millions of cells (Leonard et al. 2018). For instance, carbapenem susceptibility profiling of *Klebsiella pneumonia* could be qualitatively and quantitatively estimated within 1 h and 3 h, respectively, by flow cytometry (Mulroney et al. 2017). The Luminex xTAG® assay (Luminex) is a well-established technology used for detection of different ESBLs, carbapenemases, and cephalosporinases in bacterial strains in TAT of 5 h and is based on combination of multiplex ligation PCR and colored microsphere-based flow cytometry (Ceyssens et al. 2016). The histograms produced by 90 °C side scattering of light to fluorescent signal enabled differentiation of methicillin-sensitive and methicillin-resistant strains of *S. aureus* upon 4 h exposure to antibiotic (Shrestha et al. 2011). Similarly, Faria-Ramos et al. (2013) differentiated ESBL- and non-ESBL-producing strains on the basis of antibiograms depicting the viability status of bacteria in the presence of antibiotics (ceftazidime, cefotaxime, and clavulanic acid), using the fluorochrome $DiBAC_4$ due to its ability to penetrate into nonviable cells.

FC is also a promising approach to study the MIC in clinical specimens. The MIC of piperacillin, gentamicin, and amoxicillin in pathogens *P. aeruginosa*, *E. coli*, and *S. aureus* has been calculated based on the antibiogram obtained in 4 h, and results were found to be 100% correlated with other systems like broth macrodilution, *E*-test, and VITEK2 (Broeren et al. 2013). Similarly, comparing the decrease in intensity of fluorescent signal obtained by hydrolysis of fluorescein diacetate substrate by the enzyme esterase present in mycobacteria can be used to determine the sensitivity of *M. tuberculosis* to antituberculosis drugs (rifampicin, isoniazid, and ethambutol) (Kirk et al. 1998). However, standardized protocols and data interpretation are limited for AST at present which in turn varies between pathogen and antibiotic combination used (Schumacher et al. 2018). Recently, FC-based AST protocol for early detection and quantification of uropathogenic *E. coli* strains against antibiotics, namely, trimethoprim-sulfamethoxazole, nitrofurantoin, ceftriaxone, and ciprofloxacin, had been optimized, thereby giving results within 4 h in urine samples (Velican et al. 2020). Thus, FC approach could play a pivotal role in enhancing the therapeutic success rate by providing early interpretation on AST against 24 h time taken by most conventional AST methods.

4.3.4 MALDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry is a technique which analyzes cellular protein reflecting gene products and metabolic products in order to identify different types of microorganisms including bacteria, yeasts, and molds (Vrioni et al. 2018; March Rosselló and Bratos Pérez 2016). This technique usually analyzes highly abundant, chiefly ribosomal proteins of microbes lying in the mass range of 2000–20,000 Da (Caroll and Patel 2015). The use of this technique for detection of drug resistance offers a major advantage of filling the gap between identification and resistance status of a species.

MALDI-TOF offers rapid AST using mainly four types of method (Kostrzewa et al. 2013; Sparbier et al. 2016) including (1) detection of resistance peak pattern by comparing analysis of mass spectra of susceptible and resistant strains, (2) studying bacterial-induced hydrolysis of antibiotic by observing mass shifts during incubation of pathogen with antibiotic, (3) examination of newly synthesized proteins by detection of the amount of isotope-labeled amino acids in pathogen incubated in the presence of antibiotic (Sparbier et al. 2013), and (4) measurement of bacterial growth in the presence and in the absence of antibiotics wherein an internal standard is used to compare the mass spectra and results in low peak intensities indicating susceptibility, i.e., lack of growth and intense bacterial protein peaks indicating resistance, viz., normal growth in the presence of antibiotics (Lange et al. 2014).

Expression of enzymes, namely, β-lactamase and carbapenemases, is the most common mechanism providing resistance to pathogens against β-lactam antibiotics and carbapenems by making them inactive. MALDI-TOF has successfully been used to detect drug resistance against β-lactams (Kostrzewa et al. 2013) and carbapenems (Hrabák et al. 2011) by detecting the hydrolyzed antibiotic that remains after the β-lactamase and carbapenemase activity, respectively.

Genes vanA and vanB are the keynote players in coding for proteins providing resistance to vancomycin and teicoplanin. Presence of van A gene provides resistance against vancomycin as well as teicoplanin and vanB against vancomycin only. Many strains of *Enterococcus faecium* are vanB-positive. MALDI-TOF has been used to differentiate between vancomycin-susceptible and vancomycin-resistant enterococci on the basis of the presence or absence of some specific peaks. This showed a sensitivity of 96.7% and a specificity of 98.1% when compared to PCR, the control method (Griffin et al. 2012; Kasper 1976).

4.3.5 Biosensors

Biosensors are rapid analytical devices which consist of two components, namely, biorecognition element for sensing the presence of analyte and transducer for capturing the signal. Recently, biosensing technology has also been used to test drug resistance in various microorganisms. The presence of antibiotics results in changes

in membranes, morphology, metabolism, movements, mass, heat production, and nucleic acid content of bacteria that can be targeted for detection by biosensing technology (Vasala et al. 2020). Several attempts have been made to develop different types of biosensors using various types of biosensing elements like bacteriophages, aptamers, and transducers like electrochemical, piezoelectric, etc.

Guntupalli et al. (2013) developed a lytic phage spheroid-based sensor for detection of methicillin-resistant (MRSA) and methicillin-sensitive (MSSA) *S. aureus* species. Bacteriophage-spheroid interactions were evaluated by a quartz crystal microbalance with dissipation tracking. Bacteria-spheroid interactions result in low resonance frequency and increase in dissipation energy for both MRSA and MSSA. Another study reported the development of a label-free electrochemical DNA biosensor for determination of MDR1 gene. The working of this sensor included the hybridization of DNA by differential pulse voltammetry in terms of monitoring the changes in peak currents based on Au nanoparticles/toluidine blue-graphene oxide (Au NPs/TB-GO) modified electrode. The developed sensor was simple, rapid, and cost-effective with high selectivity and stability and showed a detection limit of 2.95×10^{-12} M (at an *S*/*N* of 3) (Peng et al. 2015). Chen et al. (2020) developed a novel multiplex loop-mediated isothermal amplification linked to a nanoparticle-based lateral flow biosensor (m-LAMP-LFB). This sensor was used to detect all *S. aureus* species and identify MRSA and MSSA strains from clinical samples. The working of LFB was based on the use of two sets of primers specific for femA gene (*S. aureus*-specific gene) and mecA gene (encoding penicillin-binding protein 2a) and analysis of products produced. A function-based biosensing approach for detection of antibiotic resistance has been developed by Mecklenburg et al. (2017). The working of this sensor was based on the identification of resistance enzymes in terms of measurement of thermal signal generated upon enzyme-mediated degradation of antibiotics. This biosensing system was able to differentiate between two AMR β-lactamase enzymes, namely, penicillinase and metallo-β-lactamase. Enzyme thermistor technology was used to create the resistance profile by analyzing a panel of antibiotics. Till date, most of the biosensing technologies reported to detect drug resistance present only proof-of-concept studies. Evaluation of their performance on clinical samples and subsequent progress for faster commercialization still remains a challenge. Currently, the exploration of biosensing technology for their application in rapid detection of drug resistance is further in progress in order to felicitate its faster commercialization.

4.3.6 Immunodiagnostics

Immunodiagnostics constitute one of the highly specific and sensitive approaches in order to felicitate identification of pathogenic bacteria (Verma et al. 2013). In the past decades, several attempts have been made by different researchers to apply immunodiagnostic concept for development of rapid methods for drug resistance detection. Poetz et al. (2018) reported the development of a highly sensitive,

reproducible, and reliable sandwich immunoassay for detection of multidrug resistance protein 1 (MDR1). MDR1 presents a prototypic 12 transmembrane domain transporter. In order to develop this assay, antibodies were produced specifically to target a proteotypic peptide derived from MDR1. The performance of this assay was cross-validated using mass spectrometry. Another study also reported the development of a strip-based immunoassay based on the principle of detection of enzyme conferring drug resistance. The working of this assay included the application of diluent containing bacteria representing antigen on the test position that has previously been loaded with specific antibody. Interaction of antigen and antibody results in the development of a colored band, thus indicating positive test. One such test has been marketed by BinaxNOW PBP2a test (Alere) to detect methicillin resistance in *S. aureus*. This test is highly sensitive and specific and has successfully been applied to blood culture with practically a sensitivity and specificity of 95.4% and 100%, respectively (Pence et al. 2013; March Rosselló and Bratos Pérez 2016). In this line, Volland et al. (2019) also developed a rapid, highly sensitive, and cost-effective lateral flow immunoassay using monoclonal antibodies selectively designed to detect colistin resistance. Colistin resistance has mainly been reported in bacteria due to a plasmid-encoded colistin resistance gene mcr-1. The working of this assay included suspension of bacterial colony in extraction buffer with subsequent dispensing on the cassette and allowing migration for 15 min. The positive results were recorded in terms of appearance of a pink color band. This assay was successfully applied to detect colistin resistance in human and animal enterobacterial isolates showed a sensitivity of 100% and specificity of 98% for detection of MCR-1.

4.3.7 Chemiluminescence and Bioluminescence

In chemiluminescence, emission of light occurs upon return of excited molecules to ground state in some chemical reactions. On the other hand, bioluminescence is a form of chemiluminescence which results from chemical reactions occurring inside living organisms such as fireflies (March Rosselló and Bratos Pérez 2016). Maity et al. (2020) developed a chemiluminescent probe for detection of beta-lactamase, the expression which is known as a major cause of drug resistance. The sensitivity reported with this probe for beta-lactamases has been found to be four times higher when compared with fluorocillin, a commercially available fluorogenic substrate. Similarly, another study carried out by Das et al. (2020) showed in their work development of a carbapenemase-sensitive chemiluminescent probe. This probe facilitates enzyme-mediated breakdown of carbapenem core and subsequently followed by a facile 1,8-elimination process leading to green light emission resulting from faster chemical excitation. Moreover, this probe has successfully been used to detect several clinically relevant carbapenemases in their work.

Chemiluminescence approach has also been applied for direct analysis of drug resistance in microbial culture. Here, menadione being permeable to bacterial membrane is added to microbial culture. After menadione enters into the microbial cells,

it gets reduced and generates several compounds that undergo self-oxidation, resulting in emission of light (March Rosselló and Bratos Pérez 2016). The working principle of this method includes determination of viability of microbial cultures incubated in the presence and absence of antibiotics by comparing their chemiluminescence signal. This approach has been used to detect intermediate and vancomycin-heteroresistant strains of *S. aureus* in 8 h. Moreover, this technique can also be used to detect mycobacteria sensitivity in 4 days (Yamashoji 2002).

Like chemiluminescence, bioluminescence approach compares the bioluminescent signal of microbial cultures incubated in the presence and absence of antibiotics. Adenosine triphosphate (ATP) bioluminescence has been used for rapid AST. Bioluminescent signal is measured in terms of extracellular, intracellular, or total ATP levels of microbial culture. In the past decades, this approach has successfully been used to test drug resistance using direct sample. This method has successfully been used on urine sample. Here, antibiogram using urine sample was made by comparing the ATP signal obtained from two different aliquots of urine sample incubated with and without antibiotic (Ivancic et al. 2008). Application of ATP bioluminescence method has also been attempted on blood culture specimens to test levofloxacin susceptibility with no false-susceptible results in a pilot study (Matsui et al. 2019). Blood corpuscles are known to have abundant intracellular ATP possibly leading to false-susceptible results. Keeping in view, some new interventions including specimen centrifugation with subsequent dilution of supernatant with broth and an ATP-eliminating reagent before addition to bacterial culture have been made to minimize background ATP in this study.

4.3.8 Colorimetric Methods

Various types of colorimetric methods chiefly employing redox indicators, tetrazolium salts, and nitrate reduction principle have been developed to rapidly detect drug resistance. Till date, various types of tetrazolium salts have been evaluated for their use in rapid detection of multiple drug resistance in causal agent of TB. Out of various salts, the compound, namely, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), has widely been used for testing of drug resistance. Mshana and colleagues first suggested its use in development of a colorimetric assay for detection of rifampicin resistance (Mshana et al. 1998; Abate et al. 1998). The working principle of this test includes the reduction of MTT which is usually of yellow color in its oxidized state to purple-colored MTT formazan crystals. This test has also been used earlier to determine the viability of cells (Mosmann 1983). In a study conducted by Mohammadzadeh et al. (2006), 2,3,5-triphenyltetrazolium chloride was used to test antibiotic susceptibility of multidrug-resistant *M. tuberculosis* isolates against isoniazid and rifampicin at a critical concentration of 0.2 μg/mL and 2.0 μg/mL, respectively. Using this method, results were obtained and recorded in terms of color change after 4.9 days.

Colorimetric methods based on the use of indicator dyes record the change in color of the indicator dye in medium with and without antibiotic. The change in color of the dyes occurs as a result of metabolic activity of bacteria during growth (Palomino et al. 2007). This concept of using redox indicators has successfully been reported to offer rapid detection of resistance. In a study carried out by Coban (2012), the crystal violet decolorization assay (CVDA) has successfully been used to test and compare susceptibility of *Mycobacterium tuberculosis* to isoniazid and rifampicin. Similarly, some other studies have shown the determination of susceptibility of pathogens against different antibiotics using resazurin. Coban et al. (2006; Coban 2012) tested the susceptibility of *S. aureus* for methicillin and vancomycin in 6 h using resazurin. The major drawback associated with resazurin is the inability of non-fermenting Gram-negative bacilli to metabolize it.

Another colorimetric method that has widely been used to evaluate multiple drug resistance is nitrate reductase assay. The working of this assay includes the measurement of metabolic activity in terms of nitrate reductase in bacterial culture in the presence of nitrate ions and antibiotics. It is a cost-effective, rapid phenotypic method and has also been recommended by the WHO for rapid detection of drug-resistant TB (Angeby et al. 2002; WHO 2009). It has also been used to test methicillin resistance in *Staphylococcus aureus* (Coban et al. 2014) and rifampin, isoniazid, and streptomycin resistance in *Mycobacterium tuberculosis* (Angeby et al. 2002).

4.4 Conclusions and Future Outlook

The methodologies covered in this chapter are the modern approaches which offer rapid detection of antimicrobial drug resistance when compared to traditional methods. The standard methods including disc diffusion and broth dilution used for determination of antimicrobial susceptibility are mainly based on microbial growth and need lengthy protocols and pure cultures. Keeping in view, in the past decades, several attempts have been made by researchers and are currently in progress to develop rapid AST. Development of new ASTs and possible improvements of existing technologies offering satisfactory performance need to well recognize the limitations of conventional and modern AST methods in order to get their faster approval and marketing. Clinical samples usually are of polymicrobial nature, and only a few growth-based ASTs can work efficiently with such samples. This indicates the need for highly sensitive measurement of microbial growth by online analysis of samples taken after short intervals using techniques like FISH and immunoassays. The WHO-approved MODA and FDA-approved Accelerate Pheno system based on FISH methods should further be validated using diversity of other clinical samples. Techniques like MALDI-TOF offer rapid formation of antibiogram. However, the interpretation of antibiogram further requires the information about bacterial species. Therefore, it is important to increase the number of strains and antibiotics under study to determine the sensitivity and specificity of such methods. Many new AST methods directly detect all types of resistance phenotypes and/or genotypes

present within a clinical sample without culturing, identification, and subsequent study of mechanism underlying resistance. This is not of much significance. Moreover, regulatory bodies like the EUCAST, the Clinical and Laboratory Standards Institute, and the FDA should lay down guidelines for developers and manufacturers of AST and give MIC breakpoints for compliance by diagnostic laboratories. The development of new AST should strictly fulfill the need of some desirable features chiefly including reduced detection time, less cost for faster implementation, and high multiplexing capacity. Approaches focusing on the prevention of infection and antibiotic stewardship should also be given attention.

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Chapter 5
The Use of Antimicrobials in Agriculture and Socioeconomic Considerations in Global Perspective

Himadri Rajput, Swati Dhiman, and Rahil Changotra

5.1 Introduction

The use of antimicrobials in agriculture at large scale is a growing concern globally. The precise measurement of antimicrobials used in agriculture worldwide is difficult; however, the studies suggest that the extent of their use in agriculture is as great as the quantity used by humans. It has been estimated that 40 million pounds of antimicrobials are used every year in the United States, out of which around 0.1% is utilized in the field of plant agriculture (Levy 1992). Also, in the United States, the use of antimicrobials for animals is far greater than in humans, as for animals 70% or even more antibiotics are used. A report by the Food and Drug Administration (FDA) in 2011 stated that in the United States, 93% of the human medically important antibiotics are administered in agriculture via feed or water (FDA 2014). It has been also studied that 75–90% of tested antibiotics are excreted unmetabolized from animals which then enter the water resources (Marshall and Levy 2011). The waste generated from animals possesses resistant bacteria strains and antibiotics that could then foster resistance in bacteria that may pose a greater risk to humans. The manure is often utilized for fertilization of crops which comes from animal farms, a major cause of resistance (Sengeløv et al. 2003). The utilization of antimicrobials in animals for meat production in the BRICS (Brazil, Russia, India, China, South Africa) countries alone could probably double between the years 2010 and 2030. Prolonged exposure to antimicrobials could cause development of drug resistance, and high-risk factors are associated with the huge consumption of

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antimicrobials such as transfer of drug-resistant strains by direct contact and transfer of these strains to humans by the food chain. Fungal diseases are more common and frequent in crops and possess huge threat. Hence, fungicides are employed in considerable quantities for crop protection. In general, they are used on grapes, cereals, and tulip production. Antifungals are widely used for the treatment of human diseases; hence, their increased use in agriculture could impose serious threat of resistance (Azevedo et al. 2015). Approximately three quarters of a million people die every year due to fungal infections. Azole-based fungicides majorly face the problem of resistance and pose threat to humans. Infections due to *Aspergillus* fungus are cured by azole-based oral treatments. If the azole resistance increases, it will highly affect the human health (Kleinkauf et al. 2013).

In the United States, antimicrobial agents of a minimum of 17 classes, including penicillin, lincomycin, tetracyclines, virginiamycin, and macrolides, have been authorized for their potential application in growth promotion or for the improvement of feed efficiency. For better understanding of the consequences due to the utilization of antimicrobials in agriculture on human health, it is important to quantify the used amount of antimicrobials in food animals. However, in the United States, there is no public health reporting system available for the quantification of antimicrobials used for food animals. The Animal Health Institute in the United States has estimated that in 1998, around 18 million pounds of antimicrobials is generated by its member companies for its application in food animals (Animal Health Institute 2000). The Union of Concerned Scientists has provided an alternative estimation in 2001 for the United States and estimated that around 31 million pounds of antimicrobials have been utilized for food animals annually. This organization also estimated that 93% of the antimicrobials which is approx. 28 million pounds used in the field of agriculture is exploited unnecessarily even if there is no disease (Mellon et al. 2001). These initial estimations provide a preliminary perspective on the huge antimicrobial quantities being used for food animals in the United States. However, more precise and accurate data is needed on the amount and motive of antimicrobials used for food animals.

For the case of human medicine, utilization of antimicrobials for agriculture possesses a selective pressure due to the problem of antimicrobial-resistant bacteria, including human pathogens, animal pathogens which have food animal reservoirs, and bacteria that are also present in food animals (Cohen and Tauxe 1986; Levy 1997). The transfer of these bacteria to humans may occur either by the food supply or through direct animal contact (Witte 1998). Once antimicrobials enter into the environment, prediction of their degradation time is very difficult as their chemical structure is very diverse, whether they come from human/animal use or from manufacturing. Some of the antimicrobials may degrade very easily, while others may react with organic matter and persist in the environment in their active form for longer periods. This opens the scope and need for further advanced studies on this serious issue.

5.2 Antimicrobials in Agriculture

The US Environmental Protection Agency (US-EPA) regulates the use of antimicrobials in agriculture, and Food and Drug Administration regulates all the other uses of antimicrobial. Streptomycin and oxytetracycline are the only two EPA-approved antimicrobials for their use in agriculture (Vidaver 2002). These two antimicrobials are grouped with fungicides and are used as prophylactic treatments. Oxytetracycline is approved for its use on four fruit crops, while streptomycin is approved for its use on 12 fruits, ornamental fruit crops, and vegetables as shown in Table 5.1.

Erwinia amylovora, a relative specie of *E. coli* and enteric bacteria, causes a major plant disease called fire blight. For the treatment of fire blight, spray treatments have been employed for 3–4 days by streptomycin and/or 4–6 days by oxytetracycline as prophylactic treatment to prevent damage during the blossom time (Johnson and Stockwell 1998). Around 53,000 hectares of antimicrobials are sprayed annually for the protection of crops (Vidaver 2001). Depending upon the type of specie, the blossom time might be extended to 6 weeks or even more by employing prophylactic treatment. The studies conducted in the past have shown that there are no streptomycin residues in detectable amount in the fruits at the time of harvest; however, the activity of streptomycin was detectable in the leaves (Shaffer and Goodman 1969). In 1992, a fact sheet was published by the EPA on streptomycin which indicated that the drug is nontoxic to freshwater invertebrates, honeybees, and birds while showing slight toxicity toward fish (warm-water and cold-water species) (US EPA 1992). In 1993, a fact sheet was published by the EPA on oxytetracycline which indicated that the drug has nontoxic effects on fishes, honeybees, birds, and aquatic invertebrates (US EPA 1993). The concentration recommended for streptomycin ranges from 50 to 200 mg L^{-1}, based on the type of crop and severity of infection. In treating fire blight-infected apples and pears, streptomycin application dose of ~2–4 L hectare^{-1} is suggested. The recommended concentration of oxytetracycline is 150–200 mg L^{-1}. For the treatment of nectarines and peaches, the application dose of 240 gallons acre^{-1} can be employed. This dose can increase for large-sized trees but shall not exceed 500 gallons acre^{-1} per application. For treating pears, 50–100 gallons acre^{-1} of solution is recommended.

For agriculture, the use of gentamicin in Latin America is of increasing concern as the antimicrobial quantity used in this region is unknown and the extent of human exposure is also not known. The US-EPA along with the American Society for Microbiology have instructed that the gentamicin-treated food products (vegetables/fruits) must not be imported as this drug plays a very important role in the human medicine. These measures have been taken as the unnecessary residue of this drug on food could cause resistance to this antimicrobial drug which is among the few economically available drugs used against bacterial infections in human. Till now, there is no data available on the use of this drug in the areas of Latin America and on the antimicrobial resistance (AMR) (Vidaver 2002). Table 5.2 represents the region wise use of drug gentamicin for agriculture. The relative utilization of antimicrobials in the field of agriculture without the implementation of better and

Table 5.1 Registered antibiotics for agricultural utilization in the United States (Agri-Mycin 17 2001; Mycoshield 2001; US EPA 1992, 1993; Vidaver 2002)

Crop	Disease	Disease-causing agent	Approved treatment	
			Streptomycin	Oxytetracycline
Food and/or feed crops				
Apple	Fire blight	*Erwinia amylovora*	√	√
Celery	Bacterial blight	*Pseudomonas cichorii*	√	–
Pear	Fire blight	*E. amylovora*	√	√
Tomato	Bacterial spot	*X. campestris* pv. *Vesicatoria*	√	–
Bean	Halo blight	*Pseudomonas syringae* pv. *phaseolicola*	√	–
Pepper	Bacterial spot	*X. campestris* pv. *vesicatoria*	√	–
Crabapple	Fire blight	*E. amylovora*	√	–
Peach	Bacterial leaf, fruit spot	*X. campestris* pv. *pruni*	–	√
Quince	Fire blight	*E. amylovora*	√	–
Nectarine	Bacterial leaf, fruit spot	*Xanthomonas campestris* pv. *pruni*	–	√
Potato	Bacterial soft rot	*E. carotovora* subspecies *carotovora*, *E. chrysanthemi*	√	–
Nonfood crops				
Tobacco	Wildfire	*Pseudomonas syringae* pv. *tabaci*	√	–
Sugar beets	Bacterial rot/ blight	*Erwinia* spp.	√	√
Ornamental herbaceous plants, *vines*, *shrubs*, and *greenhouse ornamentals*				
Roses	Crown gall	*Agrobacterium tumefaciens*	√	–
Anthurium	Bacterial blight	*X. campestris* pv. *dieffenbachiae*	√	–
Quince	Fire blight	*E. amylovora*	√	–
Cotoneaster	Fire blight	*E. amylovora*	√	√
Pyracantha	Fire blight	*E. amylovora*	√	–
Elm	Lethal yellows	*Phytoplasma*	–	√
Hawthorn	Fire blight	*E. amylovora*	√	–
Philodendron	Bacterial leaf spot	*X. campestris* pv. *dieffenbachiae*	√	√
Dieffenbachia	Bacterial stem rot	*Erwinia* spp.	√	–
Palm	Lethal yellows	*Phytoplasma* sp.	–	√

Table 5.2 Utilization of drug gentamicin for agriculture in different countries (Vidaver 2002)

Country and crop	Disease	Disease-causing agent
Mexico		
Watermelon	Bacterial spot	*Xanthomonas* sp.
Potato	Black leg	*E. carotovora* subspecies *atroseptica*
Tomato and chili	Bacterial spot	*X. campestris* pv. *vesicatoria*
Apple, pear, and ornamentals	Fire blight	*E. amylovora*
Agave	Bland rottenness of the heart of agave	*Erwinia* spp.
Chile		
Pear	Fire blight	*Erwinia amylovora*
Tomato	Bacterial canker	*Clavibacter michiganensis* subspecies *michiganensis*
Central America (Honduras, Guatemala, Costa, Rica, El Salvador)		
Cauliflower and broccoli	Bacterial soft rot	*Erwinia* spp.
Potato	Blackleg	*Erwinia carotovora* subspecies *atroseptica*
Chili	Bacterial spot	*Xanthomonas campestris* pv. *vesicatoria*
Cabbage	Bacterial black rot	*X. campestris* pv. *campestris*
Tomato	Bacterial speck	*Pseudomonas syringae* pv. tomato

appropriate policies is likely to expand sooner due to the increasing economic growth and food demand.

5.2.1 AMR due to Manure

High frequency of resistance has been observed toward several metals such as nickel, zinc, cobalt, and cadmium and toward the antibiotics like streptomycin, spiramycin, ampicillin, and olaquindox in copper-resistant isolates as compared to the copper-sensitive bacteria isolated from agricultural lands where pig manure with copper contamination had been used (Huysman et al. 1994). Pig manure is a public health hazard as it is a major source of resistance genes. In addition to the utilization of antibiotic agents, utilization of heavy metals in aquaculture and animal farming might further promote the AMR via co-selection. When the soil is fertilized with antibiotic-contaminated manure and sewage sludge, the transfer of antibiotics occurs to the soil and water (Heuer et al. 2011). The utilization of metal-contaminated fertilizers, liquid manure, and sewage sludge is a common practice followed in European countries and in other parts of the world. Due to these applications, heavy metals such as Cd, Pb, Cu, Hg, Ni, Zn, and Cr are transferred to the arable soil (Han et al. 2002). Pesticides containing Cu have been used in organic and conventional agriculture due to the bactericidal and fungicidal properties of Cu (Nemecek et al.

2011). Also, metals like iron, cobalt, manganese, Zn, and Cu have been used as nutritional supplements in animal feed for fish production and livestock farming in European countries. The European Union demonstrated that the major contributor of Zn and Cu metal pollution in the environment is the aquaculture and agriculture activities. It was estimated that the no effect amount of Zn and Cu will be exceeded in some of the water and soil systems within the coming 10–50 years (Monteiro et al. 2010). Agriculture has been also identified as a major source of Zn and Cu contamination of arable soil in Wales and England (Nicholson et al. 2003). Moreover, 30% of the Cd identified in the agricultural soil comes from inorganic fertilizers. Manure is one of the major antibiotic pollution sources existing in the environment, and China annually produces almost 618 billion kg of swine manure (Wang et al. 2006). Most of the veterinary antibiotics used for animals are poorly absorbed by them and are excreted and dispersed in soil when the manure is used in farming land as fertilizers (Alcock et al. 1999). The utilization of subtherapeutic levels of antibiotics for animal feeds leads to an increased antibiotic resistance traits in manure (Looft et al. 2012), manure-amended soils (Ghosh and LaPara 2007), and river waters and sediments (Pruden et al. 2012). Moreover, several metals are added to swine feeds for disease control and as a growth promoter which may impart a long-term pressure for antibiotic resistance (Baker-Austin et al. 2006).

5.2.2 Fate of Antimicrobials in Agriculture

Pharmaceutical drugs used for humans, animals, fragrances, sunscreens, and cosmetics are discharged in raw sewage water after their regular use. These drugs are inefficiently treated in the sewage treatment plants and hence make their entry in water resources (Verlicchi et al. 2012). It has been observed that intensive livestock activities and farming practices are majorly responsible for the entry of pharmaceuticals in the environment either in their active form or in their unmetabolized form (Bottoni et al. 2010). Worldwide, the application of recycled organic manure is increasing which also has its environmental implications (Motoyama et al. 2011). Manure is widely used in agriculture as a valuable fertilizer as it possesses essential nutrients needed by plants like phosphorus, nitrogen, potassium, organic carbon, etc. The excessive utilization of manure obtained from medicated animals in the farming lands is a major route through which pharmaceuticals enter the environment and ultimately to water reservoirs through runoff and percolation, respectively. The main concern related to the entry of these pharmaceuticals in the environment is better understanding of their final fate as once they enter in the system their fate is governed by various factors (Kemper et al. 2008). The major concern related to pharmaceuticals entered into the environment is that they may have potential biological effects on aquatic life and human health (Howard and Muir 2011). The presence of antibiotics has been observed in German soils where swine liquid manure has been applied as fertilizer, and 15 μg kg^{-1} of sulfadimidine was also detected suggesting the stability of sulfadimidine in the soil (Christian et al. 2003). In China,

the presence of pharmaceuticals has also been detected in the soils where cattle, swine, and chicken manures were employed as organic fertilizers (Wang et al. 2014). The highest concentrations of pharmaceuticals detected in the soils where pig manure was used were oxytetracycline, chlortetracycline, and sulfamethazine. Oxytetracycline was detected in the soils where cattle manure was used. However, high concentration of quinolones and tetracyclines was observed in the soils where chicken manure was used (Wang et al. 2014). Sulfachloropyridazine was detected in the soils where poultry manure was used indicating that this can be the main source of soil contamination by such antimicrobial compound (Karcı and Balcıoğlu 2009).

5.3 Antimicrobials as Growth Promoters

Since the early 1950s, antimicrobials have been utilized in the agriculture field including livestock and poultry for the treatment of infections and to promote growth and feed efficiency. There has been always a developing controversy toward the utilization of pharmaceutical drugs as growth promoters. These pharmaceuticals have been used at low concentration in animal feeds which improves the product quality with low-fat percentage and higher protein content in meat. When antibiotics are used as growth promoters, there are several other associated advantages such as control of zoonotic pathogens like *Escherichia coli*, *Salmonella*, *Enterococcus*, and *Campylobacter*. The utilization of drugs as growth promoters imparts a selection pressure for bacteria which are resistant to antibiotics and may be used in human or animal treatment. It has been observed in the 1940s that when pigs and chickens were treated with the broad-spectrum antibiotics, the size and amount of meat produced increased. Afterward, antibiotics were widely applied as growth promoters even after the detection of antibiotic resistance in farm animals in the year 1951. In countries where this practice is followed including the United States, more than half of the antibiotics administered to the animals are for increasing the growth. The utilization of antibiotics for growth promotion purpose is most common in pigs. Almost 30% of the world's pork is produced by China, and its farmers utilize antibiotics four times more than their US counterparts for producing the same amount of meat (Cully 2014). It has been reported that resistance genes have been increased up to 28,000-fold in soil and manure at the Chinese pig farms (Zhu et al. 2013). These resistance genes can easily get transferred from bacterial specie that infects animals to the species that infect humans. It was observed that overuse of antibiotics in livestock could lead to larger contribution toward antibiotic resistance. In the United States, pigs are often exposed to antibiotics like lincosamides, teracyclines, penicillins, macrolides, and erythromycin. All these pharmaceuticals are also employed in humans for the treatment of infections. Pigs in the United States are exposed to a range of compounds such as pleuromutilins, bacitracin, virginiamycin, flavophospholipol, and arsenical compounds for the purpose of growth promotion. In the United States, flavophospholipol and virginiamycin are employed as cattle growth promoters, which are also used as poultry growth promoters. Cattle are also

treated with ionophores like monensin for growth promotion purpose, and poultry are exposed to arsenical compounds for growth promotion.

The accurate quantity of used antimicrobials in agriculture is difficult to estimate; however, a considerable amount of subtherapeutic doses has been provided to food animals for disease protection and growth promotion (Mellon et al. 2001). The World Health Organization (WHO) has recommended stopping the use of antimicrobial growth promoters in agriculture, which are used in human medicine (World Health Organization 1997, 1999). The Institute of Medicine (USA) has made the same recommendation in the year 2003 (IOM 2003). Various European countries have also taken measures toward the restricted use of antimicrobial growth promoters in agriculture which are used for human treatment. Due to the problem of antimicrobial resistance, in the year 1999, farmers from Denmark willingly stopped the usage of antimicrobials as growth promoters (Aarestrup et al. 2001). This voluntary action has led to the 60% reduction (from 206 to 81 tons) in the amount of antimicrobials used for food animals annually (DANMAP 2001; Sørensen et al. 2002). The antimicrobial agents used as growth promoters which are also used for human medicine such as bacitracin, tylosin, virginiamycin, avoparcin, and spiramycin are banned by the European Union in the year 2001 (European Commission, Belgium). Also, in the European Union, the Health Ministries have agreed to stop the utilization of all antimicrobial growth promoters by the year 2006.

Several studies have been performed to assess the effect of this ban, and no negative impact has been observed in the profit of farmers and on animal health in broiler chicken. The similar conclusions were made in the case of fattening pigs; however, incidences of diarrhea in weaned piglets were observed to increase which required other modifications like changes in the feeding and weaning processes (WHO report). In 1986, Sweden completely banned the utilization of antimicrobials as growth promoters which lead to 55% decrease in the total usage without affecting the long-term productivity. This showed that competitive production can be achieved without using antimicrobial agents as growth promoters (Wierup 1998; Greko 1999). The complete elimination of antimicrobial use as growth promoters has led to a significant decrease in the resistance in humans, animals, and food products (Bager et al. 1999; Klare et al. 1999).

The National Antimicrobial Resistance Monitoring System (NARMS) has been launched in the year 1996 for monitoring the resistance in foodborne pathogens against the use of antimicrobials. The NARMS is collaboration between the US-FDA Center for Veterinary Medicine and Centers for Disease Control and Prevention (CDC) and state/local health departments. Moreover, the Foodborne Diseases Active Surveillance Network (FoodNet) performed studies in ten states on the basis of population for estimating the sources and impact of specific foodborne infections (Angulo et al. 2004). The occurrence of antimicrobial resistance in non-Typhi *Salmonella* has been monitored using NARMS since 1996. In the year 1996, the study was done on *Salmonella* isolates collected from 14 sites against 14 antimicrobials, and 164 out of 1527 isolates showed resistance toward five or more antimicrobial agents (Centers for Disease Control and Prevention 2003). The multidrug resistance (MDR) strains have posed a threat to the world which is growing day by

day. In the United States and other countries, MDR *Salmonella* serotype (DT 104) has shown its resistance toward various drugs like streptomycin, ampicillin, sulfonamides, chloramphenicol, and tetracycline (ACSSuT). This shows the increasing development of *Salmonella*-resistant clone having the ability to spread among animals and subsequently to humans. Another MDR strain of *Salmonella* which is commonly emerging is *Salmonella* Newport, which is resistant to several drugs such as chloramphenicol, tetracycline, ampicillin, sulfamethoxazole, cephalothin, streptomycin, amoxicillin/clavulanate, ceftiofur, and cefoxitin. The MDR strain possesses a decreased susceptibility toward the drug ceftriaxone, i.e., MIC $\geq$16 μg mL^{-1}, which is termed as MDR *S.* Newport Amp-C. The NARMS has been employed to monitor the extent of resistance among *Campylobacter jejuni*, which is the most common strain of *Campylobacter* and *Campylobacter coli* in the United States since 1997. In the monitoring process, isolates from five different sites were considered, and 28 isolates out of 217 were found to be resistant toward the drugs like ciprofloxacin and fluoroquinolone (Centers for Disease Control and Prevention 2003).

The use of antimicrobials in agriculture can significantly affect the treatment employed to cure human diseases. In the United States, increasing resistance has been noticed in the foodborne pathogens like *Campylobacter* and *Salmonella* (Centers for Disease Control and Prevention 2003) which limited the use of therapeutic agents and increases the probability of treatment failures and undesirable clinical outcomes (Travers and Michael 2002). Also, patients who are on antimicrobial drugs for their treatment are at high risk of developing antimicrobial-resistant foodborne infection (Barza and Travers 2002). To maintain the effectiveness of antimicrobial agents, their appropriate utilization in food animals and humans is of extreme importance. The fact cannot be denied that the utilization of antimicrobials in food animals is important for their health; however, the effectiveness of antimicrobial agents which are used in human medicine must also be preserved for their long-term effectiveness.

5.4 Clinical Implications

One of the major consequences of increased antimicrobial resistance in foodborne bacteria on human health is the increase in cases of foodborne illness. The antimicrobial-resistant foodborne pathogens may cause human infection due to the interaction between the antimicrobial treatment, the antimicrobial-resistant *Salmonella*, and the host flora. The resistant bacteria might get a short-term advantage as the treatment might suppress the activity of normal protective flora. If the infection causing pathogen strain is resistant to the antimicrobial drug used for its treatment, it may lower down the infectious dose for *Salmonella* (Barza and Travers 2002). An antimicrobial-resistant *Salmonella* outbreak analysis has suggested that the parallel subjection of antimicrobial agents could lead to increase in the number of cases if the outbreak is caused by sensitive strain (Cohen and Tauxe 1986). A

study conducted in the early 1960s showed that mice with "undisturbed" normal intestinal flora have approx. 106 organism *Salmonella* infectious doses (Bohnhoff and Miller 1962). However, when the normal intestinal flora was "disturbed" by administering streptomycin, the streptomycin-resistant *Salmonella* infectious dose was decreased to merely ten organisms. In the *Salmonella* outbreak studies, it was concluded that the antimicrobial treatment which is unrelated to *Salmonella* infections can cause human infections with either susceptible (Pavia et al. 1990) or resistant *Salmonella* (Holmberg et al. 1984; Spika et al. 1987). When the sporadic salmonellosis was studied, it was suggested that the previous treatment using antimicrobial agent imposes the risk for acquisition of antimicrobial-resistant infections, when compared to the susceptible infections (Riley et al. 1984; Lee et al. 1994).

Personnel in the field of medicine must be aware that as the resistance of foodborne pathogens increases, the treatment of patients using antimicrobial agents enhances the possibilities for patients to develop infection soon after the treatment. Based on resistance frequency and antibiotic administration frequency, the public health impact could increase illness cases and larger outbreaks (Travers and Michael 2002). In addition to causing increased illnesses in humans, the increasing resistance may lead to unsuccessful treatments when a resistant antimicrobial agent is employed for the treatment. Hence, efforts have been made to increase the optimum utilization of antimicrobial agents in human medicine, for example, the American Academy of Pediatrics suggested very conscious and selective utilization of antimicrobials for treating upper respiratory infections in children (Dowell et al. 1998). The problematic issue of resistance is faced by the antimicrobial agents generally utilized for treating major *Salmonella* infections, such as fluoroquinolones for adults. An example of treatment failure due to drug resistance has been reported in Denmark, where an outbreak of *S. typhimurium* DT104 leads to contaminated pork (Mølbak et al. 1999). The *Salmonella* isolates from pork and human samples exhibited reduced response toward the drug fluoroquinolones which lead to the death of two patients where fluoroquinolones were used for their treatment.

5.5 High-Throughput Methods in the Study of AMR in Agriculture

There is very limited knowledge available on the transmission modes of antimicrobial resistance in between the agricultural sites and to humans through food chain. Also, limited information is there concerning the health risks to humans due to agricultural release of antimicrobial resistance genes (ARG), antimicrobial agents (AMA), and antimicrobial-resistant bacteria (ARB). Food animals are one of the main modes of AMR transfer and are the endpoints in the selection, spread, and dissemination of ARG and ARB. In determining the ARG and ARB profiles of pathogenic bacteria, surveillance laboratories are using culture- and PCR-based techniques. The major limitation associated with these methods is the identification

is possible of those bacteria which are capable to grow under laboratory conditions, and prior knowledge of resistant genes is required for the primer designing. There are a large number of resistance genes present in the environment; hence, PCR runs needed to test the sample for all genes are also large which is not practically feasible and is not cost-effective either. However, with time the advancement in molecular biology has enabled the utilization of other methods for screening the total DNA of a sample for ARG, AR-carrying plasmids, and antibiotic resistance proteins. Nowadays, chip-based AR gene detection is also feasible which has significantly reduced the DNA sample screening cost for several numbers of resistance genes. This also eliminated the need of bacterial isolation resulting in reduced detection time and cost (Zhu et al. 2013). The next-generation sequencing (NDS) is capable of detecting millions of resistance genes present in a DNA sample. However, there are few limitations associated with this method such as requirement of minimum copy number of a gene needed to be detected. Pacific Biosciences has applied a method known as single-molecule real-time sequencing (SMRT) for plasmid genome sequencing which can perform the sequencing of long fragments (40 kb) in one read. PLACNET (plasmid constellation networks) tool has solved the issue of plasmid construction and has made it feasible. Other methods utilize functional metagenomics for the identification of novel resistance genes. This method needs longer time than that of metagenomics but has the advantage of identification of resistance phenotype at start. Microarray expression analysis has helped in the expression profiles of bacteria which are under antibiotic stress. The more recent and advanced versions of transcriptomics and meta-transcriptomics tools enable the identification of gene transcripts present in the sample. However, these methods are not common as whole-genome sequencing and metagenomics, but they are capable in the identification of function genes present in the sample.

5.6 Agriculture, AMR, and Socioeconomic Aspects

AMR has created a very significant economic and health burden on the society, and due to AMR, bacterial infections are untreatable (Stewardson et al. 2016). The threat of AMR is increasing due to inappropriate utilization of antibiotics (Cheng et al. 2012), poverty (Alividza et al. 2018), poor sanitation practices (Hendriksen et al. 2019), international travel (Frost et al. 2019), and increased healthcare interventions (Chatterjee et al. 2018). Efforts and future research are required to assess the contribution of AMR toward the occurrence of diseases and to develop efficient strategies. For effectively tackling the rapidly increasing issue of AMR, estimation and understanding of global burden of disease are most important. On the basis of these estimations' strategies could be decided for evaluation of interventions, decisions on resource allocation, comparisons between countries, driving research priorities, and comparisons with other diseases (Hay et al. 2018). Various studies have been reported on the national and regional burden of AMR including the estimations from the US CDC (Centre for Disease Control and Prevention (CDC) 2013) and the

European Centre for Disease Prevention and Control (ECDC) (Cassini et al. 2019) and from Thailand (Pumart et al. 2012). However, the direct comparison of these estimations is not possible as each of the study utilizes different data source, case definitions, and methods to calculate the burden of disease. A report from the UK government (2014) has estimated that approximately 700,000 annual deaths may occur globally due to AMR bacterial infections, MDR, and drug-resistant tuberculosis (Hay et al. 2018). Various good quality surveillance AMR networks are present in low- to middle-income countries (LMICs); however, there is a need to widen and deepen the surveillance. The WHO has launched the Global Antimicrobial Resistance Surveillance System (GLASS) for developing a standard approach for the collection, analysis, and sharing of data on AMR globally. This initiative taken by the WHO has established a national AMR monitoring system which provides a reliable platform for the collection of data which allows the monitoring of followed trends and estimation of future interventions. An international collaborative effort has been made with the Global Research on AntiMicrobial resistance (GRAM) study combined with Oxford University's tropical medicine and Big Data Institute expertise with the Global Burden of Disease (GBD) framework at the Institute for Health Metrics and Evaluation at the University of Washington to map and determine the burden of AMR globally (Hay et al. 2018). The main challenge is production of reliable estimates of global AMR burden which greatly depends upon the selection of methodology (Limmathurotsakul et al. 2019). Disability-adjusted life years (DALYs), excess length of stay, annual mortality, and years of life lost are all important matrices which have several calculation approaches. The global burden of disease framework utilizes a single cause of death mentioned in the death certificate for the estimations of mortality (GBD 2017 Causes of Death Collaborators 2018). This method has the advantage of being easily understandable; it can utilize data from international death certificate and also avoids double counting of deaths. However, this method is not very suitable for the estimation of AMR as the cause of death in the certificate may be different from the actual cause of death. The best approach for the estimation of AMR burden may vary for community-acquired versus hospital-acquired infection and high-income countries versus LMICs.

5.7 Conclusions

Antimicrobial resistance is an emerging public health issue in front of the world. The widespread presence of drug resistance is rapidly rising worldwide which can have serious medical implications. To avoid the problem of resistance, appropriate utilization of antimicrobials for humans and animals must be practiced which will also need the collaborative effort by partners from different fields such as public health communities, veterinary, farming, and medical. To monitor the preventive efforts, increased surveillance is needed for antimicrobial use and resistance problems. The promotion of veterinarian's education for the appropriate utilization of antimicrobial agents has been suggested. In the United States, Public Health Action

Plan outlined collaborative federal actions for the antimicrobial resistance in agriculture and published the report in 2001 (Interagency Task Force on Antimicrobial Resistance). The suggested actions mentioned in the plan includes increased surveillance rate of antimicrobial utilization and drug resistance, enhanced research and education, and refining and implementing the FDA's Framework being on the top priority item. This also proposed an improvised approval process for the utilization of antimicrobial drugs in animals (Food and Drug Administration–Centre for Veterinary Medicine). The actions were taken for ensuring the safety of humans toward antimicrobial drugs used for animals by prioritizing the utilization of these antimicrobials on the basis of their importance in human treatment. The large-scale utilization of antimicrobial drugs for food animals may lead to increased drug resistance in microorganisms, which ultimately can be transferred in humans. The potential increase in the treatment failures is the major adverse health consequence of transfer of resistant bacteria in humans. For controlling and avoiding this public health issue, the appropriate use of antimicrobials for food animals and humans must be promoted and regulated. Adherence to the guidelines is an important factor for the appropriate utilization of antimicrobials in food animals for reduction in the resistance.

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Chapter 6
Epidemiology of Microbial Infections

Kanika, Aneesh Ali, Md. Meraj Ansari, and Rehan Khan

6.1 Introduction

The emergence of infectious disease and even re-emergence of deadly infectious disease are found to be a threat to public health and welfare. Microbes and hosts that include humans are interdependent for their survival. They also represent a close connection between microbes and humans as half of human DNA originated from viruses. The skin, gut, and mucous membranes are occupied by microbes.

The germ theory of disease was found to be essential in identifying the disease that is caused by microbes and also aids in finding the ways that prevent the infection through sanitation, immunization, etc. Human civilization has a major role in the spread of microbial infection, and it has become the major cradle of humans travailing from both morbidity and mortality. Urbanization and large settlements lead to play a major role in the spread of microbial infection. Large pandemics such as smallpox, cholera, plague, and other diseases like tuberculosis and syphilis have been affecting the human throughout the ages.

The spectrum of occurrence of diseases in a particularly specified population comprises the presence of sporadic methods of occurrence: endemic indicates that there is continuous occurrence, epidemic implies that there is a considerable increase of disease, and ultimately pandemic means that occurs in several nations.

In a particular defined population, the study of determinants, distribution of health and disease, and occurrence is known as epidemiology. The study of

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epidemiology can be descriptive, analytic, and experimental. It exploits the problem-solving by developing a case; hypothesizing; analyzing the data by time, place, and person; preparing the public report; and lastly evaluating control and taking different preventive measures (Hennekens and Mayrent 1987; Beaglehole et al. 1993).

An epidemiology is a science that gives descriptive information regarding rate determination and measurement of the diseases occurring in certain populations. The major goal of this is to find out the effective measures for control of disease by defining the various parameters including risk factors, etc. MacMahon and Pugh 1970).

Microbial agents can grow into two main compartments that are intracellular and extracellular. The intracellular pathogens invade the host cell to replicate and can be divided into two parts: one that replicates freely in the cell such as viruses and some bacteria and another one that replicates in cellular vesicle such as mycobacteria. There are major unique features of a pathogen that include its mechanism of replication, its pathogenesis, and its modes of transmission that majorly affect the pathogenicity.

6.1.1 Chain of Infection

The chain of infection, i.e., how infections initiate and further spread to the human body, includes three main agents, which are the etiologic agent; the transmission method that can be through air, vector, etc.; and lastly the host:

6.1.1.1 Etiologic Agent

The agents that come under this category are microorganisms that have the ability to cause a disease. The microorganism's virulence and invasiveness depict its pathogenicity. The other characteristic that describes the agent is the infecting dose site of entrance of the organism into the host and host species. The other is its agent specificity. The site where the organism resides, metabolizes, and multiplies is known as the reservoir of an organism.

6.1.1.2 Transmission Methods

How agents spread through the source to the host is known as the method of transmission. The methods include via contact, by air, or vector:

6.1.1.2.1 Contact Transmission

In this method, the spread is through air droplets directly or indirectly. The transmission that used to take place when the transmission is via from source to host without the involvement of intermediate object also known as the person-to-person transmission is called direct contact. An example is the hepatitis virus that is transmitted by hand contact. The transmission that takes place from a source to a host via a means of an inanimate object is known as indirect transmission. An example is the transmission of *Pseudomonas* organism via shaving brush.

6.1.1.2.2 Common Vehicle Transmission

The transmission that takes place through the inanimate vehicle leads to multiple cases resulting from such exposure. This category of transmission includes the food or water that acts as the vehicle of infection. An example is waterborne *shigellosis* that results from the use of intravenous fluid that was contaminated with Gram-negative bacteria.

6.1.1.2.3 Airborne Transmission

The spread of infection is by dust or droplet nuclei. They are found to be light enough to be transmitted by more than 3 feet from the source. The major airborne disease is tuberculosis in which the source is a coughing patient who creates aerosols that contain tubercle bacilli.

6.1.1.2.4 Vector-Borne Transmission

In this transmission, arthropods act as vectors and these can be external or internal, for example, *Salmonella* organisms and malaria parasites in the mosquito vector. The transmission of infection can be by more than one route like *Salmonella* transmitted by food or human carrier.

6.1.1.3 Host

The entrance of microorganisms is through the skin, lungs, and gastrointestinal tract or may enter the fetus through the placenta. The entrance of organisms through the skin, mucous membrane, genetic factors, nutrition, and the presence of other diseases comes under the category of nonspecific defense mechanism. The other crucial factor that affects the chain of infection is temperature, air velocity, low humidity, and ultraviolet radiation that can kill the microorganism (Mandell et al. 1990; Bennett and Brachman 1992; Benenson 1995).

The major microbial causes of infection can be explained as infection can be caused by different sets of vectors that include bacteria, viruses, fungi, or parasites. The particular infection can be endogenous which means that it occurs when a microorganism penetrates the skin or mucosal barrier as a result of surgery. In contrast, where the microorganism is acquired from the environment is known as exogenous infection (Koneman et al. 1997).

6.2 Mechanism of Microbial Infection

6.2.1 Mechanism of Bacterial Infection

Many bacterial pathogens have many similar mechanisms of infection like having the ability to adhere, invade, and cause damage to host cells and establish an infection. There are various characteristics that indicate the pathogenicity of bacteria including the adherence to host cell, transmissibility, invasion of the host cell and tissues, and ability to evade the host' immune system. Numerous bacteria are found to be the vital cause of the disease which include *Streptococcus pneumoniae* and *Staphylococcus aureus*, but in contrast bacteria like *Salmonella typhi* are pathogens, but their infection became latent and the host acts as the carrier. Hence, several mechanisms have been used by the bacteria to cause disease in human hosts. The various mechanisms of bacterial infection are as follows:

6.2.1.1 A Bacterial Pathogen After Encountering A Human Host Initiates Several Mechanisms To Evade the Host defenses:

1. Various components present on the bacteria are capsules that will interact with the host even though it acts as frustrated phagocytosis that causes enhanced inflammatory response leading to more tissue damage; hence, more WBCs are recruited to the infection site. It aids in protecting the pathogen from neutrophil engulfment and macrophage. *Streptococcus pneumonia* (pneumococcus), *Neisseria meningitides* (meningococcus), and *Pseudomonas aeruginosa* are found to be capsules producing bacteria (Liu et al. 1971; García et al. 1999).
2. The cell wall of the bacteria and its toxic components play a crucial role in the pathogenesis of bacterial septic shock. Indeed, lipopolysaccharide, a large amphiphilic molecule present in the outer membrane of the Gram-negative bacteria, aids in triggering the event of septic shock that is found to be the combined action of complement components, cytokines, and coagulation cascade. Gram-negative bacteria, like *E. coli*, *P. aeruginosa*, and *Meningococcus*, and Gram-positive bacteria, like *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus,* are commonly involved in septic shock (Walker 1998).

3. Bacteria also produce toxins that act as biological weapons to destroy or damage the host cell. Toxins can be non-proteinaceous also known as endotoxins that are lipopolysaccharide; another one is proteinaceous toxins known as exotoxins that are delivered to eukaryotic cells. Several different species of bacteria contain A-B toxins including *P. aeruginosa*, *E. coli*, *Vibrio cholera*, *Corynebacterium diphtheriae*, and *Bordetella pertussis* (Finlay and Falkow 1997).
4. Adhesins are microbial adherence factors and can be made from polypeptides or polysaccharides. The main step in host-pathogen interaction is the adherence of the pathogen to the host surfaces like the skin, mucous membranes, and deeper tissues. Hence, after the adherence, pathogen would be able to start its biochemical process that includes toxin secretion, host cell invasion, and activation of host cell-signaling cascades that lastly result in disease. Gram-negative bacterial pathogens, in particular, rely on fimbriae for adherence. Examples include *E. coli* (for both urinary tract infections and gastroenteritis), *P. aeruginosa*, and *Neisseria* species (Hahn 1997; Donnenberg 2000; Merz and So 2000).

6.2.1.2 Invasion

To further perpetuate the infection cycle, pathogens gain deeper access into the host termed as an invasion. This invasion can be both extracellular and intracellular. Extracellular invasion happens while remaining outside of host cells in which the microbe breaks down the barrier of the tissue to disseminate in the host. For example, this strategy is used by group A b-hemolytic *Streptococcus* and *S. aureus*.13. In intracellular invasion, the microbe survives within the environment and penetrates the cells of host tissue. The bacteria that show this intracellular nature are Gram-negative, Gram-positive, and mycobacterial pathogens. Some pathogens have an obligate intracellular life cycle which requires a mammalian cell for growth. These include *Chlamydia* spp., *Rickettsia* spp., and *Mycobacterium leprae*. This specific biochemical cross talk has become vital for host and pathogen interaction and meant essential for the penetration of host cells. A type 3 secretion mechanism, which injects bacterial signaling proteins into the host cell, is a common technique used by pathogens to induce uptake. The pathogen may be present in the host cell cytosol, phagolysosomes (phagosomes that have fused with lysosomes), or phagosomes that have not fused with lysosomes (Walker 1998) Fig. 6.1.

6.2.2 Mechanism of Viral Infection

The process by which infection through virus leads to disease is called pathogenicity that comprises of various steps:

The steps include the entry of virus followed by replication and ultimately virus shedding where spread and multiplication to target organs take place. This mechanism of pathogenicity is affected by so many factors that are its determinant,

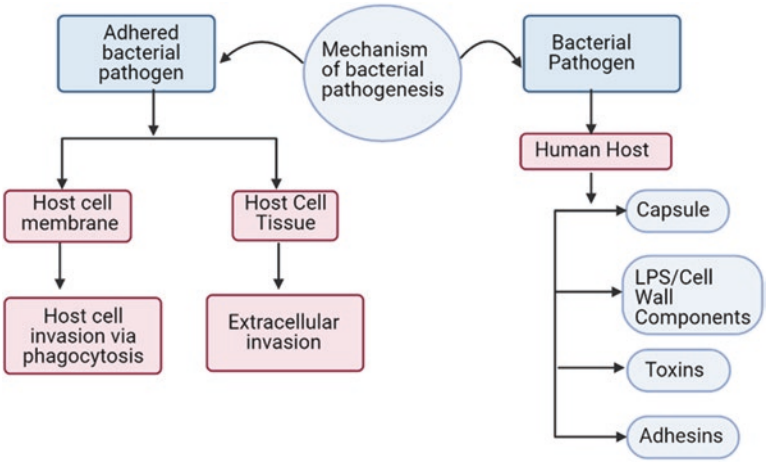

Fig. 6.1 Schematic representation of the mechanism of bacterial infection

accessibility which is affected by physical barriers and cellular susceptibility, and characteristic of the virus that makes it virulence, hence making it susceptible to get spread and target in large numbers to various specific organs (Strayer et al. 1990). The mechanism includes the following steps:

Penetration at the Portal of Entry The possible routes like air, food, bites, and contaminated objects help in carrying the virus to the body. The highest implantation is observed when the virus contacts the living cell directly.

Replication and Spread of the Virus The first step is followed by the local replication within the first initiated cell and that it may further spread to the adjacent cells can be possible by extracellular (almost all virus) or intracellular method (include paramyxoviruses that spread by both routes). Therefore, infection establishment at entry further leads to shedding of virus, causing disease.

Dissemination The most prevalent and common route for the spread of the virus is through circulation that can be in the bloodstream and nerves although the latter one was found to be less common but has been considered as the main source in the spread of many important diseases. It mainly occurs in rabies virus, herpesvirus, and, occasionally, poliomyelitis virus infections. This step is further followed by the incubation period in which the virus travels only a short distance to reach the target organ.

Multiplication in Specific and Targeted Organs During the time of virus progression, the various recovery mechanisms like local immunity, local inflammation, and interferon are activated. Nevertheless, these systemic defenses can diffuse in various degrees into target organs and thereby help retard virus replication and disease.

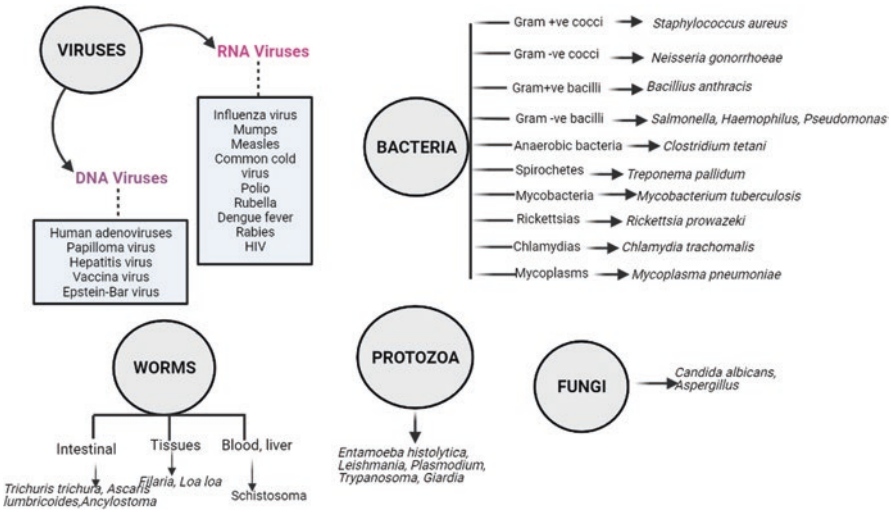

Fig. 6.2 Different types of microbial infection

Shedding of Virus Since biting insects become infected along this path, the blood and lymph are sites of shedding for arboviruses. The disease is transmitted through blood the and sperm. Viruses such as certain RNA tumor viruses (retroviruses) and cytomegalovirus shed their coats in milk. Several viruses (e.g., cytomegaloviruses) are shed around the same time from the urinary tract and other shedding locations. Herpesvirus type 2 shedding is normal in the genital tract, and the virus may be spread to sexual partners via this path (Fields 1983; Coen 1994; Grieder et al. 1995) (Fig. 6.2).

6.3 Types of Microbial Infection and Its Reservoirs

6.3.1 Bacterial Classification

Being ubiquitous in nature, bacteria play a crucial role in maintaining the environment. There has been seen a huge effect on health of public by small percentage of bacteria. Although having double-stranded DNA, they considered as prokaryotic organism. Giving the bacteria two categories named as Gram-negative and Gram-positive bacteria makes them differ in susceptibilities toward specific antibiotics. They further classified as obligate aerobes that require oxygen to sustain and another one facultative organism that can sustain in the absence or presence of oxygen (Engelkirk et al. 2020).

According to Koch's postulates, bacteria have been classified as pathogens, opportunistic pathogens, or nonpathogens. *Mycobacterium tuberculosis* and *Yersinia pestis* are pathogens, yet their presence is found to be abnormal. Other

species of bacteria are known as *Escherichia coli* that are part of the normal flora of the human but cause disease like they act as the gastrointestinal flora of normal humans but act as a major cause of urinary tract infection because of the virulence in animals or models of infection and genetic makeup that is specifically associated with the production of disease. Other bacteria mainly termed as opportunistic pathogens are *Stenotrophomonas maltophilia*, *Pseudomonas* species, and yeasts and molds and therefore cause disease in immunosuppressed persons (Falkow 1988; Bannister et al. 1996). Meningitis is caused by the bacteria *Neisseria meningitidis* that infects the meninges and further can also infect the lungs and cause pneumonia.

The reservoir of bacterial infection: The site where the survival of the pathogen is possible until it gets transfer to the host is known as its reservoir that can comprise of birds, humans, animals, etc., but the reservoirs that come under nonliving are soil, water, and air. Some infections are caused even without making the person conscious of it especially through bacteria *Salmonella typhi*. *Salmonella* diarrhea may be caused by touching turtles and contaminating one's hands with their feces, ingesting undercooked chicken infected with the bacteria, or eating undercooked or raw chicken eggs, among other items. There are also nonliving reservoirs like air, food, soil, etc. It includes various diseases, for example, the various species of *Clostridium* can be acquired from exposure of a wound to dirt or soil. Food may be contaminated by feces, or the animal itself may be infected, such as in the case of chickens with *Campylobacter* or *Salmonella*. Food can also be contaminated with the ubiquitous spores of *botulinum*, which can cause a form of paralysis called botulism. Water generally becomes a reservoir for infection when it is contaminated by soil microbes or animal or human feces and causes a disease when contaminated by *Shigella* and *Legionella*. (Evans 1976).

6.3.2 Viral Classification

The genome of the virus can be either RNA or DNA which are packed with proteins that are encoded by the genome of the virus. The virus is categorized into an enveloped and non-enveloped virus. The earlier one has a lipid membrane, and the latter one does not have a membrane. The steps included in infection are the attachment to the cell surface receptors.

Acute viral diseases including respiratory, diarrheal, exanthematous, and neurological infections will overlap and manifest as seasonal epidemics that peak in incidence every few years and overlap with the aggregation of a sufficient number of nonimmune hosts in the young population. In Southeast Asia, arboviral disease infection also correlates with arthropod vector activity, such as mosquito breeding during hot rainy seasons, and is linked to an uptick in hemorrhagic fever or neurological diseases like dengue hemorrhagic fever, West Nile virus, or Japanese encephalitis. Many lifelong viral infections, such as the human immunodeficiency virus (HIV), hepatitis A, and hepatitis B, are spread via the bloodstream. Some of

these chronic viral infections such as HBV, HCV, HIV, polyomaviruses, and papillomaviruses are also linked to the genesis of cancers. Over 70% of emerging viral infections such as the Ebola virus, severe acute respiratory syndrome (SARS) coronavirus.

Major viral families	Virus within family
Herpesviridae	HSV-1 HSV-2 VZV
Orthomyxoviridae	Influenza A Influenza B Influenza C
Filoviridae	Ebola virus Marburg virus
Picornaviridae	Poliovirus Rhinovirus
Rhabdoviridae	Rabies virus
Flaviviridae	Dengue virus, Hepatitis C virus, Yellow fever, Tick-borne virus
Paramyxoviridae	Measles, mumps virus
Togaviridae	Rubella virus, Chikungunya
Retroviridae	HIV-1, HIV-2
Hepadnaviridae	Hepatitis B virus
Hepeviridae	Hepatitis E virus
Reoviridae	Rotavirus
Coronaviridae	Human corona virus
Papillomaviridae	Human papillomavirus

Cheng et al. (2017)

6.3.3 Parasitic Classification

Intestinal parasitic infections are present nearly everywhere on the globe, with elevated rates of occurrence in many countries. The ten most common diseases on the planet include amoebiasis, ascariasis, hookworm infection, and trichuriasis. While these diseases have a low mortality rate, complications do occur often, and certain cases require hospitalization. Malabsorption, diarrhea, blood loss, decreased job ability, and reduced growth rate caused by intestinal parasitic infections are serious health and social concerns in many countries. *Giardia lamblia*, which causes giardiasis, is the most common pathogenic protozoan found in the human duodenum

and jejunum. *Leishmania donovani* spread from the site of inoculation to multiply in reticuloendothelial cells which causes kala-azar in humans, and this is usually fatal (Lange Medical Microbiology 2007).

6.3.4 Fungal Classification

Being beneficial to humankind, fungi reside in nature and are helpful in breaking down and recycling organic matter. Fungi are aerobes that can be obligate or facultative and are eukaryotic. Infection caused by fungi is called mycoses. These mycoses are classified based on the initial site of involvement and their usual portal of entry into superficial, cutaneous, subcutaneous, opportunistic, and systemic:

Superficial Mycoses It includes various infections like pityriasis versicolor. The infection caused by lipophilic yeast that requires lipid in the medium for growth is known as *Malassezia globosa* that cause chronic mild infection of the stratum corneum.

Tinea Nigra The infection caused by the dematiaceous fungus *Hortaea werneckii* that causes infection in the stratum corneum.

Cutaneous Mycoses They are the types of fungi that only infect the superficial keratinized tissues, which are the skin, hair, and nails. The fungi that participate in this type of infection are *Trichophyton*, *Epidermophyton*, and *Microsporum* but are restricted to the nonviable skin. Tinea pedis is found to be the most prevalent of all dermatophytoses.

Subcutaneous Mycoses They normally reside in soil or on vegetation and are only confined to subcutaneous tissues. Examples of this are *Sporothrix schenckii*.

6.4 Epidemiology of Microbial Infections

Epidemiology is the analysis of the spread and variables of health-related conditions, diseases, or incidents in special communities, as well as the interpretation of the findings to the management of medical issues. As we are aware, the microbial infection takes place when immune-compromised individuals are exposed to pathogens and to the host via a portal insertion site, which typically includes recognizable immune responses to the specific microbial agents. Morbidity and mortality are detected based on the incidence rate of ill persons per 100,000 persons at risk and incidences of deaths per 100,000 persons at risk. The study of the geographic spread of different microbial diseases has become a priority in public and global health. These unique infectious disease trends are critical in resolving the upcoming

infectious disease threats. It was observed in the geographic distributions of human infectious disease that mainly depict that specific rise in the disease was found in the tropics relative to higher latitude and even more diseases were found on the larger islands rather than smaller ones. It was observed in the geographical ranges of human infectious disease that a particular increase in disease was seen in the tropical regions compared to the high latitudes and even more diseases were found on the larger islands rather than smaller ones (Cliff and Haggett 1995; Guernier et al. 2004; Cliff and Haggett 2004). These biogeographical patterns have long been used to maintain, discover, and track the world's biodiversity (Diamond et al. 1976; Channell and Lomolino 2000; Myers et al. 2000). A better understanding of the huge range of biogeographical patterns of various human microbial diseases has considerable potential in managing and monitoring the risk faced by the global health community (Scheiner 2009).

6.4.1 Types of Epidemiological Study

Mainly epidemiological studies are descriptive and analytical as it purely depends on the available data. The descriptive study describes the pattern of the disease based on place and personal factors; on the contrary, analytical studies describe the hypothesis involved in the development of descriptive study. Furthermore, analytical studies are classified into three subclasses which are as follows:

1. *Cohort Study*: These experiments are used to illustrate how an infection develops over time in the absence or presence of risk factors that are assessed at the start of the study (Song and Chung 2010).
2. *Case-Control Study*: These are the observational experiments in which the researcher investigates the relationship between exposure and outcome in study subjects.
3. *Cross-Sectional or Prevalence Study*: In these types of studies, the investigator looks for the exposures which are followed by the results of the study participants simultaneously. The participant's selection for these studies is done based on exclusion and inclusion criteria (Setia 2016).

6.5 Prevalence and Temporal Patterns of Microbial Infections

In the following paragraph, we have included the few major epidemiological stats of microbial infection which are as follows:

Infectious diseases are also a major cause of the extreme poverty troubles so much of the world. These diseases kill about nine million individuals per year, most of whom are children under the age of 5, and they often inflict severe economic

burdens due to lifelong disability. In 2018, 104,017 new cases have been reported including tuberculosis, salmonella infection, Lyme disease, and meningococcal disease in the United States alone (Villano and Ogden 2017; Ilyas and Alowibdi 2018).

According to the US CDC report, from 2013 to 2018, there have been 195,203 human immunodeficiency virus (HIV) cases registered among people between the ages of 13 to 65 and above. In contrast to other age groups, we find that Black or African Americans are the main focus of the spread of the disease. In 2019, 5044 mortality was recorded per 100,000 population as per the same report. In 2019, approximately 690,000 mortalities were recorded with AIDS-related illness globally, whereas 1.7 million and 1.1 million cases were recorded in 2004 and 2010. The mortality incidence rate due to AIDS has been declined by 39% since 2010.

According to the WHO TB report of 2020, around 10.0 million people suffered from TB worldwide. India, Indonesia, China, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa account for approximately two-thirds of the global number. In 2019, 1.4 million people died of tuberculosis, and the worldwide TB incidence rate dropped by 9% from 2015 to 2019 per 100,000 population (Ahmad et al. 2021).

As per the WHO Global hepatitis report 2017, the viral hepatitis pandemic is responsible for around 1.4 million deaths per year from acute infection and hepatitis-related liver cirrhosis and cancer; these stats are very much comparable to tuberculosis and HIV. Hepatitis B virus causes approximately 47% of deaths, hepatitis C virus causes 48% of deaths, and hepatitis A virus and hepatitis E virus cause the remaining 3% of deaths. Viral hepatitis is also a rising cause of death among HIV-positive people. Hepatitis C and B coinfected almost 2.9 million HIV-positive individuals and 2.6 million with hepatitis B. Worldwide, lower than 5% of people with chronic viral hepatitis are aware of their condition.

According to the WHO COVID-19 22 March 2021 report, coronavirus disease (COVID-19) was first reported from Wuhan, China, on 31 December 2019 which is, later on, declared as a pandemic. COVID-19 confirmed cases increased for the fourth week in a row as only 3.3 million new cases reported in the previous week. Interestingly, the mortality rate of COVID-19 has been plateaued after 6 weeks as there were only 60,000 new death cases reported. Western Pacific, Southeast Asia, and European and Mediterranean regions recorded a marked spike in new cases in the last few weeks. The morbidity and mortality rate in the European region and region of the Americas is individually reported to be around 80% of all cases.

As per the WHO Global leishmaniasis surveillance (2017–2018), leishmaniasis is one of the major diseases in the following world regions: the Americas, North Africa, East Africa, Southeast Asia, and West Asia. In 2018, 253,435 cutaneous leishmaniasis cases were reported, and along with that, 17,223 new visceral leishmaniasis cases were reported by the WHO. Visceral leishmaniasis (VL) which is also known as kala-azar is a severe condition and leads to death if not treated in over 95%. The majority of VL cases are reported in Brazil, East Africa, and India. Roughly approximately 50,000 to 90,000 new cases of VL are reported globally and very less around 25–45% recorded by the WHO between 25 and 45%. It is one of

the parasitic threads which can be a pandemic outbreak and causes more mortalities.

According to the 2019 CDC report, malaria took the lives of approximately 409,000 people in sub-Saharan Africa, mostly children under the age of 5. In 2019, the WHO recorded 94 percent of all deaths in the African region alone. Over the past decades, intensive work has been undertaken to eradicate malaria, which has saved millions of lives around the world. Malaria is mainly found in rural tropical and subtropical regions of the world. From 2010 to 2019, mortality decreased by 44%, increasing expectations and plans for reduction and, finally, abolishment by the joint efforts of various agencies. Young children, pregnant women, and travelers or migrants from areas with little or no malaria transmission are prone to malaria since they are lacking immunity or have compromised immunity.

6.6 Prevention and Cure of Microbial Infections

6.6.1 Prevention and Cure of Bacterial Infections

Lower respiratory infection and diarrhea which are the third and sixth most common causes of mortality in the world are caused by bacteria. In this age of increasing antibiotic resistance, prevention is found to be crucial for survival. Interruption in chain of infection, protection of host against infection, and elimination of the main source that causes the infection were found to be the three main causes of infections. Primary prevention includes prevention via vaccine, and prevention of symptomatic infection is primarily named as secondary prevention, and to prevent further transmission, treatment is given to infected people, which is known as tertiary prevention. Using personal equipment, the use of pesticides and proper disposal of animal waste are measures of infection that help in preventing the infection. Untreated water can cause various parasitic and viral diseases (Detels et al. 2011).

In developing countries, improving water quality has been critical in reducing the burden of infection in cities. Filtration, settling, and coagulation to kill bacteria-carrying particles, aeration, and chlorination or treatment with another reactive halogen are all part of the current water treatment procedure. It's incredibly difficult to keep bacteria from spreading across the air. It is difficult to sterilize the weather. Laminar flow units are used in hospitals to ensure that air polluted by patients with airborne bacterial diseases such as tuberculosis does not spread within the facility. Quarantine, as well as the application of ionizing radiation, can be used to prevent infectious transmission from person to person (Benenson 1995).

Treatment: Antibiotics can target four major sites, which are the cell wall, the cell membrane, the nucleic acid synthetic pathway, and the ribosome. The antibacterial agent can be classified based on its chemical structure, its target site, and whether the agent is bactericidal or bacteriostatic. Some bacteria are innately resistant to certain classes of antibiotics, because they either lack the target or are

impermeable to the drug. The mechanism of resistance includes alteration in the target site, by decreasing permeability of the cell an alteration can do in target site or lastly by the production of enzymes that inactivate the antibiotic. Antibiotics can target various sites: nucleic acid synthetic pathway, cell membrane, ribosome, and cell wall. On the basis of target site and its chemical structure, the antibacterial agent can be classified as bacteriostatic or bactericidal. Resistance mechanism can work by doing target site alteration and ultimately by enzyme production that makes the inactive antibiotic (http://www.cdc.gov – Centre for Disease Control and Prevention).

6.6.2 Prevention and Cure of Viral Infections

Vaccination, which is used to avoid infections by developing immunity to a virus or virus family, is the most effective way of controlling infectious disease. Vaccines can be made of live or killed viruses, as well as viral molecular subunits. Virus vaccines that have been killed and subunit viruses that have not been killed are both unable to cause disease. The threat of using live vaccines, which are always more successful than killed vaccines, is that these viruses will return to their disease-causing state due to back mutations. The "wild-type" (disease-causing) virus is normally attenuated (weakened) in the laboratory by growing it in tissues or at a certain temperature (Ada 2001).

Antiviral agents have the potential to selectively block viral activities, making drug production impossible without harming the host. Viruses affect a wide range of infections in animals and humans, from the common cold to potentially deadly illnesses like meningitis. Antiviral medications and vaccines may cure these infections, but certain viruses, such as HIV, are capable of evading the immune system and mutating to become antiviral drug-resistant (Hawley and Eitzen Jr 2001).

6.7 Conclusions

The study of the determinants, prevalence, spread, and control of health and disease in a given population is the subject of epidemiology, which is discussed in this chapter. The incidence rate is the number of disease cases separated by the population in which the cases occurred. Before the origin of a disease is discovered, epidemiology can reliably explain it and all of the causes that influence its incidence. The relationship between the host and the microorganism is reflected in how an infectious disease presents clinically. Host immune status and microbial virulence factors affect this relationship. The signs and symptoms differ depending on where the infection is located and how severe it is. Infectious diseases disproportionately impact poorer countries in the developing world, becoming a global burden as a result. Infectious diseases are the leading cause of death for children and young adults in developed

countries, accounting for one out of every two deaths. An hour, 1500 people are expected to die from an infectious disease, with more than half of them being children under the age of 5. Respiratory infections, an acquired immunodeficiency syndrome (AIDS), diarrheal diseases, tuberculosis (TB), malaria, and measles account for 90% of all infectious disease deaths worldwide.

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Chapter 7
Socioeconomic Impact of Antimicrobial Resistance and Their Integrated Mitigation by One Health Approach

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7.1 Introduction

Microbes are generally microscopic in size and we cannot see it by our naked eye. They are about 1/10th the size of a typical animal cell and generally measured in micrometer scale. Antimicrobial agents are group of chemical compound that have been used against microbes for combating infectious disease (Becker 2013; Uchil et al. 2014; McEwen and Collignon 2018). The antimicrobial agents generally inhibit bacterial cell cycle by acting on the cell wall, DNA replication, and protein synthesis. In early times, antimicrobial agent was synthesized from plant extracts. Whereas microorganisms become resistant to drugs due to mutation in chromosomes, bacteria become resistant to drugs due to decrease in cell membrane permeability, poor influx of drugs, rapid efflux of drugs, modification in drug binding site, drug inactivation, and formation of 3D biofilm of extracellular polymeric matrix. Nowadays, pharmaceutical industries are involved in the synthesis of antimicrobial agent on a large scale. Synthesis of new antimicrobial agent is a long-term process and very expensive, whereas development to resistance takes hardly few years. Nowadays, antimicrobial resistance (AMR) has become a global threat for public health (Prestinaci et al. 2015; Founou et al. 2017). Development of drug resistance by microorganism is an evolutionary survival process, and human activities are also responsible for the development of AMR (Moo et al. 2020). Humans have major contribution in the development of antimicrobial resistance (AMR). Using

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antimicrobial agent to overcome microbial infection without proper guidelines, without advice of expert physician, and without proper diagnosis of infection and not taking antimicrobial drugs as prescribed by physician are major causes of resistance development in microorganism. Once resistance is developed, then microorganisms are able to adapt and grow in the presence of medication (Founou et al. 2017). Day by day, human practices such as misuse or overuse of antimicrobial drugs in health care, poor sanitary conditions, inappropriate food handling, and poor infection control and prevention practices in hospitals is making AMR situation worse. The World Health Organization (WHO) is extensively working on AMR, and in 2001, the WHO explains that AMR infection is most common threats for human health. In 2020, according to the WHO, AMR infection has become one of the top ten global public health threats to humanity. According to expert opinion on AMR infection impact on health, it is estimated that there would be about ten million human deaths annually by 2050 (O'Neill 2014; Garau and Bassetti 2018).

Day by day, AMR has become a challenge for human health globally. It has become a big hurdle for developing country to tackle AMR due to their social and economic impacts. According to researcher's opinion, it has expected that 7% of the world's economic GDP will lose due to AMR infection (O'Neill 2014). Infection with AMR strain causes serious health problem and prolonged hospital care that increase health-care cost, due to expensive second-line drugs and sometime treatment failures (ECDC. Surveillance of Antimicrobial Resistance in Europe 2017, Shrestha et al. 2018). For example, the European region is estimated to spend about 9 billion euro annually due to AMR infection (Llor and Bjerrum 2014; Prestinaci et al. 2015). Furthermore, the Centers for Disease Control and Prevention (CDC) has calculated about 20 billion dollars additional in direct health-care cost in the United States which may cause 35 billion dollars loss in annual productivity (Antibiotic resistance threats in the United States; 2013). Therefore, the health-care sector recently considered AMR infection as a serious health problem and needs crucial treatment with new-generation drugs which are expensive. AMR infection is responsible for public health problem in terms of rate of mortality as well as economic loss (Taneja and Sharma 2019).

The health-care system is also affected by country's economic condition as well as patient individual conditions. Generally rich or developed countries have more health budget as compared to poor or developing countries. The people of developed countries are mostly economically rich, and they can spend more money on health and hygiene, whereas people in poor countries are not economically rich and they are not able to spend more money on health and hygiene because these people are struggling for their basic needs. Therefore, these rich countries generally have less mortality rate as compared to poor countries from AMR infection. For the assessments of socioeconomic impact on AMR infection, some viewpoints are very important such as patient perspectives, physician perspectives, drug industry perspectives, health-care perspectives, and societal perspectives. These viewpoints will be helpful in exact assessments of socioeconomic impact on AMR.

"One Health" approach is essential for the integrated mitigation of AMR. One Health approach combines the efforts of multiple sector and agency action plans

on national and international level to ensure optimum health for animals, humans, and the environment. In 2001, the World Health Organization (WHO) has described AMR as a global problem. The Global Strategy for Containment of Antimicrobial Resistance has provided a framework of interventions to slow the emergence and reduce the spread of AMR bacteria (World Health Organization 2001). However, there is a lack of sufficient financial and human resources for the implementation of the strategy for the intended goal. In 2015, the Global Action Plan (GAP) for the purpose of endorsing to tackle AMR was adopted at the 68th World Health Assembly in Geneva (World Health Organization 2015). In 2020, AMR condition has become one of the top ten global public health problems (World Health Organization 2020), and till date, it has failed to gain proper attention. The proper and global implementation of the One Health approach will be relevant in the mitigation of the AMR. If actions are not taken as early as possible for integrated mitigation of AMR, their bad impact will be on socioeconomic conditions as well. The social inequality will increase, the economy will shrink, and the public health-care sector will be less sustainable. Even more serious will be the increase in rate of mortality from microbial infection. Lord Jim O'Neill and his team have published a review entitled "Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations" in 2014, and the review has estimated that there would be ten million deaths every year caused by AMR condition by 2050 (Review on Antimicrobial Resistance 2014). The European Commission (EC) is also working on the broad vision of One Health. The EC has claimed that AMR condition attributed costs approximately €1.5 billion every year (European Commission 2011).

The WHO is eagerly concerned on AMR and ways of tackling it by One Health approach; in spite of mentioning the importance of establishing AMR surveillance system at the local, national, and global level (Grundmann et al. 2011), the lack of capacity, the surveillance gap, and the integration remain to be the major challenge. The US National Action Plan (NAP) view for One Health concept is appreciable. The NAP has recommended the enhancement of a "One Health" national surveillance system, for humans, animals, and the environment, with the upgraded international association and capacity (White House 2015). However, the One Health goal also refers to extensive, system-based access to complex problems (Zinsstag et al. 2011). The socioeconomic factors are directly connected to the use and misuse of antimicrobial agents; therefore, AMR has major impact on the global socioeconomic conditions. Hence, the One Health goal is appropriate for AMR surveillance because it acknowledges the underlying structural factors which influence AMR, including the material, biological, socioeconomic, and political factors (Kock 2015). The One Health approach also introduces the analysis of context across all levels of society (Rushton 2015). The main objective of One Health approach is to make sure that the entire community has sound health. Therefore, public awareness of One Health goal and proper implementation of this approach will be useful in integrating mitigation of AMR.

7.2 Antimicrobial Agent

Antimicrobial agent is a general term for drugs, chemical substance, and other plant derivatives that either kill or inhibit the growth of microbes and most frequently has been used against infections and infectious disease (Punjataewakupt et al. 2019). Antimicrobial agents have been used since ancient times (Shukla et al. 2016) such as some specific molds and plant extracts for treatment of infections. Antimicrobials are grouped according to their action against microbe. For example, antimicrobial agents used against bacteria are called antibiotics, and those acting against fungal infection are called antifungal. Penicillin was the first antibiotic accidently discovered by Sir Alexander Fleming in 1928, and now it becomes the most widely used antibiotics in the world. Penicillin was first naturally occurring antibiotic and most frequently used therapeutically to fight against microbial infections. Penicillin was served as a gift from God during World War II because it was used to reduce the number of deaths from wound infection. In the meantime, Sir Alexander Fleming warned the misuse of antibiotics due to its unexpected adverse effect. Nowadays, people are facing unexpected adverse effect in the form of development of bacterial resistance from multiples of antibiotics, leading cause of deaths of humanity from antimicrobial resistance.

7.3 Antimicrobial Resistance

Antimicrobial resistance is a major public health concern (Bishop 2016). When a microbe developed defense mechanism against antimicrobial medicine, it is called antimicrobial resistance. Nowadays, antimicrobial resistance is a major health threat globally, and for this all we are responsible. Sir Alexander Fleming warned at the time of penicillin discovery that misuse and overuse of antibiotic will give an opportunity to microorganism to develop resistance for their survival. All we know is that the famous phrase "survival of the fittest" of Herbert Spencer describes the idea; in nature, there is very tough competition to survive and reproduce. Therefore, only the fittest can survive in any condition. The development of antimicrobial resistance is an evolutionary process for microbes to survive and reproduce. Due to the development of resistance, day by day classical drugs become noneffective against infectious disease and cause severe illnesses and deaths. Nowadays, antimicrobial resistance has become major cause of deaths globally. The World Health Organization described the development of antimicrobial resistance as a global threat for humanity in 2001 and predicted it as a leading cause of mortality by infection through antimicrobial resistance strains in the future. According to the World Health Organization, antimicrobial resistance has become one of the top ten worldwide threats to humanity in 2020. It's time to take action against the unfair means that are leading cause of development of antimicrobial resistance.

7.4 Mechanism of Resistance

Currently, we are using antimicrobial agent against infections which have mainly three sites of action to inhibit bacterial cell cycle in the course of combating infections: the bacterial cell wall, DNA replication, and translation of proteins. However, bacteria become resistant against antimicrobial agent due to induction of mutation in chromosomes or some other intrinsic factors. After resistance development, older strain of bacteria may terminate with the use of classical drugs, and mutant strain can survive and reproduce. The mutant strain become resistant to multiple drugs by some basic mechanisms such as poor drug influx by porin due to the modification in their cell wall permeability by mutation, rapid and active drug efflux from drug action site due to mutation regulatory protein, inactivation of drug molecule by covalent binding, resistance plasmid and modification in drug target site. Biofilm formation is another way of bacterial resistance against drugs. In biofilm formation, bacteria develop extracellular polymeric matrix around it and prevent from high drug concentration around it. Therefore, these resistance mechanisms of microorganism are leading cause of hindering the antimicrobial drug effectiveness against harmful microorganism.

7.5 Human Practice Accelerating Rate of AMR

From first antibiotics to latest new antibiotics, human is responsible for their synthesis as well as major contributor in drug resistance development. Some microorganism becomes resistant against antibiotics by evolutionary process for their survival (Davies and Davies 2010), and some becomes resistant by human practices as summarized in Fig. 7.1:

1. *Misuse or overuse of antimicrobial drugs in health care*: From early day of discovery of antibiotics, Sir Alexander Fleming warned the people that the high demand of antibiotic could cause their misuse or overuse (Ventola 2015; Langford and Morris 2017; Zaman et al. 2017). Misuse or overuse of antimicrobial drugs is a major problem in developing AMR. Use of antimicrobial agent against infections without proper diagnosis or without recommendation of expert physician, use of antibiotics in healthy animal for growth and production and unnecessary use of antibacterial accelerating AMR.
2. *Inappropriate food handling*: Inappropriate food handling is responsible for major infectious disease. Inappropriate food handling is responsible for foodborne infectious disease and may promote drug resistance as well as acceleration in AMR. We should take care about hygiene for combating AMR.
3. *Poor sanitary conditions*: Poor sanitation is a major cause of many infectious diseases. Open defecation and unsafe drinking water are responsible for transmission of many infectious diseases. Human practices such as poor sanitation may be responsible in the acceleration of AMR. For combating AMR, we should focus on health and hygiene.

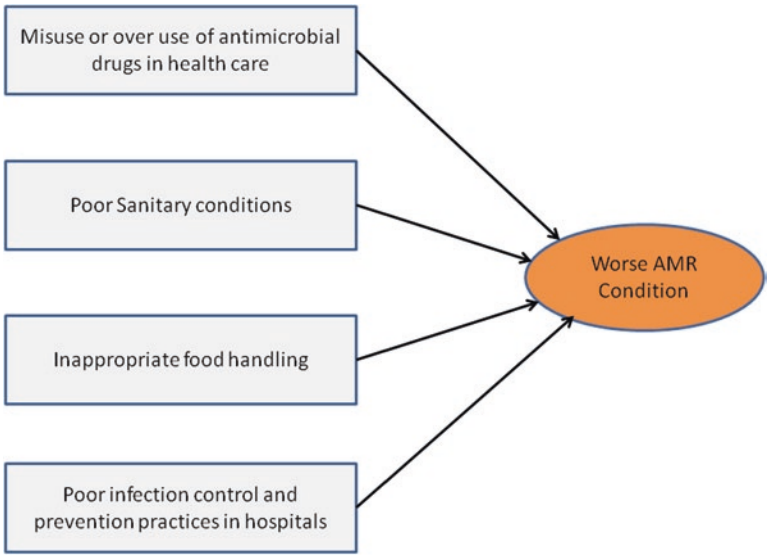

Fig. 7.1 Practices by human that are making AMR worse

4. *Poor infection control and prevention practices in hospitals*: Nowadays, healthcare system is not efficient in infection control and prevention in hospital. Human practices like this may accelerate the AMR. For combating AMR, people should observe proper hand-washing and clean the hospital surface with disinfectant, and special infected people must be at isolated place and use gloves as required.

7.6 Socioeconomic Impact

The costs of new-generation drug are generally high (Gronde et al. 2017). Treatment of AMR-infected patient day by day becomes very expensive. Nowadays, AMR infection has become the leading cause of loss in the global GDP. There are many variables that have contributed in the assessments of socioeconomic impact on AMR infection. The different viewpoints are involved in assessments of socioeconomic impact on AMR infection and very well-represented in Fig. 7.2. Understanding socioeconomic perspective on AMR infection can be better explained by another subheading such as social impact and economic impact.

7.6.1 Economic Impact

We can say that every disease and infection has their impact on global economy. AMR infection is a major health threat to galvanize political and financial investment (Chandler 2019). AMR has their global impact on economic loss. A recent

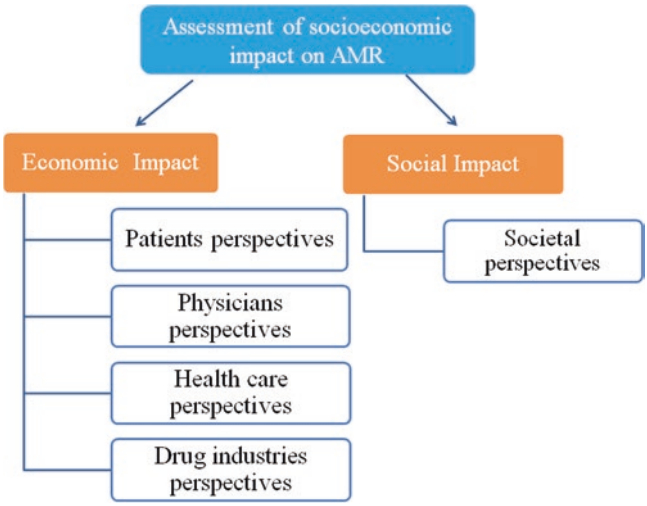

Fig. 7.2 Different viewpoints for the assessment of socioeconomic impact on AMR infection

Table 7.1 Estimated economic loss by 2050 by different medical conditions

Medical condition	Percentage loss of the world's GDP
Caesarean sections	2%
Joint replacements	0.65%
Cancer drugs	0.75%
Organ transplants	0.1%
AMR infections	7%

O'Neill (2014)

review on "Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations" by Jim O'Neill in 2014 estimated the loss in global GDP by 2050 as summarized in Table 7.1. It has been estimated the global percentage loss in GDP by 2050 with different medication conditions such as 2% loss by caesarean sections, 0.65% by joint replacements, 0.75% by cancer drugs, 0.1% by organ transplants, and 7% by AMR infection. We can understand from these data that antimicrobial resistance strain infections are the major contributor in the loss of global GDP by 2050. There will be great impact of antimicrobial resistance on global GDP, and we should take action as early as possible. There are many variables that have significant role to address economic perspectives. Therefore, exact evaluation will be very tough, but proper evaluation of economic perspectives on AMR infection, several viewpoints, and its impact include those of patient, physician, drug industry, and health-care perspectives.

7.6.1.1 Patient Perspectives

Patients suffering from AMR infection have their views and observations that may be valuable in addressing the economic perspective. There are some important consequences such as mortality and morbidity in AMR-infected patients. AMR-infected patients have double chances of development of serious health complication as compared to normal infection and triple chances of mortality (Cecchini et al. 2015). Economic impact can also be measured in terms of consequences faced by patients from day of infection till death by taking into account added cost of treatment by AMR infection because patients pay drug price and services. Patients have their own experience in paying such charges directly or indirectly. If patients have healthcare coverage, then they have to increase their premiums for treatments of such type of infections. As we know that there is three times mortality from AMR infection, this may create fear in susceptible patients that leads to more severity in AMR-infected patients (Friedman et al. 2016). From a review article by Jim O'Neill on "Antimicrobial resistance: tackling a crisis for the health and wealth of nations," the comparison between expected mortality by AMR infection and cause by other conditions by 2050 is summarized in Table 7.2. Also, in Table 7.2, the global mortality rate of AMR infection by 2050 will be ten million, and it is a matter of concern. This high mortality rate of AMR infection may have great impact on the global economy.

Nowadays, about 700,000 patients lose their lives annually due to AMR infection (Porooshat Dadgostar 2019). The mortality rate due to AMR infection in different regions of the world is summarized in Table 7.3. These data may create fears in people suffering from AMR infection and consequently cause more complication.

The United States have reported that annually two million individuals are affected due to AMR infection that leads to cause about 23 thousand deaths annually (Davis et al. 2017). In the same pattern, the European Union has about 25 thousand annual mortality due to AMR infection (WHO 2016). In the meantime, researchers have estimated that about ten million individual will die due to AMR infection globally if strong action is not taken against AMR infection (Laxminarayan et al. 2013).

Table 7.2 Mortality rates by AMR related infections and other conditions by 2050

Condition	Mortality rate
Cholera	0.1 - 0.12 million
Measles	0.13 million
Diabetes	1.5 million
Diarrheal disease	1.4 million
Tetanus	0.06 million
Cancer	8.2 million
Road traffic accidents	1.2 million
Antimicrobial resistance	10 million

O'Neill (2014)

Table 7.3 Mortality rates due to AMR infection in different regions of the world by 2050

Regions	Mortality rates
Asia	4,730,000
Europe	390,000
Africa	4,150,000
Latin America	392,000
North America	317,000
Oceania	22,000

O'Neill (2014)

7.6.1.2 Physician Perspectives

Physicians are directly linked to the infected patient, and their point of view will be very valuable during assessment of economic impact due to AMR infections. When a person gets infected by AMR and consults to the physician for the treatment, then financial condition may be affected by treatment. Physicians are highly professional and they charge money for diagnosis and treatment. AMR-infected patients take time to cure. Treatment for AMR-infected patient is very costly; latest drugs required for the treatment are very expensive for patients. In the absence of suitable drug agent, it is very difficult for physician to treat AMR-infected patient; then, the main economic problem start from this point. Therefore, the economic impact diminishing the effectiveness of a given drug depends on the availability of other potent drugs.

7.6.1.3 Drug Industry Perspectives

The pharmaceutical firms are extensively involved in the production of drugs for the treatment of and prevention from infectious disease. The production of antimicrobial agent by pharmaceutical firm is health-care business. The purposes of these industries are production and making money directly from patients or some time through the health-care sector and government. The product sales are their desire outcome. For the industries, development of bacterial resistance becomes a challenge. Their old antibacterial product day by day is becoming useless or noneffective, and production of new viable drugs for the treatment of AMR infection is very expensive process for them. The production of new-generation drug by extensive research will be very costly, and their impact will be directly on the infected patient as well as on country economic conditions. However, development of antimicrobial resistance gives opportunity to develop new-generation drugs for the treatment.

7.6.1.4 Health-Care Perspectives

Nowadays, health-care sector financial resources are more frequently controlled by administrators and financial manager as compared to doctors and nurses. These peoples are highly professional and see reduced illness and death as a reasonable goal, and their objective is to achieve the goal with fiscal efficiency (McGowan Jr 1991). AMR infections have disastrous impacts on health-care costs. According to CDC reports, in the United States alone, AMR infection treatment could add about $1400 to the treatment bill with any bacterial infection (Antibiotic resistance threats in the United States 2013; Thorpe et al. 2018). According to the researcher, AMR infection could cost from $300 to more than $1 trillion every year globally by 2050 (Chokshi et al. 2019). Extensive treatment requiring intensive care units and isolation beds to prevent the spread of infection against AMR-infected people (Friedman et al. 2016) may cause economic burden on health-care sectors. Researchers have also reported indirect health-care burden as secondary effect from AMR infection (Naylor et al. 2018). These secondary effects make more expensive treatment from AMR infection. Furthermore, organ transplants will be challenging because patients may be exposed to different infections (Li and Webster 2018). Therefore, we can say that AMR infections have economic burden on health-care sector, leading cause of loss in the global economy.

7.6.2 Social Impact

The social impact or societal perspectives are a final view and to be considered for the betterment of public health. The societal perspectives are fuelled by social good conclusively the entire population of town, village, cities, and even the world (McGowan Jr 2001). The goal of societal perspective is maximum heath for entire the population; then it will take long time. Misuse or overuse of antimicrobial drug causes drug resistance and mean time inappropriate decrease in resources. The societal perspectives have great impact on AMR. When we are treating one person causes to decrease in effectiveness in treating to another the person by same drugs, society is adversely affected. The antimicrobial resistance infection prevalence has been increased and become major health problem for the people. Nowadays, mortality rate has increased due to AMR infection. Furthermore, the global loss in economy has been observed due to AMR infection. The recovery rate in developed counties from AMR infection is generally high as compared to developing countries. The treatment cost and mortality rate of AMR infection are high which may create anxiety and psychological problem in poor people leading to complications and poor recovery rate from AMR infection.

7.7 Importance of One Health Approach

The One Health term was first used in 2003–2004, and it was related with the severe acute respiratory syndrome (SARS) in early 2003 (Mackenzie and Jeggo 2019). Nowadays, One Health approach has become a global vision for tackling several infectious diseases. The main objective of the One Health approach is to maintain best health for animals, humans, and the environment (McEwen and Collignon 2018), and it plays a very significant role to tackle most of the contagious disease (zoonotic in nature) that are affecting human health. The zoonotic contagious disease represents a significant threat to global health, economic growth, and security (Allen et al. 2017). The One Health objective can be effectively implemented for combating AMR as well as zoonotic threats. The main motive of One Health goal is represented in Fig. 7.3.

Over the past three decades, it has been observed that majority of the novel, emerging zoonotic infectious diseases originated from animals, especially the wildlife (Taylor et al. 2001). However, the key drivers of such emergence are thought to be related with human activities like changes in habitats, use of land for agricultural intensification, urbanization, trade, and travel (Jones et al. 2008; Karesh et al. 2012; Jones et al. 2013). Collaborative and multidisciplinary access, cutting across boundaries of animals, humans, and the environmental health, is necessary to explain the ecology of each budding zoonotic disease in order to start the assessment of risk and develop strategies for response and control. These assumptions are very useful to recognize the demand and importance of collaborative cross-disciplinary access for the acknowledgment of budding and resurging diseases and in particular for the addition of wildlife health as a crucial component of global disease prevention, surveillance, mitigation, and control.

The One Health definition most commonly used was shared by the US Centers for Disease Control and Prevention and is defined as a collaborative, multisectoral, and transdisciplinary approach – functioning at the local, national, and global levels – with an ultimate goal of accomplishing optimal health outcomes, perceived interconnection between plants, animals, and their shared environment (Mackenzie and Jeggo 2019). One Health Global Network suggested a definition: One Health recognizes that the health of humans, animals, and ecosystems are interconnected.

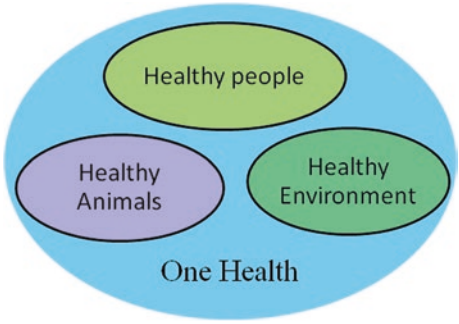

Fig. 7.3 Pictorial representation of One Health goal

It associates applying a collaborative, coordinated, multidisciplinary, and cross-sectoral approach with forwarding potential risk that originates from animals, humans, and ecosystem interface (Mackenzie and Jeggo 2019). According to One Health Institute of the University of California, Davis; One Health is a match to ensure the well-being of people, animals, and the ecosystem through combining problem-solving locally, nationally, and globally. However, the opportunity of One Health as conceptualized by many international organizations such as the WHO, UNICEF, and other organizations also clearly grasps other disciplines and domains, including environment, social sciences, ecosystem health, wildlife, land use, and biodiversity.

The American Medical Association is also working on One Health objective. The American Medical Association, WHO, and Public Health England are engaging in the incorporation of One Health objectives into the medical school curricula as a necessary part of the perspective of public health and infectious disease (Rabinowitz et al. 2017). The celebration of 3 November as One Health Day every year is important for global awareness of One Health views in public, particularly among young generation and students. The One Health Platform Foundation, the One Health Commission, and the One Health Initiative have started in 2016 to celebrate "One Health Day" by organizing One Health education sessions and public awareness events around the globe. In nutshell, we can say that the implementation of One Health approach in surveillance of AMR might be relevant in the integrated mitigation of AMR.

7.8 AMR Surveillance: Role of One Health Framework

For the integrated mitigation of AMR, the One Health approach role is significant. For integrated mitigation of AMR, we have to emphasize the way by which microorganisms develop resistance against certain types of antimicrobial agents and unify the AMR surveillance. In this section, we have focused on a collective data analysis approach from unified AMR surveillance and unified antimicrobial consumption surveillance. In the surveillance of AMR, the One Health approach framework plays a significant role. At a time, it unified and assimilates surveillance of antibiotic usage as well as utilization for humans and animals with AMR data from animals, humans, food, and the environment. The human consumption data are reuniting in the community and hospital, whereas in animals they are collected at the species level, i.e., wildlife, food-producing animals, and companion animals. AMR surveillance and conceptual framework of One Health is proposed here and illustrated in Fig. 7.4. The unified surveillance of AMR in humans include invasive clinical case sampling from community care and hospitals while noninvasive sampling from commensals. Whereas unified surveillance of antimicrobial consumption data can be collected from human sources such as hospitals and community care, the isolates can be accessed from commensals and clinical cases from the healthy animals in the food processing. In the same way, clinical case samples from wildlife can easily be

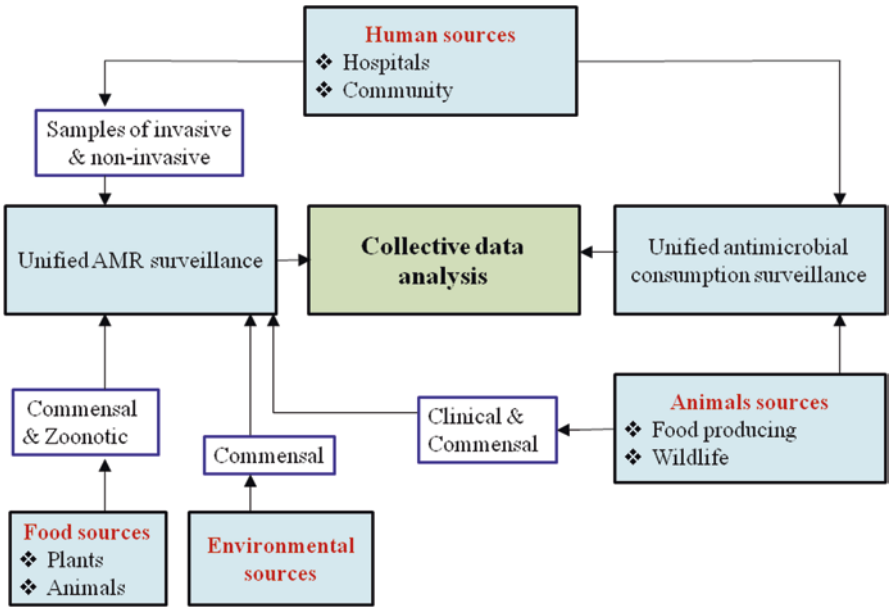

Fig. 7.4 Schematic representation of One Health framework in AMR surveillance

accessed through veterinarians and commensals from food production and wildlife. AMR surveillance in organisms isolated from food includes plants and animals, while the surveillance of zoonotic pathogen is from the commensals. However, samples from water sources and soil can be taken for the surveillance of the environment. After the collection of data from such categories, it requires a unified analysis method facilitated by a company experienced in analyzing such various sectors. In the same way, clarification of analysis requires the company of sectoral professionals who also maintain intersectoral understanding and knowledge which unify skills to work with those from other sectors, social scientists, and behavior change experts. This is to assure that the One Health concept is developing the subsequent recommendations for AMR surveillance. For surveillance purpose, a centralized program is required to set standards for the data collection from various sources for analysis and interpretation. Meantime, this approach will also improve communications and networking between sectors and disciplines via shared meetings, discussions, and report editing.

For integrated mitigation of AMR, the goal set by the One Health is global. For proper and unified AMR surveillance, guidelines given by different global agencies for the fulfillment of the One Health objective might be implemented at the global level. The antimicrobial agent consumption surveillance might be implemented at the global level for the achievement of One Health goal in a way of mitigation of AMR. A strong network recommended for global agencies are dealing with unified AMR surveillance, unified antimicrobial consumption surveillance, and their collective analysis. In nutshell, we can say that public awareness and implementation of One Health approach aim can resolve AMR.

7.9 Ten Ways to Tackle AMR

AMR is a major global health problem, and if action is not taken against AMR, then it will be the leading cause of mortality of almost ten million peoples annually by 2050 (Review on antimicrobial resistance 2016). Recently, researchers have reported that AMR is a global health problem and the expected mortality rate by 2050 in different regions will be as mentioned in Table 7.3. Consequently, One Health principles have been working globally as a tool to tackle AMR. The most important key messages to tackle AMR are adopted from the 2016 report (Review on Antimicrobial Resistance) by Jim O'Neill. This review has mentioned ten different perspectives to tackle AMR:

1. *Public awareness program*
   It is a very tough task to educate our entire societies about the side effects, misuse, and overuse of antimicrobials or antibiotics. This is because patients directly demand antibiotics from doctors or buy them directly from pharmacists for any infectious diseases. An efficient and well-delivered public awareness program can definitely improve public viewpoint and can reduce the sale of unprescribed antibiotics.
2. *Prevent the spread of infection by improving hygiene and sanitation*
   Improving sanitation and hygiene is essential to counter infectious diseases. In the twenty-first century, this is true and is very crucial to reduce the rise of multiple drug resistance. If fewer people get infected by microorganisms, the demand for antibiotics will automatically reduce, and fewer cases for drug resistance will arise. Countries must work on this common aim. The economic condition of all developing countries in the world is not good; these countries should give emphasis on improving the basics first, i.e., by expanding access to sanitation and clean water. The developed countries should focus on reducing the infections among the healthy, by preventive superbugs in hospitals. We all can contribute to this goal of controlling the infection by simply following proper sanitary measures.
3. *Restrict unusual use of antimicrobial in the agriculture sector and their dissemination into the environment*
   Nowadays, in agriculture and aquaculture to maintain the animal welfare and food security, antimicrobials are used frequently. However, the use of antimicrobial agent is not for treating the diseased animal, but it is to prevent infections or simply to promote growth and production. The majority of antimicrobial agent consumption across the world comes from aquaculture and agriculture. In the United States, around 70% (by total volume annually) of antimicrobials that are medically important are frequently used in the agriculture sector. The use of such antimicrobials for infection prevention and growth promotion should be considered unnecessary and dangerous.
4. *Improve worldwide surveillance of drug resistance and antimicrobial consumption*
   To improve global surveillance of AMR as well as antimicrobial consumption, surveillance is essential. For scientists and general physicians to explain

the mechanisms of AMR development of new drugs by monitoring the already present cases and to predict the future health threats, they need to have proper insights into the present and past data of AMR. Therefore, three main areas are required for better information and structure, i.e., (1) consumption of antimicrobial among animals and humans, (2) rate of resistance for the available drugs, and (3) scientific knowledge on AMR molecular foundations.

5. *To cut unnecessary use of antimicrobials and promote new and rapid diagnostic*

   Rapid diagnosis of any disease plays a significant role in rapid management and accurate treatment. Therefore, the development of new rapid diagnostics could transform the way of antimicrobial drug use in humans and animals, reducing the unnecessary use of antimicrobials, so that, existing drugs can last longer. Annually, about 27 million patients in the United States are taking antimicrobials unnecessarily due to misdiagnosis of the disease. Therefore, only the development of rapid and accurate diagnostic tests would be helpful for doctors to target disease with suitable antimicrobials.

6. *Encourage development and use of vaccines and other alternatives*

   Development of vaccine for infectious disease can prevent infection and can therefore decrease the demand of antimicrobial drugs automatically. This will inevitably reduce the cases of drug resistance. Although, at present, there is a scarcity of licensed vaccines against the urgent pathogens, there are some promising clinical candidates coming up against *Pseudomonas aeruginosa* and *Clostridium difficile*. However, greater investment is recommended for early-stage research of such new vaccines. Further, alternatives to antibiotics can also be opted which includes probiotics, antibodies, and phage therapy.

7. *Improve the number and recognition of people working with infectious disease*

   A qualified workforce is required for proper implementation and management of AMR. Currently, there is a shortage of expert physicians and microbiologists to deal with such infectious diseases. Therefore, countries need to invest in rewarding and training such specialists.

8. *A worldwide innovation fund for noncommercial and early stage*

   Nowadays, there is no sufficient public and private investment in research and development which focuses on tackling AMR. We need to emphasize on early-stage extensive research in the area of pharmacology or diagnostic. Therefore, we should generate a global innovation fund to support attractive research in this field.

9. *Better incentives for improving the existing ones and promote investment for new drugs*

   Developments of new antimicrobial agent for pharmaceutical companies are not attractive until widespread AMR has emerged against previous generations of drugs. It is very difficult to predict exactly when and how AMR will develop, leaving the pharmaceutical companies in an uncertainty in making business decisions. Developing new antibiotics is an expensive process for companies. The clinical trial networks and harmonized regulations can play a significant role in the regulatory aspects of new drugs for reducing the development costs.

10. *Build a global coalition for real action*

    AMR is not a problem of any one country or even anyone region, so this problem cannot be solved by any one country. AMR is a global problem, and we should plan a global action to make meaningful progress in tackling AMR. The One Health objectives are impacting in tackling AMR on the international political agenda.

7.10 Strategies of One Health in Mitigation of AMR

A strategy of One Health approach is important for integrated mitigation of AMR. In this section, we are highlighting the significance of One Health strategies in combating AMR, and the same is pictorially represented in Fig. 7.5. At present, the emphasis of One Health strategy is to reduce the usage of antimicrobial agent in food animals (White and Hughes 2019). Recently, a meta-analysis of multiple scientific studies and systematic review observed an association between reduction in antimicrobial use in food of animals and decrease in AMR in animals with limited evidence of decline in humans. Thus, by recognizing the complexity of this system, it prevents expression of highlighting research gap and clearing causal pathway (Hoelzer et al. 2017; Tang et al. 2017; Scott et al. 2018). In a report to the president in 2014 on combating AMR (President's Council of Advisors on Science and Technology 2014; World Health Organization 2017), this complexity was highlighted. The WHO is very actively involved in addressing AMR via One Health

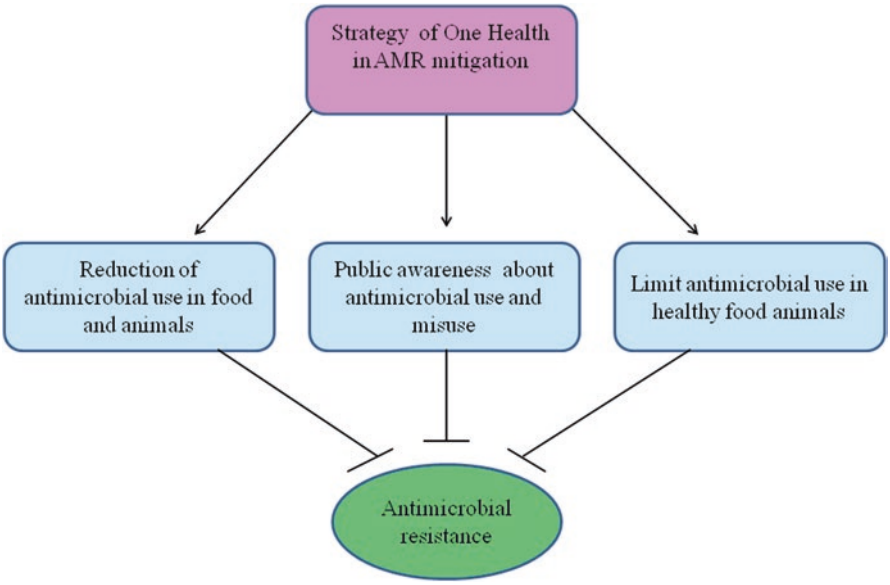

Fig. 7.5 Pictorial representation of One Health strategy in AMR mitigation

approach. The WHO has a role in making guidelines from time to time for the mitigation of AMR. Recently, the WHO published a new guideline to limit or reduce the use of antimicrobial in healthy food animals (World Health Organization 2017). This guideline on antimicrobial use in food and humans is important in combating AMR, a comprehensive approach that addresses aquaculture, wildlife, and the environment (Aga et al. 2018; Thakur and Gray 2019). The wildlife serves as a resistant gene as well as a resistant organism reservoir. For example, drug-resistant *E. coli* have been found in many wildlife species, which could spread among human and livestock (Furness et al. 2017; Dolejska and Papagiannitsis 2018; Weiss et al. 2018). The unnecessary use of antimicrobial agent is the leading cause of AMR, and One Health research gives stress on public awareness about antimicrobial use and misuse for combating AMR. The increasing AMR has an impact on wildlife too. One study revealed that the human strain of drug-resistant *Staphylococcus aureus* indicated the antimicrobial resistance in chimpanzees as well and this indicates the impact humans have on nonhuman primates (Schaumburg et al. 2012). However, there is a paucity of available data on the public health impact of the use of antimicrobial agent in aquaculture, and therefore, the administration of antimicrobial in aquatic systems may promote antimicrobial resistance development (Cabello et al. 2016). Other studies have revealed that antibacterial-resistant genes in aquatic systems can transfer the gene from aquatic microorganisms to human pathogens (Jiang et al. 2012; Tomova et al. 2015). Due to the unnecessary use of antimicrobials which are widely dispersed in aquatic system, it is becoming a containment challenge (Heuer et al. 2009). Environmental contamination related to antimicrobial administration both in food production (aquaculture, cropland, and livestock) and in humans via runoff has been identified (Kumar et al. 2005; Boxall 2012). However, a recent review on the One Health has concluded that environmental health expertise was underrepresented (Khan et al. 2018), and therefore, inclusion of environmental issues becomes necessary in the action plan for mitigation of AMR. Environmental pollution from the production of antimicrobial has been connected to contamination of the surrounding environment (Khan et al. 2013; Larsson 2014). So we can say that the unnecessary use and misuse of antimicrobial agent promote its resistance development, and for the integrated mitigation of AMR, we should limit the use of antimicrobial agent. One Health research is very focused on some basic and easy steps in the way of mitigation of AMR such as public awareness regarding the use and misuse of antimicrobial agent, reduction of their use in food and animals, and limiting their use in healthy animals and the environment.

7.11 Conclusions

Antibiotics are lifesaving drugs and have been used for treatment of infectious disease. The prevalence of antimicrobial resistance has increased. Nowadays, AMR has become global health threats. Day by day, human practices such as misuse or overuse of antimicrobial agent in health care, inappropriate food handling, poor

sanitary conditions, and poor infection control and prevention practices in hospitals make AMR worse. Due to AMR infection, the global mortality rate has increased. Therefore, for extensive treatment of AMR-infected patients, the health-care sector needs new-generation drugs. Researchers are extensively working on the synthesis of new-generation drugs. New-generation drugs are very expensive, which is leading to the cause of global economic loss. The socioeconomic perspectives have great impact on AMR infection. For exact evaluation of socioeconomic perspectives on the impact of AMR, some viewpoints such as patient perspectives, physician perspectives, health-care perspective, and societal perspectives are very useful. Generally, the annual budget for health and family welfare of developed counties is higher as compared to developing countries. Therefore, people of economically developed countries have better life and recovery rate from AMR infection compared to developing countries, whereas the rate of AMR infection in people of urban area is higher than the rural area due to sewage problem, supply of contaminated water, and biomedical waste disposal. Social stigma has great impact on AMR infection. Poor people know that AMR infection treatment is very expensive and patient becomes panic that may hamper recovery rate. Scientists, the WHO, and different health-care organizations have focused on the integrated approach for mitigation of antimicrobial resistance, and the One Health approach is one of them. The concept of One Health approach is to ensure best health for animals, humans, and the environment. The WHO and these organizations have developed guidelines to limit the misuse of antimicrobial agents for agriculture, animals, and human. Furthermore, the food chain and environment can spread antimicrobial resistance. Thus, understanding the importance of environmental, food, and agriculture sectors in developing AMR can lead us in preventing the spread and development of AMR. The integrated mitigation of AMR may reduce loss of global GDP. Therefore, we can say that AMR has great impact on global socioeconomic condition and implementation of One Health concept may reduce global economic burden.

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Part II
Mode of Action of Antimicrobials in Mitigation of AMR

Chapter 8
Regulations in Antimicrobial Drug Development: Challenges and New Incentives

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8.1 Introduction

Recent decline in the developmental rate of antimicrobial agents resulted in less frequent approval of novel antimicrobial drugs (AMDs). Due to the slow development of AMDs, the microbial population developing the resistance to existing drugs could not be counterbalanced, therefore restricting the choices of treatments. Several reasons account for this decline in developmental rate including monetary issues, trouble in designing clinical trials, and hindrances in examining the medication for acute infections. The approval of new drugs for their use in particular treatment is decided by regulatory bodies like the US Food and Drug Administration (FDA) and European Medicines Agency (EMA). Though irrespective of the regulatory bodies, the enrolment and approval of drugs are exorbitant. Several measures have been taken by the FDA to encourage the development of antimicrobial medications so that medical practitioners have access to more number of treatment choices. The collaboration of the FDA and EMA is a good initiative to exchange information and in taking decisions regarding clinical trial designs, considering that most of the drug advancement programs are delivered to both the USA and European controllers (Nambiar et al. 2014). From the last 35 years, the regulatory standards set by the FDA have been improved significantly, and newly developed regimens are more focused to accelerate the regulatory procedure to achieve the required medication for treating serious conditions (Darrow et al. 2014, 2020).

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In comparison to the pre-antibiotic era, the discovery of AMDs dramatically decreased the mortality rate resulting from acute microbial infections. In the pre-antibiotic era, 70–90% of the mortality rate was accounted for acute meningococcal meningitis, which was reduced to approximately 10% by subcutaneous use of sulfanilamides (Schwentker et al. 1937). Later, the use of antimicrobials was expanded to less severe diseases by clinicians, and this expansion was based on the selective and specific target of antimicrobials without any adverse effects on the host. However, this expansion did not consider the fact that several diseases are self-limited and get cured on their own. Due to insufficient data on the placebo-controlled trials of these antimicrobials, their efficacies for the self-limited disease are still questionable (Powers 2004).

Another question is about the safety of these antimicrobials in terms of public health. In the past, there were safety issues associated with the use of AMDs, like the use of sulfanilamides resulted in the loss of several lives due to side effects (Ballentine 1981). This incidence resulted in the enactment of the Food, Drug, and Cosmetic (FD&C) Act in 1938, under which, for the first time, the new drugs were subjected to pre-market safety evaluation (Temple 1995). Later in 1962, FD&C Act was amended due to the detrimental effects of thalidomide that was used for leprosy treatment. The amendment clearly stated that the FDA's decision on the drug's approval would rely on scientific testing, proof of safety and "substantial evidence" of a drug's efficacy. However, the majority of the antimicrobials were introduced in the market for use before this amendment. Nonetheless, this amendment led to the recognition of the fact that the adverse effects of the drugs should be compensated by their beneficial effects; otherwise, there is no point in their use as antimicrobials (Powers 2004).

Since the 1960s, new antimicrobial classes were evidently absent from the FDA-approved list of antimicrobial agents. In the 1980s and 1990s, the FDA approved 29 and 22 new AMDs, respectively (Powers 2004). Only 3% of the 225 anti-infective new molecular entities approved by the FDA were new antimicrobial agents (Spellberg et al. 2004), which showed that the AMD development would soon reach its saturation point. This decline in the development despite huge expenditure on the research and development (R&D) of AMDs suggests the inherent challenges in this field and the rigorous regulatory guidelines that pharmaceutical industries have to go through for approval.

The FDA, later, took up the testing standards for drugs previously established voluntarily by the American Medical Association's (AMA) Council on Drugs, the US Pharmacopeia, and the National Formulary. Since 1962, the FDA has overseen substantial refinements to the broad legal requirement that post-1962 new drugs be approved on the basis of "adequate and well-controlled" studies (Daemmrich 2007). In the 1980s, the FDA and Infectious Diseases Society of America (IDSA) formulated the first ever guidelines for conducting the clinical trials of AMDs for their subsequent approval (Shlaes 2010). Currently, the FDA along with the EMA, International Council for Harmonisation (ICH), and World Health Organization (WHO) are the leading international institutions guiding the evaluation of controlled clinical trials and market release of medicinal drugs (White 2008).

8.2 Drug Development Pipeline

Drug development is a complex and lengthy process involving several steps that usually take years and decades to complete. The drug development pipeline comprises five major steps comprising drug discovery, preclinical research, clinical trials, approval, and post-market surveillance (Fig. 8.1) (FDA 2021a). In the first step, researchers identify the most promising potential candidate from possibly hundreds or thousands of compounds and develop an assay for further evaluation and target optimization. The second step involves extensive preclinical studies that generally comprise in vitro, in vivo, and ex vivo screening complying with good laboratory practices guidelines led by drug regulatory agencies like the FDA and EMA. Also, detailed information about the target, ADME-Tox (absorption, distribution, metabolism, excretion, and toxicity), and dose profiles of the drugs is calculated. Based on dose and toxicity profiles obtained in the preclinical studies, the clinical trials are planned. This step may be considered as the most crucial step, as most of the drugs do not qualify the clinical trials owing to its stringent criteria and ethical issues involved (Chow and Liu 2008). The clinical trials are planned after acquiring information on the qualification criteria of participants, number of participants, duration of the study, control and target group to limit any bias, dose administration, assessment criteria, type of review, and analyses. The successful clinical trials are followed by approval of regulatory authorities if it is considered safe and effective for the proposed use in the specified target population. It is important to acknowledge that despite the rigorous drug development pipeline, few limitations exist in the process. Thus, the true scenario about the drug's safety and optimal usage evolves over a period of months or maybe years that makes up the lifetime of the product in the market. The problems and/or side effects of over-the-counter drugs are always under surveillance, and regulatory bodies like FAERS (FDA Adverse Event Reporting System) may direct to add cautions and usage information or may ask to retract the product in case of serious issues (FDA 2021a).

The developmental pipeline faces several challenges of cost and uncertainties of positive outcomes at each step. Besides, additional hurdles, like reproducibility, result validations, insufficient knowledge of the mode of action, lack of specific targets and biomarkers, high failure rate, patient heterogeneity, and regulatory challenges, pose a burden on the researchers (Norris et al. 2012). Each year, a few tens of drugs are approved for use, while several thousands of drug candidates fall by the wayside. The drug development journey of the licensed drugs begins from the

Fig 8.1 Overview of the drug development pipeline

laboratory bench and undergoes an extensive analysis at the cellular and molecular level that might have taken around 10–30 years and cost around billions of dollars (Torjesen 2015).

The modern drug history dates back to the early 1800s when J V Liebig began synthesizing organic molecules and synthesized the first sleeping drug "chloral hydrate" in 1832 (Gauillard et al. 2002). The historical drug development process took several decades for drug commercialization, for example, quinine (isolation drug) was discovered in 1820, but its synthesis mechanism was deciphered in 1944. In the early twentieth century, drug development picked up the pace, and later, toward the end of the twentieth century, more stringent norms were introduced for drug development and approval process. Several drugs like bevacizumab (anticancer), valdecoxib (anti-inflammatory drugs for rheumatoid arthritis), etoricoxib (cox-II inhibitor for rheumatoid arthritis), and alirocumab (whole antibody against high cholesterol) have been approved after 10–15 years since their discoveries (CentreWatch 2021). This time period is cut down to 1–6 years recently, and constant efforts are being made to further streamline the process.

8.3 Clinical Trials

Clinical trial is an important and specialized form of a bioassay to test the therapeutic efficacy, effectiveness, and safety of a new AMD against a specific disease (Chow and Liu 2008). Other trials like investigational trials and observational trials are conducted to define the safety as well as the effectiveness of AMDs under controlled conditions and the health-related concerns of the population in natural conditions, respectively. However, clinical trials are considered most important as these measure the therapeutic effectiveness of AMDs. For a successful clinical trial, every AMD has to pass preclinical trials that involve in vitro and animal studies subjected to a wide range of AMD doses to obtain the preliminary data on efficacy, toxicity, and pharmacokinetics of AMDs (Dudley and Ambrose 2000; MacGowan 2004; MacGowan and Bowker 2002).

The clinical trials are conducted in different phases constituting extensive treatment and observational strategies. Phase I trials are carried out to determine the pharmacokinetics, safety, and gross effects of AMDs on healthy individuals. After successful completion of phase I, phase II trials are conducted wherein pharmacokinetics, safety, and the therapeutic efficiency of AMDs are determined on selected patients. In phase III, the safety and therapeutic efficiency of AMDs are studied on hundreds of selected patients. Once a drug qualifies all three phases with good potential of minimizing the disease condition without any major side effects, it is approved by the regulatory bodies and then commercialized. Lastly, in phase IV, clinicians and medical practitioners review and evaluate the effectiveness and adverse reactions of the test drugs (Stolberg et al. 2004). In 2006, the FDA incorporated one more phase, phase 0, before the phase I trial. Phase 0 trial is meant for speeding up the promising drug development. It involves the testing of AMDs on a

comparatively smaller number of subjects to obtain the preliminary pharmacokinetic and pharmacodynamic data (Martinez and Silley 2010).

To reduce the effect of any imbalance or bias, the clinical trials are randomized and controlled. The randomized and controlled clinical trials are the simplest, yet the strongest tools for clinical studies and are considered as the gold standard for establishing the efficacy of new drugs. In the randomized controlled trials, random allocation of participants is done to receive the test AMDs, and this allocation is not decided by the clinicians or the investigators; thus, all the participants have an equal chance of being allocated to any of the test groups, minimizing the chances of imbalance and biasness (Altman and Champman and Hall 1990; Jadad 1998). Randomization of the participants rules out the possibility of any other variations except the AMDs; thus, any significant differences among the groups can be directly attributed to the AMDs. Despite being an effective tool, randomized controlled trials have some drawbacks. Due to financial constraints, low compliance expectations, or high dropout rates, random clinical trials (RCTs) are not feasible. To conduct a successful RCT, the sample size plays a key role. In order to get statistically significant differences between the groups under study, the difference in the effects should be large; otherwise, the sample size should be increased to get reliable data (Chan et al. 2001). The RCTs can be classified into several categories based on the needs of results as shown in Table 8.1.

In order to follow the strict regulatory rules and/or guidelines to reduce the imbalance and biasness in the outcomes, even the seemingly promising AMDs failed to get approval, resulting in the approval of very few drugs. Therefore, several investigators struggle to get approval for many AMDs after successful clinical trials. Thus, there is a need of few amendments in the regulatory guidelines that relax a few parameters in the trials without compromising the quality, safety, efficacy, and effectiveness of the AMD trials.

8.4 Challenges in Antimicrobial Drug Development

The decline in the development of new AMDs started in the 1980s that may be credited to several setbacks like recruitment of patients for clinical trials, spiraling costs to develop new AMDs, acceptance of the new AMDs in the market, and also the regulatory requirements.

The most frequent challenge faced in the AMD development is the slow recruitment process during the clinical trials which are mainly due to low number of eligible patients (Kadam et al. 2016). Even if sufficient patients are available to conduct the clinical trials, other factors, such as resources and stringent policies for trials, come into the picture. Regardless of every attempt to figure out and tackle the problems associated with patient recruitment, the challenge persists. To overcome these issues, Clinical Trials Transformation Initiative (CTTI) came up with effective clinical trial recruitment planning which includes (1) trial design and protocol development that focus on the information source, design elements, and activities

Table 8.1 Classification of randomized clinical trials (RCTs)

Basis of classification	Classification of trials	Description of trials	References
Treatment efficacy	Superiority trials	It shows that the new AMD is superior or better than the control or the existing AMDs	(Sackett 2014; Bokai et al. 2017; Sackett 2004; Lesaffre 2008)
	Non-inferiority (NI) trials	It shows that the new AMD is not better but also not worse than the control or the existing AMDs	(Bokai et al. 2017; Sackett 2004; Lesaffre 2008)
	Equivalence trials	It shows that the new AMD is not too different clinically than the control or the existing AMDs	(Bokai et al. 2017; Sackett 2004; Lesaffre 2008)
Evaluation of different aspects of AMDs	Explanatory	It shows whether AMD works and its mode of action	(Stolberg et al. 2004)
	Pragmatic	It shows whether AMD works and its side effects	(Stolberg et al. 2004)
	Efficacy	It shows the efficacy of AMDs, i.e., effect of AMDs under ideal conditions	(Stolberg et al. 2004)
	Effectiveness	It shows the effectiveness of AMDs, i.e., effect of AMDs under daily life conditions	(Stolberg et al. 2004)
	Phases 1, 2, 3, and 4	These trials are done for the market release of AMDs	(Stolberg et al. 2004)
Participant's exposure and response to AMDs	Parallel	Each group of participants is treated with only one AMD	(Louis et al. 1984)
	Crossover	Each group of participants is treated with all the AMDs under study in succession	(Louis et al. 1984)
	Factorial	Each group of participants is treated with one or more AMDs separately as well as in combination and against a control	(Jadad 1998)
Number of participants	N-of-one or individual patient	It considers an individual patient as the sole unit of observation to study the AMD treatment.	(Stolberg et al. 2004)
	Mega-trials	Thousands of patients from multiple centers and different countries are treated with AMD, but the data collection is limited	(Charlton 1995; Woods 1995)
	Sequential trials	The number of participants is added sequentially and continuously in the study until a clear effect of the AMD is observed or until the investigators get convinced that there is no effect of the AMD	(Stolberg et al. 2004; Altman and Champman and Hall 1990)
	Fixed trials	Either arbitrarily or statistically, investigators decide the sample size to study the effect of AMD. This method is more commonly used	(Stolberg et al. 2004)

(continued)

Table 8.1 (continued)

Basis of classification	Classification of trials	Description of trials	References
Level of blinding	Single-blinded trials	It involves blinding of participants only, i.e., they do not know which AMD treatment is given to them	(Stolberg et al. 2004)
	Double-blinded trials	It involves the blinding of participants as well as clinicians	(Stolberg et al. 2004)
	Triple-blinded trials	It involves blinding of participants, clinicians, and investigators	(Stolberg et al. 2004)
	Quadruple-blinded trials	It involves blinding of patients, clinicians, investigators, and statisticians, but this method is rarely used	(Stolberg et al. 2004)

that drive recruitment, (2) trial feasibility and site selection that mainly consider practicability of trial- and site-related issues, and (3) recruitment communication planning to keep the study team informed about the stakeholder's requirements since communication is necessary to complete trial enrolment successfully. Therefore, to improve the clinical trial recruitment process, CTTI suggested initial stage strategies for recruitment design that produces a positive impact in reducing later-stage hurdles (Huang et al. 2018). Ethical and regulatory obstacles are also important barriers in the recruitment process. Enormous time required for the ethical and regulatory review of the implementing grant sometimes leads to the expiry of grants even before the recruitment process started (Alemayehu et al. 2018).

Sample size is an important factor in the clinical trials, which if not met may incur an increase in type 2 error (erroneously concluding that there is no significant difference observed between treatment groups) (Kadam et al. 2016). In order to show the efficacy of AMDs among different races, it becomes necessary to increase the sample size with the heterogeneous population. Bigger sample size with a heterogeneous population may help in getting AMD globally accepted. However, to launch the AMD globally or across different countries, it is necessary to get the AMD patented individually in the targeted country since the rights granted by a country's patent are extendable only to that particular country and do not affect a foreign country (Saha and Bhattacharya 2011). So, companies willing to protect patents in other countries must apply for a patent in each of the other countries or regional patent offices.

Developing a new AMD and successfully launching it into the market are risky, lengthy, and expensive ventures (Atkinson and Jones 2009). Irrespective of the AMDs, the regulatory cost to launch them in the market was rising. For instance, it globally costs approximately $1581 million to develop a new AMD, excluding the postlaunch surveillance (Towse et al. 2017). Profitability is also a major challenge in the development of new AMDs. The new AMDs are often considered as a last resort in the treatment of any infectious disease where already existing AMDs failed to work, thus resulting in the sale of fewer units. Due to this, several large

pharmaceutical companies have already exited the market, and the "small- and medium-sized enterprises" (SMEs) are struggling to get finances for their efforts (Årdal et al. 2020).

Second perception was that there are already enough antibiotics in the market to tackle infectious diseases and there is no requirement of the new AMDs (Duval et al. 2019). However, there was a concern about antimicrobial resistance (AMR), but the numbers of resistance development cases were not enough to encourage the pharmaceutical and biotechnology companies to invest. At the same time, there are extensive regulatory barriers for the new AMDs which require strict validation for the potential of AMDs in fighting against the AMR. Regulatory bodies, however, relaxed some rules in order to address the issues, but they are still bound by the overarching intent of enabling legislation that is designed to protect patient safety. Also, due to the lack of regulatory guidelines for pediatric drug development and evidence-based treatments, infectious diseases are still a major cause of morbidity and death in children. The regulatory plans need to be developed to carefully evaluate the dose regimen and formulation of the new AMDs for the treatment of infectious diseases in children (Årdal et al. 2020).

In order to ensure the patient's safety, regulatory bodies have devised several guidelines. However, these guidelines pose exhaustive regulatory barriers which are big hurdles in the development of new AMDs, resulting in decreased progress in the drug development pipeline of new AMDs. In addition, the possibility of adverse outcomes from the trials is also hampering the R&D of novel AMDs (Alvan et al. 2011). It is necessary to understand the problems hindering the development of new AMDs to get effective resolutions to be executed at the R&D level. However, to address these problems, the perspectives of the R&D professionals are required (Bettiol et al. 2015). The difficulties encountered by the pharmaceutical companies are multifactorial that include (1) monetary issues, (2) the challenging research to study the therapeutics for an acute infectious disease, and (3) scientific issues with regulatory guidelines for AMDs.

There is a need for quick diagnostic tests, updated regulatory norms, as well as advanced trial designs specifically in the case of multidrug-resistant pathogens. Mittra et al. proposed some factors that impede the development of AMDs such as market feasibility and the interplay between identification of rapid pathogens and their impact on trial expenses, specifically, for narrow-spectrum AMDs (Mittra et al. 2019). It has also been reported that previously the capacity to produce commercially viable AMDs was hindered by the regulatory guidelines set for the clinical trials, but now unappealing business prospects are the primary boundary.

As per the FDA regulatory norms, the efficacy and safety drug profile of the new AMDs has to be determined before patients are exposed to them. According to the FDA, the review of AMDs under investigations should have abstained until sufficient data is obtained to justify the risk-benefit profile of investigational AMDs (Darrow et al. 2014). Conclusively, it seemed difficult to fulfill the high standards of regulatory guidelines regarding the trial conduct. To allow the successful conduct of practical and ethical trials, the FDA and EMA have tailored regulatory guidelines. To enable small trials, there is a need to modify the statistical demands together with

advances in enrolment conditions. An effort has also been made to decrease the regulatory weights for new AMDs for patients with restricted treatment alternatives. Therefore, revised guidelines are now no longer an obstruction to the pharmaceutical companies in the path of discovering new AMDs (Mittra et al. 2019).

For the first time, the clinical trials on the patients infected with the same infectious agent but at different anatomical places could be pooled. These pooled trials can show the positive profile of the drugs for the different infections caused by the same infection agent and thus made the trials more economical for such pathogens (Rex et al. 2013). To ascertain the safety and efficacy of commercialized medicines, the FDA established new strategies in July 2012, and a new class of "breakthrough therapy" was established by the Congress in the FDA Safety and Innovation Act (FDASIA). The drugs included under the breakthrough therapy were the new molecular entities that are used for the treatment of serious diseases and whose preclinical evidences showed their substantial superiority over the existing drugs (FDASIA 2012). To promote the supporters of antimicrobials and to seek after creative statistical and clinical improvement schemes, the FDA had proclaimed directions involving usage of (1) minor, shorter, or lesser clinical trials, (2) greater NI margins, (3) external controls (nonrandomized design), and (4) enhanced reliability on preclinical researches (animal studies) (FDA 2019; FDA 2017). The regulatory bodies are working hard to streamline and harmonize the regulatory policies to work toward globalization.

8.5 Harmonization of Regulatory Policies: A Collaborative Effort

The recent COVID-19 pandemic has made us realize the worth of globalization of healthcare products irrespective of physical and political boundaries. Despite rapid globalization in the development, production, and distribution of various technologies, the globalization of regulatory policies in medicines, especially AMDs, needs to be addressed on an immediate basis. The pharmaceutical industries that tend to expand in the international markets are required to obtain separate approvals for a single product from different countries. The different regulatory guidelines may require additional data, and even the cost and efforts of putting the same data in several different formats add to the total cost and delay in the release of the product. Thus, globalization and convergence of regulatory policies are necessary for early access of AMDs to patients (Bassetti et al. 2019).

The process of harmonization usually falls under three major categories, viz., regional, bilateral, and global. As the name indicates, the regional initiatives are carried out in a confined region only. For instance, the Europe Union (EU) can be considered as an example of a regional initiative; however, there exist several subgroups including the Gulf Cooperation Council, the Pan American Network for Drug Regulatory Harmonization, the Asia-Pacific Economic Cooperation (APEC),

etc. Likewise, bilateral agreements are usually made between two individual countries or groups of countries. For example, in the bilateral cooperation among the EU and the USA in April 2007, the agenda for promoting trans-Atlantic economic integration was signed which particularly targeted the simplification of administrative procedures in the regulation of medicinal drugs. The third major harmonization initiative is global and involves several establishments and countries (Lezorte 2014; EMA 2021a).

World Health Organization (WHO) and International Council for Harmonisation (ICH) To expand the horizons of global harmonization, many initiatives are being taken to achieve convergent regulatory policies like international collaborations in pharmaceutical projects, mutual recognition agreements (MRAs), and the establishment of the organizations like the WHO and ICH. Besides the WHO's key role in the pandemics and treatments across the globe, it has also given an internationally recognized classification system of medicinal drugs – anatomical therapeutic chemical/defined daily dose. The WHO has developed its own prequalification system for drug evaluation which comprises a team of evaluators from WHO staff and professionals from the national regulatory authorities (NRAs) (WHO 2013). Another international body, the ICH, was introduced in 1990 which united the pharmaceutical regulatory agencies from three countries, Europe, Japan, and the USA, to build consensual scientific and technical standards to achieve approval for medicinal products (Louis et al. 1984). At the first ICH meeting, the main criteria for the approval of new medicinal products were decided which were based on the safety, quality, and efficacy of the testing product. The ICH brought regulatory bodies and pharmaceutical companies together to analyze and review the technical and scientific issues related to pharmaceutical industries to frame comprehensive ICH guidelines addressing overall problems. The primary objective of the ICH is to accomplish better alignment in implementing necessary technical guidelines for the registration of the pharmaceutical product, which offers various profits to both regulatory agencies and pharmaceutical companies such as (1) minimizing repeated testing of the new medicinal products on humans and reducing animal testing along with maintaining the required safety standards and effectiveness, (2) decreasing the duration and resources for drug development, and (3) optimizing the regulatory evaluation for the application of the new medicinal products. Since its origin, ICH guidelines have gradually improved. Besides quality, safety, and efficacy, it extends its guideline to multidisciplinary topics like drug interactions, mutagenic impurities, etc. Recent ICH guidelines cover broad categories including clinical safety for the drug used in long-term treatment, pharmacovigilance, dose-response studies, general considerations for clinical trials, statistical principles for clinical trials, choice of control groups in clinical trials, clinical evaluation by therapeutic category, and safety data collection (I ICH 1990).

European Medicines Agency (EMA) The EMA performs a crucial role to combat AMR by promoting new developmental and treatment approaches, encouraging appropriate usage of existing AMDs, and gathering the necessary data to further

formulate policies and research. The EMA, FDA, and Japanese Pharmaceuticals and Medical Devices Agency (PMDA) are making collaborative efforts to promote a single development program for new AMDs fulfilling the regulatory standards of all three regulatory bodies. In successive meetings in the years 2016 and 2017, the decisions to harmonize data requirements and the process of clinical trials in specific ailments like gonorrhea and urinary tract infections were made. Consequently, they agreed upon comprehensive and multidirectional approaches to facilitate the common regulatory strategies (EMA 2021b). The major points addressed in these guidelines are (1) explanation of minimal inhibitory concentration of the test AMD, (2) pharmacokinetic (PK)/pharmacodynamic (PD) indices and PK-PD targets from nonclinical data using in vitro and/or in vivo PD models, (3) clinical PK data to support PK-PD analysis, (4) identifying the probability of target attainment (PTA) in order to decide dosage regimen, and (5) assessment of clinical exposure-response (E-R) relationships (EMA 2015a). Besides, several newer instructions are formulated by the EMA that emphasize on the PK/PD effects of antibiotics which provides the guidance to evaluate the development of new antibiotics especially drugs against multiple drug-resistant (MDR) bacteria. These guidelines delineate the regulatory outcomes for the application dossier and describe the scientific progress in pharmacometrics allowing better developmental programs for antimicrobial agents. Consequently, PK-PD analysis has become a key player in deciding the efficient dosage regimen for the AMDs, e.g., beta-lactamase inhibitors, and drugs against MDR pathogens (EMA 2015b).

In the addendum to the guidelines on the evaluation of medicinal products for treating bacterial infections, EMA provided meticulous guidance on the best suited study design for conducting studies in human infections. The guidance regarding the clinical evaluation of the drug administered directly at the site of infection to exert local antimicrobial action remains inadequate, for instance, topical remedy for superficial skin infection, inhalational therapy, and oral administration to exert an effect on the gut. Thus, the following indications in this addendum have presented guidance on clinical data demands: (1) indications for which NI trials are admissible, (2) indications which might need superiority study designs, (3) circumstances in which limited clinical data may be accepted, and (4) other indications requiring special attention to design the clinical trials and data interpretation, e.g., bacteremia, treatment of acute bacterial infections in neutropenic patients, eradication of carriage, and oral medication acting on the gut (EMA 2013).

Food and Drug Administration (FDA) The FDA plays a pivotal role in promoting public health and outracing MDR. The FDA is a member of several international regulatory establishments like the ICH, Pharmaceutical Inspection Co-operation Scheme, and APEC and has always encouraged the implementation of common regulatory guidelines worldwide. The FDA had identified priority areas in the years 2012–2013 and labeled them as Focus Areas of Regulatory Science (FARS). These FARS are mainly based on the following initiatives: (1) emergency preparedness and response in public health, (2) innovation strategies, (3) data sciences, and (4) patient and consumer empowerment. The FDA also

developed a report on Advancing Regulatory Science: FARS with a tendency to accommodate frequent updates and revisions. The FDA's strategic plan against AMR announced in 2019 includes comprehensive approaches to coordinate policy and external collaborations in AMR. It addresses the following four key areas: (1) product development to combat AMR with a robust pipeline, (2) promotion of antibiotic stewardship to slow down the AMR, (3) surveillance for the adequate use of AMDs and resistance against them, and (iv) scientific initiatives to support stakeholders in case of resistance development (F FDA 2021). Besides these efforts, the FDA is also actively engaged in the R&D of novel/products and methods involved in animal research with the 3Rs strategy: replace, reduce, and refine 2021 (FDA 2021b).

Central Drugs Standard Control Organisation (CDSCO) The CDSCO is the national regulatory authority of the Government of India which has its headquarter in New Delhi. It has six zonal offices, four sub-zonal offices with associated ports and laboratories that carry out pre-approval as well as post-approval assessments, and post-market surveillance. The CDSCO performs regulatory tasks for the regulation of drugs and cosmetics in the country as conferred by "Drugs and Cosmetics Act," 1940 (Press Information Bureau 2021). It is the key authority preparing national guidelines that specify the regulatory pathways for approval of AMDs and emergency drugs, as in the case of COVID. The Drugs Controller General of India, CDSCO, granted emergency approval to "Covishield" and "Covaxin" developed by Serum Institute of India and Bharat Biotech, respectively (Press Information Bureau 2021). Under the 12th 5-year plan, the Center had allocated budget support of Rs. 1750 crores to strengthen the drug regulatory system in the country, and rapid measures are being taken to divert enough funds during the public health crisis of the COVID pandemic (Hirodkar 2021).

National Medical Products Administration (NMPA) NMPA, previously known as China Food and Drug Administration, is China's national body streamlining drug regulatory policies and has its headquarter in Beijing. Under the State Council of the People's Republic of China, the responsibilities of NMPA include supervision of the safety of drugs and medical devices, developing national regulatory guidelines, encouraging drug development, maintaining a quality management system, and conducting pre-approval and post-approval surveillance (National Medical Products Administration 2021). The NMPA has also approved the use of CoronaVac developed by Sinovac as an emergency measure against COVID (Xinhua 2021).

Besides coordinated and consistent efforts toward harmonization at the international level, authorities are also reviewing and updating existing policies and are receptive for formulating new ones. This is evident from several new incentives that are being considered for implementation.

8.6 New Incentives in Regulations and Policies

The scarcity of effective AMDs in the impending health crisis calls for newer incentives to encourage R & D in the pharmaceuticals. The regulatory norms for the R & D of AMDs are constantly being updated from the past few years to meet the global health implications caused by MDR organisms. In the wake of this global health challenge, the FDA and EMA have revised their regulatory guidelines for the development of AMDs (EMA 2013). Both the FDA and EMA are working toward antimicrobial stewardship; however, this, in turn, poses an obstacle in the path. It is critically important to maintain an equilibrium between supporting general well-being by authorizing the development of novel medicines and safeguarding community health. These regulatory bodies have faced huge criticism from pharmaceutical industries because of posing restrictions and implementing strict guidelines which led to huge financial losses. Thus, several strategies are being implemented to safeguard the interests of stakeholders.

The "push and pull incentives" are designed wherein government "pushes" SMEs by providing financial injections at the entry level, thereby lowering the financial risk and reduction in cost inputs. Such incentives usually come in terms of contracts, grants, public-private collaborations, tax credits, and clinical trial target networks (Morel et al. 2020). For example, CARB-X (Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator) has invested $500 million for the year 2016–2021 for the development of novel AMDs, diagnostics of multiple drug-resistant (MDR) pathogens, vaccines, and related innovations. On the other hand, pull incentives ensure a significant reward only toward the end of the pipeline, once an effective product/process is developed. These incentives may include indirect benefits like reduction in time of regulatory review, premium pricing, and extension of patent terms. However, the pull incentives take much longer implementation times with uncertain rewards and, thus, are not of immediate interest for SMEs (Morel et al. 2020).

In addition, the licensing authorities are constantly reviewing their policies to achieve a balance between the development of innovative AMDs and public health safety. The regulators are also displaying a better understanding toward the acceptance of patients for orphan drugs (lifesaving drugs) with rigorous scientific and legal implications. Thus, alternative pathways for early-limited registration and early-patient access are also proposed for such drugs. The FDA has recommended the enlistment of more patients with more stringent enlistment measures to ensure a better assessment of treatment impacts and trial risks. Mostly, the licensing process is very bureaucratic, time-consuming, and globally inconsistent that further adds on to the cost and timeline of the drug production. Consequently, drugs are released at much economical rates into low-cost generic markets leading to a decline in profit (Wise et al. 2011; Piddock 2013). The licensing authorities conceptualized the adaptive licensing processes that incorporate scientific advice, market sanctions under normal as well exceptional circumstances, risk management plans, and

regulatory measures within the legal framework of a particular region (Bax and Green 2014).

The EMA introduced two addenda in 2012 and 2013 to its guidelines for evaluation of the medicinal products against bacterial infections. These addenda permit flexibility in regulatory measures to enable the collection of satisfactory information and hence guarantee the balance between risk and benefit ratios. These addenda specified five main indications for acceptability of NI trials and emphasized the clinical development program for antibiotics to address the requirements of AMDs especially in the case of treatment of MDR pathogens. Additionally, other studies requiring superiority study models and special considerations in study designs are also enlisted (EMA 2013, 2011).

The EMA guidelines emphasize more over the PK/PD standards, wherein only a single key study may be accepted in support of an indication. In fewer cases, data extrapolation from adults to kids may be allowed. In the case of rare diseases, distinct targeted studies and/or standard randomized clinical trials can be used to gather efficacy data. However, in the case of MDR pathogens, a minimal number of treated cases may be required to support any claim on a case-to-case basis. The phase 1 investigations in human participants are carried out only if satisfactory results are achieved by the preclinical and early safety studies. Thus, all the data derived from PK/PD predictions based on in vitro/in vivo studies and preclinical and clinical studies provides better conclusions about the drug efficacy (Bax and Green 2014).

The tiered framework with categorical indications for either disease-based or pathogen-based labels is also in consideration for internationalized standards. The adaptive strategies are more convenient for pharmaceutics in obtaining early approvals and providing adequate risk-benefit data in later stages. Several pilot projects with "safe harbor" sessions among regulators, pharmaceutics, and stakeholders are already being conducted. Also, advanced approval may also be granted to conduct studies on restricted human volunteers if promising risk-benefit ratios are attained (EMA 2017). Besides, the pharmaceutical industries have welcomed the approach of post-marketing allegiances between the industries and the regulators which will result in efficient data collection and observational studies. However, cost-determining concerns are still an issue in such cases. The regulators have also acknowledged the evidence-based performance of "controlled clinical trials" which are the gold standard till date. As per the available data, NI trials for antibiotics don't produce reliable results (Bax and Green 2014). The customizable study designs allow pharmaceutical industries to acquire limited endorsement of new treatments much quicker during the drug development process with the aim to further deliver sufficient data on risk-benefit analysis (EMA 2007).

In exceptional cases, special provisions are provided by regulatory bodies in life-threatening diseases. During the current COVID pandemic, the FDA has created Coronavirus Treatment Acceleration Program (CTAP) to accelerate and assess the possible treatment strategies for COVID patients (FDA n.d.-a). The approval of the first antiviral drug, remdesivir, was done under an Emergency

Use Authorization (EUA) plan issued in May 2020 (FDA n.d.-b). Likewise, in January 2021, under President's Emergency Plan for AIDS Relief (PEPFAR), the FDA launched an interactive database offering crucial information about antiretroviral drugs to combat HIV owing to the global pandemic (FDA n.d.-c). The guidelines are also available for emergency use of unapproved investigational drugs/test drugs against life-threatening and/or severely debilitating diseases which may be exempted from approvals by institutional review boards (IRBs) and investigators (FDA n.d.-d). The "Right to Try Act" was signed in May 2018 to get access to unapproved drugs for patients who have exhausted all approved treatment options available and can't participate in clinical trials (Shlaes 2010). In this regard, informed legal consent and legal authorization are mandatory, and the drug should meet the qualification criteria set by Right to Try Act (FDA n.d.-e).

Despite making the required adaptations, the current regulations still pose challenges in many cases and, hence, require further modifications. However, several useful impacts are observed after modifying the regulatory guidelines. As per a survey, 74% of the respondents believe that significant positive outcomes would be achieved on harmonizing worldwide regulatory needs. Additionally, 50% of respondents suggested the four following modifications: (1) establishment of completely new regulatory pathways, (2) provision of regulatory guidelines based on the pathogen-specific indication in MDR pathogens, (3) restricted use of approved existing and new antibiotics, and (4) regulatory approval of external control data. It is believed that gradual revisions of the guidelines with additional rooms for special and emergency cases will be more beneficial (IDSA 2012).

8.7 Conclusions

The AMD development pipeline has been facing many challenges from the past two decades; many of them are identified and addressed while evaluation of others is in progress. It is very important to maintain a strict balance between drug development and its safety, efficacy, and accessibility which consequently leads to equally stringent regulatory guidelines by authorities. While disease-causing pathogens see no boundaries, the availability of drugs is many a times restricted by jurisdictional boundaries, thereby posing challenges in AMD development. Coordinated global efforts toward conceptualizing the common regulatory policies are being made. The extensive debates at regular conferences and meetings during collaborations of international regulatory bodies provide room for new updates and revisions. A thorough understanding, technological advancements, strong leadership, and willingness are all that take to frame and make changes in legal policies. It is believed that harmony among drug development, drug surveillance, and market release will pave the way for early access to novel, effective, and cost-effective drugs in the future.

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Chapter 9
New Insights into and Updates on Antimicrobial Agents

Vagish Dwibedi and Santosh Kumar Rath

9.1 Introduction

Worldwide, microorganisms and their contribution towards sustainable development are obliging for advanced research in microbiology and microbial drug discovery (Kuhad 2012; Koehn and Carter 2005). Natural products and their semisynthetic analogous have played a crucial role in the identification and development of antimicrobial drug innovation programme (Wright et al. 2014; Atanasov et al. 2021; Moloney 2016). In spite of the notable impact on wellbeing, nature-derived compounds have achieved specific attention for their potential activities against various pathogens. Undoubtedly, antimicrobial agents have saved the human race from piles of microbial infectious disease burden and remain one of the most significant discoveries in the twenty-first century (Moloney 2016). However, at present, the most crucial health trouble is widely seen due to the rise and spread of antimicrobial resistance among the different microorganisms (bacteria, fungi, virus, and parasites). The mechanisms for survival of the bacterial resistance under various unfavourable and toxic environmental conditions include (i) enzymatic alteration or degradation of drug, (ii) variation or modification in target, and (iii) reduced uptake or increased efflux. These mechanisms when act together are responsible for enhanced resistance (Abreu et al. 2012; Lambert 2005). Efflux-mediated resistance is an important mechanism for bacteria to expel the

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chemotherapeutic agent out of cell to render them ineffective. Inhibition of efflux is regarded as an efficient strategy for the rejuvenation of old antibiotics again to market as finding new antibiotics is a much time-consuming and costly affair. Many microorganisms are the major sources of precious bioactive molecules considered as useful secondary metabolites to stand and fight against various microbial resistant strains (Singh et al. 2017a). Many pure natural isolates along with newly developed scattered synthetic analogues have proved their eligibility as the best alternatives as antimicrobial agents against resistant pathogens (Abdel-Razek et al. 2020; Martelli and Giacomini 2018). Furthermore, natural antimicrobial agents have gained extensive interest among young and established researchers to reinstate the potency of ineffective antibiotics. Thereby, re-evaluation approach of existing drugs with a combination of newer pharmacophore as efflux pump inhibitors (EPIs) is now considered as best alternatives against multidrug resistance strains (P Tegos et al. 2011; Lamut et al. 2019). Many heterocyclic natural alkaloids are now well accepted along with known antibacterial due to their significant role as efflux pump inhibitory activity against many infectious diseases (Y Mahmood et al. 2016). A natural piperidine-type alkaloid, piperine, isolated from *Piper nigrum* and *Piper longum* and berberine, an isoquinoline alkaloid, isolated from roots and rhizomes of *Berberis vulgaris*, *Rhizoma coptidis* and *Cortex phellodendri* were identified as effective natural EPI to overcome the multidrug-resistant pathogens and also can improve the clinical performance of various other antibiotics when co-administered (Jin et al. 2011). Piperine and many of its analogues when co-administered with ciprofloxacin were observed to inhibit the growth of a mutant *S. aureus* strain by reducing MIC values noticeably (Rath et al. 2019). Palmatine, a newer natural alkaloid, acts as EPIs in *P. aeruginosa* by lowering the MIC-MBC level of ciprofloxacin (Aghayan et al. 2017). Reserpine, another plant alkaloid, is a known inhibitor of the Bmr efflux pump of *Bacillus subtilis* used to accelerate the action of tetracycline in *Staphylococcus aureus* strains and also observed reversing NorA-conferred multidrug resistance in *S. aureus* (Shaheen et al. 2019; Rath et al. 2019). Microorganisms are considered as useful drug targets for various widespread diseases. Though the fundamental life path of microorganisms, their responses to antimicrobials and concerned biochemical pathways seem to be quite complex they need to be understood and explored using modern tools of molecular biology.

Foodborne illness due to fungal or bacterial growth is another major issue in recent times. The widespread microorganisms can easily reach food, grow by utilizing nutritious materials and produce metabolites which are the major cause for the spoilage of plentiful food and food products (Pitt and Hocking 2009; Petruzzi et al. 2017). They can survive even in adverse conditions like low temperature, vacuum packing, processing, and modified atmosphere (Carpena et al. 2021). Thereby, considering the food safety and improving shelf life of foods, many significant efforts have been made by food industries and researchers to find existing or new natural antimicrobials as food preservatives (Gutiérrez-del-Río et al. 2018;

Carpena et al. 2021). Plants, bacteria, fungus, and animals are different sources of the production and recognition of antimicrobials. Plants, the major source of natural products, have been largely used in the domain of the antimicrobial drug finding process. The plant extracts, crude drugs and different class of secondary metabolites are now considered as major opportunities to identify newer antimicrobial medicines and food preservatives. Many recently identified extracts/compounds which are showing antimicrobial activity belong to the families of Asphodelaceae, Solanaceae, Rutaceae, Berberidaceae, Anacardiaceae, Rhamnaceae, Euphorbiaceae, Myrtaceae, Zygophyllaceae, Asteraceae, Erythroxylaceae, Lamiaceae, Colchicaceae, Amaryllidaceae, Verbenaceae, Lythraceae, Podocarpaceae, Salicaceae, Apocynaceae, Zingiberaceae, etc. (Singh 2018; VasudhaUdupa et al. 2021; Swain and Rautray 2021) Altogether, several class of compounds such as alkaloids, glycosides, terpenoids, flavonoids, tannins, and phenolic or polyphenolics isolated from natural sources especially plants are now taken in major consideration towards to development of newer antimicrobials (Takó et al. 2020). Natural crude extracts of ginger, mustard, garlic, cinnamon, basil, sage, and other herbal products are typically high in terpenes such as carvacrol, eraniol, linalool, and several other phenolic compounds, which serve as food additives and antimicrobials against broad spectrum of Gram-positive and Gram-negative bacteria (Makroo et al. 2021). Citral, a main component of lemongrass essential oil, has demonstrated important antioxidant and antimicrobial activity against a variety of food pathogens (Moumni et al. 2021). Furthermore, numerous extracts from Chinese chives and cassia have been documented to dramatically reduce the proliferation of *Escherichia coli* and other bacteria during the preparation and storage of foods, juices, and dairy products. Understanding the process of antimicrobial activity of medicinal plant extracts is therefore needed for their optimum use as natural antimicrobial agents to extend shelf life and maintain food safety (Makroo et al. 2021).

9.2 Antimicrobial Agents from Natural Origin

Natural antimicrobial agents are getting major attention of researchers due to their structural diversity, safety, and nontoxic status. Plants, microbes, and fungal sources are considered as best possible alternatives in finding natural preservatives to avoid or control microbial food spoilage (Saeed et al. 2019). Majorly, plants are having rich sources of many bioactive scaffolds bearing secondary metabolites which are now the primary focus of scientists to explore them to any particular target site to prevent/cure ailments.

9.3 Plant-Derived Antimicrobial Agents

Various phenolic compounds, terpenoids, volatile oils, flavonoids, and sulphur-containing compounds have been detected in seeds, herbs, and spices. These bioactive compounds can be present in plant leaves, branches, seeds, roots, flowers, bulbs, and other pieces. Many herbal and medicinal plants have been recognized for centuries for their preservative and antimicrobial effects (Tuyen and Le 2021). The rich sources of essential oils and different classes of secondary metabolites like terpenes, flavones, aromatic and aliphatic compounds bearing functional groups alcohols, esters, ethers, aldehydes, ketones, and lactones in plants can most effectively destroy several bacterial, fungal, or microbial pathogens (Hyldgaard et al. 2012; Orey 2019). Since times of yore, essential oils like peppermint oil, eucalyptus, and lemongrass are mostly used widely in tribal areas as natural antibacterial and antimicrobial agents due to their benefic application for myriad of cleaning and cleansing function (Sarkic and Stappen 2018; Orey 2019; Desam and Al-Rajab 2021). Traditional use of peppermint essential oil for mouthwash, tea tree essential oil as jewellery cleaner, cedarwood oil for flu and cold, and lavender oil as cleaner are most customary treatments usually followed (Chouhan et al. 2017; Sarkic and Stappen 2018; Desam and Al-Rajab 2021). The essential oils like 1,8-cineole, camphor, borneol, α-pinene, oleanolic acid, β-bisabolene, longicyclene, β-pinene, limonene, β-pinene, eugenol, β-isoeugenol, caryophyllene, α-humulene, p-cymene, γ-terpinene, thymol, and methyl chavicol in many plant species are responsible for antimicrobial activity (Chouhan et al. 2017; Martelli and Giacomini 2018; Ju et al. 2020; Orey 2019). Plant-derived antimicrobials like thymol, eugenol, carvone, citral, carvacrol, linalool, etc. were identified active against *L. monocytogenes* in food model systems (Kawacka et al. 2021; Ju et al. 2020). Alongside many naturally isolated flavonoids like quercetin, kaempferol, apigenin, chrysin, epicatechin gallate, naringenin, myricetin, phloretin, genistein, luteolin, etc. are responsible for promising antimicrobial/antibacterial activity (Manzoor et al. 2020). Many of these substances have a protective function and are effective for inactivating or inhibiting a wide variety of microorganisms. Coumarins and its analogues are widely accepted among various classes of natural bioactive agents for the treatment against diverse diseases related to inflammation (Sharma et al. 2019), cancer (Küpeli Akkol et al. 2020), and additionally these are also useful to control, prevent, and destruct various microbial pathogens (Gouda et al. 2020). Cinnamic acids and coumarins are examples of a large class of phenylpropane-derived compounds with the maximum oxidation state (Gupta and Pandey 2020; Sharma et al. 2018). The increase in hydroxylation of phenolic compounds might be a cause of better effectiveness against microbial pathogenic bacteria. It was proved that hydroxylated phenolic catechol and pyrogallol, which are having two and three hydroxyl groups respectively, are found lethal to microorganisms (Lima et al. 2019; Leontopoulos et al. 2017). Phloretin, a natural bioactive flavonoid, isolated from *Malus sylvestris* has shown antimicrobial activity against a variety of microbial pathogens. Withaferin A, isolated from *Withania somnifera,* is a potential drug lead itself considered strong

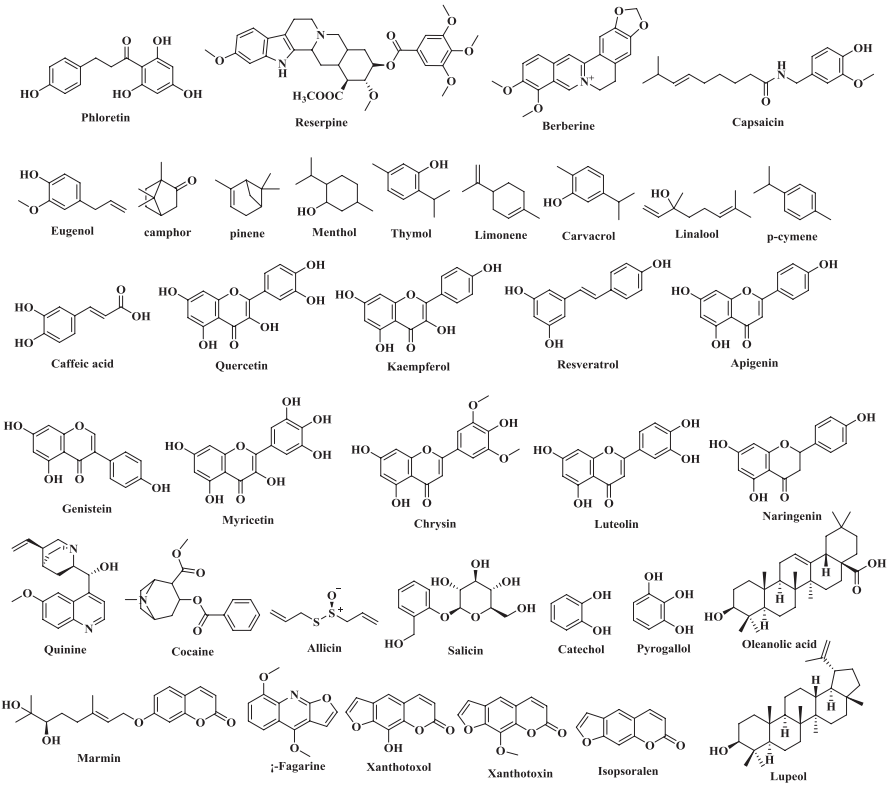

Fig. 9.1 Plant-derived antimicrobial agents

antimicrobial and useful starting material for the development of newer antimicrobials due to the presence of lactone ring and ketone containing unsaturation. Marmin, xanthotoxol, xanthotoxin, lupeol, γ-fragarin, and isopsoralen are class of alkaloids, flavonoids, and terpenoids in *Aegle marmelos* with many reported antimicrobial effects in different in vitro and in vivo assay methods (Reiter et al. 2020). Allicin, a diallylthiosulphinate bioactive defence molecule isolated from *Allium sativum* L., is useful as a broad spectrum antimicrobial agent. However, the instability issue of the molecule retards its effectiveness against microbes in normal or raise in temperature. Allicin's antimicrobial role is largely related to the thiosulphinate functional group (Leontiev et al. 2018) of the molecule. Resveratrol, a naturally occurring phenolic phytoalexin belonging to the stilbene family, has antibacterial activity against diverse Gram-positive and Gram-negative pathogens found in fruit (Dwibedi et al. 2021) (Fig. 9.1).

9.4 Bacterial Origin Antimicrobial Agents

Bacterial infectious diseases are most common in today's time especially in infants and a major cause of paediatric mortality. The antibiotics are the most widely used drugs as powerful therapeutics against various pathogenic bacterial infections (Berkley 2021). Antibacterial drugs, such as ertapenem, erythromycin, gentamycin, tobramycin (*Staphylococcus sp.*), *Aloe vera* (Ghani et al. 2019), retapamulin, periconicins A and ß-resorcyclic acid (*Staphylococcus aureus*), were all identified from natural products and are effective in treating many microbial infections (Suresh and Sona n.d.; Alter and Reich 2021). As a consequence, extensive and injudicious use of antibiotics can be a cause of development for multidrug-resistant microorganisms. The issue of resistance necessitates a renewed attempt to find antibacterial agents from natural sources that are selective against pathogenic bacteria. The 'penicillin' was discovered by Alexander Fleming in 1928. But industrial production of this antibiotic was performed only in 1940 by Howard Florey et Ernst Chain, using *Penicillium chrysogenum* (Gould 2016). Discovery of penicillin made the era of

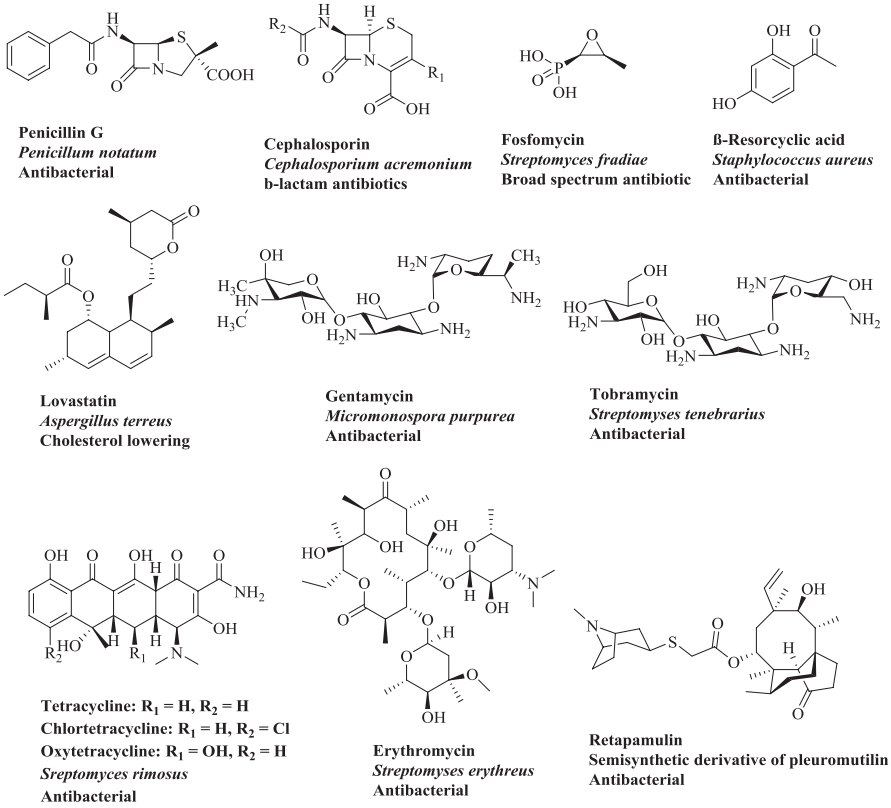

Fig. 9.2 Antimicrobial agents from bacterial source

antibiotics possible, as well as drove the modernization of new methodologies for penicillin discovery (Gould 2016). Many antibiotics used today are derived from microbial classes like β-lactams (penicillin), aminoglycins (gentamicins) and macrolides (erythirosyms), as per instructions (Fig. 9.2). Chlortetracycline, the first antibiotic of tetracycline class, was discovered in 1945 by Benjamin Minge Duggar from Streptomyces (Fig. 9.2). The oxytacycline collaboration between Pfizer and Harvard was called Terramycine (Hochstein et al. 1953; Aminov 2010).

9.5 Fungal-Derived Antimicrobial Agents

Fungi have the ability to produce toxic secondary metabolite mycotoxins which can withstand various harsh/unfavourable conditions at different stages of food chain causing many unavoidable severe health issues and also death in both humans and other animals. Even though fungi are considered as a major cause of food spoilage, still they can have the ability to produce many effective and successful antimicrobials against various ailments. The discovery of first antibiotic Penicillin, a beta-lactum drug that targets the cell wall of bacteria, was derived from the fungus *Penicillium notatum* (Guzmán-Chávez et al. 2018). For many years, *Penicillium notatum* has undergone a program of classical strain improvement (CSI) to improve antibiotics titters. This achievement allowed the lower than normal quantities of BGC-expressed natural products to be generated, resulting in a considerable reduction in the scope of BGC-associated natural product output, or, thus reduced the abundance of a diverse array of these items, which resulted in a significant increase in penicillin enzyme capability alongside the downregulation of a variety of biosynthetic gene clusters (DGCs), resulting in a smaller than usual volume of BGC-encoded DGC-enriched products (NPs). Similarly, edible fungi, such as mushrooms, have possible nutraceutical and inhibitory action against pathogenic microbes (Guzmán-Chávez et al. 2018). From the other side, the fungus *Acremonium fusidioides* (formerly *Fusidium coccineum*) produces steroidal antibiotic fusidine (fusidic acid), the biosynthetic pathway of which is quite close to cholesterol synthesis throughout the human body (Trenin 2013).

Several microbes from the aquatic ecosystem have been shown to secrete secondary metabolites, such as *E. coli*, Proteus genus, and others (Valente et al. 2020). Echinocinocandin, a particular antimycotic, was extracted from *Aspergillus nidulans* using lipo-hexosides as the carbon source (Hu et al. 2020). Interest in the pleuromutilin class has exploded in the modern century, as shown by the production of new human derivatives. Patients with impetigo and untreated superficial lacerations, abrasions, or sutured wounds caused by *Staphylococcus aureus* and *Streptococcus pyogenes* were given Retapamulin, a medicinal antibiotic (Paukner and Riedl 2017).

Trichothecium cinnamon was found to be stable in the organic fraction of the fungus and was also tested for antifungal activity against filamentous fungi. It also reported anti-tumour activity against breast cancer cells, MDA-MB-231 and HeLa, and against MDA- lines B10F and MDA-MB231. (Taware et al. 2015; Silva et al.

2017). Silva et al. isolated and characterized three new isoaigialones, A, B, and C, as well as an aigialone from the endophytic fungus *Phaeoacremonium sp*., and measured them against the phytopathogenic fungi *Cladosporium cladosporioides* and *C. sphaerospermum* (Silva et al. 2017). Curvularine was found in a leaf of the Murraycian tree (*Hyloomsantia myrmexella*) and produced the antifungalecycol products: murolide A, murolide A, and murop acid, along with six previously unknown compounds: mupiranol A, murasin, mursan, and muran-6, all together with the well-known components muracidin, and murin (Mondol et al. 2017). Acetolide compound (2-amino-1-1-acetapregnadicapramide) 3-ben-β-ol-C and ergosylan-7,22(2,5,6), obtained from ethyl acetate extract of *Anvillearcium chasteriinense*, was used to characterize ethyl acetate extract a novel fusario acetohydide (2-AG:ET:1O) while three known compounds (1-acetolan, 8:3β-diol:7, 6:6-d) and epichlororenone (6:3β-dihydroxymide) were used to complete the characterization of the fused amide. Disc diffusion assay was used to monitor the antibacterial and antifungal efficacy of compound fusarithioamide A against various microorganisms. It demonstrated antibacterial activity against *B. cereus*, *S. aureus*, and *E. coli*, with inhibition zone diameters (IZDs) of 19.0, 14.1, and 22.7 mm, and MICs of 3.1, 4.4, and 6.9 g ml1, respectively.

One additional antifungal toxin out of three new examples of triovirabacinolides and three new triovirividines from the endophytic fungus *Penicillium raccum* (Kajula et al. 2016) found in the literature by expounding upon three ways of looking at this genus of fungus was noted for antifungostase and anti-inflammation, the terms described above (Kajula et al. 2016). The recognized compounds of seven different species of fungi ((R)-3-Hydroxybutynonine isolated from the endophyte fungus *Aspergillus* sp.) have bioactive dianospermine as the seventh in their list of isolated bioactive compounds. These secondary metabolites were screened against fungi that are phytopathogenic (*Botrytis cinerea*, *Gibberella saubberti*, *Colletrix gloeoproides*, and *Magnipeniella grossi*). A test was performed against *Phytophthora capsici, Escherichia coli*, *Rhizoctonia solani*. Compounds R-3-hydroxybutanonil were effective against all of the phytopathogenic fungi studied, with the exception of those with a minimum inhibitory concentration (6.25–50 μM) and below MIC level of 6.25 μM, although less active on viruses, antimicrobial compounds less than 25-fold inhibitory concentrations, outosaminic acid had a MIC in the range of 25–100 μM, but was ineffective, or outamin C was active against all pathogenic bacteria, but active down to the low MIC values (of 25-fold) less than 25-to-mM concentrations were ineffective (Xiao et al. 2014). Similarly Trichodermin is a strong antifungal bioactive compound isolated from endophytic fungus *Trichoderma brevicompactum* with EC_{50} of 25 μg/mL fraction possessed significant ability to hinder growth of the plant pathogen *Phenacoccus solani*. Also, it had minimal ability to influence *B. cinerea* at EC_{50} of 25 mg/L (Shentu et al. 2014). A fifth mammalian antifungal that was sourced from *Bignonia magnifica*, evaluated for their anti-pityriasis properties on walnut and mediterrane fungus species *O. fragariaeola*, *O. cinereoxys*, *C. glosum* were also tested. (Silva-Hughes et al. 2015).

It was found that four compounds in Wang's study include cladosin, isocladoside, and 5-hydroxyaspeona. Additionally, Wang et al. (2013) discovered an additional one that foundcladoside, isocladoside, and 5-hydroxyaspeona could be extracted from the endophytic fungus *Cladosioquinidium*. In the presence of this weed, both the synthetic growth inhibitors were found to be effective against *Colletodothis viti* (weed) and the natural relatives (the synthesized and natural kinase inhibitors) (Wang et al. 2013). Altenusin showed activity against clinical strains of *Aspergillus* fungi, and some other *Aspergillus* and *Penicillium* molds. Endophytic *Alternaria alternata* extract shows strong antifungal activity against *Staphylococcus aureus, Escherichia coli* and *Chlamydia trachomatis*. (Johann et al. 2012). Two amides called trimethynilic [also known as tetramic] and tetraethlyinic were obtained from the endophytic (or graminophilic) fungus *Bimucidula* MU34. IC50 μg/mL against plant pathogen, 1.6 mg per gm/ml, 3.2 mg/ml, and 1.6 mg/gm per millilitre of bacteria, which proved to be useful antifungal compounds, in addition to the beneficial for the fungi *Cladosporina*, *Gylezymea*, which has a MIC of 16 mg/mL, and *Gylezymea* which has a MIC of 32 mg/gm and bacteria, that has a 1.5 mg/g 3 g Tiyzin, which can be utilized as anti fungicide (Siriwach et al. 2014). An endophyte-derived phioprothine (an inhibitory one, phorbininophoreinorein compound, used for Giberella root rot), with an IC50 of 15.9 mm was found for *Pestalopsis* sp. a new PC 50-82, also from the root system of an endophytic fungus (Liu et al. 2013).

Chemical investigation of an acetonitrile fraction led to isolation of novel product 2-hydroxyethylol and monoglycolate, along with cytochalasins J and H and 5'-epialtenuene, and the mycotoxins alternariol monomethyl ether, alternariol and cytosporone C from the endophytic fungus *Phomopsis* sp. Furthermore, the antioxidant, anti-inflammatory, antifungal and cytotoxic activities of these compounds, which were isolated from *Phomopsis* sp., *were calculated. C. globosum* and *clostridium extracts* was proven to be strong antimicrobial activity against the human pathogenic bacteria such as *Salmonella* sp., *Staphylococcus* sp. and *Streptococcus* sp. (Chapla et al. 2014). The novel marine bacterium CN-328 grows a novel fungal extract made in coculture was treated with an antifungaliotic, *Potia* sp., as commonly found in this medium. It showed a strong antimicrobial potency (or activity) in the human microdilution assay against methicillin-resistant *Staphylococcus* with a MIC of 37 ng/mL and against vancomyceicm *Antophysomonas endocarditium* with a MIC of 78 ng (Cueto et al. 2001). Strong antimicrobial activity was found in Emerrla red from *Proteus fragilasens*, which had a MIC of 12.5, and bioactive collagen extracts from *S. geliferum* produced excellent antimicrobial activity against S. a that had a MIC of 12 μg (Bugni and Ireland 2004). Periconin-forming diterpenic A and B, which was created by endophytic bacteria *Klebsiella*, *Staphylococcus*, and *Salmonella*, tested in the same range (measured in millilitres per litre), had bacteriocins *Klebsiella*, *Staphylococcus*, and *Salmonell*a typhi with a 3.12–12.5 micomol per litre resulting in measurable bacterization, *Staphylococcus microclo* and *Salmonella* (Kim et al. 2004).

9.6 Animal Origin Antimicrobial Agents

Studies into the antibiotic resistance of animals (whether terrestrial or marine) have been made considerably less in comparison to their use in plants and microorganisms, as well as in smaller animals, which includes amphibians and molluscs (Wang et al. 2018). Quite a small number of the roughly 7.77 million animal species living in different habitats have been evaluated for their antimicrobial efficacy (Jiravanichpaisal et al. 2007). The incredible competency of complex fauna to thrive in difficult environments provides a road on which to discover their survival causes for decades. Because several groups of animals, such as fish, amphibians, reptiles, and rodents, are exposed to changing habitats, it is believed that they have built-in defence against pathogenic threats. Many animals, for the development of new antimicrobial drugs, are ubiquitous and have a significant and mostly underutilized supply (Wang et al. 2018; Jiravanichpaisal et al. 2007).

A novel cecropins B-derived peptide with potent antimicrobial activity against Grame-negative bacteria such as *Micrococcus luteus*, *Aerococcus viridans*, *Bacillus megaterium*, and *Bacillus subtilis*, as well as low toxicity in human cells (Wang et al. 2018). This particular compound generally found in insects was isolated from the *Musca domestica* (Wang et al. 2018). It is also the duty of them to safeguard the crayfish against in the marine world, diverse fish pathogens. The antimicrobial peptide astacidin was derived from the freshwater crayfish *Pacifastacus leniusculus*, and it has a large spectrum of bactericidal potential against both Gram-positive and Gram-negative bacteria (Jiravanichpaisal et al. 2007; Ennaas et al. 2016). In an In vitro study Ennaas et al. (2016) extracted and characterized Collagencin, a bactericidal peptide with good action against multidrug-resistant *Staphylococcus aureus* (Ennaas et al. 2016). Dermaseptin is a brand-new linear peptide with antimicrobial effects. It was first discovered in amphibian skin secretions. Dermaseptin was produced by Ying et al. (2019), and it demonstrated high antimicrobial potential against planktonic bacteria *M. luteus*, *S. aureus*, *S. epidermidis*, *S. enterica*, *Aeromonas hydrophila*, and *E. coli*, which were extracted from cystic fibrosis (CF) patients (Ying et al. 2019). Squalamine is a compound polycationic aminosterol obtained from the shark *Squalus acanthias*. Squalamine has shown to be successful against multidrug-resistant Gram-negative and Gram-positive bacteria. Squalamine's membranolytic efficacy and outstanding biocompatibility render it one of the most powerful antibiotics against nosocomial pathogens including *Acinetobacter baumannii* (Nicol et al. 2019).

Crocodiles and alligators are recognized for their longest life span, and they experience many infectious agents, toxicants, contaminants, carcinogens, etc., during their lives, but they survive under these circumstances (Leelawongtawon et al. 2010). *Siamese crocodile* (*Crocodylus siamensis*) serum has been purified into different antimicrobial agents and has been shown to be effective against so many pathogenic bacteria, including *S. typhi*, *E. coli*, *S. aureus*, *S. epidermidis*, *K. pneumoniae*, *P. aeruginosa* and *V. cholerae* (Leelawongtawon et al. 2010). Birds, such as crows, chicken, ostrich, vulture, turkeys with antimicrobial peptides that regularly

feed on tainted food. Janecko et al. (2018) reported in their study a strong antimicrobial peptide with MDR activity against *Escherichia coli* and *Klebsiella* sp. isolated from *Corvus corax* in Canada (Janecko et al. 2018). Pancreatic juice, which is present in the intestine of mammals such as rabbits, guinea goats, pigs, dogs, and livestock, was discovered to have antibacterial action against *Micrococcus pyogenes*, *E. coli*, *Shigella* sp., *Salmonella* sp., *K. pneumoniae*, *Staphylococci*, and *Pseudomonus aeruginosa* (Pierzynowski et al. 1993). L-lysophillic peptides have antimicrobial efficacy against Gram-positive and Gram-negative microbes, such as *Streptococcus* and *Pseudomonas* (Szponder et al. 2018) (Table 9.1).

9.7 Mechanism of Antimicrobials

Due to the immense chemical diversity available in bioactive compounds, the mode of action of all these molecules are not well understood (Wink 2015). Numerous studies have shown that different bioactive molecules target different levels of organization, varying through cellular to individual scale and population scale, and also in some instances, including such biofilms (Wink 2015; Singh et al. 2017b; Abushaheen et al. 2020). The complexity of mode of action posed by bioactive natural products appears to become very encouraging in combating the development of multidrug resistance often seen in pathogens responsible for various infectious diseases. At the cellular level, different antimicrobial phytochemicals react with different biomolecules present at the target site and thus alter themselves chemically and physically to the degree whereby they drop their bio functionality whether partially or fully. During these interactions, bioactive natural compounds bind to different biomolecules, including such protein and nucleic acid, through various bond formation. Many of these bioactive components contain very active sites, like C=O and R-S-R′, RCO_3H, double bonds with anion configuration, and triple bonds in their framework, which can form covalent bonds with proteins and sometimes the DNA of microorganisms (Abushaheen et al. 2020; Singh et al. 2017b; Wink 2015). For example, during defined circumstances, the reactive aldehyde group of these molecules may create a Schiff base with amino/imino groups that occur in amino acid residues and protein and DNA nucleotide bases, respectively.

On the one side, a number of bioactive compounds such as polyphenols have the potential to minimize ROS generation via their strong antioxidant potential, whereas on the other hand, some bioactive compounds induce ROS generation. ROS tends to contribute significantly in the inducing of programmed cell death. After which the O_2-generated in mitochondria by aerobic cellular respiration is changed to H_2O_2 by superoxide dismutase, which then in turn reacts with ferrous ions and produces highly reactive OH-radicals. OH-radicals interact wantonly with various macromolecules, such as unsaturated fatty acids, proteins and DNA, and thus induce apoptosis induction (Le et al. 2017; Memar et al. 2018). The mechanism of the antimicrobial

Table 9.1 Antimicrobials from natural sources

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
(A) Plant-derived antimicrobial agents					
1.	Aloe vera (*Aloe barbadensis*)	Latex (Complex mixture)	*Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*)	Inhibition zone on *S. aureus* bacterium (26.33 mm) was larger than *E. coli* (21 mm)	Hilmi et al. (2019)
2.	Apple (*Malus sylvestris*)	Phloretin (Flavonoid derivative)	Oral-toxicity test in Kunming mice	The total bacterial and *Pseudomonas* sp. counts suppressed by 2 and 1.5 logarithms	Wei et al. (2020)
3.	Ashwagandha (*Withania somniferum*)	Withaferin A (Lactone)	*Leishmania donovani*-infected peritoneal macrophages and BALB/c mice	Withaferin-A (1.5 μM) reduce amastigote count in peritoneal macrophages	Chandrasekaran et al. (2017)
4.	Bael tree (*Aegle marmelos*)	Essential oil (Terpenoid)	*S. aureus, C. diphtheriae, B. cereus* and *C. diphtheriae*	Inhibition zone was 21.4 mm, 17.2 mm, lowest MIC was against *B. cereus* and *C. diphtheriae* (MIC = 125 μg/ml)	Mahomoodally et al. (2018)
5.	Barberry (*Berberis vulgaris*)	Berberine (Alkaloid)	Influenza A/FM1/1/47 (H1N1) in vivo and in vitro	Berberine strongly suppressed viral replication in A549 cells and in mouse lungs	Yan et al. (2018)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
6.	Brazilian pepper tree (*Schinus terebinthifolius*)	Terebinthone (Terpenoids)	*Candida albicans*; *Candida krusei*; *Candida glabrata*; and *Candida tropicalis*	There were no significant differences regarding the different strains of *Candida* tested	Torres et al. (2016)
7.	Buchu (*Agathosma betulina*)	Essential oil (Terpenoid)	*Trichophyton rubrum* and *Trichophyton mentagrophytes*	Fungal growth index of 2.3% against *Trichophyton rubrum*	Fajinmi et al. (2019)
8.	Cascara sagrada (*Rhamnus purshiana*)	Tannins (Polyphenols)	*Staphylococcus aureus*, *Bacillus subtilis* and *Streptococcus mutans*	Biofilm formation up to 99.9% and reduced planktonic cell growth up to 10 log units relative to untreated controls	Campbell et al. (2019)
9.	Cashew (*Anacardium pulsatilla*)	Anacardic acid (Polyphenols)	*E. multilocularis*, *E. granulosuss*	Anacardic acid exerted a better efficacy against both pathogen in vitro, and in vivo compared to positive control	Yuan et al. (2019)
10.	Castor bean (*Ricinus communis*)	Volatile organic compounds (VOCs)	Plant-parasitic nematode *Meloidogyne incognit*a	Immobility (>97.3%) and death (>96.9%) of M. *incognita*	Pedroso et al. (2019)
11.	Chamomile (*Matricaria chamomilla*)	Anthemic acid (Phenolic acid)	*M. tuberculosis*, *S. typhimurium*, *S. aureus*	— —	Khameneh et al. (2019)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
12.	Chapparal (*Larrea tridentata*)	Nordihydroguaiaretic acid (Lignan)	Larvae of *Haemonchus contortus*	The effective concentration of the *L. tridentata* extract for 50% larvae mortality (EC_{50}) was 36 mg/mL	García et al. (2018)
13.	Chili peppers, Paprika (*Capsicum annuum*)	Capsaicin (Terpenoid)	Clinical MRSA strains	MIC values ranging from 8 to 256 mg/L against effluxing MRSA strains SA1199B (NorA), XU212 (TetK) and RN4220 (MsrA)	Oyedemi et al. (2019)
14.	Clove (*Syzygium aromaticum*)	Eugenol (Terpenoid)	Several proinflammatory biomarkers VCAM-1, IP-10, I-TAC, MIG	Eugenol significantly inhibited VCAM-1 and collagen III at both protein and gene expression levels	Han and Parker (2017)
15.	Coca (*Erythroxylum coca*)	Cocaine (Alkaloid)	Gram-negative and Gram-positive cocci	__ __	Tsuchiya (2017)
16.	Coriander, Cilantro (*Coriandrum sativum*)	Essential oil (Terpenoid)	*Staphylococcus aureus*	Essential oils have excellent activity against both growing and stationary phase *S. aureus*	Xiao et al. (2020)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
17.	Eucalyptus (*Eucalyptus globulus*)	Tannin (Polyphenol)	Various Gram-negative and Gram-positive bacteria	*E. camaldulensis* is active against many Gram-positive (0.07–1.1%) and Gram-negative bacteria (0.01–3.2%)	Sabo and Knezevic (2019)
18.	Fava bean (*Vicia faba*)	Fabatin (Thionin)	Food spoilage caused by pathogenic and nonpathogenic bacteria	__ __	Kraszewska et al. (2016)
19.	Garlic (*Allium sativum*)	Allicin, Ajoene (Sulphoxide)	Inhibits DNA gyrase activity in *E. coli*	Cys_{433} in DNA gyrase subunit A (GyrA) was 6% oxidized in untreated bacteria. After allicin treatment the degree of Cys_{433} oxidation increased to 55% in susceptible Pf0–1 but only to 10% in tolerant PfAR-1.	Reiter et al. (2020)
20.	Glory lily (*Gloriosa superba*)	Colchicine (Alkaloid)	MRSA, *S. pyogenes*, and DR extended-spectrum beta-lactamase (ESBL)	The results suggest that the nanoemulsion of colchicine effective against DR bacteria, and acts by inhibiting the drug efflux mechanism of DR strains.	Krishnamoorthy et al. (2018)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
21.	Goldenseal (*Hydrastis Canadensis*)	Berberine, Hydrastine (Alkaloids)	Bacteria, *Giardia* duodenale, Trypanosomes	__ __	Mandal et al. (2020)
22.	Green tea (*Camellia sinensis*)	Catechin (Alavonoid)	*Shigella*, Vibrio, *S. mutans*, Viruses	Catechins, especially epigallocatechin-3-gallate (EGCG), have antiviral effects against diverse viruses and microbes	Xu et al. (2017)
23.	Hemp (*Cannabis sativa*)	ß-Resercyclic acid (Organic acid)	*Staphylococcus aureus* (MRSA)	__ __	Karas et al. (2020)
24.	Henna (*Lawsonia inermi*)	Gallic acid (Phenolic acid)	*S. aureus*	__ __	Karas et al. (2020)
25.	Hops (*Humulus lupulus*)	Lupulone, Humulone (Phenolic acids)	*Bacteroides fragilis* or *Clostridium perfringens*	MIC and MBC values ranging from 15–107 µg/mL	Cermak et al. (2017)
26.	Lantana (*Lantana camara*)	Squalene, β-ionone, Caryophyllene oxide, β-caryophyllene	*Leishmania amazonensis*; *Leishmania mexicana*	IC_{50} value ranging from 12.02 ± 0.36 µM	Delgado-Altamirano et al. (2019)
27.	Lavender-cotton (*Santolina chamaecyparissus*)	Essential oil (Terpenoid)	Nine strains of bacteria and fungi	IC_{50} value ranging from 0.10/0.30 µl/ml for lavender	Mesic et al. (2021)
28.	Onion (*Allium cepa*)	Allicin (Sulphoxide)	*S. aureus*, *Candida albicans*	__ __	Fuchs et al. (2018)
29.	Oregon grape (*Mahonia aquifolia*)	Berberine (Alkaloid)	*Plasmodium falciparum*, *Trypanosoma cruzi*	__ __	Karas et al. (2020)
30.	Periwinkle (*Vinca minor*)	Reserpine (Alkaloid)	*S. aureus*, *Candida*	__ __	Fuchs et al. (2018)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
31.	Peppermint (*Mentha piperita*)	Menthol (Terpenoid)	ATCC 25922, ATCC 27853, ATCC 14452, ATCC 29213, ATCC 6633, *S. typhimurium*, *B. cereus*	MIC ranging from 0.062% to 0.5% (v/v)	Marwa et al. (2017)
32.	Quinine (*Cinchona sp.*)	Quinine (Alkaloid)	(MDR) Gram-negative bacteria in vitro and in vivo	Significant improvement in the inactivation of MDR *P. aeruginosa* and *A. baumannii* (planktonic cells and biofilms) when aBL was illuminated during Q-HCL exposure	Leanse et al. (2020)
33.	Chandra (*Rauvolfia serpentina*)	Reserpine (Alkaloid)	*Pseudomonas aeruginosa* PAO1 biofilms	Reserpine reduced biofilm formation, cell motility, virulence factor production, and QS-controlled gene expression	Parai et al. (2018)
34.	Rosemary (*Rosmarinus officinalis*)	Essential oil (Terpenoid)	*Candida* sp. and *Streptococcus pneumoniae*	The results showed that the essential oil from *Rosmarinus officinalis* was the most active on all the tested microorganisms	Akroum (2020)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
35.	Tarragon (*Artemisia dracunculus*)	Caffeic acids (Terpenoid)	Nine strains of bacteria, both gram-negative and Gram-positive	MIC ranging from 0.09 mg/mL to 47 mg/mL	Majdan et al. (2020)
36.	Thyme (*Thymus vulgaris*)	Caffeic acids (Terpenoid), thymol (phenolic alcohol)	ATCC 25922, ATCC 25923	The highest inhibition zone was obtained by Tv extracts at 20 µL loading (22 mm)	Akin and Saki (2019)
37.	Tree bard (*Podocarpus nagi*)	Totarol (Flavonol)	*P. acnes*, other gram-positive bacteria	__ __	Hou et al. (2020)
38.	Tua-Tua (*Jatropha gossyphiifolia*)	__ __	Different strains of bacteria and fungi	The seeds and fruits can be used against influenza and as a sedative, analgesic or anti-diarrheal agents	Wu et al. (2019)
39.	Turmeric (*Curcuma longa*)	Curcumin (Terpenoids)	*Escherichia coli* O157:H7 and *Listeria innocua*	Enhancement in antimicrobial activity reduced the time required for the inactivation of 5 log CFU mL^{-1} of *E. coli* O157:H7 from 10 min to 2 min of treatment	de Oliveira et al. (2018)
40.	Willow (*Salix alba*)	Salicin (Phenolic glucoside)	Human peripheral leucocyte cells and human hepatoma cell line HepG2	The results using trypan blue staining test showed viability decreases (viability less than 70%) for concentrations of SA extract equal and higher to 200 µg/ml.	Maistro et al. (2019)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
(B) Bacterial-derived antimicrobial agents					
1.	*Streptomyces antibioticus*	Actinomycin	Antibacterial activities against *Sarcinalutea*, *Bacillus mycoides*, *Bacillus subtilis*, *E. coli*, *Aerobacter aerogenes* and *Brucella abortus*	Transcription inhibitor which preventing RNA polymerase elongation	Genilloud (2014)
2.	*Streptomyces lavendulae*	Streptothricin	Various Gram-positive and Gram-negative bacteria	A protein synthesis inhibitor with miscoding activity	Mander and Liu (2010)
3.	*Streptomyces erythraeus*	Erythromycin	*Streptococcus*, *Staphylococcus*, *Haemophilus* and *Corynebacterium*	Inhibits protein synthesis by binding to the 23S rRNA molecule of the bacterial ribosome and hence blocking the exit of the growing peptide chain	Majer et al. (1977)
4.	*Streptomyces aureofaciens*	Tetracycline	*E. coli*, *Haemophilus influenzae*, *M. tuberculosis*, *Pseudomonas aeruginosa*	Inhibits protein synthesis by binding to the 30S ribosomal subunit in the mRNA translation complex	Nelson and Levy (2011)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
5.	*Micromonospora*	Aminoglycosides	Aerobic, Gram-negative bacteria and *M. tuberculosis*	Binding specifically to the 30S ribosome of the bacteria, preventing attachment of the aminoacyltRNA to the RNA-ribosome complex	Mingeot-Leclercq et al. (1999)
6.	*Streptomyces lincolnensis*	Lincomycin	Gram-positive bacteria	Inhibits protein synthesis	Spížek and Řezanka (2004)
7.	*Amycolatopsis orientalis*	Vancomycin	Gram-positive bacteria particularly methicillin-resistant *Staphylococci*	Inhibition of peptidoglycan synthesis	Levine (2006)
8.	*Bacillus* sp.	Mersacidin	MRSA	— —	Kruszewska et al. (2004)
9.	*Bacillus amyloliquefaciens*	Surfactin, Iturin, Fengycin, Plipastatin, Bacitracin, Amylolysin	*M. luteus*, *Micrococcus roseus*, *Bacillus anthracis*, *Bacillus mycoides*, *Corynebacterium pseudodiphthericum*, *S. aureus*, *L. monocytogenes*, *B. cereus*, *Serratia marcescens*, and *Pasteurella haemolytica*, *Proteus vulgaris*	Inhibitory activity	Benitez et al. (2010); Awais et al. (2007)
10.	*Amycolatopsis rifamycinica*	Rifamycin	*Mycobacterium intracellulare* and *Mycobacterium avium*	Inhibition of bacterial DNA-dependent RNA synthesis	Lin et al. (2017)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
11.	*Dactylosporangium aurantiacum*	Tiacumicins	Gram-positive bacteria	__ __	Hochlowski et al. (1987)
12.	*Streptomyces roseosporus*	Daptomycin	MRSA	__ __	Baltz (2009)
13.	*Streptomyces tenebrarius*	Tobramycin	Gram-positive *Staphylococcus aureus* and various Gram-negative bacteria.	<0.25 μg/mL - 92 μg/mL and 0.5 μg/mL - >512 μg/mL	Meylan et al. (2017)
14.	*Streptomyces cattleya*	Thienamycin	Have a high specificity for PBP2 of gram-positive and Gram-negative microorganisms	Hydrolysis by metallo β-lactamases and other β-lactamases	Bennett et al. (2014)
15.	*Streptomyces clavuligerus*	Clavulanic acid	Three mammalian species, hamsters, rats and cotton-top tamarin monkeys in a series of behavioural assays	Enhance the activity of antibiotics by blocking bacterial beta-lactamases	Kim et al. (2009)
16.	*Burkholderia pseudomallei*	Malleobactin A	*B. pseudomallei*	MICs in the range 0.004–0.016 μg/mL	Mima et al. (2011)
17.	*Burkholderia cepacia*	Burkholderic acid	MRSA	MIC of 0.125–0.5% (v/v)	Vasireddy et al. (2018)
18.	*Janthinobacterium lividum*	Janthinocin A	Gram positive bacteria in vitro and in vivo	__ __	Johnson et al. (1990)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
19.	*Janthinobacteriumag aricidamnosum*	Jagaricin	*Candida albicans*	Jagaricin shows haemolytic effects, at which the growth of prevalent phytopathogenic fungi is inhibited	Fischer et al. (2019)
20.	*Pseudomonas syringae*	Pseudomycins	*Candida albicans*	— —	Kumari Chikkode Narayana et al. (2017)
21.	*Pseudomonas viridiflava*	Ecomycins	*Cryptococcus neoformans* and *C. Albicans*	— —	Kumari Chikkode Narayana et al. (2017)
(C) Fungal-derived antimicrobial agents					
1.	Penicillin	*Penicillium notatum*	Mainly against gram-positive bacteria	Cell wall of bacteria	Guzmán-Chávez et al. (2018)
2.	Fusidic acid	*Acremonium fusidioides*	Methicillin-resistant *S. aureus* and Vancomycin-resistant *Enterococcus faecium*	Sterol biosynthesis	Trenin (2013)
3.	Griseofulvin	*Penicillium griseofulvum*	Mainly against different species of ringworm	Griseofulvin binds to tubulin, interfering with microtubule function thus inhibiting mitosis	Valente et al. (2020)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
4.	Echinocandin B	*Aspergillus nidulans*	Antifungal drug	Inhibits the synthesis of glucan, a major component of the fungal cell wall, via noncompetitive inhibition	Hu et al. (2020)
5.	Retapamulin	*Pleurotusmutilus* sp.	Gram-positive and fastidious Gram-negative pathogens as well as against mycoplasmas	Inhibit bacterial translation	Paukner and Riedl (2017)
6.	Cephalosporin	*Cephalosporium acremonium*	Gram-positive cocci, *Citrobacter*, *Enterobacter*, *Haemophilus influenzae*	Inhibit synthesis of the bacterial cell wall, causing cell lysis	Hu and Zhu (2016)
7.	*Trichothecium* sp.	Trichothecin	*S. cerevisiae*, *C. albidus* var diffluens (NCIM 3371), *C. albidus* var *diffluens* (NCIM 3372), *F. oxysporum*, *P. expansum*, *T. viride*, *P. varioti* and *A. niger*	MIC of 6.0, 20.0, 12.0, 10.0, 30.0, 40.0, 20.0 and 12.0 μg/mL respectively	Taware et al. (2015)
8.	*Phaeoacremonium* sp.	Isoaigialone B and C, aigialone	*C. cladosporioides* and *C. sphaerospermum*	7 exhibited antifungal activity, with a detection limit of 5 μg nystatin, positive control, showing a detection limit of 1 μg	Silva et al. (2017)
9.	*Curvularia* sp., strain M12	Murranolide A, Murranopyrone, Curvularin, Pyrenolide A, Modiolide A	*Phytophthora capsici*	IC_{50}: 50–100 μg/mL	Mondol et al. (2017)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
10.	*Fusarium chlamydosporium*	Fusarithioamide A	*C. albicans*	MIC 2.6 µg/mL	Ibrahim et al. (2016)
11.	*Penicillium raciborski*	Outovirin C	*F. oxysporum*, *B. cinerea*, and *V. dahlia*	Compound active against *B. cinerea* (57% inhibition) and slightly less effective against *V. dahliae* (45% inhibition)	Kajula et al. (2016)
12.	*Aspergillus sp.* KJ-9	Fonsecinone A	*G. saubinetti*, *M. grisea*, *B. cinerea*, *C. gloeosporioides* and *A. solani*	MIC range of 6.25–50 µM	Xiao et al. (2014)
13.	*Trichoderma brevicompactum* 0248	Trichodermin	*R. solani*, *B. cinereal*, *C. lindemuthianum*	EC_{50} of 0.25, 2.02 and 25.60 µg/mL respectively	Shentu et al. (2014)
14.	*Biscogniauxia mediterraneaOhu* 19B	5-methylmellein	*C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *F. oxysporum*, *B. cinerea*, *P. obscurans*, and *P. viticola*	__ __	Silva-Hughes et al. (2015)
15.	*Cladosporium cladosporioides*	Cladosporin, Isocladosporin	*C. acutatum*, *C. fragariae*, *C. gloeosporioides* and *P. viticola*	At 30 µM compound exhibited 92.7, 90.1, 95.4, and 79.9% growth inhibition	Wang et al. (2013)
16.	*Alternaria sp*. UFMGCB 55	Altenusin	Eleven strain of *P. brasiliensis*	MIC values ranging between 1.9 and 31.2 µg/mL	Johann et al. (2012)
17.	*Bipolaris sp*. MU34	Bipolamide B	*C. cladosporioides*, *C. cucumerinum*, *S. cerevisiae*, *A. niger* and *R. oryzae*	MIC values of 16, 32, 32, 64 and 64 µg/mL, respectively	Siriwach et al. (2014)
18.	*Pestalotiopsis fici*	Ficipyrone A	*G. zeae*	IC_{50} 15.9 µM	Liu et al. (2013)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
19.	*Phomopsis sp.*	Cytochalasin H	*C. cladosporioides* and *C. sphaerosphermum*	MIC 10.0 and 25.0 µg, respectively	Chapla et al. (2014)
20.	*Pestalotiopsis sp.* when cocultured with marine bacterium, strain CNJ-328	Pestalone	Methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium*	MIC = 37 ng/mL and 78 ng/mL	Cueto et al. (2001)
21.	*Emericella variecolor*	Varixanthone	*E. coli*	MIC values of 12.5 µg mL^{-1}	Bugni and Ireland (2004)
22.	*Periconia sp.*	Periconicins A and B	*Bacillus subtilis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*	MIC in the range of 3.12–12.5 µg/mL	Kim et al. (2004)
(D)animal origin antimicrobial agents					
1.	*Musca domestica*	Cecropins	*M. luteus*, *Aerococcus viridians*, *Bacillus megaterium*, *B. subtilis*	Cecropin DH has potential as a therapeutic agent for both antibacterial and anti-inflammatory applications	Wang et al. (2018)
2.	Crayfish	Astacidin	Gram-positive and Gram-negative bacteria	Bactericidal activities	Jiravanichpaisal et al. (2007)
3.	*Larimichthys crocea*	Collagencin	*S. aureus*	The peptide completely inhibited the growth of *S. aureus* at 1.88 mM and non-toxic at 470 µM	Ennaas et al. (2016)

(continued)

Table 9.1 (continued)

S.N.	Sources	Antibiotic compound	Tested system	Biological activity response	References
4.	*Phyllomedusa distincta*	Dermaseptin	*M. luteus*, *S. aureus*, *S. epidermidis*, *S. enterica*, *Aeromonashydrophila* and *E. coli*	Rapidly killed planktonic bacteria isolated from cystic fibrosis (CF) patient	Ying et al. (2019)
5.	*Squalus acanthias*	Squalamine	Anti-persister activity against *Acinetobacterbaumannii*	Squalamine at 100 mg/L (below its haemolytic concentration) was able to kill dormant cells	Nicol et al. (2019)
6.	*Crocodylus siamensis*	Antimicrobial peptides	ATCC-registered strains of nine bacterial species and two fungal species	MIC was in the range of 25.00–100.00 mg/ml	Leelawongtawon et al. (2010)
7.	*Corvus corax*	Peptides	*Escherichia coli* and *Klebsiella*spp	Specific target site not determined	Janecko et al. (2018)
8.	Pancreatic juice of rabbit, Guinea pig, rats, pig, sheep and cattle	Heat-stable proteinaceous substance with molecular weight of <4000	*Micrococcus pyogenes*, *E. coli*, *Shigella sp.*, *Salmonella sp.*, *K. pneumoniae*, *Staphylococci* and *Pseudomonus aeruginosa*	The antibacterial activity remained unchanged after heating to 65 degrees C and upon dilution to 1:10	Pierzynowski et al. (1993)
9.	Rabbit granulocytes	Antimicrobial peptides	Gram-positive and Gram-negative bacteria	These peptides exhibit microbicidal activity due to increased acidity and ionic strength	Szponder et al. (2018)

agent is primarily due to two pathways, namely chemical interaction with the synthesis or function of essential bacterial components and/or circumvention of traditional antibacterial resistance mechanisms. Multiple targets for antimicrobial agents include microbial protein biosynthesis; microbial cell-wall biosynthesis; microbial cell membrane destruction; microbial DNA replication and repair; and metabolic pathway inhibition. Cell wall is an ultra-dynamic structure in some microbes, such as fungi and bacteria, which protects the body from environmental osmotic shocks which are also essential for the distinctive phenotypes of different species. Any alteration triggered by an antimicrobial triggering an organizational or functional disruption of the cell wall will lead to the death of the microorganism (Timofeeva and Kleshcheva 2011; Le et al. 2017; Memar et al. 2018) (Fig. 9.3).

In the case of microbial antibiotics such as penicillin which inhibit cell synthesis, the mechanism of cell wall disintegration is well understood. Two types of family enzymes, including transglycosylases and transpeptidases, have critical roles in the creation of this sheet, while their functionality has been defined previously. Bifunctional enzymes containing both the transpeptidase and transglycosylase

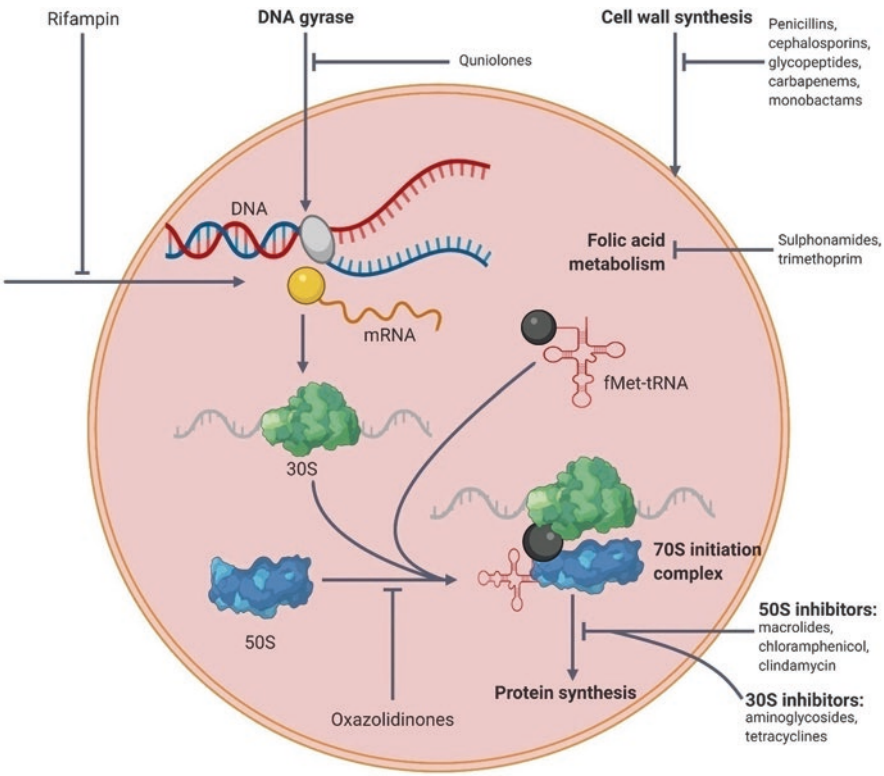

Fig. 9.3 Antimicrobial drug target; in microbes, there can be five major antimicrobial drugs targets: cell-wall synthesis, DNA gyrase, metabolic enzymes, RNA polymerase directed by DNA, and protein synthesis

domains are appropriate targets for bactericidal antibiotics such as penicillins and cephalosporins (Memar et al. 2018; Le et al. 2017; Timofeeva and Kleshcheva 2011). The glycopeptide antibiotics group, like vancomycin, has often been identified to attack the peptidoglycan layer in some other way inside the cell-wall assembly. These antibiotics are capable of binding to the peptide substrate of the peptidoglycan layer, thus preventing enzyme reactions from occurring. However, the net result is very similar, reducing peptidoglycan cross-linkage and thereby weakening the cell wall (Wink 2015; Singh et al. 2017b).

The cell membrane is an essential element of the lipid bilayers that includes integrated extrinsic and intrinsic proteins that serve the roles of enzymes, signalling protein and transport proteins. Owing to their lipophilic nature or bonding to some particular membrane part, numerous bioactive compounds can trigger membrane degradation, leading to loss of membrane stability and functionality (Ibrahim et al. 2000; Chongsiriwatana et al. 2008). Multiple antibiotics including polymyxins may bound to the lipid a constituent of lipopolysaccharide and thus cause substantial modifications through phospholipid interchange, which might lead in osmotic disturbance and, eventually, lead to microbial death. In the case of microbial biosynthesis, there seem to be a significant number of molecular steps involved in the initiation, elongation and termination of microbial ribosome protein assembly. Inhibiting protein synthesis by targeting ribosomal subunits is also an efficient way to fight microbial infections. Significant groups of antibiotics, such as macrolides, tetracycline's, aminoglycosides and oxazolidinones, demonstrate antimicrobial activity through this particular mechanism (Chongsiriwatana et al. 2008; Ibrahim et al. 2000).

9.8 Current Antimicrobial Therapy and Drug Resistant

Microorganisms had evolved on universe more than four billion years ago. During that period, a wide variety of naturally occurring antibiotics are encountered, including those created by other bacteria, such as *Penicillium notatum*, which produces penicillin (Yim et al. 2017). In order to sustain, microbes have established a seemingly inexhaustible repertoire of antibiotic resistance mechanisms (Mulani et al. 2019). This is not shocking that they rapidly became immune to all the antimicrobial agents which have been produced throughout the last five decades. For this reason, there is a lot of variability in antimicrobial responses; even the best of antibiotics have differing effects on the level of resistance. Mode of operation, if an antimicrobial compound is a dose or time-dependent killing agent, effectiveness against pathogenic bacteria, and the magnitude and duration of the available serum concentration are all variables that affect whether resistance arises (Petchiappan and Chatterji 2017). For example, the resistance of β-lactam within streptococci class a still has not been established. But at the other hand, certain antimicrobial agents,

like rifampicin, are easily selected for resistance. Antimicrobials that target single enzymes, such as rifampicin, are thought to be the most resistant to resistance production, while agents like penicillin, which irreversibly inactivates several targets, may build resistance more steadily. Because pathogens have been exposed to natural antibiotics such as ß-lactams and macrolides in the environment, it is rational to believe that susceptibility determinants to natural products have formed and spread horizontally. While it was anticipated that resistance to synthetic antimicrobial agents such as fluoroquinolones and linezolid will be sluggish to develop, resistance to synthetic agents developed rather rapidly. It seems that if an antibacterial agent is widely employed in the human community, tolerance can develop rapidly, at least in some microbe populations (Buehrle et al. 2017; Laws et al. 2019).

The development and dissemination of resistant pathogens is a significant concern as the main trigger of antimicrobial drug resistance (Juárez-Verdayes et al. 2012; Iino et al. 2012). The pathways entail modification of drug targets or enzymatic inactivation of antimicrobial agents like ß-lactams, macrolides, tetracyclines and fluoroquinolones. Many antibiotics were discovered to be efflux pump substrates, resulting in medication extrusion from cells. Problem becomes more serious due to intensive use of antibiotics which result in clonal selection of efflux pump overexpressing strains for which chemotherapeutic agents are good substrate. Moreover, hyper expression of naturally occurring multidrug efflux transporters plays an ubiquitous type of resistant element which could use chemical energy (e.g. ATP, Na + or H+ gradients) to expel a set of dissimilar molecules or antibiotics from the cytoplasm through an antiport mechanism (Campion et al. 2004; Stavri et al. 2007; Abdali et al. 2017). Protein architecture has distinguished between five families, i.e. Multidrug, Multid, MATE, ABC, the resistance-family, and the main facilitator superfamily. Secondaryly these have been studied at present in significant amounts including NorA, NorB, MdeA, and LmrP pump. NorA among these has been found overexpressed in nearly half of resistant clinical isolates as compared to other efflux pumps (Abdali et al. 2017; Jang 2016). As a consequence of the intense battle against MDR pathogens, efflux pump inhibitors (EPIs) are potentially effective as adjunctive therapies with an antibiotic to obstruct the activity of such efflux proteins and could be a better approach to improve antibacterial potency at low concentration and help in decreased virulence of bacterial infection (Patkari and Mehra 2013; German et al. 2008). Capsaicin is shown to alter fluoroquinolone pump tolerance in clinical isolates of *Staphylococcus aureus*. Similarly, polystyryladines, for example, dihydropanamidic polyamine esters with amino acid esters, have recently been discovered as antibacterial agents against NorA-overexp bacterium strains. It's worth exploring whether these drug-intermediate infections can even be treated with non-EPIs, which could have new therapeutic benefit for obsolete antibiotics (Fig. 9.4).

Ampuse from available drugs may come from the organism's intrinsic properties, or due to genetic transformation. Resistance is likely to occur in the commensal microflora as well, and the more likely it is (Buehrle et al. 2017; Laws et al. 2019).

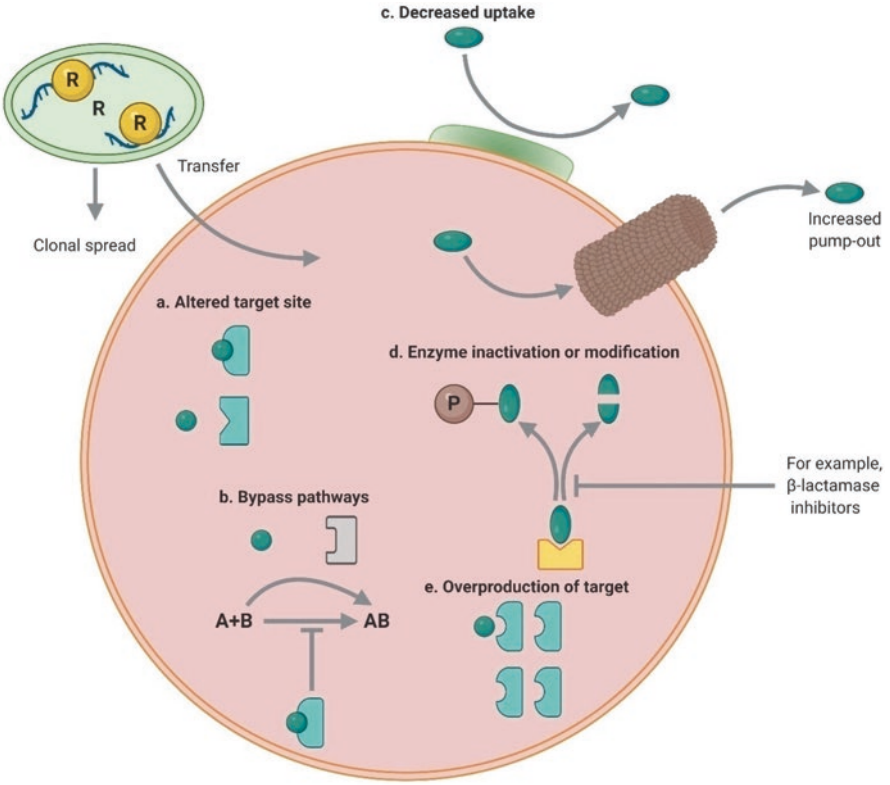

Fig. 9.4 Mechanisms of genetic resistance to antimicrobial agent

9.9 Future Opportunities

There have been a change in the way the drugs/lead molecules used in experimental trials to clinical studies, as researchers began to use advancement in the techniques of synthesizing them from the results of in vitro study. Bioavailability is a challenge because certain bacteria can not only move through the skin but also because of tissue penetration, so when using bioactive products is mixed with the natural antimicrobials. According to that theory, phenolic compounds are said to profoundly influence the body's ability to enter both the liver and the blood. A significant challenge to effective therapy of pathogenic microorganisms has been the emergence of antibiotic-resistant microorganisms. As of now, there is an urgent need to establish a new drug resistance strategy. Bioactive moieties with different chemical structures and modes of action are promising therapeutic platforms for the discovery of novel bioactive compounds in the years to come. However, more study should be done to properly completely comprehend mechanisms as well as the pharmacokinetic and pharmacodynamics characteristics of the bioactive compounds. Although conducting more research on combinations of antibiotics to improve their duration of action

would further the duration of these compounds, This class of multidrug-resistant bacteria is a true to life origins, so more research on them must also be performed to reduce resistance in normal flora. Currently, checks are needed to ensure the efficacy of any pathogens that are still in the sample. Since many antibiotics in modern treatments lack specificity, this could yield medications that are less effective when combined with the conventional antimicrobials that can mitigate environmental pathogens that do not have established resistance to these days. If these potential advantages are combined, then a more compliant patient-friendly and cost-conscious approach to antibiotic therapy is taken, such resistance could be prevented, longer durations of use could be achieved, and so less resistance to medications could be developed.

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Chapter 10
Polyphenols as Emerging Antimicrobial Agents

Ashraf Ali, Antonio Parisi, and Giovanni Normanno

Abbreviations

AMR	Antimicrobial resistance
CHQA	3-*p*-trans-Coumaroyl-2-hydroxyquinic acid
EGCG	Epigallocatechin-3-gallate
GTE	Green tea extract
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
PACs	Proanthocyanidins
RMA	Resistance modification agent
STEC	Shiga toxin-producing *Escherichia coli*
UTI	Urinary tract infection
WHO	World Health Organization

10.1 Introduction

Polyphenols represent one of the largest groups of natural compounds obtained from the plant kingdom which have several medicinal properties (Di Lorenzo et al. 2021; Durazzo et al. 2019; Mehany et al. 2021). Until now, about 8000 phenolic compounds have been characterized starting from simplest bioactive phytochemicals containing single substituted phenyl rings to highly polymeric substances (Di Lorenzo et al. 2021; Cheynier 2005; Daglia 2012; Ciulu et al. 2018; Aliaño-González et al. 2020). They are important secondary metabolites in plants and are considered as some of the most beneficial natural molecules due to their bioactive properties (Durazzo et al. 2019). They are extracted from fruits, vegetables, seeds, nuts, stems, and floral parts of the plants. Polyphenols are found universally in a

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variety of foods, medicinal supplements, and products of cosmetic use, and have gained most attention in the last two decades due to growing indication of their helpful health outcome (Durazzo et al. 2019; Cheynier 2005; Crozier et al. 2009; Castro-Barquero et al. 2020). Increased consumption of polyphenol-rich plant products including whole grain cereals, vegetables, and fruits leads to a decreased risk of several severe health complications and pathologies associated with reactive oxygen species (oxidative stress), including cardiac complications, diabetes, severe inflammation, cancers, and several degenerative pathologies (Castro-Barquero et al. 2020; Tsao 2010; Vetrani et al. 2018; Giacco et al. 2020; Grosso et al. 2017; Kopustinskiene et al. 2020; Van de Velde et al. 2019; Csepregi et al. 2020; Tajner-Czopek et al. 2020). Oxidant properties of polyphenol can be classified as either pro-oxidant or antioxidant depending on the design of specific polyphenol and the cellular redox setting, which may enhance oxidant scavenging proteins or reduce the level of oxidized proteins and lipids (Tajner-Czopek et al. 2020; Chen et al. 2014; Yu et al. 2012; Julibert et al. 2019). Plant polyphenols are scavengers of metal chelators as well as free radicals. They are very effective in inhibiting the peroxidation of lipid molecules and display numerous physiological and biological actions as antioxidants. These molecules contribute to the defense mechanism in plants and take part in adaptation and pigmentation activities (Fischer et al. 2018; Ma et al. 2018a). In addition to health benefits against various diseases, several extracts rich in these polyphenols can serve as excellent inhibitors of several pathogenetic bacteria (Julibert et al. 2019; Gutiérrez-Del-Río et al. 2018; Redondo-Blanco et al. 2020; Makhuvele et al. 2020; Simonetti et al. 2020).

Many bacteria can form biofilms and can cause serious contamination and infection. Cell-to-cell chemical communication in bacteria is performed by quorum sensing, which plays decisive role in antibiotic resistance, formation of biofilm, proliferation, survival, and production of toxins (Abisado et al. 2018). Disruption of this signaling pathway can make way to control these pathogenic organisms and prevent them from causing infection and disease (Tsao 2010; Defoirdt 2018; Mion et al. 2018, 2019). These bioactive compounds can be obtained by various physical and chemical extraction methods. Some of the effective methods include enzyme-associated extraction techniques with application of carbohydrase active microorganisms or enzymes and solid-phase fermentation (Yu et al. 2012; Aherne and O'Brien 2002; Cowan 1999; Brglez Mojzer et al. 2016; Iglesias-Carres et al. 2019; Mesquita and Monteiro 2018; Heng et al. 2017; Capriotti et al. 2014). Overall, the effectiveness of the polyphenol and its derivatives depends on the target microorganism, their membrane and cell structure, the time and duration of treatment. The antimicrobial effect of these polyphenols also depends on the type of solvent used and which extraction method was applied and on the plant cultivars. The compound can also affect efflux pump expression and their functions besides causing down-regulation of genes linked with the virulence factor of microorganism.

The discovery of penicillin revolutionized the treatment of bacterial diseases but the overuse of antibiotics has created an opening for potential future pandemic leading to the emergence of antibiotic resistance pathogens (AMR), which is now the greatest threat to public health around the globe. The microbes display antibiotic

resistance either through the expression of resistance phenotype or due to the inherent resistance genes inside them. The appearance of multidrug-resistant bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) is giving more nightmares to clinicians than resistance to a single antibiotic (Abreu et al. 2012). Studies on resistance modification agents (RMAs) which can counter the problem of AMR have been explored and polyphenolic compounds can provide one such alternative. The synergistic approach between resistance modification agents (RMAs) like polyphenols and antibiotics can be a more effective strategy to control the phenomenon of AMR.

An interesting area of future research is the interaction of polyphenol and gut microbiota. It is hypothesized that polyphenolic compounds favor the growth of beneficial bacteria while doing reverse with harmful bacteria (Marín et al. 2015). Some research indicates that there is positive correlation between blueberry drink and bifidobacteria (Vendrame et al. 2011), while extracts obtained from green tea modulate activities of bacteria such as *Salmonella typhimurium, Clostridium difficile*, and *Escherichia coli* (Lee et al. 2006; Pacheco-Ordaz et al. 2018). There is also an indication that phenolic extracts may stimulate the beneficial effects of probiotics (de Souza et al. 2019). The efforts are underway to exploit polyphenols as treatment option for COVID-19 and other viruses (Paraiso et al. 2020; Annunziata et al. 2020).

Polyphenols can be classified based on source of origin, the structure of the chemicals, and their biological function (Di Lorenzo et al. 2021; Durazzo et al. 2019; Singla et al. 2019) (Figs. 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, and 10.7). Chemically, one aromatic ring and presence of one or more hydroxyl (–OH) groups are characteristic of phenolic compounds which are further divided into flavonoids and non-flavonoids (Figs. 10.1, 10.2, 10.3, 10.4, 10.5, 10.6, and 10.7). Flavonoids are defined as cluster of low molecular weight polyphenolic compounds obtained from various plant sources. Their synthesis is done by phenylalanine and acetic acid and combination of their derivatives. Flavonoid structure is composed of flavonoid nucleus, consisting of three phenolic rings designated as A, B & C (Tsao 2010;

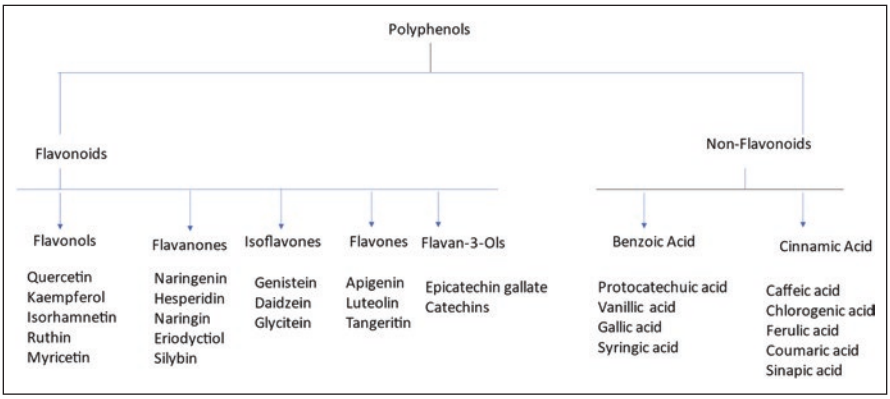


Fig. 10.1 Polyphenols classification with some examples

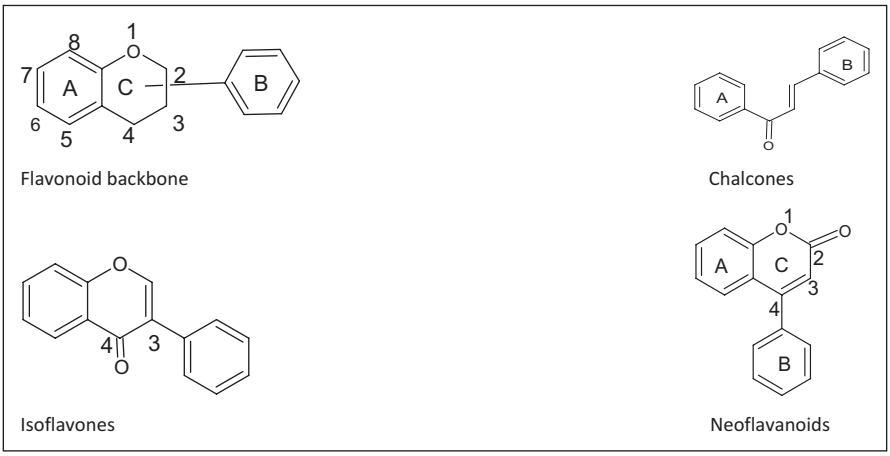


Fig. 10.2 Basic flavonoid structures

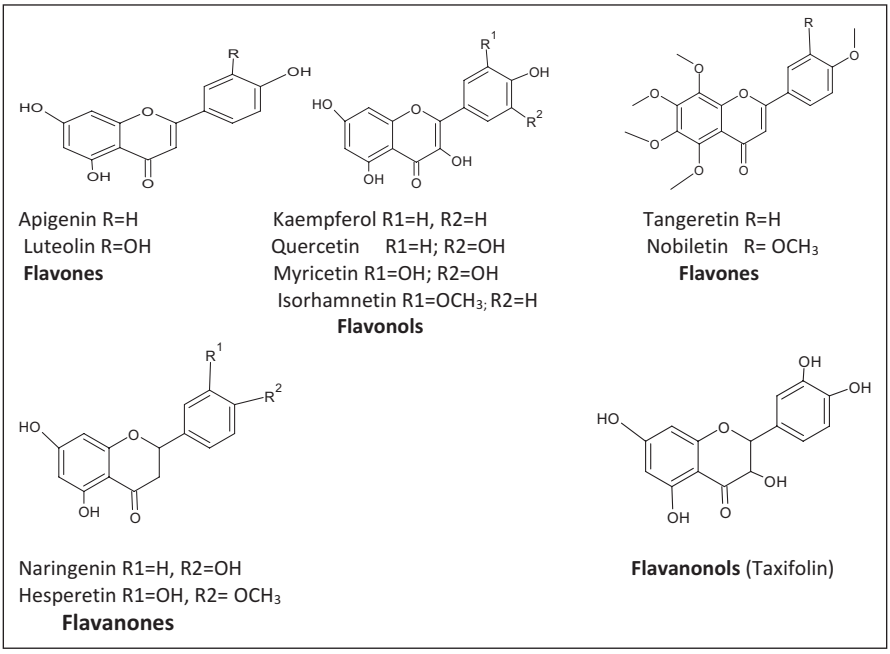
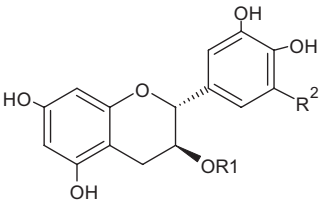


Fig. 10.3 Structure of flavones, flavonols, flavanones, and flavanonols

Aherne and O'Brien 2002) (Fig. 10.2). Flavonoids can be further differentiated into different groups based on the presence or absence of double bonds between carbon atom C2 and C3 and a hydroxyl group (–OH) at C3 of ring C. Based on these structural modifications, they are further subdivided into different subfamilies. The

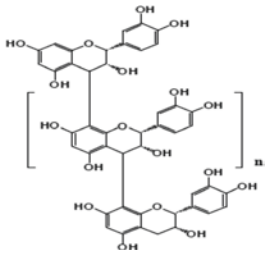


(+)-Catechin: R1=R2=H R1

(+)-Catechin gallate: R1=gallyl, R2=H

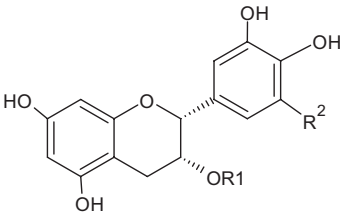
(+)-Gallocattechin: R1=H; R2=OH

(+)-Gallocatechin gallate: R1=gallyl, R2=OH



Procyanidins: a> 0

Oligomeric procyanidins: n=0-7



(-)-Epicatechin: R1=R2=H

(-)-Epicatechin gallate: R1=gallyl, R2=H

(-)-Epigallocatechin: R1=H; R2=OH

(-)-Epigallocatechin gallate: R1=gallyl; R2=OH

Fig. 10.4 Flavanols and procyanidins

subfamilies are classified as flavonols, flavones, isoflavones, flavanones, and flavan3-ols (Singla et al. 2019; Yu et al. 2020; Křížová et al. 2019) (Figs. 10.2, 10.3, 10.4, and 10.5). The position and number of hydroxyl groups on the phenol ring play a major role in their biological properties. It is hypothesized that these groups are responsible for their relative toxicity toward different pathogens, and there is a positive correlation between hydroxylation (no of –OH groups) and toxicity of the compounds (Cowan 1999).

Flavonols are classified as a cluster of polyphenolic compounds having two benzene rings attached by a linear three carbon chain (Figs. 10.3 and 10.4). Flavonols are derivatives of flavonoids with a ketone group. Flavonols are present in abundant quantities in several fruits and vegetables. Apples, grapes, berries (Blueberries), onions, kale, lettuce, and tomatoes are rich sources of flavonols. Due to their antioxidant activity, they possess several benefits to health, which are suggested to be

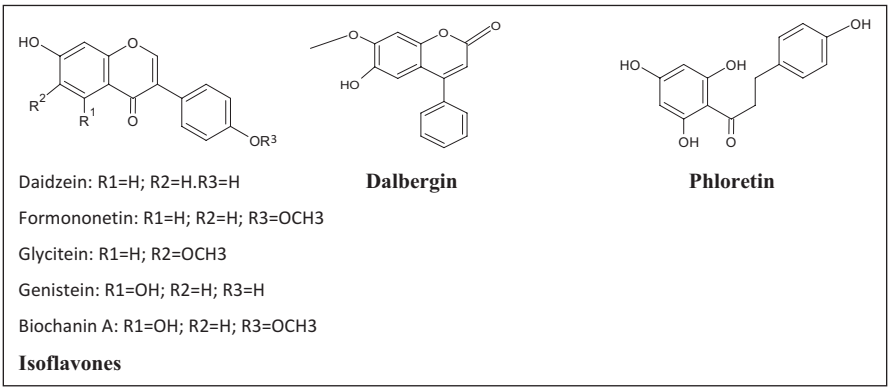


Fig. 10.5 Typical isoflavones, neoflavones found in plants

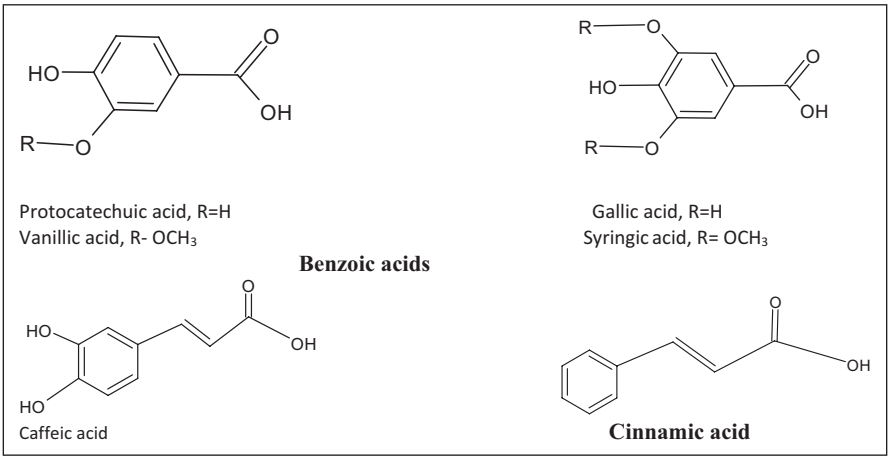


Fig. 10.6 Typical phenolic acids (non-flavonoids) in plants

due to the presence of phenolic hydroxyl functional group (OH) on the B ring (Kim et al. 2006).

Flavones are almost structurally like flavonols except that there is presence of oxygen at C3 (Figs. 10.2, 10.3, and 10.4) (Di Lorenzo et al. 2021; Daglia 2012; Das 2020). Isoflavones (Fig. 10.5) have the B ring attached at C3 rather than at the C2 position. They are mostly found in leguminous family of plants like soybean. Genistein and daidzein are two examples of isoflavones found in soy along with glycetein, biochanin A, and formonetin. Red clovers are also good source of this compound. In neoflavanoids Ring B is attached at C4 position of ring C. Neoflavanoids are rarely found in many plants, but Dalbergin is the most frequent and commonly circulated in the plant world. In flavanones, D2,3 double bond is absent and there is presence of a chiral center at C2. Flavan-3-ols, also called catechin, are a complex

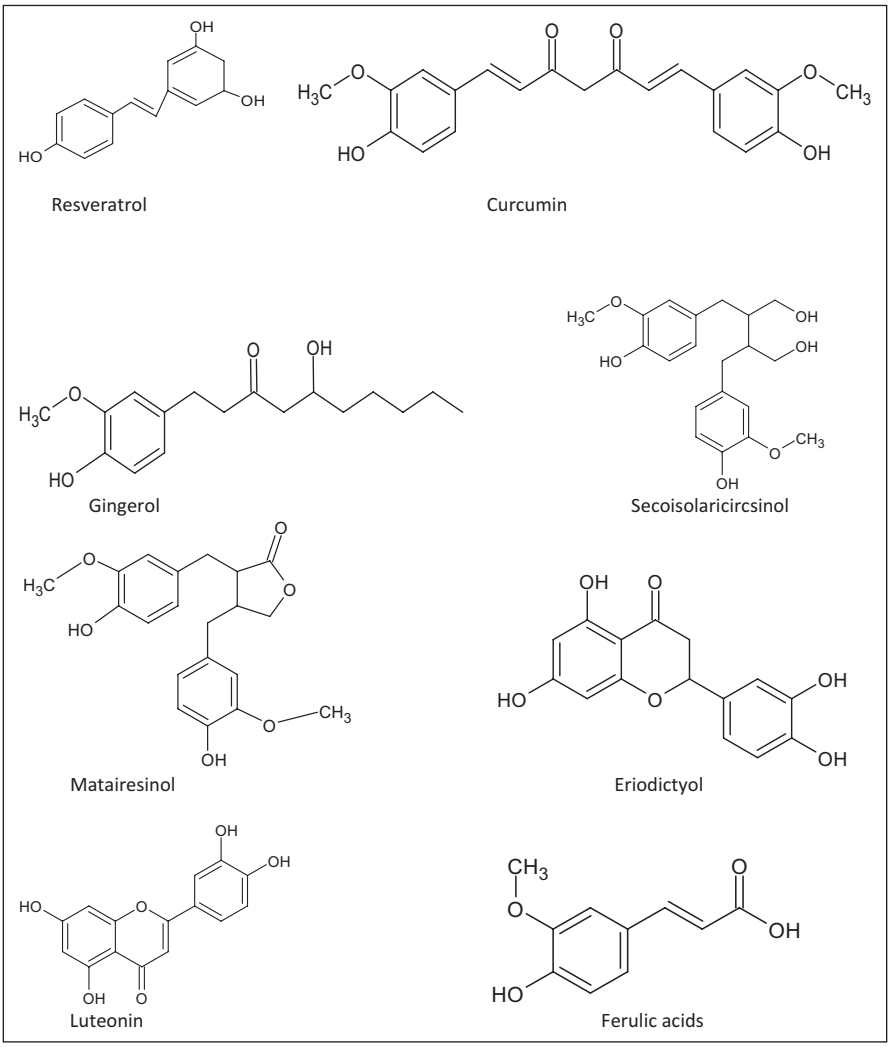


Fig. 10.7 Other important polyphenols

subclass of flavonoids. In their structure, the two chiral carbons at C2 and C3 of the monomeric flavan-3-ol produces four isomers for each level of B ring hydroxylation (Figs. 10.3 and 10.4). Isomer with *trans* configuration is named catechin while one with *cis* configuration is called epicatechin.

Proanthocyanidins are polymers of catechin and epicatechin because anthocyanidins are produced by acid-catalyzed cleavage of the polymeric chains. Although chalcones lack the heterocyclic ring at C they are still counted as part of the flavonoid family (Fig. 10.2). Grapes, apples, and blueberries are rich sources of flavanols. Monomeric flavanols like catechin and epicatechin and their byproducts (e.g.,

gallocatechin) are the major flavonoids found in tea leaves, cacao beans, and chocolates (Tsao 2010).

Tannins are another class of polyphenols with mol weight ranging from 500 to 3000 Da but some tannins have been found with mol weight ranging from 3000 to over 30,000 Da (Serrano et al. 2009; Fraga-Corral et al. 2021). They can be classified based on their solubility and extractability and on the basis of their chemical structure. Tannins can be further categorized into two major groups, either as proanthocyanidins or condensed tannins and hydrolyzable tannins depending upon the structure of monomer (Giacco et al. 2020).

The classical example of non-flavonoid polyphenolic compounds is phenolic acid. Phenolic acids are further sub-divided into two main groups, benzoic acid and cinnamic acid derivatives depending upon C1–C6 and C3–C6 backbones (Fig. 10.6) (Tsao 2010). Some polyphenolic compounds with their target microorganism are mentioned in Tables 10.1 and 10.2. Some other polyphenols are depicted in Fig. 10.7 (Tsao 2010).

10.2 Type of Flavonoids and Their Antimicrobial Activity

Flavonoids are one of the most widely researched polyphenols (a type of phytochemical) due to their numerous health benefits. They belong to a class of secondary metabolites with polyphenolic structure. They provide enormous health benefits, and they are part of various pharmaceuticals, nutraceutical, cosmetic, and medicinal products. They can modulate important cellular and enzymatic functions. They can inhibit activities of several enzymes like cyclo-oxygenase (COX), xanthine oxidase (XO), lipoxygenase, and phosphoinositide 3-kinase. They are available in abundant quantities in fruits, vegetables, certain beverages, and green tea extract (GTE). Since ancient times flavonoids from plant and herbal extracts were utilized as folk medicine across the world. They display several health benefits, for example, as protective agents for peptic ulcer, cancers related to stomach, and treatment of rheumatic pain (Di Lorenzo et al. 2021; Rupasinghe 2020; Kozłowska and Szostak-Wegierek 2014; Samec et al. 2021; Ullah et al. 2020). Flavonoids display antioxidant and anti-inflammatory properties besides their effectiveness as antibacterial agents. The antimicrobial potential of flavonoids has been increasingly explored and many studies across the world have reported identification of different chemicals having antibacterial characteristics (Marín et al. 2015; Sarbu et al. 2019).

There are different mechanisms through which flavonoids can target bacterial growth. Inhibition of nucleic acid synthesis and targeting the function of cytoplasmic membrane are the most widely used strategies adopted by flavonoids, especially by flavonoids with B ring hydroxylation (Sarbu et al. 2019; Farhadi et al. 2019). A natural phenolic compound, 3-*p*-trans-coumaroyl-2-hydroxyquinic acid (CHQA) from needles of *Cedrus deodara*, has been exploited as strong antibacterial agent. Extraction of 3-*p*-trans-coumaroyl-2-hydroxyquinic acid

Table 10.1 Polyphenolic compounds and their target microorganisms

Compound	Target bacteria	References
Galangin	*K. pneumoniae*	Chen and Huang (2011)
Kaempferol	*K. pneumoniae* *S. aureus*	Shao et al. (2016), Randhawa et al. (2016), Wu et al. (2013)
Quercetin	*K. pneumoniae* *S. aureus* *S. haemolyticus* *E. coli* O157:H7 *S. pyogenes*	Chen and Huang (2011), Liu et al. (2010), Siriwong et al. (2015)
Myricetin	*K. pneumoniae* *S. aureus*	Chen and Huang (2011), Huang et al. (2015)
Luteolin	*S. pyogenes* *S. aureus* *E. coli*	Liu et al. (2010), Siriwong et al. (2015)
Chrysin	*P. lachrymans* *B. subtilis* *S. haemolyticus* *S. aureus* *E. coli* *A. tumefaciens* *B. subtilis* *S. haemolyticus*	Liu et al. (2010), Siriwong et al. (2015)
Camellia sinensis polyphenols	*S. aureus* *S. marcescens* *P. aeruginosa* *H. pylori* *E. coli*	Ankolekar et al. (2011), Thakur et al. (2016), Yi et al. (2014), Radji et al. (2013)
ECGC	*S. aureus* *S. mutans* *S. pyogenes* *E. faecalis* *E. coli* *S. pyogenes* *S. typhi* *P. gingivalis* *P. aeruginosa* *K. pneumoniae*	Cui et al. (2012), Cho et al. (2011), Lee et al. (2009a), Betts et al. (2015), Asahi et al. (2014)
Naringenin	*S. typhimurium* *P. aeruginosa*	Vandeputte et al. (2011), Vikram et al. (2011)
Hesperetin	*S. aureus* *A. hydrophila*	Abuelsaad et al. (2013), Bakar et al. (2012)
Malvidin	*K. pneumoniae*	Gopu et al. (2015)
Daidzein	*Vancomycin-resistant* *E. faecalis*	Chin et al. (2012)
Gallic acid	*E. coli* *S. mutans*	Shao et al. (2015, 2016), Dwivedi et al. (2016)

(continued)

Table 10.1 (continued)

Compound	Target bacteria	References
Resveratrol	*V. cholerae* *P. mirabilis*	Ma et al. (2018b), Klančnik et al. (2017), Ferreira et al. (2014)
Coumarins	*H. pylori*	Basile et al. (2009), Takeda et al. (2007)
Lacinartin	*P. gingivalis*	Marquis et al. (2012)
Furocoumarins	*E. coli O157:H7* *S. typhimurium*	Girennavar et al. (2008)
Blueberry proanthocyanidins	*L. monocytogenes* *H. pylori* *S. typhimurium* *E. coli*	Joshi et al. (2014), Lacombe et al. (2013), Park et al. (2011)
Proanthocyanidins	*E. coli* *P. aeruginosa* *S. epidermidis*	Zeng et al. (2020)

(CHQA) from *Cedrus deodara* has demonstrated effective antimicrobial activity towards 11 food-borne pathogens like *S. aureus*. The MIC values obtained against such pathogens are between 2.5 and 10 mg/mL. CHQA is responsible for damaging the cytoplasmic membrane of bacteria and induces seepage of intracellular components. It can act as a future natural antibacterial candidate against various food pathogens (Wu et al. 2016). Sophoraflavanone B is a prenylated flavonoid extracted from the roots of *Desmodium caudatum*. It can directly target the peptidoglycan layer and inhibit biosynthesis of cell wall inhibiting MRSA (MIC observed between 15.6 and 31.25 μg/mL) (Mun et al. 2014). Urease, a major virulence factor of *H. pylori,* is the target of many phenolic compounds such as 4-deoxy analogs of flavonoids which acts as a potent inhibitor of urease (Xiao et al. 2013). Curcumin isolated from turmeric has displayed beneficial activity by altering the cell membrane of *S. aureus* and *E. coli*. It inhibits sortase A from *S. aureus* (IC50 13.8 μg/mL) (Tyagi et al. 2015). Haloemodins are classified as semisynthetic natural anthraquinone derivative having antimicrobial properties. They target DNA gyrase in MRSA and vancomycin-resistant *Enterococcus faecium* showing effective inhibition (Duan et al. 2014). In a recently conducted study, the molecular characteristics exploring antibacterial activity of prenylated (iso)flavonoids against MRSA are evaluated (Kalli et al. 2021). Phloretin, a kind of flavonoid, obtained from apple was able to block biofilm formation in *E. coli O157:H7*. It was successful in limiting curli genes, i.e., csgA and csgB, responsible for the production of fimbriae (Lee et al. 2011).

Table 10.2 Polyphenolic compounds with their target microorganisms with MIC and source of extraction

Name of plant	Common name	Target pathogens	MIC (mg/mL)	Polyphenol derivatives	References
Eugenia caryophyllata	Cloves	*B. subtilis*	1.6	Flavonoids Terpenoids	Shehadi et al. (2014)
Mentha piperita	Mint	*B. subtilis*	0.5×10^2	Terpenoids Flavonoids	Shehadi et al. (2014), Othman et al. (2019)
Rosemarinus officinalis	Rosemary	*B. subtilis*	2.96	Phenolics Flavonoids Terpenoids	Shehadi et al. (2014), Othman et al. (2019)
Prunus avium	Cherry	*B. subtilis*	4	Phenolics	Othman et al. (2019)
Matricaria aurea L.	Golden chamomile	*B. subtilis* *S. pyogenes* *S. aureus* *K. pneumoniae*	0.4 0.4 50 0.4	Phenols Phenolic acids	Rizwana (2016)
Annona squamosa L.	Custard Apple	*S. aureus* *E. faecalis* *E. coli*	50	Quinones Flavonoids	Mohamad et al. (2017)
Premna resinosa	Mukarakara	*S. aureus* *B. subtilis* *E. coli* *S. typhimurium* *E. faecalis* *S. flexneri*	0.01–1	Quercetin Kaempferol	Albadawi et al. (2017)
Premna resinosa	Mukarakara	*MRSA* *M. tuberculosis*	0.031 0.006	Quercetin Kaempferol	Njeru et al. (2015)
Pheonix dactylifera L.	Date palm	*P. aeruginosa*	0.06	Flavonoid Glycosides Quercetin Apigenin Luteolin	Selim et al. (2012)
Thymus vulgaris *Boswellia carterii*	Thyme Myrrh	*S. aureus* *B. cereus* *A. flavus* *F. oxysporum*	2–4% (v/v)	Rosmarinus acid Caffeic acid Carnosol Flavonoids	Al-Juraifani (2009)
Olea europaea	Olive	*S. aureus* *B. cereus*	0.08 0.04	Flavonoids	Malik (2015)
Eruca sativa	Arugula	*S. aureus* *B. cereus*	0.06 0.02	Flavonoids	Malik (2015)

(continued)

Table 10.2 (continued)

Name of plant	Common name	Target pathogens	MIC (mg/mL)	Polyphenol derivatives	References
Azadirachta indica	Neem	*B. subtilis* *S. aureus* *M. roseus*	0.05–0.2	Flavonoids Saponins Anthocyanins	Othman et al. (2019)
Zingiber officinale	Ginger	*S. aureus,* *B. subtilis*	0.21–1.33	Tannins	Shohayeb et al. (2013)
Curcuma Longa	Turmeric	*P. gingivalis*	0.012	Tannins	Sha and Garib (2019)
Thymbra spicata L.	Mediterranean thyme	*MDR S. aureus* *K. pneumoniae*	0.006–0.012 0.012	Alkaloids	Haroun and Al-Kayali (2016)
Salvadora persica L.	Miswak	*S. aureus* *S. mutans* *S. faecalis* *S. pyogenic* *L. acidophilus* *P. aeruginosa* *C. albicans*	3.12 1.56 0.781 3.12 6.25 6.25 6.25	Saponins Alkaloids Terpenoids Flavonoids	Al-Bayati and Al-Mola (2008), Al-Bayati and Sulaiman (2008), Sher et al. (2011)
Origanum majorana	Oregano	*ESBL E. coli* *K. pneumoniae*	0.0025–0.08	Rosmarinic acid	Abdel-Massih et al. (2010), Daoud et al. (2011), Hossain et al. (2014)
Trigonella foenum-graecum	Fenugreek	*ESBL E. coli* *K. pneumoniae*	0.0025–0.08	Stilbenes	Abdel-Massih et al. (2010), Daoud et al. (2011)
Quercus infectoria	Aleppo oak	*B. cereus* *Y. enterocolitica*	0.00256 0.00512	Flavonoids	Shariatifar et al. (2014)
Galla rhois		*Salmonella* *E. coli*	0.004–0.125 0.03	Methyl gallate	Choi et al. (2014), Madikizela et al. (2013)
Boletus edulis	Wild polish mushroom	Gram-positive bacteria	0.156–5	Flavonoids	Nowacka et al. (2015)
Kaempferia pandurata Roxb	Temu kunci	*S. aureus*	0.06–2	Natural chalcone	Cushnie and Lamb (2011), Rukayadi et al. (2009)
Helichrysum italicum (Roth)	Curry plant	*S. aureus*	0.001–0.004	Phloroglucinol	Taglialatela-Scafati et al. (2013)

(continued)

Table 10.2 (continued)

Name of plant	Common name	Target pathogens	MIC (mg/mL)	Polyphenol derivatives	References
Hypericum beanie	St John's wort	*MRSA*	0.016–0.032	Acylphloroglucinols	Shiu and Gibbons (2006)
Cedrus deodara	Cedar	*S. aureus*	2.5–10	Natural phenolic compound	Wu et al. (2016)
Desmodium caudatum	Salparni	MRSA	0.016–0.031	Flavonoid	Mun et al. (2014)
Citrus bergamia Risso	Bergamot	*E. coli* *P. putida* *S. enterica*	0.25–0.08	Flavonoid	Mandalari et al. (2007)
Polymnia fruticose	Ming aralia	*H. pylori*	0.087	Flavanone	Zhang et al. (2008)
P. fruticose	*Potentilla* species	*P. aeruginosa*, and a fungus *Candida albicans*	0.78–6.25	Caffeic acid Ferulic acid	Wang et al. (2013a)
Vaccinium virgatum	Blueberry	*S. epidermidis* *P. aeruginosa*	4	Chlorogenic acid	Zimmer et al. (2014)

10.2.1 Flavonols and Their Antimicrobial Activity

Flavonols are derivatives of flavonoids having a ketonic group in their structure, and they are building blocks of proanthocyanins. The most common example of flavonols is kaempferol, quercetin, myricetin, and fisetin which are abundantly found in a variety of fruits and vegetables. The rich sources of flavonols are lettuce, tomatoes, onions, kale, apples, grapes, and berries besides tea and red wine. Flavonols are found in large varieties of plants, apart from algae and fungi. Some of the most frequently found flavanols are galangin and quercetin which possess good antimicrobial activity (Rauha et al. 2000; Basile et al. 2000; Arima et al. 2002; Rizzo et al. 2014). Flavonols like myricetin, morin, rhamnetin, and quercetin show inhibitory activity against the growth of *Chalamydophyla pneumoniae*, an obligate intracellular Gram-negative bacterium responsible for community and nosocomial pneumonia. Quercetin and apigenin, the most abundant flavonoids can block D-alanine by blocking D-alanine ligase, an important enzyme in the assembly of peptidoglycan precursors of the cell wall in *H. pylori* and *E. coli* (Wu et al. 2008).

Resveratrol, a natural polyphenol, is a promising candidate against different pathogenic bacteria like *Campylobacter jejuni, M. smegmatis*, and *Arcobacter cryaerophilus* (Ma et al. 2018b; Klančnik et al. 2017; Ferreira et al. 2014; Lechner et al. 2008). Resveratrol, at 40 μM concentration in combination with quercetin at 20 μM, was able to inhibit the growth of *C. pneumoniae.* In some reports, rhamnetin was more potent than quercetin, probably due to its hydrophobic nature (Alvesalo et al. 2006). Application of clarithromycin, a macrolide, and ofloxacin, a

fluoroquinolone, was beneficial in the treatment of chlamydial infections although the therapy was not completely successful (Rizzo et al. 2014). A synergistic approach with quercetin and ofloxacin or clarithromycin may provide better alternative in treating this type of infection (Rizzo et al. 2014). Some important enzymes including beta-ketoacyl acyl carrier protein synthase (KAS) II and III are responsible for generation of precursor of bacterial cell membrane and they are targets of phenolic compounds. The phenolic derivatives also target enzymes involved in elongation cycle of fatty acids and biosynthesis such as FabG, FabI, and FabZ. 3,6-Dihydroxyflavone, a flavonol, can also bind effectively to KAS III and KAS I and can inhibit *E. coli* with MIC at 512 μg/mL(Farhadi et al. 2019).

Propolis, containing main components like flavonoids and phenolic acid esters, is a non-toxic natural extract having multiple pharmacological characteristics and is found in several parts of the world, including Europe, China, and North America (Patel 2016; Rivera-Yañez et al. 2020). The main polyphenolic components of propolis analyzed by HPLC are galangin, pinocembrin, apigenin, quercitrin, myricetin, kaempferol, caffeic acid, and its phenylethyl ester (Koru et al. 2007; Barrientos et al. 2013; Santos et al. 2020). They displayed various antimicrobial activities against several bacteria, parasites, viruses, and fungus (Bankova et al. 1995; Kujumgiev et al. 1999; Sforcin et al. 2000; Orsi et al. 2005; Bosio et al. 2000; Almuhayawi 2020). The main component participating in these antimicrobial activities are galangin and pinocembrin (Bosio et al. 2000; Hegazi et al. 2000). The antimicrobial property of propolis depends on the solvent used for extraction as ethanol and acetone extracts were observed to be more potent against the most microorganism and this action was most inhibitory in a slightly acidic (pH-6) environment (Ivančajić et al. 2010).

Propolis and its various polyphenolic extracts are very effective in inhibition of biofilms and caries development by *Streptococcus mutans.* Inhibitory activity of various propolis components was studied on several oral microorganisms like *Veillonellaparvula, Actinomycescesnaesundii, Prevotella oralis*, and *Lactobacillus acidophilus.* Gram-positive anaerobic were more susceptible toward these extracts compared to Gram-negative ones. Propolis isolates from Chile were found to inhibit *Candida* spp. The extracts are very potent against isolates from the oral cavity of removable dentures and from cariogenic bacteria *Lactobacillus fermentum* (Koru et al. 2007*;* Przybyłek and Karpiński 2019*;* Saavedra et al. 2011). The exact mechanism by which propolis displays its antibacterial activity is still not clear. Some researchers suggest that the polyphenolic compounds present in propolis such as flavonoids probably act at a site on the cytoplasmic membranes or cell wall of bacteria causing structural and functional changes affecting their pathogenic mechanism (Almuhayawi 2020; Przybyłek and Karpiński 2019).

Kaempferol (3,4′,5,7-tetrahydroxyflavone) is a natural flavanol and a regular flavonoid which can be extracted from different plants and plant-derived foods, including spinach, kale, beans, tea, and broccoli. It is characterized as a yellow strong glasslike structure that has a low dissolvability rate in water but is profoundly soluble in ethanol, ethers, and dimethyl sulfoxide. It has a high liquefying point (276–278 °C) and is utilized in different drug items for its medical advantages and

in food items for nutritional benefits. Kaempferol is exceptionally utilized in dietary enhancements because of its micronutrients, which help in treating chronic diseases and type 1 diabetes. Also, it finds applications in the therapy of sicknesses and infectious diseases caused by viruses and bacteria. Effectiveness of kaempferol against different pathogenic microorganisms, including fluconazole-resistant *C. albicans* and MRSA, is already well demonstrated (Shao et al. 2016; Randhawa et al. 2016; Holler et al. 2012a). A natural glycoside product of kaempferol (kaempferol rhamnoside) isolated from *Persea lingue* has potential to increase the antimicrobial activity of ciprofloxacin in a NorA overexpressed *S. aureus* (Holler et al. 2012a).

Another rich source of flavonols are white and red onion (*Allium cepa*), which contain very high amounts of quercetin-4″-glucoside and quercetin-3,4″-*O*-diglucoside (Sidhu et al. 2019). Extracts of white onion displayed higher inhibitory activity against Gram-negative bacteria such as *E. coli* and moderate activity against *S. aureus.* Compared to white onion, red onion is less effective in inhibition of pathogens as it displayed no activity against *S. aureus* (Benmalek et al. 2013). Different range of antimicrobial activity was shown by isolated dry buds of *A. cepa*. as *S. aureus* was found to be most susceptible, while *Pseudomonas aeruginosa* and *Salmonella typhi* are the most resistant (Bakht et al. 2014). Quercetin is one of the most studied flavanols and a major bioflavonoid is also quite effective against *E. coli*. It probably inhibits DNA gyrase and probably binds to β subunit of *E. coli* DNA gyrase and blocks the ATPase activity (Plaper et al. 2003). Many factors are responsible for potential gyrase inhibition, such as altering the permeability and pharmacokinetics of the membrane. The inherent mechanisms of gyrase inhibitors lie in the activity against the target enzyme (Hilliard et al. 1995). The binding mechanism of some flavanols including ellagic acid, quercetin, and 4-quinolons were studied by Hillmond et al. (Hilliard et al. 1995) against *E. coli* DNA gyrase.

10.2.2 Flavones and Isoflavone and Their Antimicrobial Activity

Citrus fruits like orange and lemons are rich sources of flavones such as tangeritin, luteolin, and apigenin. In citrus fruits, these polyphenols are found as conjugated polymethyl flavones while in the diet they appear as O-glycosides and C-glycosides (Hollman and Arts 2000; Hostetler et al. 2017). They show antimicrobial activity against several bacteria and pathogens (Liu et al. 2013a; Stojanović et al. 2005). Soyabean and other leguminous plants are a rich source of isoflavones. Soyabean contains ample amount of isoflavones like daidzein and genistein (Osawa et al. 1992; Leuner et al. 2013). Antimicrobial activity of genistein and other isoflavones was tested against several bacteria like *Vibrio harveyi, E. coli*, and *Bacillus subtilis* (Ulanowska et al. 2006). Among them *Vibrio harveyi* was most sensitive while *E. coli* did not show any inhibition. Genistein was able to alter cell morphology at a

final concentration of 0.1 mM. Baicalein, a flavone extracted from roots of plant *Scutellaria Lateriflora, Thymus vulgaris*, and *Scutellaria baicalensis*, has displayed a range of antimicrobial activities. Extract isolated from *Scutellaria baicalensis* has been tested against several bacterial strains (Lu et al. 2011).

Baicalein has the potential to reverse anti-AMR of β-lactam antibiotics against MRSA by inhibition of NorA efflux pump. Baicalein shows greater antimicrobial activity with a combination of tetracycline against *E. coli* (Fujita et al. 2005). Biochanin A, an isoflavone, was found to be quite effective against a range of pathogens like MRSA, chlamydia, and mycobacterium (Zou et al. 2014; Hanski et al. 2014; Cannalire et al. 2017). Two methoxylated flavones from *Artemisia annua,* Chrysosplenol-D and chrysoplenetin, have shown inhibitory activity against NorA efflux pump (Stermitz et al. 2003). Two other classes of phenolic compounds, isoflavonoids and flavonolignans, are able to inhibit Nor A efflux pump and can enhance the potency of norfloxacin and berberine (Morel et al. 2003). Orobol from *Lupinus argenteus* and silybin, a flavonolignan from the famous medicinal plant *Silybum marianum*, was effective against *S. aureus* (Morel et al. 2003; Stermitz et al. 2001). Morin (a type of flavonol), a compound isolated from the bark of *Rhus verniciflua*, was able to inhibit *S. aureus* by targeting sortase A and B (Kang et al. 2006).

10.2.3 Flavanones and Their Antimicrobial Activity

Citrus fruits like oranges, lemons, and grapes are the exclusive source of flavanones such as naringenin, eriodictyol, and hesperetin. Grapefruit contains a higher amount of naringenin than hesperetin (Testai and Calderone 2017; Barreca et al. 2017). Finnish plant rich in Naringenin displayed potent antibacterial activity mainly toward Gram-positive bacteria like *Micrococcus luteus, S. epidermidis, S. aureus,* and *B. subtilis,* while against Gram-negative bacteria like *E. coli* and *P. aeruginosa* there was moderate or little activity (Rauha et al. 2000*;* Radulescu et al. 2020). In one study, flavanones isolated from peel of bergamot (*Citrus bergamia Risso*) were tested for their antimicrobial activity against different bacteria and eriodictyol displayed the highest antimicrobial activity (Observed MIC 250–800 μg/mL) (Mandalari et al. 2007). Several in vitro studies showed antibacterial activity of hesperetin and naringenin against *Helicobacter pylori* (Bae et al. 1999; Fukai et al. 2002; Tombola et al. 2003; Burger et al. 2002). Naringenin displays its antibacterial effects by disruption of the cytoplasmic membrane and targeting DNA of *Staphylococcus aureus* (Wang et al. 2017, Wang et al. 2018; Duda-Madej et al. 2020). In a recent study, it was demonstrated that Pompia and lemon juices have inhibitory and antibiofilm activity against pathogenic bacteria like *Streptococcus aureus, Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Barberis et al. 2020). A methoxy derivative of the flavanone naringenin extracted from *Polymnia fruticose* called Sakuranetin successfully blocked FabZ in *H. pylori* with MIC 87.3 μM (Zhang et al. 2008).

10.2.4 Flavan-3-ols as Antimicrobial Agent

Fresh leaves of green tea (*Camellia sinensis*) are a major source of polyphenols, particularly catechins, a monomeric flavanols. High number of flavan-3-ol monomers are obtained from green tea, the main component found is (−)-epigallocatechin-3-gallate (EGCG), containing around 60% of the total catechin content followed by (−)-epigallocatechin (EGC) containing around 20% catechin. The other components include (−)-epicatechin-3-gallate (ECG) with approximately 14% and (−)-epicatechin (EC) with approximately 6% catechin content (McKay and Blumberg 2002; Khan and Mukhtar 2018). Catechins can cause damage to the bacterial membrane and appear to have more potency against Gram-positive bacteria. The presence of lipopolysaccharide makes Gram-negative bacteria more resistant to these chemicals. Like other polyphenols, tea polyphenol also displays a wide range of health benefits due to its antioxidant property. It also shows anticancer properties as well as being beneficial in cardiovascular complications (Navarro-Martínez et al. 2006; Hemaiswarya et al. 2008). McNaught in mid-1990s first showed antimicrobial activity of tea when he observed that brewed black tea could kill *B. melitensis* and *Typhi* (McNaught 1906). The extract obtained from green tea displayed anti-adhesive properties as it can inhibit the adherence of several microorganisms on the host cell membrane (Lee et al. 2009b). The extract obtained from green tea is found to be promising against the inhibition of MRSA (Taylor 2020). Cho et al. explored the antibacterial effect of polyphenols obtained from green tea on 30 clinical isolates of *S. aureus,* including 13 MRSA isolates (Cho et al. 2008). He observed MIC in the range of 50–80 μg/mL for both the MRSA and the non-MRSA strains (Cho et al. 2008). It is also found that green tea extract was a potent inhibitor of *E. coli* in cases of urinary tract infections (UTI), EGC compounds which are oozed in urine prevented 99% of strains tested at MIC concentration of ≤4.0 mg/mL (Reygaert and Jusufi 2013; Yang et al. 1998). The antimicrobial potential of green tea extract was also studied against *P. aeruginosa* (Radji et al. 2013; Taylor 2020; Jazani et al. 2007; Yi et al. 2010). Green tea extract displayed significant inhibitory activity against clinical MDR isolates of *P. aeruginosa* (MIC and MBC 2.06 ± 1.76 and 2.54 ± 2.22 mg/mL, respectively). ECGC has shown inhibitory potential against 18 clinical isolates of nosocomial pathogen *Stenotrophomonas maltophilia* by inhibiting dihydrofolate reductase. The compound has the potential to replace trimethoprim in patients who cannot tolerate the side effects associated with this antibiotic (Mun et al. 2014).

In one interesting activity, the antimicrobial effect of Chinese green tea beverages was analyzed. The study was performed through different parts of the digestive system with events after duodenal, gastric, and gastroduodenal parts were analyzed. After passing through different digestive processes green tea has shown significant activity against *Staphylococcus* with different MIC concentrations at each step. The anti-*Staphylococcus* activity was performed by different chromatographic methods like RP HPLC and mass spectrometry. Catechin concentration of green tea was resistant to decomposition under gastric condition while it was reversed in the case

of duodenal and gastroduodenal processes. Galloxyl catechin, not native catechin, is the component responsible for antibacterial behavior of green tea which is generated after decomposition of duodenal digestion (Marchese et al. 2014).

EGCG is considered the most potent among the various tea catechins due to its broad range and increased antimicrobial activities (Reygaert and Jusufi 2013). It shows effective antibacterial activity against *E. coli O157:H7, P. aeruginosa, S. aureus,* and *S. mutans* (Cui et al. 2012). Various morphological changes are induced by EGCG in *S. aureus.* EGCG binds with the peptidoglycan layer of bacterial membrane making the cell surface wrinkled and aggregated. The compound can also make structural changes in bacterial proteins and peptides by disruption of H-bonding. The effect is induced by the hydroxyl group or galloyl group of the compound (Oneda et al. 2003; Zhao et al. 2001; Yoda et al. 2004). EGCG also effects temporary change to the cell wall of *E. coli O157:H7,* a foodborne pathogen responsible for hemorrhagic colitis and uremic syndrome. Pore-like lesions were induced by EGCG in bacteria which also show concentration-dependent effect. Hydrogen peroxide production was mainly responsible for damage to the cell wall of Gram-negative bacteria (Ikigai et al. 1993). Transcription of the key virulence gene was also altered by this compound as it also inhibited biofilm formation and swarming motility to this food-borne pathogen. Extracellular release of Shiga toxin from *E. coli O157:H7* was also hindered by EGCG (Sugita-Konishi et al. 1999). This compound is also effective in inhibition of biofilm and blocking the swarming motility of other Gram-negative bacteria like *Burkholderia cepacian* and staphylococcal isolates (Huber et al. 2003). Several Gram-negative bacteria show resistance to carbapenems, a β-lactam, having broad-spectrum antibacterial activity. The resistance mechanism involves increased assembly of carbapenem hydrolyzing β-lactamases(Livermore 1992). *Klebsiella pneumoniae* is also resistant to imipenem and EGCG is very effectively used as inhibitor and stressor to these disease-causing bacteria with observed MIC between 300 and 650 μg/mL. EGCG also shows synergistic activity with imipenem against such bacteria (Cho et al. 2011). Green tea catechins, especially EGCG, is also very effective against different viruses (Xu et al. 2017; Yang et al. 2014; Carneiro et al. 2016; Raekiansyah et al. 2018).

10.3 Tannins and Its Antimicrobial Activity

Tannins are also a type of phenolic molecule found in several parts of the plant, barks, leaves, fruits, wood, and roots (Serrano et al. 2009; Chai et al. 2018). The oxygenation pattern and degree of polymerization determine the biological properties of tannins. Several researchers indicate that tannins are very effective against filamentous fungi, yeast, and other microorganisms (Guo et al. 2018). The antimicrobial effect of tannins is well-defined against several pathogens (Chung et al. 1993). They can act by several mechanisms such as by inhibition of extracellular enzymes and targeting oxidative phosphorylation in bacteria (Guo et al. 2018; Olchowik-Grabarek et al. 2020; Schofield et al. 2001). Chebulinic acid, a kind of

tannin isolated from *Terminalia chebula*, has inhibitory potential against quinolone-resistant *M. tuberculosis* DNA gyrase (Patel et al. 2015). The importance of chebulinic acid as DNA gyrase inhibitor and antituberculosis agent can be explored further as it can be a promising candidate for AMR mitigation.

10.3.1 Proanthocyanidins

Proanthocyanidins (PACs) are polymers of flavan-3-ol, having a common C6–C3–C6 flavone skeleton (Esquivel-Alvarado et al. 2021). The source includes fruits, berries, leaves, and seeds (Zeng et al. 2020; Rauf et al. 2019). Most of the research about PACs is conducted on berries, which are rich sources of dietary polyphenols, particularly they contain a considerable number of PACs with structural and compositional diversity depending on the type of berry species. Besides PACs, berries are also rich source of many flavonols (especially blackcurrant, cranberry, lingonberry, and whortleberry), anthocyanidins (especially bilberry and black currant), hydroxycinnamic acids, and hydroxybenzoic acid (Puupponen-Pimiä et al. 2005a; Hellström et al. 2007; Kähkönen et al. 2001). Other sources of PACs include beer, wine, beverages, and some common fruit juices. Proanthocyanidins and flavan-3-ols are effectively used in the prevention and treatment of pathogens associated with periodontitis (Nawrot-Hadzik et al. 2021a, b).

Proanthocyanidins which comprise A-type linkage have been extracted from cranberry and these PACs display potent antibacterial and antiadhesive properties (Baranowska and Bartoszek 2016; Narwojsz et al. 2019; Luís et al. 2017). They are effective in inhibiting *E. coli* attachment to uroepithelium (Reid et al. 2001; Raguzzini et al. 2020). *E. coli* is responsible for more than 80% of all urinary tract infections acquired in the community and it is found that frequently taking cranberry juice can reduce the risk of urinary tract infections (Luís et al. 2017; Singh et al. 2016; Barbosa-Cesnik et al. 2011), also its exposure can also dislodge pre-attached *E. coli* (Singh et al. 2016; Howell et al. 2005; Guay 2009). In one meta-analysis, cranberry intake was recommended for the treatment of urinary tract infection, particularly in patients having recurrent episodes (Das 2020; Luís et al. 2017; Gbinigie et al. 2020). It is evident that plant tannins are effective toward *E. coli O157:*H7 and this potency relies on the resources of tannins (Wang et al. 2013b; Bumunang et al. 2020; Min et al. 2007), particularly purple prairie clover condensed tannins prevented *E. coli O157:H7* growth at concentrations as minimum as 20 μg/mL (Štumpf et al. 2020; Liu et al. 2013b).

Cranberry (*Vaccinium macrocarpon*), a rich source of proanthocyanidins, has been extensively explored as natural weapons against periodontal diseases (Feghali et al. 2012). In one interesting study, Orophenol (a mixture of polyphenols from different wild berries) displayed interesting anti-caries properties against *Streptococcus mutans* (Souissi et al. 2021).

Low bush wild blueberry is a rich source of polyphenols like PACs, anthocyanins, and monomeric phenolic acid. They are very effective in reducing the growth

o*f E. coli O157:H7,* depending upon the equivalent content of gallic acid used. Low bush wild blueberry significantly increased permeability of *E. coli* cell membrane making it susceptible for inhibition (Lacombe et al. 2013). Virulence of *S. mutans* was slowed down by PACs as they can inhibit bacterial adhesion to apatitic surface, and it also inhibited the growth of biofilm by oral bacteria in vitro (Souissi et al. 2021; Yamanaka et al. 2004; Singhal et al. 2020; Sánchez et al. 2020). Isolates obtained from proanthocyanidins were able to reduce the acidogenicity of *S. mutans* biofilms in in vitro conditions (Yamanaka et al. 2004; Gregoire et al. 2007). According to some reports, cranberry juice provides a promising therapeutic option for diseases related to biofilm formation (Souissi et al. 2021). Tannin extracts from plants like *Anadenanthera* and *Commiphora* in Brazil were able to inhibit the formation of biofilm as they disrupt bacterial membrane and inhibit matrix production; they are used as folk medicine in Brazil (Trentin et al. 2013). Tannin extract particularly proanthocyanidins have bacteriostatic property and they also exhibit antiadhesive property against *P. aeruginosa* with observed MIC between 1 and 4 mg/mL (Trentin et al. 2013).

10.3.2 Hydrolyzable Tannins

Hydrolyzable tannins are made of complex monomeric or oligomeric polyester molecules of phenolic acid and α-glucose or related sugar molecule or polyol. They are synthesized by large varieties of plants and trees and are found in barks, wood, leaves, and galls of such plants. Esters with hexahydroxydiphenic acid are named as ellagitannins while the compound is named as gallotannin when the acidic part of Hydrolyzable tannins is gallic acid ester. These compounds display several useful biological properties such as anticancer, antimicrobial, and antioxidant activity (Yang and Liu 2014; Valverde Malaver et al. 2019; Smeriglio et al. 2017; Ekambaram et al. 2016). A promising inhibitory activity was displayed toward Shiga toxin-producing (STEC) *E. coli O157:H7* in murine model by ellagitannins extracted from *Quercus infectoria.* These compounds can provide alternative therapeutic option against infection caused by this enterohemorrhagic organism as these compounds can remove the colonization o*f E. coli O157:H7* in the intestinal tract of mice (Voravuthikunchai et al. 2012).

Antibacterial and antiviral potency of various other berries and their phenolic extracts were also studied toward many pathogens (Puupponen-Pimiä et al. 2005a, b; Nohynek et al. 2006). Strawberry containing little amounts of ellagitannins displayed moderate activity while cloudberry and raspberry isolates were highly effective against *Staphylococcus* spp. and *Salmonella* spp. (Nohynek et al. 2006). Extracts from Finnish berry rich in ellagitannins were found to have potent antibacterial activity, the highest antimicrobial activity was observed from berries from genus Rubus (cloudberry and raspberry abundant in ellagitannins) (Rauha et al. 2000). Another rich source of hydrolyzable tannins is methanol extract of pomegranate containing high amount of ellagic acid and gallic acid, punicalins, and

punicalagins (Reddy et al. 2007; Saeed et al. 2018). Oligomeric ellagitannins are most potent antimicrobial component extracted from pomegranate, although it also contains anthocyanins and flavanols showing good activity alone or in combination with other compounds (Reed et al. 2005; Fourati et al. 2020; Elshafie et al. 2021; Das et al. 2021). Compound extracted from pomegranate show activity against several enteric pathogenic bacteria including *E. coli* O157:H7 (Pagliarulo et al. 2016; Voravuthikunchai et al. 2004), *Listeria monocytogenes* (Lucas and Were 2009), *V. cholerae* (Guevara et al. 1994; Mathabe et al. 2006), *Salmonella* spp. (Wafa et al. 2017; Rani and Khullar 2004), and some viruses (Moradi et al. 2020). Additionally, methanolic peel extract of pomegranate also displayed excellent inhibitory activity against *S. mutans, L. acidophilus*, and *S. salivarius* (Fourati et al. 2020; Abdollahzadeh et al. 2011). Peel extract of *Punica Granatum* is also very effective against oral candidiasis (Madugula et al. 2017; Kumar et al. 2020; Paul et al. 2018).

10.4 Non-flavonoid Polyphenols and Their Antimicrobial Activity

Non-flavonoid polyphenolic compounds include phenolic acids. Many free phenolic acids are found in fruits and vegetables, although they mostly occur in the bound form in grains and seeds (Veloso et al. 2020; Chandrasekara and Shahidi 2010). Some polyphenolic compounds like (−) epicatechin and oleuropeinglucoside, caffeic acid, gallic acid, ferulic acid, phloridzin, etc., act as anti-quorum sensing molecules (Asfour 2018). Inhibitory potential of such molecules was confirmed by disk diffusion assay based on pigment inhibition in *Chromobacterium violaceum* (CV12472) by Borges et al. (2014). They observed that this polyphenolic compound can alter the quorum sensing mechanism of *C. violaceum* CviI/CviR homologs of LuxI/LuxR systems (Borges et al. 2014).

Caffeic acid displayed antibacterial activity against *S. aureus* and *Escherichia coli* having MIC equal to 1600 μg/mL (Lim et al. 2016). Gallic acid another kid of phenolic acid was found to be quite effective against *Enterococcus faecalis* (Aires et al. 2013), and it also showed good inhibitory activity against the bacteria *Campylobacter* with MIC equal to 125 μg/mL (Sarjit et al. 2015). Gallic acid also showed inhibitory activity against *P. aeruginosa* (MIC equal to 500 μg/mL), *S. aureus* (MIC equal to 1750 μg/mL), *E. coli* (MIC equal to 1500 μg/mL), and *Listeria monocytogenes* (MIC equal to 2000 μg/mL). Ferulic acid was also effective against bacteria *E. coli* and *P. aeruginosa* (MIC equal to 100 μg/mL), *L. monocytogenes* (MIC equal to 1250 μg/mL), and *S. aureus* (MIC equal to 1100 μg/mL). The target of gallic acid and ferulic acid was bacterial cell wall in *E. coli, S. aureus*, and *P. aeruginosa* generating local damage and leading to leakage of cellular material (Borges et al. 2013).

Many respiratory pathogenic bacteria were the target of phenolic compounds like gallic acid and ethyl gallate isolated from wine (Cueva et al. 2012; Friedman

2014; Lima et al. 2019). Several respiratory pathogens like *E. faecalis, S. aureus, Moraxella catarrhalis*, and *P. aeruginosa* are susceptible toward these compounds. *M. catarrhalis* is the most sensitive bacteria toward some non-flavonoid molecules. Also, these phenolic compounds were found to be more potent toward Gram-negative bacteria compared to Gram-positives ones (Cueva et al. 2012). Gallic and caffeic acid inhibited the synthesis of α-hemolysin displaying anti-staphylococcal activity. They worked with different mechanisms as gallic acid targeted the adhesive property of *S. aureus*, and caffeic acid made interference with the structure of cell membrane (Dos Santos et al. 2018; Luís et al. 2014). Some phenolic compounds like cinnamic acid and *p*-coumaric acid displayed anti-adhesive property against pathogenic bacteria *L. monocytogenes.* Other effective inhibitors against bacterial pathogens are ferulic acid and caffeic acid. These studies showed that there is a positive correlation between hydroxylation of the cinnamic acid with antibacterial activity (Pernin et al. 2019; Vasconcelos et al. 2018). Some phenolic compounds isolated from blueberries like chlorogenic acid and caffeic acid were able to inhibit biofilm formation in pathogenic bacteria like *S. epidermidis* and *P. aeruginosa*. Chlorogenic acid displayed the antibacterial activity at the concentration of 4 mg/mL, against *S. epidermidis* and *P. aeruginosa*. Caffeic acid was also able to inhibit biofilm formation in *S. epidermidis*, but it was not effective in inhibition of growth of this bacteria (Zimmer et al. 2014). In one study, it was observed that coffee can inhibit biofilm formation in *S. mutans* and chlorogenic acid; trigonelline and caffeine were the major parts showing highest anti-adhesive activity (Daglia et al. 1998). Antibacterial activities against bacteria causing periodontitis were shown by catechol (MIC 4.9–312.5 μg/mL) and pyrogallol (MIC 2.4–2500 μg/mL) (Shahzad et al. 2015). In one study antimicrobial activity of 22 polyphenolic compounds was observed against several Gram-positive and Gram-negative bacteria and they found pyrogallol-based compounds were more effective compared to catechol or resorcinol (Taguri et al. 2006).

Wild polish mushroom is found to be quite rich in polyphenols such as protocatechuic acid, vanillic acid, syringic acid, 4-hydroxybenzoic acid, caffeic acids, and *p*-coumaric and ferulic acids. These compounds displayed intermediate antibacterial activity with MIC ranging from 156 to 5000 μg/mL against a variety of bacteria showing stronger activity against Gram-positive bacteria (Nowacka et al. 2015). *Galla rhois*, a plant from Korea, is a rich source of methyl gallate that showed antibacterial activity against *Salmonella* with MIC between 3.9 and 125 μg/mL (Choi et al. 2014). It also demonstrated effective antimicrobial activity against *E. coli* and *Shigella flexneri* with MIC of 30 μg/mL (Madikizela et al. 2013). Another rich source of polyphenol is *Potentilla* species which is quite rich in compounds like caffeic acid, ferulic acid, rutin, hyperoside, (+)-catechin, and ellagic acid. *P. fruticose* displayed the highest effect against *P. aeruginosa*, and a fungus *Candida albicans* with MIC ranging from 0.78 to 6.25 mg/mL (Wang et al. 2013a). The synergistic activity of isoquercitrin (10 μg/mL) with gallic acid (10 μg/mL) was quite effective in inhibiting the growth of *S. aureus* as their combined MIC was found to be 10 times less compared to their individual activities (Soberón et al. 2014).

The ethanol extract of *Searsia chirindensis* containing myricetin-3-*O*-arabinopyranoside, myricetin-3-*O*-rhamnoside, methyl gallate, kaempferol-3-*O*-rhamnoside, and quercetin-3-*O*-arabinofuranoside was effective against many bacteria like *E. coli, S. flexneri, Campylobacter jejuni,* and *S. aureus* (Madikizela et al. 2013). The seed kernel of *Mangifera indica* L. was a rich source of phenolic acids like methyl gallate, gallic acid, and pentagalloylglucopyranose. The extracts displayed inhibitory activity against MRSA, and they also show better synergistic activity with penicillin G against MRSA (Jiamboonsri et al. 2011). A compound extracted from *Kaempferia pandurata* Roxb, called Panduratin A (a natural chalcone), displayed inhibiting activity against *S. aureus* with MIC ranging from 0.06 to 2 μg/mL (Cushnie and Lamb 2011; Rukayadi et al. 2009). Aerial part of the plant *Hypericum olympicum* L. contains acylphloroglucinols (Olympicins A–E) which showed antibacterial activity against MRSA and multidrug-resistant *S. aureus* (Shiu et al. 2012). Arzanol is a very effective strong anti-staphylococcal drug that is isolated from *Helichrysum talinum* sp. and is also very potent against different *S. aureus strains* (MIC equal to 1–4 μg/mL) (Taglialatela-Scafati et al. 2013).

Two potent acylphloroglucinols, 1,5-dihydroxy-2-(2′-methylpropionyl)-3-methoxy-6-methylbenzene, and 1,5-dihydroxy-2-(2′-methylbutanoyl)-3-methoxy-6-methylbenzene isolated from *Hypericum beanie* were found to be inhibitory against multidrug-resistant *S. aureus* with MIC between 16 and 32 μg/mL (Shiu and Gibbons 2006). β-Lactamase and penicillin-binding protein 2a (PBP2a) inhibitory activity was shown by epicatechin gallate, catechin gallate, and epigallocatechin gallate as they show antibacterial activity against *S. aureus* and MRSA (Yam et al. 1998; Hu et al. 2002; Sudano Roccaro et al. 2004). *Epigallocatechin gallate* displayed its activity by inhibiting Tet(K) pump and upgrading intracellular concentration of tetracycline and ultimately its activity. These compounds also reduced the MIC of norfloxacin four times against *S. aureus* with NorA (Gibbons et al. 2004). Bromelain is a protein-degrading enzyme and found in large amounts in fresh pineapples applied tenderizing meat. It has been found that that bromelain targets the outer membrane by disintegrating the surface membrane protein in Gram-negative bacteria, causing leakage, swelling, and damage to the cells (Zharfan et al. 2017).

10.5 Synergistic Antimicrobial Activities of Polyphenols and Antibiotics

Beta-lactam antibiotics having a β-lactam nucleus in their chemical structure is one of the most recommended drugs for the treatment of bacterial infections. But regular use of these antibiotics has resulted in bacteria developing resistance to these antibiotics. The drug-resistant bacteria are emerging as a global public health problem. The potential of modulating β-lactam resistance in *S. aureus*

through polyphenolic compounds is quite promising (Shiota et al. 1999; Stapleton et al. 2004a; Hu et al. 2001). Several studies have documented the anti-MRSA activity of EGCG, a type of catechins. Different hypotheses have been proposed regarding synergistic activity between EGCG and β-lactam antibiotics. The target site of EGCG is the same as the target site for antibiotics as both targets the peptidoglycan layer (Zhao et al. 2001; Yam et al. 1998), and has been shown to inhibit penicillinase production (Zhao et al. 2001). According to some report, EGC is more potent than EGCG in modulating β-lactam resistance. The effect is more powerful when used in combination with oxacillin against *Staphylococcus.* The occurrence of two rather three hydroxyl groups on the B ring of catechin is a possible cause of this synergistic effect (Shiota et al. 1999; Stapleton et al. 2004b). The combination therapy of cefotaxime (a β-lactam antibiotic) and EGCG is found to be very effective toward extended-spectrum beta-lactamase (ESBL) *E. coli* as a topical application to skin. ECGC was very effective in reducing tetracycline resistance in staphylococci by preventing tetracycline efflux pump, Tet(K) (Sudano Roccaro et al. 2004).

Another synergistic activity of polyphenols was displayed against amoxicillin-resistant *E. coli* isolates by application of luteolin (80 μg/mL) in combination with amoxicillin (70 μg/mL). After 6 h of treatment the mixture of amoxicillin and luteolin reduced the bacterial cell count to 1×10^3 CFU mL^{-1}. Luteolin can work by reduction of β-lactamase activity, by disrupting protein and peptidoglycan synthesis, and it can alter permeability of outer and inner bacterial cell membranes (Eumkeb et al. 2012a, b). Ceftazidime in combination with flavones, apigenin, and naringenin displayed antimicrobial effects against ceftazidime-resistant *Enterobacter cloacae* in vitro. Apigenin was quite useful in reversing resistance activity by affecting the cell structure and the blockage of β-lactamase activity (Eumkeb and Chukrathok 2013). Other flavanols such as galangin and kaempferide also displayed similar activity and inhibited the hydrolysis of benzylpenicillin (Sudano Roccaro et al. 2004). Chalcones are another class of phenolic compound that show a synergistic effect in mitigating AMR. Chalcones extracted from *Dalea versicolor* inhibited NorA efflux pump and effectively decreased the MIC of erythromycin against *S. aureus* (Belofsky et al. 2004). Holler et al. studied library of 117 natural and synthetic chalcones for their antimicrobial activity. Among them the potency of two chalcones was equivalent to reserpine, an alkaloid with compelling NorA efflux pump-repressive activity (Holler et al. 2012b). Synergistic activity between various green and black tea extracts in combination with several antibiotics was studied toward some enteropathogens by Tiwari et al. who reported better antibacterial activity by green tea as compared to black tea and more effectiveness against enteropathogenic *Shigella dysenteriae.* Tea extract also displayed superior synergistic activity when applied in combination with chloramphenicol (Tiwari et al. 2005).

10.6 Conclusions

The discovery of penicillin as the first antibiotic was an outstanding scientific breakthrough in medicine which was responsible for mankind gaining tremendous advantage and an important tool to treat and control bacterial infections. It also spawned a whole new area of research for development of new and more effective antibiotics creating several new milestones in treating infectious diseases. However, the emergence of life-threatening drug-resistant pathogens has become a very important problem that can impact and impair the global public health in a serious way. The development of new effective antimicrobials with a different mechanism of action against infectious pathogens remains an urgent task. Scientists are looking to plant defense molecules as a new ray of hope and more focus is on the combination of plant extracts and antibiotics for their synergistic activity against such pathogens. Phenol and phenolic acid compounds are the potential plant origin molecules that can provide a novel preventive and therapeutic pathway in combination with antibiotics against such pathogens. Recently, research on bioactive compounds, particularly polyphenols, has gained momentum. Polyphenolic compounds like flavonols and tannins isolated from different plant sources have displayed antimicrobial, antiviral, and antifungal activities. Green tea, berry extracts, mushrooms, curcumin, and propolis are rich source of polyphenolic compounds and found to display potent activity against several bacteria. Pyrogallol-based extracts are showing better results than other polyphenol derivatives such as resorcinol, gallic acid, and ferulic acid and in destabilizing the cell wall of *S. aureus, E. coli, P. aeruginosa,* etc., causing leakage of cellular contents.

Plant polyphenols exert antibacterial activity by targeting the physiology of bacteria by various mechanisms. They can alter bacterial membrane affecting membrane proteins and permeability. They also affect various virulence factors associated with the pathogenicity of microorganisms. They can downregulate bacterial enzymes and toxins and affect the signaling receptor molecules. The other important mechanisms include the inhibition of biofilm. Polyphenols are more potent when used in combination with different antibiotics reducing the phenomenon of AMR.

Several methods are available to evaluate the antimicrobial activity of polyphenols and their derivatives. The assays include disk diffusion and agar dilution method and micro and macro dilution method. The size of the inoculum, the size of the well or proper disk as well as the incubation period are variables, which must be considered in various assays. Although polyphenols are promising targets for their antimicrobial properties and AMR, there are many discrepancies available, mainly in the application of different assays and solvents used. Many *in vitro* susceptibility tests and marks are needed as tentative breakpoints for the most encouraging polyphenolic compounds. More such studies are needed to confirm pathogen as susceptible or resistant to an environmental agent, and the authenticity of the susceptibility method is fundamental and important to confirm the in vitro and in vivo activities. In summary, polyphenols represent possible alternatives as antimicrobial agents that

can be exploited either alone or in combination with antibiotics for treatment and management of infectious diseases. However, much research further is needed in this area to exploit this opportunity, especially, in an era of antibiotic-resistant bacteria which can be considered as a future looming public health crisis.

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Chapter 11
Antimicrobial Peptides and Small Molecules as Antibiotics Substitute

Nidhi Sethi

11.1 Introduction

With the incessant emergence of resistance, there has been an ever-rising demand for antibiotics. However, the development of new antibiotic molecules has reached an impasse, thereby posing an increased threat to public health. This crisis could have been easily averted or at the least be delayed by prudent use of the existing antibiotics, i.e., by limiting the usage of antibiotics only when required and with appropriate dosing for treatment. A large fraction of antibiotics are being administered to livestock these days. Although pharmaceutical giants consider investing in development of new antibiotics a non-profitable enterprise, scientists around the world have been exploring different perspectives toward their endeavor to find alternatives to the current overexploited antibiotic regime. Keeping in mind the urgency to find new antibiotics, it becomes equally important to strategize upon preserving the existing ones (by correct adjuvant selection) for their prolonged use. Numerous substitutes to classic antibiotics treating specific diseases have been identified. These include antimicrobial peptides, bacteriocins, SMAMPs (synthetic mimics of antimicrobial peptides), IDR peptides (innate defense regulatory peptides), antibacterial oligonucleotides foldamers, antibacterial nucleic acids, and immune stimulation by P4 peptide each one offering its own advantages and disadvantages.

Antimicrobial peptides (AMPs) are endogenously produced small molecules accounting for a large group of diverse peptides. AMPs, alternatively known as "host defense peptides", "alarmins", or "defensins" have not only gained significance since their discovery in the latter half of the twentieth century but also have researchers reconsider the way they have been reflecting upon the immune defense mechanisms and physiology of human diseases. These peptides have been isolated

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from a variety of multicellular and unicellular organisms such as animals (vertebrates and invertebrates), plants, fungi, and bacteria. Blood cells (macrophages and neutrophils), epithelial cells, reproductive tract, hemocytes, body fat, etc., have been the warehouses for AMPs. Hundreds of AMPs have been identified, mostly containing amino acid residues ranging between 10 and 50 units. AMPs have shown a broad spectrum of activity against a number of Gram-positive and Gram-negative bacteria, fungi, protozoa, and viruses; also exhibiting cytotoxic effects toward human sperm and tumor cells. Most AMPs are cationic in nature, having an amphipathic structure which facilitates their binding to the membrane interface and likely to be their mechanism of action against various microbes. AMPs have been of therapeutic importance having been indicated for their use as topical antiseptics, anti-inflammatory, oral mucositis, candidiasis, impetigo, chronic respiratory infection, cystic fibrosis, acute bacterial skin infections, and inflammatory bowel disease; also encompassing to overcome the challenge of resistance.

11.2 AMPs from Diverse Natural Origin

Sundry antimicrobial peptides have been identified and isolated from almost all types of organisms such as insects (ants, flies, spiders), plants, animals (including humans), and even bacteria themselves. AMPs are composed of different types and sizes of amino acid residues forming the primary and secondary structure. The unifying factor in these diverse peptides is the N-terminal domain and a cationic C-terminal domain exhibiting antimicrobial activity. Their occurrence in human skin has provided ample revelation on the vitality of AMPs to mammalian defense system and has unlocked a new era for the admiration of these peptides in human health and disease. In this section, several examples of antimicrobial peptides occurring naturally from various sources have been discussed.

11.2.1 AMPs Isolated from Insects

Since the unearthing of AMPs from cecropia silk moth, *Hyalophora cecropia*, a number of peptides have been recognized in various insects. Named as cecropins, these molecules are linear, amphipathic in nature, and exert activity against parasites, bacteria, and fungi. A breakthrough in the discovery of insect AMPs can be attributed to the septic injury in fruit fly, *Drosophila melanogaster*, resulting in an immune response by producing AMPs such as drosomycin, drosocin, cecropins, attacin, diptericin, and metchnikowin possessing antifungal and antibacterial properties (Reddy et al. 2004). Venom of ponerine ant, *Pachycondyla goeldii*, has been found to be rich in 15 types of peptides displaying insecticidal and antibacterial properties. As the name suggests, these peptides are called ponericidins and have properties similar to cecropins and dermaseptins (Peravali et al. 2013). Other

examples of insect AMPs include defensins (from flesh fly, *Sarcophaga peregrina* and from larvae of black-brown fly, *Phormia terranovae*), thanatin (from hemipteran insect, *Podisus maculiventris*), acaloleptins (from the hemolymph of beetle, *Acalolepta luxuriosa*), lycotoxins and cupiennin-1 (from the venom of spiders, *Cupiennius salei* and *Lycosa carolinensis*), etc. (Gabay and Almeida 1993; Kuhn-Nentwig et al. 2002; Reddy et al. 2004; Vetterli et al. 2018). These peptides demonstrate good insecticidal, antimicrobial, and mild hemolytic activity.

11.2.2 AMPs Isolated from Invertebrates

In addition to insects, vertebrates and invertebrates also contribute to a large number of AMPs. For instance, tachyplesins (from immune cells of Japanese horseshoe crab, *Tachypleus tridentatus*) and polyphemusin (from hemocytes of horseshoe crab, *Limulus polyphemus*), both are small in size, cysteine-rich, have intramolecular disulfide bridging patterns and exhibit carboxy-terminal amidation (Edwards et al. 2017; Powers et al. 2004). Other peptide, tachycitin, a 73-amino acid peptide also isolated from *T. Tridentatus*, has shown specific binding affinity for chitin (essential constituent of fungal cell wall); defensin, has also been purified from the hemocytic granules of *T. tridentatus*, displaying activity against both Gram-positive and Gram-negative bacteria; penaeidins isolated from the hemolymph of shrimp, *Penaeus vannamei*, show activity against filamentous fungi only; mytilin and mytimycin obtained from mussel, *Mytilus edulis*, are cysteine-rich with activity against Gram-positive bacteria and filamentous fungi respectively; androctonin, a 25-residue molecule, obtained from scorpion, *Androctonus australis*, forming two intramolecular disulfide linkages is active against fungi, Gram-positive and Gram-negative bacteria (Mandard et al. 1999; Sperstad et al. 2011).

11.2.3 AMPs Isolated from Vertebrates

Vertebrates have also served as abundant sources of AMPs. These include defensins, cathelicidins, bactenecins, histatins, protegrins, etc. α-defensins, are small peptides varying from 29 to 35 amino acids with three intramolecular disulfide bonds. So far, six β-defensins have been identified in humans, four from granulocytes and lymphocytes, also called human neutrophil peptides (HNP 1–4) and two expressed by the paneth cells in intestine (Human defensin, HD-5 and 6). β-defensins contain approximately 45 amino acid residues and their synthesis can be induced by cytokines (Schneider et al. 2005). Cathelicidins (CAP-18 and LL-37) are composed of highly conserved cathelin domain, stored in neutrophils as propeptides and are released upon proteolytic stimulation (Dijksteel et al. 2021; Kang et al. 2017). Bactenecins (Bac-5 and Bac-7) are cathelicidin-derived peptides isolated from bovine cattle, exhibit antimicrobial activity (Kościuczuk et al. 2012). Histatins are a

family of histidine-rich proteins which have been isolated from human saliva and exhibit moderate activity against *Candida albicans* (Reddy et al. 2004). Protegrins are antimicrobial peptides, having 16–18 residues and are isolated from neutrophils of pig (Kosikowska and Lesner 2016).

11.3 AMPs of Synthetic Origin

Advances in the field of organic chemistry, computational chemistry, and pharmacology have streamlined the process of unwinding the amino acid sequence, size, primary, secondary, and 3D (three-dimensional) structure along with finding the mode of action and therapeutic potential of the naturally occurring AMPs and design strategies to synthesize their derivatives. The designing of a synthetic AMP postulated to be used therapeutically must consider length of amino acid chain (minimum 7–8 residue), net charge, helicity, hydrophobicity, amphipathicity, solubility as well as ease of synthesis, involving least steps, producing desired effectiveness against pathogen of interest with high therapeutic index and minimum or no adverse effects as important physicochemical parameters before synthesis (Bahar and Ren 2013; Park and Hahm 2005). Various methodologies have been developed for the synthesis of new antimicrobial peptides of known or foreseen sequences. These include alteration of known AMP sequences or biophysical modeling comprehending peptidic activity, e.g., WR12 is a novel 12-residue peptide composed of arginine and tryptophan has been found to exhibit activity against multidrug-resistant staphylococci, MRSA (methicillin-resistant *Staphylococcus aureus*), linezolid-resistant *S. aureus*, vancomycin-resistant *S. aureus*, and methicillin-resistant *Staphylococcus epidermidis* infections (Mohamed et al. 2016). D-IK8 is another 8-residue peptide having a β-sheet structure and has an activity profile similar to WR12 (Mohamed et al. 2016). MSI-78 or pexiganan is a 22-amino acid derivative synthesized from magainin II and has been found to be more potent than parent molecule magainin II against both Gram-positive and Gram-negative bacteria (Dijksteel et al. 2021). CP-11, a derivative of indolicidin, contains an additional disulfide bond and a trp-trp (tryptophan-tryptophan) cross-linking than indolicin and has higher protease stability without any change in antimicrobial action (Kang et al. 2014). P60.4 is another synthetic AMP, a derivative of LL-37, synthesized by removal of asparagine and glutamine amino acids. Addition of two cationic arginine units into the parent sequence displayed diminished cytotoxic effects on eukaryotic cells but has been successfully used in nasal formulations acting against MRSA (Bahar and Ren 2013). AMP72, AMP126, and AMP2041 are three peptides synthesized from arenicin, protegrin, and thanatin respectively possessing low cytotoxicity compared to the parent AMPs and showed dose-dependent antimicrobial potency against many Gram-negative bacteria (Bahar and Ren 2013). Tet-20 is a synthetic peptide exhibiting broad spectrum of antimicrobial activity and inhibits biofilm formation without any toxic effects (Gao et al. 2011). A synthetic histatin derivative dhvar4 has been investigated against oral flora and it decreased the number of viable

biofilm cells (Ricci et al. 2020). Other examples include MUC17 12-mer-L and MUC17 20-mer (derived from MUC7, a humans saliva AMP) which inhibit biofilm formation against *Streptococcus mutans* (Bahar and Ren 2013); NRC-16 is a pleurocidin-like cationic AMP which inhibits *Pseudomonas aeruginosa* biofilm formation (Gopal et al. 2013); $(RW)_4$-NH_2 (a cationic peptide) inhibits formation of biofilms and kill *Escherichia coli* HM22 persister cells in planktonic cultures (Bahar and Ren 2013).

11.4 Classification of AMPs

Most AMPs are amphipathic in nature having clusters of positively charged and hydrophobic chains of amino acids. Due to the amphipathic nature, AMPs interact with the phospholipids (negative charge) and fatty acids (hydrophobic) embedded in the microbial plasma membranes. These act by forming a pore on the membrane and thus leading to lysis and release of components of the cytosol (Wimley 2010). Isolation and characterization of these peptides through chromatography and NMR (nuclear magnetic resonance) spectroscopy respectively has helped understand their 3D structure and function. Based upon the type of amino acid present, conformational and sequence analysis, these peptides have been divided into several categories as tabulated under Table 11.1 (Reddy et al. 2004).

11.4.1 α-Helical AMPs

These are highly amphipathic in nature, having helical structure with hydrophobic and cationic charged surfaces. Cecropins was the first α-helical AMPs isolated from cecropia moth, *Hyalophora cecropia* and have the propensity to attain helical form in various organic co-solvents. Another group of α-helical AMPs includes magainins. Magainins (I and II) have been isolated from skin glands of African clawed frog, *Xenopus laevis,* are 21–27 residues long. These have been documented to be broad-spectrum antimicrobial agents, potent against a number of pathogenic microorganisms and cancer cells (murine and human cancer cells), exhibiting cidal activity. Magainins are easy to synthesize, selective in action, are less prone to bacterial resistance and hence, have been developed as therapeutic agents (Jacob and Zasloff 1994; Reddy et al. 2004).

11.4.2 Cysteine-Rich AMPs

Defensins were the first cysteine-rich AMPs to be isolated from human granules. These are the human neutrophil peptides, HNP-1, HNP-2, and HNP-3, composed of 30 amino acids rich in cysteine residues and are found in numerous organisms such

Table 11.1 Classification of AMPs based upon structural features (amino acid type, conformation, and sequence)

S.no.	Type	Characteristic features	Example	Activity potential
1.	α-Helical	Highly amphipathic, helix with hydrophobic and cationic charged surfaces	Cecropins	Antimicrobial, anticancer
			Magainins	Antimicrobial, anticancer, wound healing
2.	Cysteine-rich	Rich in cysteine residues, pairs of cysteine residues forming intramolecular disulfide linkages	Defensins (HNP-1, HNP-2, HNP-3)	Antimicrobial, immune stimulation
			Drosomycin	Antimicrobial
3.	β-sheeted	Form a single β-hairpin structure, approximately 20 residues long, one or two disulfide linkages	Tachyplesins	Antimicrobial, antitumor
			Polyphemusin II	Antimicrobial
			Thanatin	Antimicrobial
			Lactoferricin B	Antimicrobial
4.	AMPs rich in regular amino acids	High content of regular amino acids	Histatin	Antimicrobial
			Cathelicidins	Antimicrobial, immunomodulation, wound healing, angiogenesis
			Indolicidins	Antimicrobial, antiprotozoal, hemolytic
			Tritripticin	Antimicrobial, hemolytic
			Bactenecins	Antimicrobial
5.	AMPs with rare modified amino acids	Composed of amino acids such as lanthionine, 3-methyllanthionine, dehydroalanine, dehydrobutyrine, DH-amino acids	Leucocin A	Antimicrobial
			Gramicidins	Antimicrobial

as plants, insects, and mammals. Apart from neutrophils, human defensins are also present in intestinal paneth cells, keratinocytes, genitourinary, pulmonary, and reproductive epithelia. Usually defensins contain six cysteine residues linked to each other through three disulfide bonds between C1-C4, C2-C5, and C3-C6 position. Defensins have a broad spectrum of activity and possess cytotoxic potential against parasites, bacteria, virus, fungi, and host cells. In addition to antimicrobial activity, defensins also tend to increase TNF-α (tumor necrosis factor-α) and IL-1 (interleukin-6) levels, promotes PG-D2 (prostaglandin-D2) production, stimulate histamine release, and exhibit antiviral potency against adenoviruses. Drosomycin, another cysteine-rich AMP, has been isolated from drosophila and possesses four disulfide linkages made up of three β strands which are antiparallel and form an α helix between the first two strands (Koczulla and Bals 2003; Reddy et al. 2004).

11.4.3 β-Sheet AMPs

β-Sheet AMPs contain approximately 20 amino acid residues with one or more disulfide bonds, acquiring a stable single β-hairpin-like structure. Some of the examples include tachyplesins and polyphemusin II found in horseshoe crab, thanatin, lactoferricin B, etc. Both tachyplesins and polyphemusin II form β-sheets which are antiparallel to each other and are connected to a β-turn bridged through two disulfide linkages. Thanatin has been isolated from the spined soldier bug, *Podisus maculiventris* and has an antiparallel β-sheet retained by one disulfide linkage (Andreu and Rivas 1998; Reddy et al. 2004). Lactoferricin B is a lactoferrin (milk protein) derivative possessing 25 amino acid residues adopting a β-sheet structure maintained by a single disulfide linkage. It is also present in tears and saliva. Studies have suggested that it also exerts antiviral effect against HIV (human immunodeficiency virus) and neutralizes endotoxic activity by bacteria in addition to antimicrobial activity (Hwang et al. 1998; Reddy et al. 2004).

11.4.4 AMPs Rich in Regular Amino Acids

Certain AMPs are majorly composed of regular amino acids such as histidine, arginine, and proline in their structure. These peptides orient themselves into α-helical or β-sheet conformations. This class of AMPs includes histatin, cathelicidins (CAP-18, protegrins, prophenins, PR-39, and porcine myeloid AMPs), indolicidins, tritripticin, bactenecins (Bac-5 and Bac-7), etc. (Koczulla and Bals 2003). Histatin has first been isolated from human saliva and is abundant in histidine amino acid residues. It has been documented to be active against *Candida albicans* (Andreu and Rivas 1998). Cathelicidins unlike histatins are rich in proline residues; indolicidins and tritripticin in tryptophan residues while PR-39 in arginine residues. Cathelicidins embody a large family of peptides which are structurally diverse. These have been found in the skin from wound fluid and were found to be components of machinery responsible for wound repair inside our body. Later, these have been isolated from rat, mouse, goat, pig, rabbit, guinea pig, monkey, and sheep as well. Cathelicidins enclose a cathelin protein precursor domain (conserved) and a C-terminal antimicrobial domain (variable). Apart from being potent antimicrobials, cathelicidins have been reported to be efficacious in angiogenesis, wound healing, and certain immune mechanisms in human body (Izadpanah and Gallo 2005; Reddy et al. 2004).

Indolicidin is a tridecapeptide isolated from bovine neutrophils and has been documented to exert antimicrobial activity against a number of bacteria, HIV-1 virus, fungi, and protozoa through membrane permeabilization (Vergis et al. 2019). Tritripticin is a 13 amino acid long AMP member of the cathelicidin family residing in neutrophils. Apart from being a good antimicrobial agent acting against various bacteria and fungi, it also exhibits hemolytic activity (Sharma et al. 2013). Bactenecins (Bac) are a group of small molecule cationic antimicrobial peptides (23

amino acid residues) found in ruminant cattle, containing arginine, cysteine, and hydrophobic residues. These have a folded structure because of the disulfide linkage formation. Bactenecins have been reported to show antimicrobial activity against Gram-negative bacteria (Munshi et al. 2020; Radermacher et al. 1993; Sun et al. 2019; Wu and Hancock 1999).

11.4.5 AMPs with Rare Modified Amino Acids

Certain AMPs are composed of atypical or uncommon amino acids unlike the ones discussed in previous four classes. These are composed of rare modified amino acids such as 3-methyllanthionine, lanthionine, dehydrobutyrine, dehydroalanine, and DH-amino acids. Examples include leucocin A and gramicidins (Reddy et al. 2004). Leucocin A is a non-lanthionine peptide isolated from lactic acid bacterium, *Leuconostoc gelidum* and is composed of 37 amino acid residues. It is a ribosomally synthesized antimicrobial peptide exhibiting activity against Gram-positive bacterial strains from different genus such as *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Listeria*, and *Clostridium* (Etayash et al. 2014). Gramicidins are linear polypeptides composed of 15 alternating D,L-amino acids obtained from *Bacillus brevis* (aerobic soil bacterium). Gramicidins are active against a wide variety of bacterial species. They are composed of DH-amino acids that help them to acquire an unusual cyclic β-hairpin (Killian 1992).

11.5 Structure of AMPs

Due to diversity and complexities in the structure of proteins, there is insufficient data available on the three-dimensional structure of the currently used AMPs. Hence, it becomes cumbersome to generalize a single model deciphering the structure of AMPs. Usually, antimicrobial peptides contain basic amino acids such as arginine and lysine which impart a net positive charge on them and certain hydrophobic amino acids. Three-dimensionally, these peptides are stabilized by disulfide linkages or fold themselves into α-helices or β-sheets of amphiphilic molecules. The amphiphilic arrangement of peptides having a segregated hydrophilic and hydrophobic portions interact electrostatically at the interface where the negatively charged bacterial membrane engages with the positively charged peptides. The hydrophobic groups however, assist in their penetration into the lipophilic section of the bacterial membrane. Structure-activity relationship studies on β-sheet AMPs have shown that the disulfide bridges do not contribute to the antimicrobial activity but are requisite for membrane translocation (Baltzer and Brown 2011; Kang et al. 2017).

11.6 Mechanism of Action for Antimicrobial Peptides

11.6.1 Most Validated Mechanisms of Action for AMPs

Continuous efforts have been made by the researchers around the world to deduce a precise mechanism of action for antimicrobial peptides based upon their size, sequence, 3D structure, cationic nature, and hydrophobicity. Although there is not much clarity on the exact mechanism, a few key features necessary for the antimicrobial activity of these peptides have been figured out. These include the amphipathic conformation of peptides, positive charge due to basic amino acids present, disulfide bonds for membrane translocation, and negatively charged phospholipid head groups on bacterial cell membrane play a vital role in the peptide-membrane interactions. However, there has been complete agreement that the antimicrobial action proceeds through cell membranes disruption. Shai-Matsuzaki-Huang (SMH) model was the first of its kind which could efficiently explain the interactions between the bacterial cell membrane and antimicrobial peptides, elucidating their antimicrobial mechanism (Baltzer and Brown 2011). The model suggests that there occur electrostatic interactions between the anionic heads of the bacterial membrane and the cationic residues of AMPs. Then the AMPs fold into amphiphilic entities which tend to acquire a 3D structure upon interaction with the bacterial membrane. This leads to displacement of lipids, followed by thinning of bacterial membrane outer leaflet, peptide aggregation, and channel formation which facilitate peptide transport from surface to the interior of the target cell. However, scientists have not reached a consensus on the steps involved in pore formation and translocation of the peptides. Because of the diversity in the structure and sequencing of AMPs, no single model is adequate to fit all peptides. So far, four plausible mechanisms mediating through membrane permeabilization and explaining the method of channel formation have been proposed (Fig. 11.1). In the barrel-stave mechanism, there is a voltage-gated transmembrane channel formation wherein the non-polar elements of the peptide are at interface with the bacterial membrane lipids; thereby leading to formation of hydrophilic pore/channel traversing through the membrane. Like barrel stave model, the peptide in toroidal model has a transmembrane topology followed by formation of toroidal pores driven by electrostatic interactions. These pores are covered by head groups of the membrane phospholipids leading to changes in membrane curvature. In the sinking-raft model, AMPs rest on the membrane surface and initiate increase in membrane curvature. The peptides self-aggregate on the membrane, leading to sinking of AMPs into it and creating transient pores. As the name suggests, the last mechanism involves formation of a carpet of AMPs around the target cell membrane, thereby affecting the membrane integration. The lipid bilayer gets bent backward, leading to formation of micelles and final collapse of the membrane (Dijksteel et al. 2021; Mahlapuu et al. 2016). Operating through these mechanisms, AMPs successfully tamper the membrane permeability, transport of ions and metabolites and loss of membrane potential, hence, facilitating loss of membrane function. It has also been reported that the slight differences in the

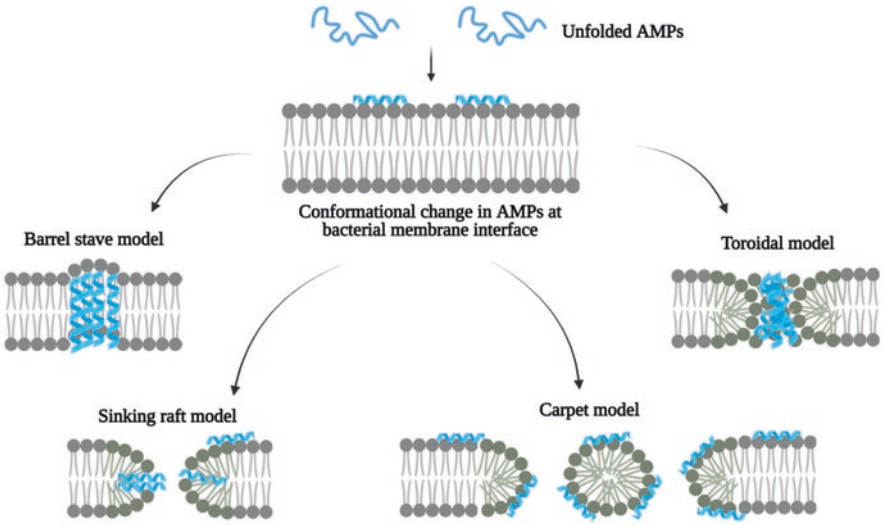

Fig. 11.1 Mechanism of action for antimicrobial peptides (AMPs) mediated through barrel stave model, toroidal model, sinking raft model, and carpet model

composition of the microbial cell membranes and the structure and sequence of the individual peptide are responsible for the selectivity of AMPs (Andreu and Rivas 1998; Dijksteel et al. 2021; Kang et al. 2014).

11.6.2 Other Reported Mechanisms for Antimicrobial Peptides

There is sizeable amount of data showing that antimicrobial peptides act not just through membrane permeabilization but there are various other mechanisms responsible for the antimicrobial activity. For instance, their interference to host protein production (e.g., PR-39); inhibition of specific membrane protein synthesis (e.g., attacins or gloverin); inhibition of protein and RNA synthesis (e.g., Bac-5 and Bac-7); interference with DNA without inhibiting its synthesis (buforins); breakdown of single strand of DNA (defensins); halting of DNA synthesis (PR-39), and production of hydrogen peroxide or synthesis of stress proteins (Andreu and Rivas 1998).

11.7 Multi-functional Roles of AMPs

With the aim of improving the selectivity and efficacy, a lot of researchers in recent years have devoted their resources in the development of new antimicrobial peptides and exploring the multifaceted roles of the existing ones. In addition to antimicrobial potential, AMPs are involved in a myriad of other functions such as histamine

release, induction of syndecan synthesis, inhibition of steroid synthesis, inflammatory mediators having impact on various cellular processes like wound repair, cytokine release, cell proliferation, chemotaxis, immune induction, and protease-antiprotease balance. The currently known AMPs have been found to exhibit broad range of antimicrobial activity against all types of pathogenic microorganisms, e.g., human lactoferrin B on oral administration, effectively help reduce *E. coli* caused inflammation and infection in the urinary tract; cecropins and indolicidin have been found to be active against *P. aeruginosa* and have been documented to be co-administered with antibiotics like clarithromycin and polymyxin E for clinical use; ranalexin and buforins-1 have greater efficacy against methicillin-resistant strains of *S. epidermidis* than rifampicin; cathelicidins display potential against oral *Fusobacterium* species and *Candida* species; defensins and protegrins help reduce colonization of *Mycobacterium tuberculosis*; cecropins have shown to exert fungicidal action against fungi like *Aspergillus* species and *Fusarium* species and dermaseptins show activity against *Cryptococcus neoformans* fungi (Andreu and Rivas 1998; Koo and Seo 2019). Some of the wider applications of AMPs have been discussed in this section.

11.7.1 Wound Repair

Among numerous applications of AMPs, healing of wound is one of the earliest known mechanisms contributing to their discovery in mammalian skin. Studies suggest that AMPs have been found to be expressed in a variety of skin disorders. For example, cathelicidin (PR-39) stimulates fibroblast present in dermis which promotes the synthesis of proteoglycans syndecan-1 and syndecan-4. Syndecans are a small family of transmembrane domain proteins acting as co-receptors and interacting with a variety of ligands. These are composed of a proteinaceous core and 3–5 glycosaminoglycan side chains essential for action of numerous growth factors. Similarly, cathelicidin (LL-37) is also involved in wound repair mediated through keratinocyte proliferation and angiogenesis. Mechanistically, the process of wound repair by AMPs involves killing of bacteria and stimulation of host repair phenomena. AMPs tend to curb the proliferation of pathogens in the skin. This can be proven by inactivation of cathelicidins in pig lesions or by deletion of cathelicidin gene in mice both leading to increased and long-lasting bacterial growth (Izadpanah and Gallo 2005).

11.7.2 Skin Infections

Granulysin, a T-cell AMP, has been found to be significantly mediating the immune response (through association with macrophages) against leprosy pathogen, *Mycobacterium leprae* as it is evident from the analysis of tissue samples of leprotic patients. It has also been reported that they have six times higher expression in the skin (predominantly in CD-4*1*T cells) of patients suffering from tuberculoid leprosy

than those with lepromatous type. The expression of granulysin in leprosy is further confirmed by the non-involvement of perforin (helps pore formation), proving it to be an effective indicator for cell-mediated immunity. AMPs have also been documented to play a vital role in another skin condition such as atopic dermatitis (AD) or eczema. AD is generally associated with periodic reoccurrence of skin infections. The patient is prone to attack by all types of pathogenic organisms such as bacteria, virus, and fungi. Evidences have suggested that production of AMP (LL-37 and HBD-2 in particular) was reduced during inflammation. However, on comparison between AD and psoriasis, the production of AMPs (cathelicidin and HBD-2) is decreased in AD while in case of psoriasis, AMPs have elevated levels during inflammation. This indicates that although both AD and psoriasis involve inflammation of skin and it is expected to have greater levels of AMPs, these behave distinctively in different conditions. The decrease in levels could be attributed to the elevated levels of Th-2 cytokines which suppress the expression of AMPs. Furthermore, AMPs have shown very good antimicrobial potential against *S. aureus*, herpes simplex virus (HSV), and vaccinia virus (VV), all three implicated to be affecting AD patients, which is evident from inflammation of keratinocytes. Thus, explaining the lack or deficiency of AMPs being associated with the susceptibility of AD patients to pathogens (Izadpanah and Gallo 2005).

11.7.3 Antiviral Activity

Evidences suggest that AMPs have protective effects against certain viral strains. For instance, cathelicidins induction is observed during human papilloma virus (HPV) infections such as verruca vulgaris and condyloma acuminatum development. Cathelicidins are also reported to have been expressed during the cutaneous proliferation of vaccinia virus (VV) infection. However, there is no indication that cathelicidins prevent the spread of virus or are involved in the process of resistance. Hence, AMPs can further be explored for their exploitation in skin infections involving viral intervention (Andreu and Rivas 1998; Izadpanah and Gallo 2005).

11.7.4 Antitumoral Activity

Several studies have shown that tumor cells unlike non-specialized cells offer greater liability toward antibiotic peptides. For instance, cecropins and magainins have displayed activity toward various human cancer cell lines (Andreu and Rivas 1998).

11.7.5 AMPs Against Sexually Transmitted Infections

Investigations on AMPs have shown them to be active against sexually transmitted pathogens. For example, defensins prevent entry and spread of syphilis bacterium *Treponema pallidum*, HIV, HSV-2 (herpes simplex virus type-2) viruses; protegrins act against *Neisseria gonorrhoeae, Chlamydia trachomatis*; cathelicidins have also shown activity against syphilis bacterium; magainins exhibit inhibitory effect against a number of pathogens responsible for sexually transmitted infections (Andreu and Rivas 1998).

11.7.6 Anti-malarial Activity

Dermaseptin-derived peptides have been found to show activity against malaria parasite *Plasmodium falciparum* in human erythrocytes, suggesting the likelihood of their use in intracellular infections. Possibly, these act by lysis of infected host cell, thereby destroying the intraerythrocytic malarial parasites. Aminoheptanoyl and isobutyryl dermaseptin derivatives have been documented to display better selectivity toward the parasite than the host cell (with reduced hemolysis). Plant antimicrobial peptides thionins have also been found to exhibit in vitro activity against *Leishmania donovani* (Mirski et al. 2018).

11.7.7 Spermicidal Agents

Among the broader spectrum of magainins' activity, they have been documented to display effects on spermatozoids by bringing morphological and functional changes in them. Studies show that dermaseptins affect motility of sperm in a time and dose-dependent manner by absolute stoppage of motility. Thus, there is sufficient evidence suggesting that AMPs can be potential candidates for use as contraceptive agents (Andreu and Rivas 1998; Zairi et al. 2009).

11.7.8 Buco-Dental Infections

A few examples of AMPs active against buccal cavity infections have been discussed. Histatins present in human saliva act as natural defenses for oral cavity with cidal action against *Candida* species and *Bacteroides gingivalis*. Similarly, human defensins are active against a number of oral Gram-negative bacteria and protegrins kill bacteria such as *Actinobacillus actinomycetemcomitans* and *Eikenella corrodens* (Andreu and Rivas 1998).

11.7.9 Ocular Infections

Antimicrobial peptides such as magainins, defensins, cecropin, and its derivatives have been proposed for their use in preserving contact lens disinfectants, cornea storage medium, and ocular antiseptics. Cecropin A has been found to be as efficacious as gentamycin toward *P. aeruginosa* infection clearance (Andreu and Rivas 1998).

11.7.10 Kostmann's Syndrome

Kostmann's syndrome is an autosomal recessive disorder characterized by a severe congenital neutropenia. Children born with this condition are deficient in neutrophils (a type of white blood cells helping in fighting infections) and are always vulnerable to recurrent infections and periodontal disease. Investigation into the neutrophils of the affected patients has revealed that there was a decrease in α-defensin levels and miniscule amount of cathelicidins with a normal oxidative burst; thereby implicating the role of functional AMPs in Kostmann's syndrome (Izadpanah and Gallo 2005).

11.7.11 Food Preservatives

In today's modern world, when packaged food has reached every nook and corner of our households in one form or another, retention of the freshness, taste, and shelf life of food product gains utmost significance. Food industry uses a number of natural and synthetic chemicals for preservation. The pre-requisite for any chemical to be used as preservative is its safety and stability profiling. The current regime of preservatives faces the biggest challenge of being vulnerable to microorganisms. Therefore, there has always been a never-ending quest to synthesize or isolate newer preservatives which are not susceptible to microbial attack. A number of AMPs have been recognized which have shown potential for their use in food industry and preventing food spoilage. Presently, pediocin has been approved by USFDA (United States Food and Drug Administration) for its commercial use. Certain AMPs have been tested and have displayed good activity at varying pH and temperature with no adverse effects. Hence, AMPs can be designated as promising candidates for their use as additives/food preservatives and development of newer peptides with enhanced activity and safety (Mor 2000; Piper et al. 1998; van Kan et al. 1998).

11.7.12 Miscellaneous Uses

Exploiting the AMPs to be fighting against an array of microorganisms, these peptides can also be used to prevent contamination/infections in therapeutic medical equipments such as catheters and orthopedic devices. Literature reports have shown that some cationic antimicrobial peptides tend to bind to bacterial lipopolysaccharides (endotoxins) when bound to a polymer/solid support resin and help the removal of these endotoxins (Mor 2000).

11.8 AMPs as Targets for Therapy

As discussed in the previous section, although numerous peptides, natural or synthetic have been identified to be efficacious against all types of pathogenic microbes, there has always been a constant quest to explore the existing ones for their potential and find or synthesize new derivatives which are small in size, easy to synthesize, have broad range of activity, fast acting, lesser adverse effects, lesser risk of developing resistance and having wider applications (Bahar and Ren 2013). Studies suggest that AMPs show promising results in vitro, indicative of their in vivo efficiency. Prior for use of any substance or chemical compound in therapy, it should be capable of overcoming the barriers of stability, bioavailability, route of administration, enzymatic breakdown, undesirable toxicity, and lower production cost. Advances in spectroscopic techniques have helped deduction of secondary peptide structure, thereby making it easier to understand their mode of action. Few AMPs and their synthetic analogues have been approved by USFDA for their commercial use. These include smaller peptides like pediocin, magainins, and its derivatives (in wound healing), gramicidin S and polymyxin B (topical application), defensins (synergistic effect), and many others (Dijksteel et al. 2021). These peptides are not only potent when used alone but also show synergy in combination with other antibiotics. Since their discovery, although a number of peptides have been successfully developed, a substantial amount of effort is still required to explore their therapeutic potential.

11.9 Challenges Faced by Antimicrobial Peptides

Since the discovery of first antibiotic, Penicillin by Alexander Fleming, antibiotic resistance has emerged to be a serious hazard for antibiotics in therapy. However, this has left researchers and pharmaceutical industry a little disenchanted toward antibiotics but the emphasis still lies on the development of antibiotics capable of enduring resistance. Several mechanisms unraveling antibiotic resistance in microbes have evolved. Interactions between bacterial membrane and peptides play

key role in the process of resistance. Studies have shown that changes in bacterial membranes are responsible for resistance. These modifications include reduction in negative charge at the interface of interaction, thereby preventing AMP entry into cell mediated through inhibition of peptide aggregation. One such example is the bacterium *S. aureus* which operates through reduction of anionic charge on the bacterial membrane, leading to the repulsion and development of resistance. Another mechanism contributing to resistance is the modification of lipopolysaccharide composition through integration of fatty acids, phosphoethanolamine, and aminoarabinose followed by reduced membrane permeability in bacterium, *Salmonella enterica* (Fjell et al. 2012). In addition to these protective modifications, bacteria can also develop resistance through alteration in their membrane fluidity and hydrophobicity. Other mechanisms to resistance can be membrane-bound efflux pumps (in case of *Neisseria gonorrhoeae*) which expel AMPs from the cell and attack by proteases in the outer membrane with subsequent cleavage (in case of *S. enterica*; Baltzer and Brown 2011).

Similar to conventional antibiotics, AMPs also have a tendency to develop resistance among bacteria when used therapeutically. The frequency of exposure of a therapeutic peptide decides its progression toward resistance. Bacteria use their capability to alter cell membrane and develop resistance. Several lineages of bacteria such as *Pseudomonas fluorescens* and *E. coli* have evolved the mechanisms of resistance (Baltzer and Brown 2011). It has been hypothesized that the diverse existence of quite a number of AMPs in almost all types of organisms can be an indication that these may have co-evolved with bacteria as a response to their attack, thereby explaining their high specificity. Hence, limited exposure or judicious use of these peptides can be an effective way of combating acquired resistance.

Cross-resistance is a consequence of bacteria developing/evolving a mechanism that is efficient not just against one type of antibiotic but also maintains effectiveness against a variety of other antimicrobial molecules. Similar to currently used antibiotics, cross-resistance raises grave concern over the therapeutic or clinical use of AMPs as an alternative to the present ones. Cross-resistance poses a serious threat to these peptides as they are vital part of the innate defense system of plants and animals. Certain studies have displayed emergence of cross-resistance to both human and non-human AMPs but at very low levels. Hence, one should not over speculate about the concept of cross-resistance in AMPs, rather more research in this area can be enlightening (Baltzer and Brown 2011).

11.10 AMPs and Analogues Under Clinical Trials and in Clinical Use

Several approaches have been employed to assess AMPs and their derivatives for their clinical expediency. A few have already entered various phases of clinical trials and some of these have been successfully approved for their therapeutic usage. For

instance, Pexiganan (magainin derivative) is in phase III clinical trials for treatment of mild infections associated with diabetic foot ulcers (Koo and Seo 2019); PAC-113 (histatin-5 derivative) is a human salivary peptide, undergoing phase II trials for treatment of oral candidiasis in immunocompromised HIV-infected patients (Cheng et al. 2018); hLF1–11 (human lactoferrin derivative) is undergoing phase I/II testing for treatment of infections in hematopoietic stem cell transplantation (HSCT) recipients (Costa et al. 2019); iseganan (protegrin-1 derivative) is undergoing phase III clinical trial for use in ventilator-associated pneumonia caused by *Klebsiella pneumoniae* and *P. aeruginosa* infections (Dijksteel et al. 2021); rBPI-21 (human neutrophil peptide, bactericidal permeability increasing protein, BPI derivative) is undergoing phase III trials to treat Crohn's disease and severe pediatric meningococcaemia (Dijksteel et al. 2021); omiganan (indolicidin derivative) is undergoing phase III clinical testing for the treatment of topical skin antisepsis and rosacea and phase II trial for vulvar intraepithelial neoplasia, AD and acne (Koo and Seo 2019); LL-37 (human cathelicidin) is presently in the phase IIb trial conducted for the treatment of chronic leg ulcers (Koo and Seo 2019); OP-145 (LL-37 derivative) is currently undergoing phase II clinical trial to treat chronic middle ear infection (Koo and Seo 2019); LTX-109 (synthetic tripeptide) is a phase I/II clinical trial for treatment of MRSA impetigo (Dijksteel et al. 2021).

At present, a great deal work is ongoing to improve AMP development and production (natural and synthetic), aiming to be cost-effective with therapeutical potential against all types of bacterial or viral infections. As of now, the application of AMPs has been limited to the treatment of local infections but scientists are hopeful of achieving promising results with wider therapeutic application from some of the peptides. Some of these peptides under investigation and in different stages of clinical trials have been discussed (Table 11.2) while those approved for clinical use have been enlisted in Table 11.3.

11.11 Other Small Molecule Alternatives to Conventional Antibiotics

The current state of affairs for antibiotics provides overwhelming evidence toward their diminishing effectiveness rendered by bacterial resistance. Therefore, the focus has to be shifted toward devising alternative therapies to combat pathogenic attack and use them clinically. With advances in synthetic organic chemistry, biotechnology, and genetic engineering, scholars have been successful in finding new areas that can be used in substitution to antibiotics. These promising strategies include use of small molecules such as antimicrobial peptides, bacteriocins, SMAMPs, IDR peptides, antibacterial oligonucleotides, foldamers, antibacterial nucleic acids, and immune stimulation by P4 peptide (Table 11.4). As already discussed, broad spectrum, small molecule antimicrobial peptides have drawn significant attention. A few of them are in clinical use and many in the clinical trial

Table 11.2 AMPs and their derivatives under investigation and in clinical trials (Costa et al. 2019; Dijksteel et al. 2021; Koo and Seo 2019)

AMP	Source	Postulated clinical use	Trial phase	Company/collaborator
Pexiganan	Magainin analogue	Diabetic foot ulcers infection	III	Dipexium Pharmaceuticals
PAC-113	Histatin analogue	Oral candidiasis in HIV-seropositive patients	II	Pacgen Biopharmaceuticals
Lactoferrin	Histatin analogue	HIV infection and nosocomial infection in critically ill patients	II	Jason Baker/Ventri Bioscience/ Minneapolis Medical Research Foundation Queen's University
Bovine lactoferrin	Histatin analogue	Neonatal sepsis Necrotizing enterocolitis	III III	Aga Khan University/ University of Sydney/ United States Agency for International Development
hLF1-11	Human lactoferrin derivative	Complications among HSCT recipient infections Bacteremia caused due to *Staphylococcus epidermidis*	I/II I/II (failed)	AM-Pharma
Talactoferrin	Human lactoferrin derivative	Oral solution for nosocomial infection in preterm infants	I/II	Agennix/NIH
PXL01	Lactoferrin analogue	Postsurgical adhesions	II	ProMore Pharma
Iseganan	Protegrin-1 derivative	Oral mucositis (patients undergoing radiation therapy for cancer of head and neck) and ventilator-associated pneumonia	III (failed)	IntraBiotics Pharmaceuticals
POL7080 (murepavadin)	Iseganan derivative	Ventilator-associated pneumonia and non-cystic fibrosis-associated bronchiectasis	II	Polyphor, Lda
Neuprex (rBPI-21)	Human neutrophil peptide, BPI derivative	Crohn's disease and severe pediatric meningococcemia	III	Xoma Ltd.

(continued)

Table 11.2 (continued)

AMP	Source	Postulated clinical use	Trial phase	Company/collaborator
Omiganan (MBI 226 or MX 226)	Indolicidin derivative	Rosacea Severe inflammatory acne vulgaris Central venous catheter infection	III II III	Cutanea Life Sciences Cutanea Life Sciences Mallinacckrodt
LL-37	hCAP18 derivative	Hard to heal venous leg ulcers	II	Lipopeptide AB
OP-145	LL-37 derivative	Chronic suppurative otitis media	II	OctoPlus BV
AP-214	α-MSH derivative	Post-surgical organ failure	II	Action Pharma A/S
SGX942 (Dusquetide)	IDR-1 analogue	Oral mucositis	III	Soligenix
Delmitide (RDP58)	HLA derivative	Inflammatory bowel disease	II	Genzyme
LTX-109	Synthetic tripeptide	MRSA/impetigo	I/II	Lytix Biopharma
EA-230	Oligopeptide	Renal failure and sepsis protection	II	Exponential Biotherapies
Surotomycin (CB-315)	Cyclic lipopeptide	*Clostridium difficile* infections	III	Cubist Pharmaceuticals
NVB-302	Lantibiotic	*Clostridium difficile* infections	III	Novacta
Ramoplanin (NTI-851)	Glycolipodepsipeptide	*Clostridium difficile* infections, VRE	III	Nano-therapeutics
LFF571	Semisynthetic thiopeptide	*Clostridium difficile* infections	II	Novartis Pharmaceuticals Co.
Friulimicin	Cyclic lipopeptide	MRSA/pneumonia	I	MerLion Pharmaceuticals
DPK-060	Kininogen derivative	Acute external otitis	II	ProMore Pharma
XOMA-629 (XMP-629)	Derivative of BPI	Impetigo/acne rosacea	III	Xoma Ltd.
PMX-30063 (Brilacidin)	Defensin mimetic	Acute bacterial skin infections	II	PolyMedix
XF-73 (Exeporfinium chloride)	Porphyrin derivative	*Staphylococcal* infection	II	Destiny Pharma Ltd.
CZEN-002	α-MSH derivative	Antifungal activity	II	Zengen
Ghrelin	Endogenous peptide	Chronic respiratory infection	II	University of Miyazaki; Papworth Hospital
Wap-8294A2 (Lotilibcin)	Produced by *Lysobacter* sp.	Gram-positive bacteria	I/II	aRigen
Novexatin (NP213)	Cyclic cationic peptide	Fungal nail infection	II	Novabiotics

(continued)

Table 11.2 (continued)

AMP	Source	Postulated clinical use	Trial phase	Company/collaborator
p2TA (AB103)	Synthetic peptide	Necrotic tissue infection	III	Atox Bio Ltd.
C16G2	Synthetic peptide	*Streptococcus* mutans	II	C3 Jian Inc.
D2A21	Synthetic peptide	Burn wound infections	III	Demegen
GSK1322322	Synthetic hydrazide	Bacterial skin infections	II	GlaxoSmithKline
PL-5	Synthetic peptide	Skin infections	I	China Food and Drug Administration (CFDA)

HIV human immunodeficiency virus, *HSCT* hematopoietic stem cell transplantation, *MSH* melanocyte stimulating hormone, *IDR* innate defense regulator, *HLA* human leukocyte antigen, *MRSA* methicillin-resistant *Staphylococcus aureus*, *VRE* vancomycin-resistant *Enterococci*, *BPI* bactericidal permeability increasing protein

pipeline. Several other approaches are also being applied to find appropriate antibiotic.

11.11.1 Bacteriocins

The first among these are bacteriocins, a subcategory of AMPs offering advantage over AMPs by being non-toxic to mammalian cells and selective against particular bacterial strains (narrowing the spectrum for bacteriocins). Bacteriocins are diverse, ribosomally synthesized small peptides produced by bacteria for their survival (kill other bacterial population), active against multidrug-resistant pathogenic microbes. They have mechanism similar to AMPs, acting through membrane pore formation. Another mechanism of action for bacteriocins involves inhibition of peptidoglycan biosynthesis, e.g., Nisin is a lantibiotic produced by Gram-positive bacteria, *Lactococcus lactis*, containing rare amino acids such as lanthionine, 3-methyllanthionine, dehydrobutyrine, and dehydroalanine. It is active against Gram-positive bacteria and acquires β-turn conformation on association to dodecylphosphocholine (Koczulla and Bals 2003). Nisin has been approved for commercial use in food industry and acts by inhibiting peptidoglycan biosynthesis. Lactococcin A is another bacteriocin, acting through pore formation in the membrane; thiopeptides and bottromycin target translation; Microcin B17 (MccB17), Microcin J25 (MccJ25), and Microcin C7-C51 (MccC7-C51) bacteriocins acting by traversing through the membrane of Gram-negative bacteria, thereby affecting DNA, RNA, and proteins metabolism; thuricin is selective toward *Clostridium difficile* and does not affect commensal bacteria. An added advantage to bacteriocins is slow development of resistance. For instance, nisin has been a perfect case of

Table 11.3 FDA-approved AMPs in clinical use (Costa et al. 2019; Usmani et al. 2017)

AMP	Characteristic features	Clinical use	Route of administration
Bacitracin	Mixture of cyclic peptides obtained from *Bacillus subtilis*; the side chain thiol of Cys residue undergoes intramolecular condensation with the foregoing peptide bond, resulting in thiazolidine ring formation	Skin and eye infections; use restricted to infants with *S. aureus* pneumonia and purulent pleuritis	Topical application Intramuscular
Polymyxin B	Mixture of cyclic lipopeptides obtained from *Bacillus polymyxa*	Last line treatment for MDR Gram-negative bacterial infections	Parenteral administration (intramuscular, intravenous, intrathecal, ophthalmic)
Tyrothricin	Mixture of cyclic polypeptides obtained from *Bacillus brevis*	Local treatment; skin and oropharyngeal mucous membrane infections; active against Gram-positive bacteria	Topical application only; parenterally (highly toxic)
Polymyxin E (colistins)	Mixture of cyclic lipopeptides from *Bacillus colistinus*	GIT infections caused by *E. coli* and *Salmonella* spp.; MDR gram-negative bacteria such as *P. aeruginosa, K. pneumoniae*, *Acinetobacter* spp. and not responding to other antibiotics	Parenteral administration (intramuscular, intravenous)
Gramicidin D or gramicidin	Mixture of linear pentadecapeptides obtained from *Bacillus brevis* with formylated N-terminal residue and peptide bond with ethanolamine at C-terminal tryptophan residue	Skin lesions, surface wounds, and eye infections	External use only
Gramicidin S	Cyclic peptide biosynthesized from gramicidin in *Bacillus brevis*; contain two identical pentapeptides coupled head to tail	Effective against gram-negative and positive bacteria and fungi; treat genital ulcers caused by STD	Topical application only
Daptomycin	Cyclic lipopeptide obtained from *S. roseosporus*, acylated with decanoic acid at N-terminal and contains unusual residue kynurenine at C-terminal	Complex skin infections caused by susceptible strains of Gram-positive bacteria	Intravenous injection

S. aureus: Staphylococcus aureus, *GIT:* gastrointestinal, *E. coli: Escherichia coli*, *MDR:* multidrug-resistant, *P. aeruginosa: Pseudomonas aeruginosa*, *K. pneumoniae: Klebsiella pneumoniae*, *STD:* sexually transmitted disease, *S. roseosporus: Streptomyces roseosporus*

antimicrobial remaining unaffected by resistance after decades of use (Allen et al. 2014; Ghosh et al. 2019). Bacteriocins have also been reported to be resilient toward extreme environmental conditions like temperature and UV radiations (van der Helm 2014). These advantages of bacteriocins establish them as potential alternatives to classical antibiotics.

11.11.2 Innate Defense Regulatory (IDR) Peptides

It is well known fact that AMPs modulate the innate immune system for defense against pathogenic bacteria. Certain peptides lacking in direct antibacterial activity but exhibit immunomodulatory and antiendotoxin activities against pathogens are called "innate defense regulatory" (IDR) peptides. Investigations have shown that these peptides do not have any direct antimicrobial activity in mice but activate cellular machinery and suppress harmful inflammation and protect from succumbing to severe bacterial and malarial infections. SGX 942 is one of such peptides undergoing phase I clinical trials, indicated in the treatment of bacterial infection (Ghosh et al. 2019). IDR peptides can hence be projected as promising candidates to be used over conventional antibiotics.

11.11.3 Synthetically Designed Strategies Synthetic Mimics of Antimicrobial Peptides (SMAMPs)

An attempt has been made to simplify the complexities in the structure of AMPs and preserve the ensemble of pharmacophoric elements in their chemical structure and retain the biological properties of AMPs. This has led to the design of polymeric mimics of AMPs, novel biomimetic/peptidomimetic oligomers, SMAMPs, or small molecules which mimic the essential properties of antimicrobial peptides. The objective is to develop approaches that can trounce over toxicity, enzymatic degradation (protease lability), and high cost of manufacture of AMPs. Alternative molecular designing and synthetic strategies have helped develop small molecules mimicking the properties of AMPs. In this approach, facial amphiphilicity is integrated into small molecules by using H-bonding motifs. For instance, Brilacidin is a defensin mimetic undergoing phase II clinical trials for use in acute bacterial skin infections. In this global COVID-19 pandemic, brilacidin like many other compounds has also been evaluated for its antiviral properties against SARS-CoV-2 and it has been found that brilacidin strongly decreases the viral load when the virus is pre-incubated with brilacidin. It demonstrates early viral inhibition in vitro against Washington and Italian strain (Bakovic et al. 2021). LTX-109 is another synthetic tripeptide, currently in phase I/II clinical trial for treatment of MRSA impetigo (Dijksteel et al. 2021). Ceragenins are cholic acid derivatives possessing

Table 11.4 Small molecule alternatives to conventional antibiotics (Czaplewski et al. 2016; Ghosh et al. 2019; van der Helm 2014)

Strategy	Characteristics	Advantages over conventional antibiotics	Possible disadvantages
Antimicrobial peptides	Endogenously produced large group of diverse peptides made up of 10–50 units Proceed through cell membranes disruption	Resilient to resistance development Broad-spectrum activity	Expensive large-scale production Susceptible to proteolytic degradation Toxicity
Bacteriocins	Ribosomally synthesized Selective to bacterial strains Mechanism similar to AMPs	Specificity toward infective bacteria Resistant to heat and UV	Expensive large-scale production Susceptible to proteolytic degradation
IDR peptides	Act through immunomodulation and antiendotoxin activity	No resistance development as no direct antimicrobial activity	Expensive large-scale production Susceptible to proteolytic degradation
SMAMPs	Small molecules mimicking essential properties of antimicrobial peptides	Ease of synthesis Good tissue distribution Resilient to resistance development Broad-spectrum activity	Toxicity Route of administration
Antibacterial oligonucleotides	Oligomeric backbone modified with morpholino or phosphorodiamidate moieties Utilize antisense technology Work by inhibiting expression of resistance causing genes	No resistance development as no direct antimicrobial activity	Relatively new approach, require extensive research
Foldamer	Short sequence-specific oligomers based on arylamide and β-amino acid backbones Fold into well-defined secondary structures	Structural simplicity and relative ease of synthesis Less toxicity Low cost of manufacture	Relatively new approach, require extensive research
Antibacterial nucleic acids	Utilize nucleic acids to kill bacteria directly	Ease of synthesis Resilient to resistance development	Expensive method

(continued)

Table 11.4 (continued)

Strategy	Characteristics	Advantages over conventional antibiotics	Possible disadvantages
Immune stimulation by P4 peptide	28 amino acid residue synthetic peptide Enhance phagocytic killing of bacteria	No resistance development as no direct antimicrobial activity	Relatively new approach, require extensive research

AMPs antimicrobial peptides, *IDR* innate defense regulatory peptides, *SMAMPS* synthetic mimics of antimicrobial peptides

antimicrobial properties which are in preclinical stage of investigation. Binaphthyl-based dicationic peptides and xanthone derivatives have been synthesized by addition of amino acids or short peptides to the hydrophobic groups and show significant antimicrobial activity (Ghosh et al. 2019). Ghosh et al. have synthesized several aryl-alkyl-lysine compounds which have shown bactericidal properties, activity against fungi, malaria parasite, and Ebola virus. Synthetic simple lipidated and biphenyl-based lysines have shown potent activity against intracellular bacteria in vivo. A library of norspermidine-based compounds have been synthesized, documented to exhibit potential against Gram-positive and Gram-negative bacteria (Ghosh et al. 2019). Further development and extensive clinical testing of these small molecules can pave way for their therapeutic use as alternative antibiotics.

11.11.4 Antibacterial Oligonucleotides

Antisense technology has proven to be a useful tool in tackling the problem of antimicrobial resistance. A number of oligonucleotides and their analogues have been generated by this technique which involves inhibition of the expression of mutant genes or in this case the resistance-causing genes. It therefore becomes important to identify and select the undesirable gene before silencing it. Diverse antisense oligonucleotides are hybridized with mRNA, thereby inhibiting the expression of genes responsible for resistance, mediating through different mechanisms (Jani et al. 2021). The oligomeric backbone is first modified with morpholino and phosphorodiamidate groups also known as phosphorodiamidate morpholino oligomers (PMOs) to prevent resistance by nuclease enzymatic degradation and then conjugated with cell-penetrating peptides (PPMOs) to facilitate transport of oligomers across the bacterial membrane. The PPMOs have displayed in vitro and in vivo antibacterial activity against *E. coli, S. enterica*, and *Acinetobacter baumannii*. Another oligonucleotide, phosphorothioate oligodeoxynucleotides (PSODNs) have been found to activate RNase H activity leading to mRNA degradation followed by antibacterial potential. The PPMOs exhibit bacterial growth inhibition when used along with colistin and polymyxin B. On combining with β-lactams and tobramycin, PPMOs disrupted biofilms and inhibited *P. aeruginosa* (Ghosh et al. 2019).

Hence, oligonucleotides embrace a diverse group of molecules capable of fighting against a number of pathogens (bacteria and virus), cancer, and genetic disorders.

11.11.5 Foldamers

Foldamers are short, sequence-specific oligomers that fold into well-defined secondary structures, postulated to act as antimicrobial agents. Similar to AMPs, foldamers typify the positively charged amphiphilic structures responsible for the activity of AMPs. In the past decade, several novel small molecule foldamers with specificity for bacterial membranes and greater activity have been synthesized and have shown excellent potency toward multidrug-resistant bacterial infections in animal models. A variety of phenylene-ethynylene, arylamide, acrylate, and hydrocarbon-based foldamers have been developed by various researchers and investigations on deducing their activity potential and mechanism of action are still in process. Guichard et al. have synthesized urea-based enantiomerically pure antimicrobial foldamers (Violette et al. 2006). While several others are undergoing human clinical trials for use as antibiotic in the treatment of multi drug-resistant *S. aureus* (Tew et al. 2010).

11.11.6 Antibacterial Nucleic Acids

Studies are being conducted by researchers to investigate certain nucleic acids that can kill bacteria directly. However, it is too early to be sure about nucleic acids exhibiting antibacterial action as studies are still at preliminary stages. Advances in microbial genetics techniques can help develop greater number of nucleic acid antibiotics (Czaplewski et al. 2016).

11.11.7 Immune Stimulation by P4 Peptide

P4 peptide is a synthetic 28 amino acid residue peptide derived from the functional site at the surface of bacterium, *Streptococcus pneumoniae* on exposure to virulence factor PsaA (pneumococcal surface adhesion) and has been found to stimulate opsonophagocytic uptake and killing in mice invasive disease experimental models for *S. pneumoniae* infection. This peptide enhances phagocytic killing of bacteria. When P4 (intranasal) and IgG (immunoglobulin G, intraperitoneal) are administered in mouse, there was 100% survival and significant reduction in bacterial burden (Czaplewski et al. 2016). Hence, P4 peptide and many more such peptides acting through immunostimulation can provide an alternative to antimicrobial therapy as it is devoid of resistance.

11.12 Conclusions

Immense focus lays on the development and building of libraries of new novel classes of small molecule antimicrobials competent of surmounting the growing problem of microbial resistance. In order to combat antimicrobial resistance and fight infections caused by pathogenic microorganisms, numerous alternative classes of antibiotics have been developed. Some of the approaches have advanced to clinical trial phases while others are still nascent at the laboratory stage. With the advent of large number of antimicrobials from our microbiome and their diverse actions, peptide-based antimicrobials have drawn considerable attention from the scientific community as well as from the pharma sector to further explore their potential applications in various fields. Studies have shown encouraging results for natural and synthetic peptides for their use in bacterial, mycobacterial, sexually transmitted, buco-dental, ocular and viral infections, as anticancer agent, wound repair, Kostmann's syndrome, as food preservatives, contraceptive, etc. AMPs have also shown desirable efficacy when used alone or in synergy with conventional antibiotics. Cumulative data from clinical studies conducted on several AMP molecules entering different phases further consolidate the claims to their therapeutic utility. Despite all the optimism toward AMPs, there are certain challenges associated with them *viz a viz* unwanted toxicity, lack of selectivity, stability of the peptide or its formulation and cost-effectiveness for large-scale production. Therefore, the focal point remains to be the optimization of strategies for candidate identification, having appropriate pharmacodynamic and pharmacokinetic properties (short half-life, high binding affinity, and high therapeutic index) and analyzing their biological functions. Peptide or non-peptide small scaffolds that can efficiently integrate these principles can be strongly considered for the future development of new and improved generations of peptides; making their employment safer for end user and a lucrative enterprise for the manufacturer.

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Chapter 12
Molecular Mechanisms of Antimicrobial Resistance in *Staphylococcus aureus* Biofilms

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12.1 Biofilms: Introduction

Biofilms are multicellular complex systems which can attach to biotic and abiotic surfaces. Bacterial biofilms were first described by Antony van Leeuwenhoek in 1684. Leeuwenhoek scraped plaque biofilm off his own teeth and studied the sample (Valen and Scheie 2018). Biofilms are a common phenomenon in nature. Arthur Henrici in 1933 reported that the majority of aquatic

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microorganisms were not found in the form of individual cells swimming freely but clustered on solid submerged surfaces (Henrici 1933). Nevertheless, it was not only until 1970s that scientists investigate the role of bacterial biofilms. Costerton and Høiby reported that bacteria should not only be considered as planktonic cells but also as sessile cells (Høiby 2014). The term biofilm was introduced in 1978 by Costerton referring to heterogenous bacterial aggregates surrounded by a matrix that allows their attachment to inorganic or organic surfaces (Costerton et al. 1978). Bacteria can grow as free-living planktonic cells or as sessile cells that attach to surface within biofilms. Biofilm formation is a developmental process which consists in the aggregation of planktonic cells and their transition to a surface-associated lifestyle (Chua et al. 2014). In nature, bacteria rarely grow as planktonic cultures. Instead, they predominantly exist as communities of sessile cells (Hobley et al. 2015; Berlanga and Guerrero 2016). The presence of biofilms can affect negatively several different areas, such as the food, industry, environment, and biomedical sectors. For instance, one of the most documented effects of microbial biofilms in the industrial processes is the biofouling which has a negative impact since it leads to corrosion (Mehrabi et al. 2020). Biofouling can affect several different fields such as drinking water systems, marine aquaculture, agricultural industry, food, beverage, and milk industries, among others (Flemming 2020). In the medical setting, biofilms (including both bacterial and fungal biofilms) are responsible for around 80% of all human infections (Fox and Nobile 2012; Dunn et al. 2020). Biofilms are usually associated with medical devices such as catheters, mechanical heart valves, joint prostheses, and orthopedic devices but can also be related with other infections such as endocarditis and osteomyelitis (Kwiecinski et al. 2019; Balaure and Grumezescu 2020). Nosocomial infections, either biofilm-associated or not, have a tremendous social and economic impact. In the United States, it is estimated that 1.96 million cases of biofilm-related infections occur, leading to 268,000 deaths, with more than $US18 billion spent in treatment costs (Rumbaugh and Sauer 2020). In the European Union and European Economic Area, it is estimated that 8.9 million healthcare-associated infections occur each year (Carl et al. 2018). Thus, biofilms formed by pathogenic and multidrug-resistant organisms create a major public health concern. In fact, biofilm-related infections are extremely difficult to eradicate since biofilms have high cell densities and the antimicrobial resistance of biofilms is up to 1000-fold higher than that of planktonic cells (Olsen 2015). Biofilm-forming bacteria display characteristics, activities, patterns, and behavior that are distinct from those of planktonic cells (Corning 2002).

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The bacterial community can respond non-homogeneously and adaptively to external physicochemical conditions which may vary depending on the requirements of the bacterial community (Stubbendieck et al. 2016). Within a biofilm there are microenvironments that favor the survival of these communities that can be modified according to their metabolic needs. For instance, anaerobic bacteria may live within biofilms in a modified microenvironment despite the presence of dissolved oxygen in the medium (Kato et al. 1997). Biofilms also express novel phenotypic characteristics due to genetic variations that may occur through mutations or recombination (Penesyan et al. 2019). Although most studies on biofilms have been performed with a single strain, in nature, biofilms are usually formed by different species of bacteria and/or fungi that can form a single community in symbiosis. This synergism seen among the bacteria living in biofilm brings some advantages, such as metabolic cooperation, passive resistance, quorum-sensing system, and a larger pool of genes that allow them a high adaptation and survival (Wolcott et al. 2013; Madsen et al. 2016). Consequently, this cooperation generates more severe infections such as those reported in biofilm-related infections caused by *Staphylococcus aureus* together with *Pseudomonas aeruginosa* (DeLeon et al. 2014; Hotterbeekx et al. 2017). Nevertheless, neutral or competitive interactions may also exist between some species which leads to significant effects of interaction or a decrease in productivity, respectively (Ballén et al. 2020). Another important feature of the biofilm-living mode is the capacity that all bacteria have to communicate among themselves which is known as Quorum-sensing (QS). QS is a type of cell-cell communication that regulates the expression of genes in a medium with high bacterial density allowing them to carry out a wide range of complex social activities (Müller et al. 2006). Bacteria that bind to the biofilm produce a signal molecule (self-inducer) that sends the message to the other bacteria nearby and the close proximity between cells of a biofilm facilitates the communication by QS. QS is necessary for the biofilm maturation stage and for the expression of virulence factors (Kong et al. 2006). Finally, one of the most important biofilm characteristics is the bacterial resistance conferred by this community mode of living. Biofilms have an organizational structure that shields them from host defense mechanisms and external antibiotics (De la Fuente-Núñez et al. 2013). They have a physical barrier, mainly constituted by the extracellular matrix, that prevents the action of the host defense mechanisms (De la Fuente-Núñez et al. 2013). The bacterial resistance of a biofilm may be due to low metabolic activity of bacteria in a biofilm, existence of "anaerobic niches" in the depth of a biofilm and slow or incomplete penetration of the antibiotic. All of these aspects are covered in more depth in the next topics.

As mentioned before, the majority of biofilm development studies are conducted using single-species biofilms. Several genus and species of bacteria are known to be strong biofilm producers. Among them we can highlight *S. aureus*, *S. epidermidis*, *P. aeruginosa*, and *Klebsiella* pneumoniae (Mohammed et al. 2018). In this chapter, we will focus on *S. aureus* biofilms.

12.2 *Staphylococcus aureus*

S. aureus is a Gram-positive, catalase, and coagulase-positive organism that colonizes the nostrils and mouth of several mammals including humans (Silva et al. 2020). However, when there is a breakdown of this usual balance of the organism, *S. aureus* is able to cause a wide range of infections from mild skin and soft tissue infections to life-threatening ones such as endocarditis and osteomyelitis (Silva et al. 2020). *S. aureus* belongs to the ESKAPE family of antimicrobial resistance pathogens that includes other bacteria that are responsible for the majority of nosocomial infections. Due to the fact that antimicrobial resistance is spreading rapidly it is estimated that it may cause ten million deaths per year by 2050 (O'Neill 2016). *S. aureus* has the ability to easily acquire antibiotic resistance determinants which, together with the wide range of virulence factors they produce, make its infections even harder to treat (Silva et al. 2020). *S. aureus* is a notable example of a human pathogen that is able to cause biofilm-related infections which are recalcitrant to clearance by antimicrobials and the host immune system (Suresh et al. 2019). These bacteria are a leading cause of biofilm-related infections associated with catheters and implanted medical devices, such as heart valves, pacemakers, and prosthetic joints (Hogan et al. 2016; Zhang et al. 2020). In most cases, the biofilm-related infection becomes chronic and the treatment requires a second surgery to remove and replace the implant (Hogan et al. 2016). Biofilm formation impairs the action of the host immune system and antibiotics due to *S. aureus* phenotypic characteristics and the biofilm matrix (Silva et al. 2020).

12.3 *S. aureus* Stages of Biofilm Development

Biofilm formation is a dynamic and cyclic process. Although many studies have reported different biofilm architectures, it is known that, regardless of the bacterial species, biofilm formation comprises the same developmental stages and general features (Rumbaugh and Sauer 2020). Based on in vitro research on single species biofilms, biofilm formation was classically described in 3 phenotypically distinct stages: attachment, maturation, and dispersion (Fig. 12.1), although recently a few authors consider two more stages: multiplication and exodus (Miao et al. 2017; Moormeier and Bayles 2017). The development of biofilm formation is triggered by unfavorable conditions such as external attack, physical conditions, or nutrient limitations (Liu et al. 2015). For the process of biofilm formation, there are a variety of physical and metabolic interactions necessary for adhesion, growth, and survival, in addition to increasing the resistance of these groups to hostile environments for their development.

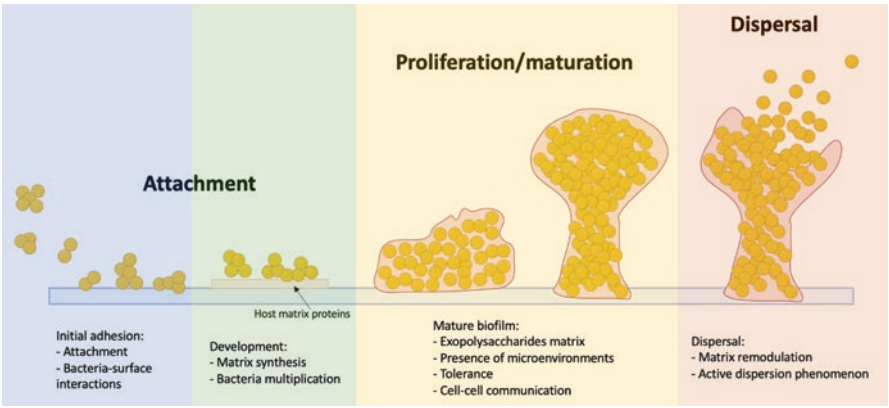

Fig. 12.1 Schematic representation of a biofilm formation. Attachment: reversible attachment of the planktonic cells followed by the adhesion to the surface. Proliferation and maturation: maturation and accumulation of bacteria in multilayered clusters surrounded by extracellular polymeric substances. Dispersal: dissemination of bacterial cells from the aggregate biofilm and reactivation of the planktonic state through dispersal

12.3.1 Attachment

Biofilm formation of *S. aureus* starts with the initial attachment of planktonic bacterial cells to biotic or abiotic surfaces, followed by the formation of bacterial aggregates (Schilcher and Horswill 2020). This step depends on physical factors such as surface charge, roughness, topography, and temperature; and on chemical factors such as pH, substrate, dissolved oxygen, and medium composition. The initial attachment may be reversible or irreversible. The reversible adhesion to the surfaces or subtracts is characterized by a weak interaction which depends on environmental factors and the genetic regulation of bacteria (Armbruster and Parsek 2018). Moreover, in this stage, the bacterial film is more susceptible to the action of antimicrobials than in the next stages. Reversible cell adhesion to surfaces is accomplished by van der Waals forces, steric interactions, electrostatic interaction, and hydrophobic forces (Van Loosdrecht et al. 1987; Gilbert et al. 1991; Carpentier and Cerf 1993). These forces are not strong enough to hold the cells together, since the bonds between cells and substrate are weak. Therefore, for cell adhesion to become permanent, the extracellular components of bacterial cells are needed (Liu et al. 2004). Cell adhesion becomes irreversible due to the presence of exopolysaccharides (EPS) on the outer surface of cells which act as adhesives, producing strong bonds between bacteria or between bacteria and the substrate. EPS includes proteins, phospholipids, nucleic acids, and polysaccharides which bond to bacteria and surfaces by stronger forces such as covalent, hydrophobic, hydrogen bonds, and

dipole-dipole interactions (Liu et al. 2004). After the irreversible adhesion stage, biofilms become very difficult to remove. Attachment is mediated by series of specific staphylococcal cell wall-anchored (CWA) proteins which can be classified based on structural and functional properties (Geoghegan and Foster 2015). *S. aureus* may exhibit up to 24 CWA proteins and this number varies with *S. aureus* strains and environmental conditions (Foster 2019). The functional diversity of these CWA proteins allows *S. aureus* to adapt to various microenvironments (Speziale and Geoghegan 2015). All CWA proteins have similar features among them such as a N-terminal secretory signal sequence, a C-terminal LPXTG motif, and a positively charged tail (O'Brien et al. 2002). Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are the main family of CWA proteins that are anchored in *S. aureus* cell wall. Many MSCRAMMs are not only involved in attachment and biofilm formation but also in invasion of host cells and tissues, evasion of immune responses, and virulence (Kang et al. 2013). MSCRAMMs are responsible for the initial attachment to native tissues, being able to bind to one or several different host extracellular matrix proteins such as fibronectin, fibrogen, laminin, and elastin; to indwelling medical devices and plasma-coated biological surfaces (Seo et al. 2008). The MSCRAMMs family includes many proteins such as fibronectin binding proteins, clumping factor proteins, biofilm-associated protein, surface-anchored proteins, elastin binding protein, fibrinogen binding protein, laminin binding protein, and collagen binding protein. *S. aureus* has two main fibronectin binding proteins, FnBA and FnBB, which are encoded by the *fnbA* and *fnbB* genes, respectively. *S aureus* may have only one or both *fnb* genes (Peacock et al. 2000). Both proteins are involved in bacterial invasion of the endothelial cells (Peacock et al. 2002). The N-terminal A region of FnB proteins has the ability to bind to fibrinogen and elastin (Murai et al. 2016). Fibrinogen/fibrin is one of the major plasma proteins accumulated on implanted medical devices. The ability of *S. aureus* to adhere to fibrinogen results in instantaneous clumping of bacterial cells. Two receptors for fibrinogen, ClfA and ClfB, promote binding of *S. aureus* to fibrinogen (Ní Eidhin et al. 1998; Hall et al. 2003). ClfA and ClfB have all the traditional features of the CWA proteins and are also members of the serine-aspartate repeat (Sdr) protein subfamily (Josefsson et al. 1998). Sdr proteins are cell wall-anchored proteins with large serine-aspartate repeat domains and are also involved in adhesion (Josefsson et al. 1998). In *S. aureus*, SdrC, SdrD, and SdrE are the known proteins belonging to the Sdr protein subfamily (Pi et al. 2020). All three share a conserved structural organization; however, unlike SdrD, and SdrE, SdrC is essential for biofilm growth via homophilic interaction (Barbu et al. 2014). The biofilm-associated protein (Bap) was first described as a large, multi-domain, cell surface-anchored protein that promotes biofilm formation. This protein has never been found in humans and was described as a cattle-specific pathogenic factor of biofilm formation being highly associated with bovine mastitis (Shukla and Rao 2017; Naushad et al. 2020). Apart from this, the prevalence of Bap among staphylococci is very low and its occurrence is significantly higher in coagulase-negative strains (Shukla and Rao 2017). The elastin binding protein is encoded by the *ebps* gene. This protein is responsible for attachment to

host cells via binding to elastin promoting colonization of mammalian tissues by *S. aureus* (Kot et al. 2018). However, the presence of *ebps* does not seem to be essential for biofilm formation since the inactivation of *ebps* has minimal effect on binding of *S. aureus* to elastin (Roche et al. 2004). The *S. aureus* ability to bind to fibrinogen is granted by the fibrinogen binding protein (encoded by the *fib* gene) which is an important factor in promoting wound infection, foreign body infection, and endocarditis (Hartford et al. 2001). *S. aureus* strains may also harbor the *eno* gene that encodes for the α-enolase. α-Enolase is able to bind laminin and can also mediate the binding of *S. aureus* to laminin which may contribute to the dissemination of *S. aureus* cells by blood leading to tissue invasion (Kot et al. 2018). Finally, the collagen-binding protein, encoded by the *cna* gene, is a mediator of the staphylococci adhesion to collagenous substrates and tissues. The presence of this protein is also necessary for the adhesion of *S. aureus* to cartilaginous tissues (Valotteau et al. 2017). Another important CWA protein family is the near-iron transporter (NEAT) motif family. These proteins are essential for *S. aureus* survival against the host immune system and are responsible for promoting biofilm formation under iron starvation (Hammer and Skaar 2011). One of the most recognized CWA protein is the staphylococcal protein A (SpA) which is also used for *S. aureus* molecular typing. Spa protein is essential for the pathogenesis of staphylococcal infections and significantly contributes to biofilm-associated infections development (Merino et al. 2009). It is known that *S. aureus* has the ability to attach to abiotic surfaces, such as plastic surfaces. The particular attachment can be mediated by teichoic acids and/or the surface protein SasC which are not involved in *S. aureus* attachment on biotic surfaces (Gross et al. 2001; Reffuveille 2017). Many of these proteins and factors play a role both in attachment and accumulation. After initial attachment to either biotic or abiotic surfaces, mediated by all these proteins and factors, the adhesion becomes irreversible. This step is the is the first to the maturation of a future biofilm.

12.3.2 Maturation/Proliferation

After the attachment, *S. aureus* maturates and accumulates in multilayered clusters surrounded by an extracellular matrix (ECM). The initial biofilm comprises a thin layer that thickens until it forms a mushroom or tower-shaped structure in the mature biofilm (Balaure and Grumezescu 2020). These structures, and also channels, form to facilitate nutrient delivery to deeper layers of the biofilm. Moreover, the architecture of biofilms derives from a division of labor between cells within a biofilm since several factors, such as morphology, phenotype, gene expression, and differentiation stage, may vary between biofilm cells (McDougald et al. 2012). The biofilm structural integrity depends upon the ECM which is produced by the organisms that make up the biofilm. In general, ECM in *S. aureus* biofilms is a complex mixture of polysaccharides, teichoic acids, extracellular DNA (eDNA), lipids, proteins, and other compounds; and the individual components present within the biofilms depends on the *S. aureus* strains and external conditions (Schwartz et al. 2016;

Sugimoto et al. 2018). Generally, ECM is responsible for protecting bacteria against desiccation, oxidation, action of antibiotics, metals, and the action of elements of the immune system (Kostakioti et al. 2013). Also, the ECM allows physical and social interactions which enhanced the rate of gene exchange and an increased tolerance to antimicrobials (Kostakioti et al. 2013). Microorganisms in biofilms are metabolically and functionally integrated consortia that, due to the three-dimensional structure of the biofilm, present differences in the metabolic and gene expression patterns based on the position of the cells in the ECM (Berlanga and Guerrero 2016). The incorporation and exchange of genetic material through cell-cell interaction is also facilitated within biofilms due to the presence of DNA strands trapped in the ECM and also to the three-dimensional structure that allows the proximity between cells. The ECM is also the first line of defense since, due to its physical and biochemical properties, prevents the diffusion of antimicrobial agents (Kumaran et al. 2018). Polysaccharides play a fundamental role in the biofilm matrix since they provide architectural support and protection. In staphylococcal biofilms, several polysaccharides have been described; however, the first and main determinant of the attachment and multiplication phases is the polysaccharide intracellular adhesion (PIA) which consists of polymeric *N*-acetyl-glucosamine (PNAG) (Cue et al. 2012). PIA production is dependent upon four genes, *icaADBC*, encoded by the *ica* operon. The operon i*caADBC* encodes IcaA and IcaB proteins (N-acteylglucosamine transferases), IcaC (predicted exporter) and IcaD (deacetylase) (Miao et al. 2019). IcaA together with IcaD have been shown to produce an N-acetylglucosamine oligomer. IcaC is responsible for chain growth and for the transport of IcaAD-synthesized oligomers across the cell membrane. IcaB is responsible for the deacetylation of PIA which results in a positively charged PIA promoting the interaction of PIA with cell surface (Fig. 12.2) (Gerke et al. 1998; Vuong et al. 2004; Cue et al. 2012). Furthermore, an atomic force microscopy study reported that PIA charged positively promotes cell-cell adhesion between *S. aureus* and polyanionic teichoic and lipo-teichoic acids (Formosa-Dague et al. 2016). *icaR* gene is a member of the TetR family of regulatory proteins and is located upstream of the *ica* operon. IcaR is one of the most important factors involved in *icaADBC* regulation since by binding to the *icaA* promoter region it represses the *ica* operon expression (You et al. 2014). Therefore, a significant increase in *icaADBC* expression occurs when *icaR* is inactivated leading to a higher PIA production (Conlon et al. 2002). Yu et al. (2017) reported a new repressor gene that recognizes and binds to the 5-nucleotide motif, located within the *icaR-icaA* intergenic region, decreasing biofilm PIA productions (Yu et al. 2017). Recently, several studies have reported that environmental conditions including temperature, low oxygen, osmotic pressure, glucose, and sub-inhibitory concentrations of some antibiotics influence the expression and production of PIA (Mhatre et al. 2014; Weidenmaier and Lee 2015; Miao et al. 2019). Initially, it was thought that PIA was essential for staphylococcal biofilm formation; however, in recent years, an increased number of studies have shown that some strains of *Staphylococcus* produced biofilm independently of the presence and expression of *ica* operon responsible for PIA synthesis (Hennig et al. 2007; O'Gara 2007; Lauderdale et al. 2009). These studies have demonstrated that PIA-independent

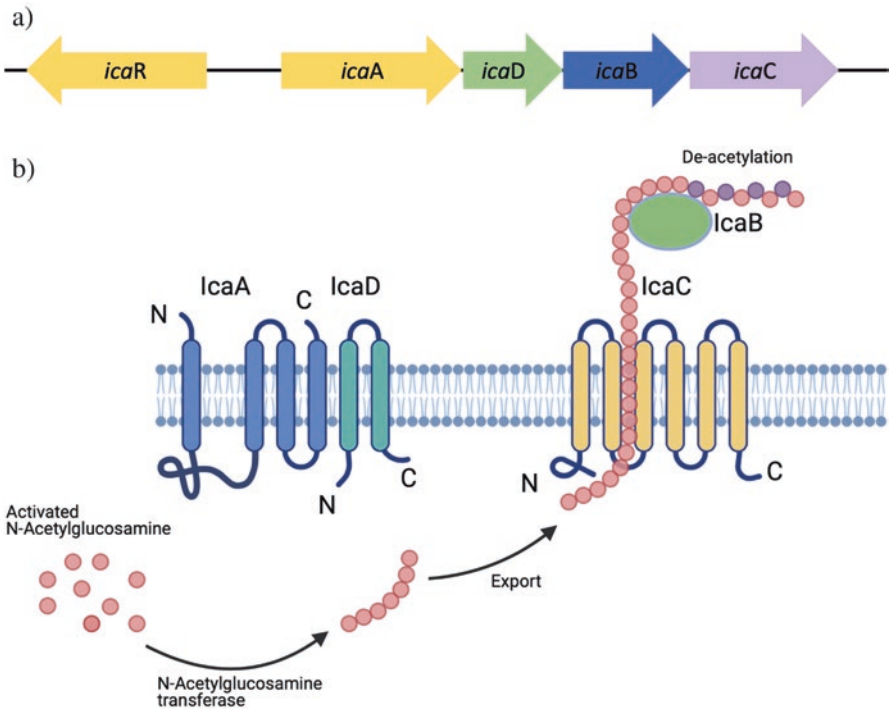

Fig. 12.2 PIA production is dependent upon four genes, *ica*ADBC, encoded by the *ica* operon. (**a**) The *ica* operon (**b**) The production of exopolysaccharide poly-N-acetylglucosamine (PNAG; also known as PIA) (adapted from Otto 2009)

biofilm formation is promoted by several cell wall-attached proteins discussed in the previous section showing that these proteins can replace the PIA function in *ica*-deficient *S. aureus* strains during biofilm formation and development. The Bap protein in bovine mastitis, FnBPs in human methicillin-resistant *S. aureus* (MRSA), and accumulation-associated protein (Aap) in *S. epidermidis* were among the biofilm-related proteins in the first studies reporting PIA-independent biofilm formation (Cucarella et al. 2001; Rohde et al. 2005; O'Neill et al. 2008). Mempel et al. reported a relationship between β-lactam susceptibility and the biofilm phenotype. In this study, it was shown that PIA production varied with the different levels of β-lactam susceptibility in several *S. epidermidis* isolates (Mempel et al. 1994; Mempel et al. 1995). *ica*-dependent biofilm formation was associated with methicillin susceptibility in *S. aureus* with PIA production found to be essential for biofilm formation in methicillin-susceptible *S. aureus* (MSSA) but not MRSA (O'Neill et al. 2007). In fact, the capacity of biofilm formation was suppressed by the deletion of the *ica* locus (Valle et al. 2003; O'Neill et al. 2007). It has been shown that *ica* operon expression is activated by the presence of NaCl in media. Unlike MSSA strains, and since *ica* locus was found not to influence MRSA biofilm formation, NaCl does not induce biofilm formation of MRSA strains, yet, the addition of

glucose to growth media seems to enhance biofilm formation of MRSA strains (O'Neill et al. 2007). Indeed, glucose and NaCl were shown to promote biofilm formation by MRSA and MSSA strains through acidification of culture media and induction of PIA production, respectively (O'Neill et al. 2007; Boles and Horswill 2008). Although PIA is a major component in the biofilm ECM, studies have been showing that some *S. aureus* strains were able to form biofilm PIA-independent being composed by proteins and eDNA (Sugimoto et al. 2018). Sugimoto et al. (2018) have shown that eDNA has different impacts on the development and structural integrity of both PIA-dependent and PIA-independent biofilms. Furthermore, the authors reported that the quantity of eDNA does not seem to influence the development of robust biofilms (Sugimoto et al. 2018). In fact, studies have shown that eDNA may play a more important role in biofilm formation than CWA proteins since *S. aureus* mutant strains deficient in the production of MSCRAMMs grew biofilms indistinguishable from those grown by the wild type (Moormeier et al. 2014; Kavanaugh et al. 2019). In contrast, in a *S. aureus* mutant strain lacking the murine hydrolase AtlA, the biofilm formation was reduced, which is consistent with the fact that AtlA can also function as an autolysin that is responsible for the release of eDNA that incorporates into the matrix (Bose et al. 2012). Therefore, it seems that eDNA plays a crucial role in biofilm maturation. Other proteins, such as extracellular adherence protein (Eap) and beta toxin (Hlb), that are secreted proteins, also play a role in biofilm maturation. Nevertheless, the level of importance of these proteins vary largely between strains (Lister and Horswill 2014). The accessory gene regulator (*agr*) system is also implicated in biofilm formation. The deletion of *agr* enhances biofilm formation by MRSA strains while its deletion does not seem to influence biofilm formation in MSSA strains (O'Neill et al. 2007). Accordantly, *agr* deletion does not significantly alter *ica*ADBC expression or PIA production (Vuong et al. 2003). Furthermore, activation of the *agr* system was shown to initiate biofilm dispersal (Boles and Horswill 2008).

12.3.3 Dispersal

The dynamic equilibrium of a biofilm is reached when its outermost layers begin to generate planktonic cells that are metabolically active and capable of dividing. Following biofilm maturation/proliferation, cells within the biofilm can reactivate to a planktonic state through dispersal. Nevertheless, studies have shown that dispersal cells have a unique phenotype, different from sessile or planktonic cells (Guilhen et al. 2017). Furthermore, biofilm-dispersed cells seem to overexpress common virulence factors when compared with planktonic or sessile cells (Hay and Zhu 2015; MacKenzie et al. 2015). Biofilm dispersal can comprise three stages: detachment of cells from the biofilm, movement of bacterial cells to a new site, and attachment of the cells to a surface/substrate in the new site (Kaplan 2010). Biofilm dispersal represents a critical step in bacterial lifestyle involving coordination of several environmental signals, signaling messengers, and molecular effectors

(Guilhen et al. 2017). Biofilm cell dispersal can be promoted by several cues, such as changes in oxygen concentration and cell density, lack of nutrients, and intense competition, resulting in total or partial biofilm dispersal (Balaure and Grumezescu 2020). Cue-specific sensory proteins detect these changes and stimulate a response that leads to production of effectors within the biofilm that are responsible for the bacterial escape from the biofilm structure. Studies have reported a wide range of signals regulatory pathways and effectors (Guilhen et al. 2017). The dispersal of cells from a biofilm can lead to new and subsequent infections in the host since cells can disseminate from the original site of infection into the host and recolonize other available host sites. Indeed, biofilm cells dispersal plays an important role in chronic infections (Speziale and Geoghegan 2015). Biofilm dispersal may also be an important approach to treat persistent *S. aureus* infections since intentional dispersal of a biofilm together with antibiotic treatment can lead to the exposure and death of metabolically active bacterial cells (Lister and Horswill 2014). No single mechanism of biofilm dispersal is used by all bacteria. The main biofilm dispersal mechanism used by *S. aureus* is the production of several exo-enzymes and surfactants to degrade the ECM. The effectiveness of the different biofilm dispersal mechanisms is dependent on the *S. aureus* strain and the matrix composition (Kiedrowski et al. 2011; Lister and Horswill 2014). In fact, in PIA-dependent biofilms, the use of proteins to induce enzymatic self-destruction, is less effective in dispersing biofilm cells and, contrarily, mechanisms specifically targeting PIA have no effect on the dispersal of PIA-independent biofilms (Lister and Horswill 2014). The different stages of *S. aureus* biofilm formation can be controlled by several different mechanisms, such as phenol-soluble polypeptides, several surface proteins, pH change, protease, DNase, and quorum sensing, among others (Boles and Horswill 2011). Notably, the activation of the *agr* quorum-sensing in *S. aureus* inhibits its own biofilm formation and dispersed the established biofilms. In fact, Boles and Horswill (2008) showed that the *agr* system triggers a dispersal pathway, detaching cells from the biofilm and reverting them to a planktonic state and consequently to a more antibiotic susceptible state (Boles and Horswill 2008).

12.4 Impact of Quorum Sensing on *S. aureus* Gene Expression and Biofilm Formation

Studies suggest that certain bacteria use the production, release, exchange, and detection of signaling molecules to measure population density and control their behavior in response to variations in the number of cells. However, for a long period of time, the phenomenon of chemical signaling between cells was considered to be limited to a few bacterial species. Fuqua et al. (1994) described the phenomenon that bacteria know their concentration in a given environment and decide when to start the expression of a certain set of genes to develop a specific response simultaneously in response to eventual changing conditions (Fuqua et al. 1994). Currently,

it is known that intercellular communication is not exclusive to certain species but that is common in the bacterial ecosystem and this process known as quorum sensing (QS) is fundamental in the formation of biofilms (Schauder and Bassler 2001). The occurrence of QS has been demonstrated in Gram-negative and -positive bacteria and it involves intra- and intercellular signals that generate benefits for groups of microorganisms. Nevertheless, cell-cell interactions are facilitated by the close proximity between bacteria within the biofilm (Flemming and Wingender 2010). QS plays an important role in biofilm development. Each bacterium that binds to a surface generates a signal molecule, and the more bacteria that bind the more the signal increases until it reaches a threshold leading to the bacterial differentiation in the biofilm. Through QS, bacteria regulate or synchronize changes in their genetic expression based on their population density, such as the production of antibiotics, toxins, pigments, exopolysaccharides (formation of biofilms), motility, and other virulence factors. Bacterial cells lacking these signals produce biofilms with a weak architecture (Lazar 2011; Khan et al. 2019). QS is based on the diffusion and subsequent detection of molecules that act as a signal called "autoinducers". Bacteria also possess a receptor that can specifically detect the respective auto-inducer. When it binds to the receptor, it activates the transcription of certain genes, including those for inducer synthesis (Zhang and Li 2016). Gram-negative bacteria diffuse low molecular weight compounds called acyl-homoserine-lactones (AHLs) into the intercellular space whereas in Gram-positive bacteria, such as *S. aureus*, the auto-inductive-modified oligopeptides produced by these bacteria act as a signal for other cells (Abraham 2006). Two QS systems have been identified in *S. aureus:* the *agr* system that is common in Gram-positive bacteria and the *LuxS*/AI-2 system that is expressed in both Gram-positive and -negative bacteria (Kong et al. 2006). The *agr* system is one of the most important factors and it has been characterized as an operon in *S. aureus*. Agr system has great relevance in the control and regulation of cell density and the expression of virulence genes. The promoters P2 and P3 are included in *agr* operon and regulate the transcription of RNAII and RNAIII, respectively (Morfeldt et al. 1995). The *agr* locus consists of four genes: *agr*B, *agr*D, *agr*C, and *agr*A. *agr*B encodes a membrane protein responsible for translocation and modification of AgrD. The *agr*D gene encodes the precursor of autoinducing peptide (AIP). *agr*C and *agr*A genes are located in the RNAII encoding region. *agr*C encodes a membrane receptor protein of the AIP signal. AIP binds to *agr*C which activates the phosphorylation of *agr*A which, in turn, binds to P2 and P3 inducing the expression of RNAII and RNAIII (Queck et al. 2008). The increasing cell density leads to the accumulation of AIP which activates the *agr* system. RNAIII is the key effector of *agr* since its concentration increases with the increase in cell density. RNAIII has been associated with the increasing in virulence factors and surface proteins, such as SpA, FnBPA, FnBPB, α-hemolysin, and Panton-Valentine leukocidin (Queck et al. 2008; Cheung et al. 2011). Due to the increase in the prevalence of antimicrobial resistance among clinically relevant bacterial strains, their high capacity to form biofilms and the failure of vaccines to prevent infection, new alternatives to antibiotics are required. Therefore, much attention has been given to chemical inhibition of QS, particularly the *agr* system in *S. aureus* (Sully et al.

2014). In fact, it has been shown that *agr*-negative strains display intermediate resistance to glycopeptides such as vancomycin (Howden et al. 2010). Due to the increase in the prevalence of antimicrobial resistance among clinically relevant bacterial strains, their high capacity to form biofilms and the failure of vaccines to prevent infection, new alternatives to antibiotics are required. Despite the fact that chemical inhibitors of *agr* have been detected and identified, it seems that none of them have demonstrated efficacy in vivo (Sully et al. 2014).

12.5 Biofilm Resistance, Tolerance, and Persistence

Biofilm cells can be between 10 and 1000 times more resistant than planktonic cells corresponding to a large number of broad-spectrum antibiotics and biocides oxidants such as chlorine, iodine, or ozone (Govan and Deretic 1996). In biofilm, the mechanisms of antibiotic resistance are altered and bacteria showing susceptibility to a given antibiotic become quiescent, increasing their tolerance to that compound (Singh et al. 2017). Nevertheless, resistance to antibiotics and disinfectants in biofilms is also dependent on the species and strain of microorganisms, on the physiological characteristics of the biofilm, on the phenotypic changes in the cells themselves, and on the age of the biofilm. Biofilm eradication is a very difficult process, since biofilm inactivation and removal are distinct processes, that is, biofilms may be inactive but remain attached to the surface (Wolfmeier et al. 2018). Furthermore, antibiotics may diminish the number of bacterial cells within biofilms, but cannot completely eradicate the pathogen which leads to refractory/relapsing infections (Dufour et al. 2010). Antimicrobial resistance is defined as the ability of bacteria to grow in the presence of high concentrations of antibiotics or an increase in the minimum inhibitory concentration (MIC) to the antimicrobial agent, necessary to stop cell growth. Biofilms, on the other hand, present various resistance mechanisms that enable the growth under antimicrobial therapy. Currently, it is thought that the high antimicrobial resistance in biofilms is the combined result of different mechanisms, such as presence of persister cells, starvation-induced slow growth, reduced metabolic rate, and the restricted penetration and sequestration of antimicrobials through the matrix. Furthermore, it has been shown that biofilm cells express genes that encode various xenobiotic efflux and export systems (Rumbaugh and Sauer 2020). Studies have shown that there are several mechanisms associated with this phenomenon of resistance (Brown et al. 1988; Costerton et al. 1999; Anderl et al. 2000; Brooun et al. 2000; Gilbert et al. 2002; Walters et al. 2003; Lewis 2007): delayed penetration of the antimicrobial agent due to the exopolysaccharides in the matrix; lower growth rate inducing physiological changes; phenotypic changes within biofilms; quorum-sensing; the expression of efflux pumps; and the development of dormant persister cells (Dufour et al. 2010). Although the primary function of the ECM is protection, it has been shown that the ECM can act as an impermeable barrier to limit antimicrobial penetration. The delaying of the antimicrobials diffusion is due to the exopolysaccharides decreasing the rate of transport

of the molecule into the biofilm and changing the reaction of the antimicrobial agent with the matrix (Sutherland 2001; Donlan 2002). The ECM protection can also be due to the direct binding of the antibiotics by the EPS matrix. After antibiotic treatment, the cells located in the outer layer of the biofilm die due to their closer exposure whereas cells embedded deep inside the biofilm are able to survive (Dufour et al. 2010). The ECM can also be considered a chemically active barrier. Diffusion obstruction is probably effective against small antimicrobial peptides, toxic cationic heavy metals, and positively charged antibiotics, such as aminoglycosides, due to the negative charge of exopolysaccharides (Nichols et al. 1988). On the other hand, the ECM has no effect on the diffusion of uncharged antibiotics such as β-lactams (Zheng and Stewart 2002). In fact, although the ECM protects biofilm cells against penetration of antimicrobials, it may not be a general protection mechanism since many antibiotics are able to penetrate into the biofilm. Studies have shown that ECM of *S. aureus* decreases the activity of oxacillin, cefotaxime, vancomycin, teicoplanin, moderately reduce the activity of linezolid and quinupristin/dalfopristin but susceptibility to rifampicin, amikacin, ranbezolid, and ciprofloxacin remains unaffected (Mathur et al. 2005; Kostenko et al. 2007; Singh et al. 2010). Other studies showed that phenotypic changes within biofilms, such as adoption of a distinct and protected phenotype, may occur contributing to the antimicrobial resistance (Dufour et al. 2010; Corona and Martinez 2013). The fact that the biofilm contains a great diversity of environmental niches, presenting different nutrients and pH in different zones, contributes for such resistance. However, this phenotypic heterogeneity is mainly due to different concentration gradients experienced within biofilms. Cells located deep inside the biofilm have a lower growth rate, due to limited nutrients and oxygen, which is similar to the stationary phase in planktonic cells. Consequently, the decreased metabolic activity of bottom layer cells contributes to antibiotic tolerance. Contrarily, cells located in the external layers have a growth rate similar to the exponentially growing planktonic cells (Stewart and Franklin 2008). Nevertheless, in general, organisms organized in biofilms have a lower growth rate than planktonic cells, thus taking on antimicrobial agents more slowly. Indeed, all antibiotics are more effective in growing cells. For instance, penicillin does not have an effect on non-growing cells and the effect rate is proportional to the growth rate. However, other antibiotics such as cephalosporins, aminoglycosides, and fluoroquinolones have an effect on cells in stationary phase (Lode et al. 1998; Rosenberg et al. 2020). Oxygen concentration and pH gradient established within the biofilms, although indirectly, also contribute to decreased growth rate.

Bacteria may show antimicrobial resistance in the planktonic state which is also responsible for the biofilm antimicrobial tolerance. However, the antimicrobial resistance by itself is not sufficient to explain the higher antimicrobial tolerance of the biofilms nor the recurrence of biofilm infections. Some studies showed that environmental changes, such as temperature, low oxygen availability, and DNA damage, among others, trigger gene expression and the transfer of genes between bacteria leading to resistance and survival in the presence of certain antibiotics (Beloin and Ghigo 2005; Resch et al. 2005). Other mechanisms have been described, such as the action of reactive agents located in the superficial layers of the biofilm

which deactivate antibiotics. Another mechanism responsible for antibiotic tolerance of biofilm is the presence of drug efflux pumps. Studies have shown that sub-inhibitory concentrations of antibiotics induce membrane-bound drug efflux pumps both in Gram-positive and -negative bacteria (Lynch and Robertson 2008). These drug efflux pumps can have several functions and can have specificity for a single antibacterial agent and a broad specificity for structurally unrelated antimicrobials (known as multidrug efflux pumps) (Dufour et al. 2010). Five families of efflux pumps associated with antimicrobial resistance are known. Four of those families use proton motive force as an energy source: multidrug and toxin extrusion, small multidrug resistance, major facilitator superfamily, and resistance-nodulation-division; while ATP-binding cassette uses ATP hydrolysis as an energy source (Pao et al. 1998; Jack et al. 2001; Lubelski et al. 2007; Kuroda and Tsuchiya 2009; Nikaido and Takatsuka 2009). The resistance-nodulation-division efflux pumps have only been found in Gram-negative bacteria (Alav et al. 2018). The major facilitator superfamily of efflux pumps are the most known among Gram-positive bacteria (Alav et al. 2018). Several studies have been conducted to better understand the role of efflux pumps on *S. aureus* biofilm tolerance. An early study conducted by Beenken et al. (2004) reported a difference in the expression of several efflux and transporter genes in biofilm cells when compared to the planktonic cells (Beenken et al. 2004). The lipoprotein streptococcal adhesion PsaA homolog (SA0587) gene and multidrug resistance protein B (SA2142) gene encode for the ATP-binding cassette and major facilitator superfamily, respectively. In a study by Resch et al. (2005), the expression of genes during biofilm formation was analyzed. After just 8 h of growth, SA0589 and SA2142 genes were expressed at least five-fold more in biofilm than in planktonic cells (Resch et al. 2005). Another study reported the presence of multidrug resistant gene cluster including the macrolide efflux pump *msr*A in *S. aureus* (Weigel et al. 2007). The expression of NorB and NorC, which are multidrug-resistant pumps conferring resistance to quinolones, tetraphenylphosphonium, cetrimide, and other antimicrobial agents, was detected during *S. aureus* biofilm growth (He and Ahn 2011). Furthermore, *norB* is expressed in *S. aureus* in response to acid environments and reduced oxygen which are conditions that are found in deep layers of *S. aureus* biofilms since cells switch to anaerobic respiration and the environment has a low pH (Costa et al. 2013). QS and intracellular signaling molecules are also implicated in biofilm tolerance. The *agr* system in *S. aureus* is related to the QS system as reported above. The Agr expression in *S. aureus* biofilms has been associated with antimicrobial resistance. The RNAIII production of *agr*-positive staphylococci is induced by sub-inhibitory levels of ciprofloxacin, mupirocin, and rifampin (Yarwood et al. 2004). Hamamelitannin has been shown to block QS through the TraP QS system and to decrease *S. aureus* biofilm tolerance to vancomycin (Kiran et al. 2008; Brackman et al. 2013). Another mechanism of antibiotic tolerance in biofilms is the presence of persister cells. These cells enter into a state of dormancy which allows their survival in stress conditions, namely during antimicrobial treatment, since antibiotics target cell growth (Jayaraman 2008; Lewis 2010). This resistance mechanism is very successful since, in general, resistance

mechanisms prevent the binding of the antibiotic to the target and, in contrast, in persister cells the targets are shut down (Dufour et al. 2010).

12.6 Conclusions

S. aureus is a relevant pathogen for both human and animal health. *S. aureus* has the capacity to easily acquire antimicrobial resistance which, together with its wide range of virulence factors that supports its evasion of the host immune system, turn it into a worrisome pathogen. Furthermore, *S. aureus* strains have a high ability to form biofilms which leads to recurrent and chronic infections. The relation between antibiotic resistance and biofilm phenotypes is of much interest since both contribute to the success of *S. aureus* infections. Many transcriptomic and proteomic studies have been conducted in order to better understand the mechanism underlying *S. aureus* biofilm formation, dispersal, and tolerance to antimicrobial agents. Given the importance of improving biofilm-related infections treatment, the eradication of biofilms, and eventually the development of a new vaccine, additional studies are necessary to fill specific knowledge gaps.

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Chapter 13
Antimicrobial Resistance: Meaning and Developing Realization

Atul N. Chandu, Samsher Singh, and Santosh Kumar Rath

13.1 Introduction

Antimicrobial resistance (AMR) is the capacity of a microorganism to resist the growth-inhibitory or killing activity of an antimicrobial to which the specific bacterial species is normally susceptible. The availability of antibiotics to treat infectious diseases has significantly improved the health and life prospects of humans, as well as the health and welfare of animals. However, use of antibiotics results in selection for antimicrobial resistance in bacteria (Verraes et al. 2013).

AMR is a fast-growing public health issue overshadowing modern medicine worldwide. The development of pathogens with a variety of resistance mechanisms is making infection control and treatment strategies increasingly ineffective. However, it is still difficult to accurately estimate the extent of AMR (Mattar et al. 2020). Development of antimicrobial resistance has added significantly to the impacts of various infectious diseases, as well as overall health care costs. Although we have a large number of antimicrobial agents, this is paralleled by continuously increasing evidence of antimicrobial resistance in the present era (Beceiro et al. 2013; Aslam et al. 2018). This continuous increase in antimicrobial resistance is a

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global health issue, accounting for about 700,000 deaths annually (World Health Organization 2019). Diseases caused by drug-resistant microbes—the majority of which are respiratory infections, sexually transmitted infections and urinary tract infections (UTIs)—are very hard to treat and are increasing all the time. So-called wonder drugs are becoming ineffective; the effect of this is currently being witnessed by the world and will become even more serious in the future. In an attempt to help control them, the World Health Organization (WHO) has classified resistant infections as one of the top ten global health threats (Prestinaci et al. 2015).

AMR spreads as a result of antimicrobial selection pressure (ASP) and clonal dissemination. Dissemination of resistant microbes can occur following selection pressure and is the main cause of the spread of AMR in settings of close personal contact, such as hospitals and day care centres (Davey et al. 2010).

Inappropriate and extensive use of antimicrobials has led to the present scenario, which is posing serious threats for the future. It is evident that human, animal, food and environmental factors play significant roles in microbial evolution, and all of these are interconnected, demonstrating the need for a coordinated, multisectoral 'one health' approach (Wang and Schaffner 2011; Newell et al. 2010).

A return to the pre-antibiotic era is fast becoming a reality in many parts of the world (Aminov 2010); it is clear that we are entering an era in which the impact of antibiotics will be minimal. It is therefore highly relevant to know the details of AMR mechanisms and the routes of resistance so that therapies, practices, surveillance and research can be adjusted to minimize the increase of resistance in the future (Larsson et al. 2018; Brown and Wright 2016).

Principally—and contrary to the dogma promulgated a few years ago—the increase and spread of resistance are not caused by or restricted to hospitals and excessive use of antibiotics. The presence of resistant bacterial strains in sewage, in water, in food, in soil and elsewhere demonstrates the participation of environmental pressures on these strains, and by associating these data with the presence of antibiotics in the environment and their use in veterinary therapy and agriculture, researchers from all over the world are expanding their studies beyond hospitals. This reflects the concept that the involvement of environmental factors is somehow partly responsible for antibiotic resistance (Lupo et al. 2012; Balsalobre et al. 2014). It is recommended that countries prioritize national action plans (NAPs) to scale-up financing and capacity-building efforts; put in place stronger regulatory systems and support awareness programmes for responsible and prudent usage of antimicrobials by professionals in human, animal and plant health; and invest in ambitious research and development of new technologies to combat antimicrobial resistance (Chua et al. 2021; Bungau et al. 2021).

Because of the continuous increase in drug-resistant infections, there is an urgent need for new antimicrobials. AMR is defined as inefficiency of an antibiotic for killing the same type of pathogenic bacteria that were previously killed by the same antibiotic, which may be due to genetic inheritable changes followed by clonal propagation or phenotypic adaptations to survive under antibiotic pressure (Vincent and Uphoff 2020).

The continued increase in antimicrobial resistance has led to fewer treatment options being available for patients, and it is believed to increase morbidity and mortality. In the present scenario where common infections are becoming resistant, we are facing more severe infections needing more extensive treatment, longer courses of illness often requiring extended periods of hospitalization, and huge increases in treatment costs (Reygaert 2018; Michael et al. 2014).

To prevent AMR, it is necessary first to understand what causes it. In simple terms, random mutations can lead to microorganisms becoming immune to a certain type of drug, while uncontrolled or unnecessary ambient use of these drugs creates transformative pressure making resistant microorganisms proliferate (Rodríguez-González et al. 2019; Liu et al. 2021).

13.2 Overview of Current Antimicrobial Resistance

Common infections were previously cured with frequently used antibiotics globally, but now resistance is common among UTIs, sepsis, sexually transmitted infections and some forms of diarrhoea (Micoli et al. 2021; Ahmad 2021). According to the Global Antimicrobial Resistance and Use Surveillance System (GLASS), the rate of resistance to ciprofloxacin for treatment of UTIs is 8.4–92.9% in cases caused by *Escherichia coli* and 4.1–79.4% in those caused by *Klebsiella pneumoniae* (Cheong et al. 2021; Veeraraghavan et al. 2018). Worldwide, there have been reports of resistance to carbapenems, an antibiotic class that includes drugs of last resort for curing intestinal infections caused by *K. pneumoniae* bacteria, which are now considered life threatening and are among the most common types of hospital-acquired infections (Chen et al. 2021). As a result of this resistance scenario, carbapenems are unable to cure more than 50% of such cases in some countries (Veeraraghavan et al. 2018). A similar scenario is now seen with fluoroquinolones, which are losing their effectiveness to treat UTIs caused by *E. coli*, although they were previously the drugs of choice in such cases (Nazir et al. 2021; Langner et al. 2021). To treat *E. coli*–related UTIs and carbapenem-resistant Enterobacteriaceae, colistin is now considered the drug of last resort, but resistance reports from several countries indicate that these infections are becoming harder to cure, and, at present, we have no alternative drugs that are effective in such cases (Ara et al. 2021; Kelly et al. 2017).

Another major concern is resistance of *Staphylococcus aureus* to multiple classes of antibiotics (Rath et al. 2019). These bacteria are among those that live commensally on the skin and are responsible for a plethora of infections in the community, as well as hospital-acquired infections (Foster and Geoghegan 2015). Methicillin (methicillin) was previously effective against them, but increasing resistance has resulted in evolution of methicillin-resistant *S. aureus* (MRSA), which has now spread worldwide and is associated with a 64% greater risk of mortality than drug-sensitive infections (Romandini et al. 2021; Lee et al. 2018). Over time, MRSA has evolved resistance to every antibiotic, and this has consequently made such

infections very hard to treat. Selection pressure from increasing antibiotic use has provided the pathogen with opportunities to develop complex survival mechanisms (Craft et al. 2019). Furthermore, the problem is become more complex because of decreased interest in antibiotic development by pharmaceutical industries, given the high levels of investment that are required and the risk that increasing resistance will rapidly make the results of such huge efforts redundant anyway (Årdal et al. 2020).

Resistance in some variants of *Neisseria gonorrhoeae* makes them hard to eradicate with sulfonamides (sulphonamides), penicillins, tetracycline, macrolides, fluoroquinolones and some old cephalosporins (Unemo and Shafer 2014; Bodoev and Il'Ina 2015). The only currently viable option for treatment of *N. gonorrhoeae* infections is monotherapy with the injectable extended-spectrum cephalosporin (ESC) ceftriaxone (Kularatne et al. 2018; Unemo and Shafer 2014).

Tuberculosis is a leading cause of mortality among infectious diseases. Antibiotic-resistant strains are hindering control of the tuberculosis epidemic around the globe (Bendre et al. 2021). Rifampicin is considered a surrogate marker of multidrug resistant tuberculosis and there were around half million (range 400000–535000) cases having rifampicin/multidrug resistant tuberculosis in 2019 (WHO, 2020; Neimz et al. 2012; Smith et al. 2012; Denkinger et al. 2014). Treatment for tuberculosis is very lengthy (6–18 months long for MDR or extensively drug-resistant (XDR) tuberculosis) and less effective than treatment of susceptible tuberculosis (Mesfin et al. 2018; Cheng et al. 2020). It has been estimated that fewer than 60% of MDR/rifampicin-resistant tuberculosis cases are treated successfully. Resistance to drugs used for treatment of MDR/XDR tuberculosis is continuing to rise and poses a major threat (Shivekar et al. 2020).

There are increasing reports of morbidity and mortality caused by variant strains of nosocomial Gram-negative pathogens such as carbapenem-resistant *Acinetobacter baumanii* (CRAB) (Isler et al. 2018). The current treatment for infections caused by CRAB relies on cefiderocol, a cephalosporin that is active against many MDR Gram-negative pathogens and was recently approved by the US Food and Drug Administration (FDA) for clinical use against CRAB with a variety of resistance. However, there have been reports of resistance to cefiderocol too, which poses a risk of increasing in the future as clonal propagation and selection will encourage these resistant variants to spread (Isler et al. 2018).

13.3 Status of Antimicrobial Drug Discovery and Development, and Evolution of Antimicrobial Resistance

The earliest uses of antimicrobials had a dramatic impact in reducing mortality from serious and life-threatening bacterial diseases in comparison with the absence of such therapy in the pre-antibiotic era. Subcutaneous use of sulfanilamide (sulphanilamide) lowers the mortality rate associated with acute meningococcal meningitis by 70–90% (Heselpoth 2014). With the approval of an increasing number of agents

within the same scaffold classes and the lack of new drug classes, it is perhaps not surprising that development of antibacterial agents would reach a saturation point (Shlaes and Bradford 2018; Heselpoth 2014).

Today, antimicrobials are the third most profitable class of drugs for pharmaceutical companies, surpassed only by central nervous system drugs and cardiovascular drugs. A recent study showed that the top five disease categories (including heart diseases, pulmonary conditions, mental disorders, cancers and hypertension) accounted for 31% of the increase in health care expenditure between 1987 and 2000 (De Oliveira et al. 2020; Ng 2015).

The development of newer antibiotics and further evolution of pathogens resistant to the same antibiotics have had significant impacts on the discovery of modern antimicrobial agents. Between about 1930 and 2005, various new antibiotics were developed (Fig. 13.1). However, from 1940 onwards, pathogens started to be

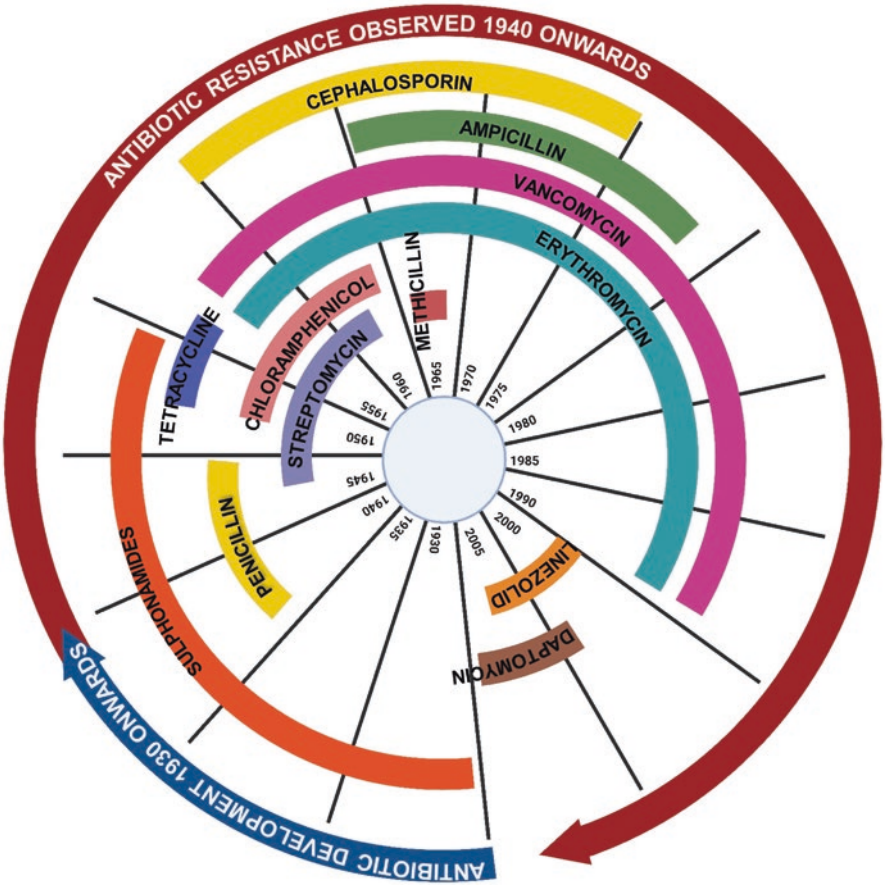

Fig. 13.1 History of antibiotic discovery and development of antibiotic resistance from 1930 to 2005

identified that were resistant to antibiotics that had previously been effective against those species. Federal agencies are aware of the needs to address the present challenges related to antimicrobial resistance and to simultaneously give more focus to stimulating antimicrobial drug development. These agencies have been using their combined resources to attempt to address these needs.

A strategy of modification of existing antimicrobials was initiated and successfully implemented during a period when the rate of discovery of novel drug classes suddenly slowed in the 1970s, and growing resistance problems forced researchers to look into possible modifications of the existing arsenal that could confer improved antimicrobial activity, improved sensitivity towards resistant organisms and less toxicity or adverse effect towards host. Possible approaches to tap into novel antimicrobial diversity include exploring ecological niches other than soils (such as the marine environment (Hughes and Fenical 2010)), borrowing antimicrobial peptides and compounds from animals and plants (Hancock and Sahl 2006), mimicking natural lipopeptides of bacteria and fungi (Aminov 2010; Makovitzki et al. 2006), accessing uncultivated portions of microbiota through a metagenomic approach (Gupta et al. 2018) and, finally, using the complete synthetic route pioneered during the early years of the antibiotic era.

The generally observed mechanisms of bacterial resistance to various antibiotics mainly include the following (Rao et al. 2018) (Figs. 13.2 and 13.3):

1. Enzymatic degradation leads to inactivation of antibiotics.
2. Enzymatic modification can alter the cellular targets of antibiotics, making them unable to bind effectively to the target site.

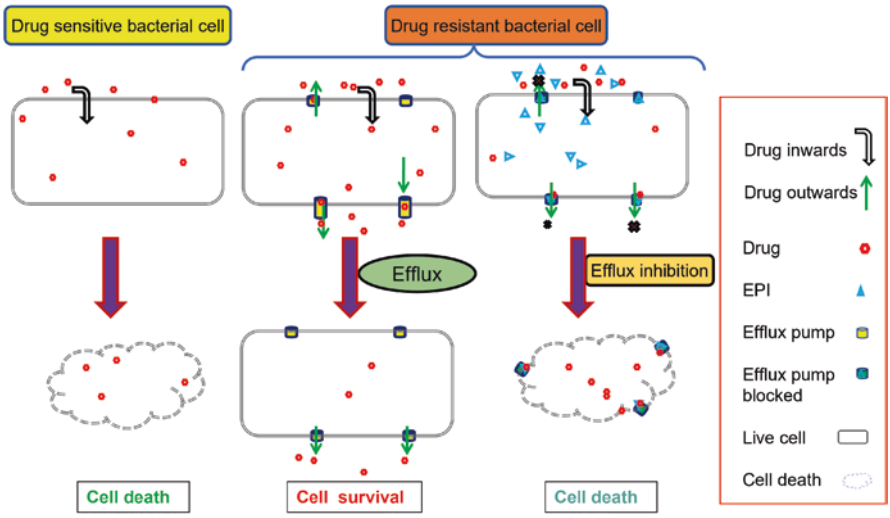

Fig. 13.2 Evolution of antibiotic resistance in bacterial cells and efflux inhibition. *EPI* efflux pump inhibitor

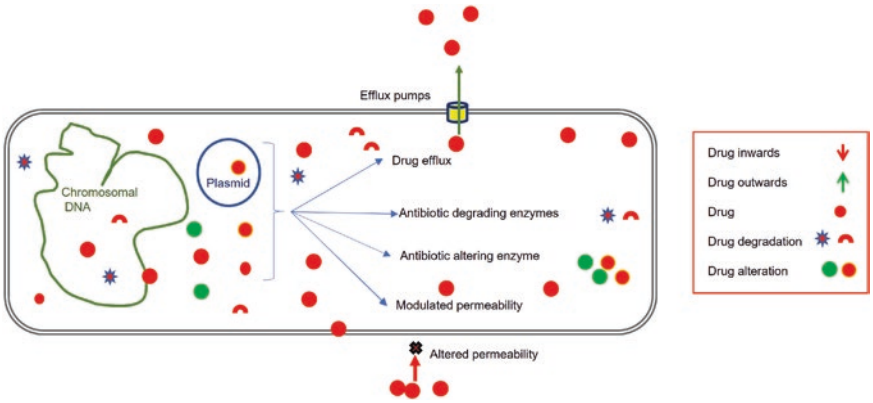

Fig. 13.3 Mechanisms behind antibiotic resistance

3. Alteration of cell membranes leads to abridged permeability of the antibiotic into the bacterial cytoplasm.
4. Active extrusion of antibiotics by membrane efflux pumps reduces cellular concentrations of antibiotics (Rao et al. 2018; Rath et al. 2019; Blair et al. 2015).

Cloning, polymerase chain reaction and gene expression techniques have been applied to detect natural genes in random recombinant clones derived from bacterial DNA libraries from soils and sediments (Mukherjee and Reddy 2020). A potential problem is that identification of functional resistance requires gene expression (transcription and translation) of the cloned genes in a heterologous host. To date, only *E. coli* has been used (Olivenza et al. 2019). Many questions remain; the roles of these environmental reservoirs in clinical resistance development are still hypothetical, and the primary metabolic functions of microbial populations are, as yet, unknown (Davies and Davies 2010).

Most bacteria were initially assumed to be commensal organisms and were considered harmless to the human system (Méthot and Alizon 2014). This is contrary to the current scientific evidence in which disease-causing bacteria have been identified alongside use of drugs in treating disease conditions. Despite the challenges emanating from clinical use of antibiotics, there is growing use of these molecules in bacterial infection treatment (Gupta and Datta 2019). However, various resistant strains have evolved and threatened the effectiveness of most antibiotics in clinical management of disease conditions. Molecular understanding of bacterial existence and its activities is key in preventing bacterial resistance (Ghai and Ghai 2018). In recent times, bioinformatics has played a vital role in drug discovery, gene sequencing, gene alignment and genera proteomics study. Adequate knowledge of the processes of bacterial resistance can precipitate the bioinformatics approach needed to unravel such resistance cases (Kaushik et al. 2018). Recently, new approaches to research involving bacterial resistance to antibiotics have appeared; these involve combinations of molecular understanding of bacterial systems with knowledge of bioinformatics. Consequently, many molecules have been developed to curb

resistance associated with different bacterial infections. Because of increased emphasis on the clinical relevance of antibiotics, the synergy between in silico study and in vivo study is well cemented, and this facilitates the discovery of potent antibiotics (Ndagi et al. 2020). Transfer of genetic traits through DNA is known to be the most common method of bacterial resistance; it involves acquisition of foreign DNA material through horizontal gene transfer (Munita and Arias 2016; Ndagi et al. 2020).

13.4 The Drug Discovery Pipeline and the Future

In respect of innovation in the development of new drugs and therapeutic biological products, the US FDA Center for Drug Evaluation and Research (CDER) reinforces the activities of the pharmaceutical industry at every step of the work. The closeness of new drugs and new biological products frequently offers new treatment options. Advances in health care achieved through previous historical innovations and approvals of antimicrobial drugs by the US FDA between 2012 and 2020 are listed in Table 13.1.

13.5 Drug-Resistant Microbes

In 1994, a global drug resistance surveillance programme was started in order to monitor trends in drug resistance. Its first report was published in 1997 and included data from 35 geographical settings for the period of 1994–1996 (Pablos-Méndez and Raviglione 1998).

After the first antituberculosis drugs—streptomycin, *para*-aminosalicylic acid and isoniazid—were introduced, resistance to particular drugs was noted in clinical isolates of *Mycobacterium tuberculosis* (Crofton and Mitchison 1948). This brought about a need to measure resistance accurately and easily. To control the drug resistance epidemic, it is necessary to gain insight into how *M. tuberculosis* develops drug resistance. This knowledge will help us to grasp how to avert the threat of drug resistance in addition to identifying genes associated with resistance to new drugs. *M. tuberculosis*, an aetiological agent of life-threatening disease, remains a major health problem, causing 1.5 million human deaths in 2019. Because *M. tuberculosis* has an ability to reside and replicate inside host tissue, it requires months-long treatment, which is difficult and often requires drug combination therapies, but these have a low success rate. Pervasive use of antibiotics has resulted in MDR, XDR and totally drug-resistant (TDR) infections, in addition to latent or persistent *M. tuberculosis* infections, for which antibiotic treatment is ineffective.

Acinetobacter species are non-fermentative, Gram-negative coccobacilli. There are at least 21 different *Acinetobacter* genospecies, 9 of which have been given formal species names (Lim et al. 2007). Risk factors for colonization or infection

Table 13.1 Antimicrobial drugs approved by the US Food and Drug Administration from 2012 to 2020 (Andrei et al. 2018; Bhutani et al. 2021; Ebied et al. 2020)

Generic drug names	Approval date	Brand name	Targeted microorganisms	Source	Purposes
Cefiderocol	November 14, 2019	Fetroja	Gram-negative bacteria	Siderophore cephalosporins	To treat complicated urinary tract infections in cases with only limited or no alternative treatment options
Imipenem, cilastatin and relebactam	July 16, 2019	Recarbrio	Gram-negative bacteria	β-Lactam antibiotic	To treat complicated urinary tract and complicated intra-abdominal infections
Rifamycin	November 16, 2018	Aemcolo	Mycobacteria	*Amycolatopsis rifamycinica*	To treat complicated diarrhoea
Omadacycline	October 2, 2018	Nuzyra	Gram-positive and Gram-negative bacteria	Semisynthetic compound derived from tetracycline	To treat community-acquired bacterial pneumonia and acute bacterial skin and skin structure infections
Plazomicin	June 25, 2018	Zemdri	*Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Enterobacter cloacae*	Derived from sisomicin	To treat complicated urinary tract infections in adults
Secnidazole	September 15, 2017	Solosec	*Atopobium vaginae*	Nitroimidazole	To treat bacterial vaginosis
Meropenem and vaborbactam	August 29, 2017	Vabomere	*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* species		To treat complicated urinary tract infections in adults

(continued)

Table 13.1 (continued)

Generic drug names	Approval date	Brand name	Targeted microorganisms	Source	Purposes
Delafloxacin	June 19, 2017	Baxdela	Gram-negative and gram-positive bacteria	Fluoroquinolone antibiotic	To treat acute bacterial skin infections
Bezlotoxumab	October 21, 2016	Zinplava	*Clostridium difficile*		To reduce recurrent *Clostridium difficile* infections in patients aged ≥18 years
Isavuconazonium sulfate	March 6, 2015	Cresemba	*Aspergillus* and Mucorales	Prodrug of isavuconazole	To treat invasive aspergillosis and invasive mucormycosis
Luliconazole	2014	Luzu	*Trichophyton rubrum*, *Microsporum gypseum* and *Epidermophyton floccosum*	Imidazole antifungal	To treat athlete's foot, jock itch and ringworm caused by dermatophytes
Pneumococcal 13-valent conjugate vaccine	2013	Prevnar-13	*Pneumococcus* sp.	*Streptococcus pneumoniae*	For vaccination against pneumococcus
Tobramycin	2013	Tobrex	*Pseudomonas* sp.	Aminoglycoside antibiotic derived from streptomycin	To treat exacerbations of chronic infection with *Pseudomonas aeruginosa* in patients with cystic fibrosis

with MDR *Acinetobacter* species include prolonged hospitalization, intensive care unit admission, cessation of mechanical ventilation, colonization pressure, exposure to antimicrobial agents, recent surgery, invasive measures and fluctuations in illness severity. AMR among *Acinetobacter* species has increased substantially in the past decade. MDR *Acinetobacter baumannii* is a opportunistic pathogen in the health care setting, where it causes infections such as bacteraemia, pneumonia, meningitis, UTIs and wound infections. This organism's ability to endure a wide range of environmental conditions and to survive on surfaces for prolonged periods of time make it a frequent cause of sudden infections and an endemic health care–associated pathogen. The resistance mechanisms used by *Acinetobacter* species are similar to those used by *Pseudomonas* species, but *Acinetobacter* species have not been studied as extensively. The mechanisms of resistance can generally be classified into

four categories: (1) activity of antimicrobial-inactivating enzymes, (2) reduced access to bacterial targets, (3) mutations that change targets or cellular functions, and (4) reduced drug uptake or drug efflux.

Pseudomonas aeruginosa is a leading cause of opportunistic infections. It can grow on a wide variety of substrates and can change its properties in response to changes in the environment. The identification of the entire genome sequence of *P. aeruginosa* and application of powerful DNA array techniques to reveal microbial gene expression under in vivo conditions should provide us with a clearer insight into the mechanisms that are involved (Eliopoulos et al. 2008; Lambert 2002). Inappropriate empirical use of antibiotic therapy for MDR/XDR *P. aeruginosa* infections has been associated with increased mortality and increased hospital costs. Antibiotic combinations are often used for treatment of these infections, although the value of such combination therapy in comparison with that of monotherapy is debatable. Use of amikacin and colistin in combination with antipseudomonal antibiotics offers the greatest coverage against MDR/XDR *P. aeruginosa,* but they are both associated with side effects and toxicity. In this scenario, new antibiotics with activity against MDR/XDR *P. aeruginosa* have been developed, and they represent a viable alternative option for treatment of infections caused by this organism (Asempa et al. 2019).

N. gonorrhoeae is an intracellular Gram-negative diplococcus. It exclusively infects human mucosa and can spread secondarily to other tissues. New strains of *N. gonorrhoeae* have now appeared that are resistant to the first-line treatment, ceftriaxone. The wide variety of gonococcal surface antigens and other factors in the immune response against *N. gonorrhoeae* weaken the likelihood of further development of this therapeutic resource (Salmerón et al. 2020).

The development and spread of resistance in *N. gonorrhoeae* have occurred mainly by addition of new DNA via conjugation and transformation, and determinants of resistance may be located on the chromosome or on extrachromosomal elements (plasmids). In contrast to Plasmid-mediated resistance can spread or transmit inter as well intraspecies levels, while chromosome-mediated resistance remains confined to the same species or descendants. Both forms of resistance can occur in one organism, and resistance to multiple antibiotics is common (Salmerón et al. 2020; Keese 2008).

Antifungal resistance can be innate (known as primary resistance) or acquired (known as secondary resistance). Many such resistance mechanisms have been described, such as biofilm formation (especially in *Candida albicans*), failure of intracellular drug accumulation or drug target alterations. The genome plasticity of human fungal pathogens is strongly associated with their ability to acquire resistance to antifungals. Some researchers have recommended use of other pharmacological classes and repurposing of old drugs used either as single antifungal agents or in combination with other known antifungal drugs (Gulati and Nobile 2016; Nobile and Johnson 2015).

The resistance of *Enterobacter* species to third-generation cephalosporins—such as cefizox (ceftizoxime), claforan (cefotaxime), tazimide (ceftazidime) and vantin (cefpodoxime)—is greater and is mostly due to widespread use of AmpC

β-lactamases. Treatment with these antibiotics may cause selection of mutants for AmpC overproduction. Modulation of the phenotype by host bacteria makes gene transmission less obvious, and this may, in part, explain why tracking and control of carbapenem resistance in Enterobacteriaceae has been especially difficult (Iredell et al. 2016; Blair et al. 2015). The evolution and spread of resistance in Enterobacteriaceae complicate the treatment of serious infections. Approximately 20% of health care–associated infections by *K. pneumoniae* and 31% of infections by *Enterobacter* species are due to strains that are resistant to third-generation cephalosporins. Resistance of *K. pneumoniae* to third-generation cephalosporins is typically caused by acquisition of plasmids containing genes encoding broad-spectrum β-lactamases, and these plasmids often also carry other resistance genes (Chong et al. 2011).

13.6 Global Approaches for Control of Drug Resistance

A global action plan (GAP) provides a framework for controlling the spread of antimicrobial resistance. At a meeting in 2015, the WHO World Health Assembly (WHA) adopted a GAP with five objectives to control the spread of antimicrobial resistance: (1) to improve awareness and understanding of antimicrobial resistance, (2) to strengthen knowledge through surveillance and research, (3) to reduce the incidence of infection, (4) to optimize use of antimicrobial agents and (5) to ensure sustainable investment in countering antimicrobial resistance (World Health Organization 2015).

To reap effective outcomes from the GAP, a multisectoral coordination action involves a ‘one health’ approach. This requires coordinated action among numerous international sectors and national agencies, including human and veterinary medicine; the agriculture, finance and environmental sectors; and well-informed consumers. The GAP recognizes the various efforts that nations need to make in order to combat antimicrobial resistance. It also discusses the economic factors that nations should keep in mind to encourage the development of alternative novel products by pharmaceutical companies (World Health Organization 2015).

13.7 The Indian Scenario in Brief

Following the WHA meeting in 2015, many countries agreed on and implemented their NAPs. In April 2017, India published its NAP for 2017–2021, documenting its priorities and implementation strategies for curbing AMR in India. The report was submitted to the 70th meeting of the WHA, held in Switzerland in May 2017 (Government of India 2017; Ranjalkar and Chandy 2019). The objectives of the NAP include improved awareness, enhancement of surveillance measures, strengthened infection prevention and control, research and development, promotion of

investment and collaborative activities to control AMR. On the basis of the NAP, various states of India have taken initiatives to make their own state action plans.

13.8 Conclusion

Antimicrobial resistance is one of the major causes of the current decline in public health and is increasing the challenges posed by disease in human society. This problem has also been accelerated by inappropriate prescribing and usage of antimicrobials; overconsumption of medicines; prolonged hospitalization; treatment failures; food spoilage; antimicrobial resistance among stakeholders; and improper control of, and regulations for, antimicrobial use in animals and food production, with consequent environmental exposure—as well as lack of newer drug development. However, proper functioning of public health care systems, promotion of good pharmacy practices, timely identification of diseases and adherence to standard treatment guidelines, rational use of antibiotics and immunization can be considered successful examples of implementation of appropriate action plans to help avoid antimicrobial resistance problems in the world. A global, collaborative and healthy research environment for new drug discovery; public commitment; and appropriate awareness about use of the available antibiotics are crucial factors in minimization or avoidance of bacterial resistance–related problems. We are still far from reaching that goal, but continued efforts, bit by bit, may lead to success in the coming decades.

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Chapter 14
Chemical Diversity in Fungi: Strategies to Mitigate Antimicrobial Resistance

Neha Kapoor, Richa Bhardawaj, and Lokesh Gambhir

14.1 Introduction

The emergence of antibiotic resistance is a swiftly evolving phenomenon in microorganisms owing to the excess utilization of antibiotics. In the current scenario, antimicrobial resistance (AMR) is considered as the prime threat to public health worldwide because of the increasing emergence of resistance observed in pathogenic bacteria. Acquiring AMR at a fastidious pace by many pathogenic microorganisms, which are no longer susceptible to the available antibiotics, requires an urgent attention for the development of effective preventive and treatment regimes. There exists an imperative need to cater to the threat of growing AMR emerging as a global crisis in healthcare system. The World Health Organization (WHO) has long been documented the need for an improved and synchronized comprehensive approach to combat the AMR. *Staphylococcus aureus* is a major pathogen contributing to the escalation in AMR incidence with the emergence of multidrug-resistant strains such as methicillin-resistant (MRSA) and vancomycin-resistant (VRSA) *S. aureus* capable of causing various infections viz. bacteremia, pneumonia, skin, soft tissue, endocarditis, and osteoarticular infections (Kumar and Schweizer 2005; Tong et al. 2015). In the context of hospital-acquired infection, *Enterococcus faecalis*, MRSA, *Klebsiella pneumonia, Pseudomonas aeruginosa*, and extended-spectrum β-lactamase-producing *Escherichia coli* (ESBL-producing *E. coli*) are reported to secrete a diverse array of resistance and virulence factors.

A significant rise in antibiotic-resistant Gram-negative bacteria, *Pseudomonas aeruginosa*, isolated from clinical setup (Fridkin and Gaynes 1999) has been well

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documented for being responsible for 10% of total hospital-acquired infections (Nagaveni et al. 2011). There are many routes via which microorganisms confer resistance against various antimicrobial agents. The main cornerstone treatment method entails the utilization of β-lactam class of antibiotics against a range of infectious diseases. Many pathogenic counterparts such as *Enterobacterales (*Paterson 2008*) are exhibiting resistance to* β-lactam antibiotics which can be channelized through efflux pumps, distorted antibiotic binding sites, and enzyme β-lactamases activity (Peirano and Pitout 2019). Cephalosporins and carbapenems are inactivated by many pathogenic microorganisms due to the cleavage of β-lactamase-mediated β-lactam ring (Pfeifer 2010). AMR has also found to be acquired through increased expression of certain genes and novel mutational events in few microbes (Barlow and Hall 2002).

According to the report of the Center for Disease Control and Prevention (CDC), a sharp increase in the mortality rate of approximately 23,000 deaths per year on an average was observed due to MDR-based infections. The growing burden of AMR has prompt the implementation of comprehensive, coordinated national and international action plans. The foremost financial report deciphering the impact of AMR gave a frightening picture depicting the exponential rise in AMR-related deaths from 700,000 to 10 million annually by 2050, if the issue remains unaddressed (O'Neill 2014, De Kraker et al. 2016). It would cost trillions of USD in the healthcare industry. The World Bank estimates that an additional 28 million people could be forced into extreme poverty by 2050, through shortfalls in economic output, unless resistance is contained. It is hypothesized that drug-resistant diseases could kill more people than cancer. As per the population modeling analysis to ascertain the burden of infections caused by antibiotic-resistant bacteria among EU and European Economic Area (EEA) in 2015, the overt effects of infections with MDR bacteria predominantly crop up in hospitals (63.5% infection cases) and other healthcare settings claiming approximately 72.4% of attributable deaths and 74.9% of DALYs per 100,000 population (Cassini et al. 2019). The inappropriate exploitation of antimicrobial agents for purposes other than treatment of infections has contributed in the selection for AMR in food production environments.

The inadequacy of appropriate strategies to mitigate growing AMR prevalence is posing adverse healthcare effects among patients. Hence, conventional treatment remedies of MDR infections are facing enormous challenge and are a matter of serious concern among healthcare facilities. Keeping in consideration the challenges being faced by existing antibiotics against MDR, the healthcare setup is in dire need of the development of novel, broad-spectrum, and effective scaffolds for their probable exploitation as antibiotics or innovative strategies to surmount antibiotic resistance.

14.2 Strategies for Antimicrobial Resistance Management

The rapid emergence of antibiotic-resistant bacteria in the last few decades seeks immediate, innovative research and development strategies. After the serendipitous discovery of penicillin by Sir Alexander Fleming, the golden age

for the development of many more antibiotics against deadly pathogens was marked. However, all of these amazing discoveries can impose adverse effects because microbial pathogens gradually evolve new ways to dodge the antibacterial drugs. AMR bacteria were categorized into three classes viz. critical, high, and medium based on their extent of resistance, mortality rate, and treatability as per the list proposed by WHO 2017. An alarming and frightening condition persists in infections caused by the Gram-negative bacteria (ESKAPE) viz. *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa*, and *Enterobacter.* These bacteria display resistance against numerous antibiotic drugs of the current pipeline including carbapenems and third-generation cephalosporins, being considered as last-resort antibiotics. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, is also included in the category of MDR bacteria and is being considered a top global health priority. In the last two decades, antibiotics therapy is facing major setbacks because of the absence of any antimicrobials in the pipeline. It is imperative to conserve the efficacy of antibiotic therapy and to refuel the current pipeline of antibacterial drugs. Further, the development of new drugs is very slow due to a lack of investment. Researchers are continuously investigating ways to reduce the incidence of antimicrobial resistance. Detailed insight into the exact mechanism by which bacteria confer resistance can provide a foundation for the development of novel treatment strategies. The researchers are in persistent search to reinforce the existing antimicrobial arsenal to conquer MDR bacterial defenses either by advocating fresh approaches/sources or by exploration of novel activity profiling of old drugs. However, the development of newer drugs seems to face both economic and scientific obstacles due to reduced profit and being derivative of previous drugs faces resistance from bacteria respectively.

Based on the above facts, it has become vital to hunt for novel and more effective antimicrobial drugs with potent scaffold and mechanism of action. Across the globe, scientific community is investing in huge efforts to unravel the new resources for the probable discovery of new moieties to combat antimicrobial resistance. In order to address the problem of AMR, World Health Organization (WHO) in agreement with members of Food and Agriculture Organization (FAO) and World Organization for Animal Health endorsed a joint Global Action Plan on Antimicrobial Resistance in year 2015. Furthermore, Natural products still remain the most vital reserve for the discovery of new and potential drug molecules (Newman and Cragg 2020, Strobel and Daisy 2003). For many decades, microorganisms are reported to manufacture diverse antibiotics that function in an antagonistic capacity in nature and most of the antibacterial agents in clinical or preclinical trials are either microbial products or analogs (Huang and Lin 2017, Pham et al. 2019). Henceforth, exploration of antimicrobial compounds from microorganisms is a promising way to convene the mounting risk of drug-resistant strains of human and plant pathogens.

14.3 Fungi as a Goldmine of Novel Chemistries for Curbing Antimicrobial Resistance

For more than a decade, numerous antimicrobial compounds have been discovered from fungi, with many more antimicrobials still being put ahead for clinical investigation every year (Saxena et al. 2019). Fungi are an enormously diverse group of organisms, with about 230,000 species distributed widely essentially in every ecosystem. Fungi are considered to be a persuasive reservoir of novel bioactive compounds with antimicrobial potency against currently drug-resistant strains of pathogenic microorganisms (Table 14.1). Five decades ago, a fungal metabolite, Aspergillomarasmine A was discovered which was more recently repurposed based on its capacity to combat the antibiotic resistance associated with metallo-β-lactamase7. Two Azaphilone compounds viz. penicilazaphilone B and C, purified from *P. sclerotiorum* M-22 inhabiting rotted leaf was reported to exhibit potent antibacterial activity ESBL *K. pneumoniae* ATCC 700603 with MIC values of 500 and 15.63 μg/ml respectively (Zhou et al. 2016). Another study highlighted the antimicrobial action of xylitol and oxysporone recovered from *Pestalotia* spp., associated with the mangrove plant, *Heritiera fomes* against MRSA with MIC value of 32–128 μg/ml (Nurunnabi et al. 2018).

Three new cerebrosides, alternarosides A–C, and new diketopiperazine alkaloid alternarosin A, purified from ethyl acetate extract of halotolerant *Alternaria raphani* exhibited antibacterial activity against *Escherichia coli, Bacillus subtilis*, and *Candida albicans*, with MIC values ranging from 70 to 400 μM (Wang et al. 2009). As per the study conducted by Nogueira et al. 2006, ethyl acetate fraction of phytopathogenic fungi *Diplodia maydis* and *Sclerotium rolfsii* recovered from the culture of mays and soya respectively exhibited potent antibacterial activity on the multidrug-resistant bacteria *Acinetobacter baumannii, Enterococcus cloacae, Klebsiella pneumoniae, Proteus mirabilis*, and *Staphylococcus aureus.* Of both the pathogenic fungi, *S. rolfsii* was observed to exert much potent activity against MDR *Acinetobacter baumannii, Klebsiella pneumonia,* followed by *Shigella* spp. and *Staphylococcus aureus.*

In the study documented by Arivudainambi et al. (2014a, b), ethyl acetate extract of endophytic fungus *Pestalotiopsis virgatula* VN2 and *Alternaria alternata VN3* isolated from the medicinal plant *Vitex negundo* L exert promising antibacterial activity against multidrug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. A partially purified fraction E from ethyl acetate extract of *P. virgatula* was found to inhibit *S. aureus* strain 4 with a zone size of 19.8 mm. Similarly, ethyl acetate extracts of three endophytes, including *Guignardia*, and *Cladosporium* recovered from the leaves of mangrove plant *Aegiceras corniculatum* displayed promising inhibitory activity against MDR bacteria under in vitro conditions. *Colletotrichum* spp. and *Guignardia* spp. inhibited MDR, *K. pneumoniae* with MIC of 4 and 8 μg/ml respectively. Further, *Colletotrichum* also exhibited promising inhibitory activity against *A. baumanii* with MIC of 0.5 μg/ml (Bin et al. 2014).

Table 14.1 Brief listing of natural potent bioactive compounds potent in mitigating the antimicrobial resistance isolated from diverse fungi

S. No	Name of compound	Source fungi	Target organism	References
1.	Sphaeropsidin A	*D. corticola*	• Methicillin-resistant *S. aureus* (MRSA) • *P. aeruginosa*	Roscetto et al. (2020)
2.	Fonsecinone A	*Aspergillus* spp. *Z120*	• ESBL-producing *E. coli* • *P. aeruginosa* • *E. faecalis*	He et al. (2016)
3.	Gliotoxin	*Aspergillus fumigatus*	• Methicillin-resistant *S. aureus* (MRSA) 2.08 • ESBL-producing *E. coli* 4.09 • Vancomycin-resistant *Enterococcus faecalis* VRE van A V4932	Stefan Svahn et al. (2012)
4.	• Diaporthin • Orthosporin	*Diaporthe terebinthifolii* LGMF907	Methicillin-sensitive *Staphylococcus aureus*, and methicillin-resistant *S. aureus*	de Medeiros et al. (2018)
5.	• Siccayne • Deacetoxyyanuthone A	*Penicillium* spp.	• Gram-positive bacteria • MRSA • Multidrug resistant	Li et al. (2005)
6.	Naphtho-γ-pyrones	*Penicillium* spp. HK1-22	MRSA strains	Zheng et al. (2019)
7.	Chlorohydroaspyrones A and B	*Exophiala* spp.	*S. aureus,* methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus.*	Zhang et al. (2008)
8.	Penicillstressol, isopenicillstressol, and 0Z-isocitreoviridinol (0.5–1 μg/ml)	*Penicillium* spp.	MRSA	Auckloo et al. 2017
9.	Chaetoglobosin A Chaetoglobosin C	*Chaetomium globosum* *Aspergillus fumigatus* isolate AF3-093A	MRSA ATCC 33591 *S. aureus* ATCC 43300 *Staphylococcus aureus,* methicillin-resistant *S. aureus*, and *Mycobacterium tuberculosis* H37R	(Dissanayake et al. 2016; Flewelling et al. 2015)
10.	((3S)-3,8-dihydroxy-6,7-dimethyl-a-tetralone (3))	*Cladosporium* spp. JJM22	MRSA CMCC(B) 63303	Wu et al. (2018)
11.	(22E, 24R)5, 8-Epidioxy-5a, 8a-ergosta-6,22E-dien-3ß-ol	*Cytospora* spp.	MRSA GIM1.771	Deng et al. (2018)

(continued)

Table 14.1 (continued)

S. No	Name of compound	Source fungi	Target organism	References
12.	Penicipyrrodiether A Pyrrospirone J	*Penicillium* spp. ZZ380	MRSA ATCC 43300 MRSA	Song et al. (2019)
13.	(*Z*)-Octadec-9-enamide (oleamide)	*Penicillium* spp. ArCSPf	MRSA	Farha and Hatha (2019)
14.	Versicolorin B Averufin	*A. versicolor* MF180151	MRSA	Hu et al. (2019)
15.	-cytochalasa-5(6),13-diene-1,21-dione-7,18-dihydroxy-16,18-dimethyl-10-phenyl-(7S*, 13E, 16S*, 18R*)	*Daldinia eschscholtzii* HJ001	MRSA ATCC 33591	Yang et al. (2018)

Two novel compounds Xanalteric acids I and II along with 11 known secondary metabolites purified from an endophytic fungus *Alternaria* spp. from the mangrove plant *Sonneratia alba* were evaluated for antimicrobial efficacy. Xanalteric acids I and II were observed to exert weak antibacterial activity against multidrug-resistant *Staphylococcus aureus*, whereas alternusin displayed broad-spectrum antimicrobial activity against several additional multidrug-resistant bacterial and fungal strains (Kjer et al. 2009). A bioactive compound namely, altechromone, was isolated from endophytic fungi *Alternaira brassicicola* ML-P08, which displayed antimicrobial activity against *B. subtilis, E. coli, Pseudomonas fluorescens*, and *C. albicans*, with MIC values of 3.9, 3.9, 1.8, and 3.9 μg/ml, respectively (Gu 2009). An endophytic fungus *Chaetomium cupreum* inhabiting ornamental plant *Mussaenda luteola* was reported to produce resorcinol type of lipid with potent antibacterial activity against *Mycobacterium, E. coli* (ATCC 25922), and *S. aureus* (ATCC 25923) with MIC of 6.3 μg/ml (Shylaja et al. 2018).

There exist numerous reports documenting the immense potential of various fungal endophytes against multidrug-resistant bacteria: *Aspergillus* neobridgeri (Sadrati et al. 2020) and *Aspergillus tubingensis* (Yadav et al. 2016), *Diaporthe terebinthifolii* LGMF907 (de Medeiros et al. 2018), *Phomopsis prunorum* (Qu et al. 2020), *P. griseofulvum* TPL25 (Luo et al. 2015), *Alternaria* GFAV15 (Yadav et al. 2020), *Alternaria tenuissima* OE7, *Pestalotia* spp. (Nurunnabi et al. 2018), Penicillium spp. CPCC400817 (Qi et al. 2019) and *Trichoderma koningiopsis* QA-3 (Shi et al. 2020). Fungal endophytes namely *Aspergillus terreus* and *Trichoderma virens* were found to secrete butyrolactone I and 9-epiviridol respectively. Both of the compounds displayed antibacterial activity against methicillin-resistant *S. aureus* (MRSA) with MIC values in the range of 128–256 μg/ml (Ratnaweera et al. 2018).

Mushroom, a fruiting body of any fungus, is being given considerate importance as food commodity and due to its medicinal attributes worldwide (Hobbs 1996). There are many reports documenting the antibacterial potency of various mushrooms against MDR bacteria. Methanolic extracts of frozen fungus *Amanita virosa* (Fr) Bertill and *Cortinarius praestans* Cordier collected from Slovenia exhibited the strongest antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Janes et al. 2007) The methanolic extract of fruiting bodies and mycelial

biomass of *Coprinopsis cinerea* (C2), coprophilous basidiomycetous mushroom, recovered from horse dung showed significant antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and carbapenemase-producing *Klebsiella pneumonia*e with a zone of inhibition ranging between 10 and 14 mm in diameter (MohanKumar and Savitha 2019). Two farnesyl hydroquinones viz. ganomycins A and B from *Ganoderma pfeifferi* were also reported to exhibit potent antibacterial action toward *S. aureus* and *MRSA* (Mothana et al. 2000). Methanolic extract of the fruiting bodies of *Tapinella atrotomentosa* yielded osmundalatone, 5-hydroxy-hex-2-en-4-olide and spiromentin C with strong inhibiting activity against MRSA (SZMC 6270) with MIC value of 250 μg/ml (Beni et al. 2018). Similarly, fruiting body extracts of *Pleurotus sajor-caju* (Fr.) Singer also displayed antistaphylococcal activity against MSSA and MRSA with a MIC value of 10 mg/ml.

Furthermore, the potential of fungal nanoparticles against multidrug-resistant microorganism has been embroiled in the literature. In the study reported by Neethu et al. 2018, silver nanoparticles of algicolous endophytic fungus, *Penicillium polonicum*, isolated from the marine green alga *Chetomorpha antennina* was found to exert strong bactericidal potential against multidrug-resistant, biofilm-forming *Acinetobacter baumanii* with MIC as well as MBC of 15.62 μg/ml and 31.24 μg/ml respectively. In a similar kind of study, silver nanoparticles of *Penicillium italicum* isolated from wasp nest soil were reported to exhibit antibacterial and antifungal activity against multidrug bacterial pathogens like *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *E. coli*, and *Shewanella putrefaciens* (Nayak et al. 2018). A recent study reported the biocidal activity of silver, copper, and zinc nanoparticles of *Fusarium solani* KJ 623702 against multidrug-resistant (MDR) *P. aeruginosa* and *S. aureus* as well as the mycotoxigenic *Aspergillus awamori, A. fumigatus,* and *F. oxysporum* based on the diameter of inhibition zone. The potent antibacterial effect was exerted by spherical AgNPs (13.70 nm) against *P. aeruginosa* with MIC of 21.33 μg/ml, whereas zinc oxide nanoparticles were found to be highly effective against *F. oxysporum* with MIC value of 24.7 μg/ml (El Sayed and El-Sayed 2020).

14.4 Strategies Adopted to Explore the Chemical Diversity of Fungi

14.4.1 Genome Mining

Genome mining refers to identifying the gene arrays responsible for producing secondary metabolites as natural products in the genomes of sequenced microorganism (Lee et al. 2020). Genome mining is entirely dependent on bioinformatics tools and analysis of the enzyme coding gene clusters with experimental verification of the expression in terms of product (Chavali and Rhee 2018). Initially, the concept of genome mining was validated in *Streptomyces coelicolor* and *Streptomyces avermitilis*, where a lot of unexplored hidden potential was explored which led to the

extension of the genome mining-based chemical prospecting to other microbes (Gross 2009). Various approaches of genome mining were put forward to accomplish this task (Fig. 14.1); novel technologies including single-cell genomics and metagenomics generated enormous data to be analysed. Multiple tactics are being used at present for big-data mining in microbial communities like:

Classical genome mining: It is the explicit exploration for genes that code for enzymes that play a vital role in the biosynthesis of secondary metabolites in (meta) genomic sequences. Aided by the analysis using high-end softwares, classical genome mining revolutionized the discovery of natural products in the past decade.

Amplicon sequencing which details the composition of the targeted microbial community uses specific marker genes including bacterial 16S rRNA and fungal internal transcribed spacer (ITS). Amplicon sequencing assigns and analyses the presence of different strains or types residing in uncultured microbial community. Its disadvantage lies in its disability in functional annotation. Therefore, metagenomics sequencing is used for resolving phylogeny and functions of strains present in the microbial biome (de Fátima Alves et al. 2018). Metagenomics sequencing can still have certain disadvantages like losing the genomes of low abundant microbes and closely related microbes can be read as repeats. Solution to such issues can be answered by metagenomics synergistically with single-cell sequencing. The approach may narrate the DNA compartmentalization into cells and may connect functions to their matching species (Embree et al. 2014).

Single-cell sequencing advantageously can differentiate and authenticate the individual functions in the community. Connecting the authenticated functions to specific species also helps in generating high-quality genome for species with low abundance. It has proven pivotal in identifying bacteria with alternative genetic codes. Due to all above said advantages single-cell genomics proved to be substantial to allow meaningful data mining and its interpretation of single-cell RNA sequence. Single-cell protein analyses are now a routine in analyzing transcriptomics of the cell (Hwang et al. 2018). The relationship between transcriptomic variation and genomic variation among a population of single cells was

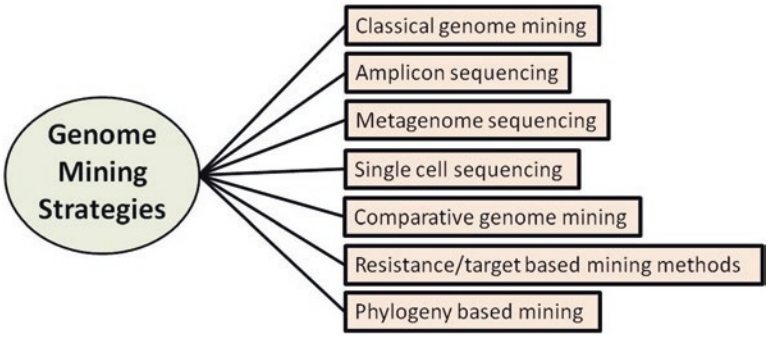

Fig. 14.1 The scheme representing the strategies adopted across the globe in genome mining-based prospecting of microbial flora for medicinal chemistries against AMR

demonstrated by multiomics single-cell sequencing—DR-seq and G&T-seq. A positive correlation was shown between gene expression and variation in the DNA copy number in individual cells. On the other hand, analysis of G&T-seq data corroborated the association among (sub) chromosomal copy number and expression level of genes located in variable regions in single cells. Both DR-seq and G&T-seq are capable in deciphering the single nucleotide variants (SNVs) in matched single-cell genomes and transcriptomes (Macaulay et al. 2017).

Metagenome sequencing: To uncover the wealth of novel genes so as to reveal the answers and understanding the processes of various biosynthetic pathways. Microbial-host metagenomics mining has proven to be significant to generate the hypothesis that can be validated experimentally. Metagenomic data mining can be used to uncover the correlations between the presence of functional genes and chemical diversity in various biosynthetic pathways (Delmont et al. 2011). The approach can also be used to retrieve the enzymes for biocatalysis and industrial use. By building an in silico metagenomic library from high-throughput sequencing data a resource is created that can be swiftly mined for enzymes (Jeffries et al. 2016; Guazzaroni et al. 2010).

Comparative genome mining: Microbial chemical diversity of high therapeutic gain needs no introduction. Genome comparison can highlight the genera which are diverse and profound producers of therapeutically and industrially important compounds. Gene networking, genome comparison can give a significant insight into the abundance of unique (unique to a single strain) and "extended families" (present in multiple strains) sequences (Shi et al. 2020). It can also identify new biosynthetic gene clusters as producers of natural compounds with potent antimicrobial activity. Comparative genomic approaches can be employed to recognize the signature genes present in the microorganism-producing antibiotic. Various algorithms have been developed to identify the gene clusters for several fungal antibiotics including searching for secondary metabolite genes of interest, like anti-SMASH, BAGEL, NP searcher, and PRISM. Mining for duplicated or altered housekeeping enzymes has also been used as strategies to predict novel biosynthetic clusters (Williams et al. 2020; Tran et al. 2019). Genome mining in combination with tools of molecular biology has paved the way for multiple novel proteins, including lantipeptides, cyanobactins, microviridins, and lasso peptides. Violaceae (violet), Rubiaceae (coffee), Cucurbitaceae, and Apocynaceae have also been mined for cyclopeptides which are head-to-tail cyclized peptides with a knotted arrangement of disulfide bonds (Hetrick and van der Donk 2017). Their structural complexity grants them bioactivities such as antibiotic, anti-cancer, and anti-inflammatory properties (Vassaux et al. 2019). Horizontal and vertical gene transfer is the underlining mechanism in the attainment and maintenance of precious secondary metabolites as confirmed by comparative genome mining which distinguished core and hypervariable regions in *Amycolatopsis* genomes (Adamek et al. 2018).

Resistance/target-based mining methods: Several potent bioactive compounds are toxic in nature that in turn results in impairment of the producing microorganism. Therefore, a self-defense mechanism is essential for the survivability of the organism. To detect secondary metabolite gene clusters based on the self-resistant

mechanisms of an antibiotic, producing organism forms the basis of resistance-based genome mining. A classical example of self-resistance mechanism is the duplication of the target gene that leads to resistance to the compound. This second resistant duplicated gene is most often recognized as the culprit producer of toxic compound. FRIGG (fungal resistance gene-directed genome mining) algorithm uses homology patterns for identifying biosynthetic gene cluster (Kjærbølling et al. 2019). Resistance mechanism may vary which include efflux pumps, removal of toxic compounds by enzyme degradation, and mitigation of the binding between the antibiotic and its target (D'Costa et al. 2011; Alvarez-Ortega et al. 2011). Resistance-based genome mining approach has also been successful in cancer and antibiotic therapy (Panter et al. 2018).

Phylogeny-based mining methods: Continuum of evolution in biosynthetic gene clusters led to structural diversities in natural products. Molecular phylogeny is a frequently used technique to demarcate the evolutionary history of a gene sequence. A major advantage of the phylogenetic approach is its fastidious analysis. A single gene is used by phylogeny-based mining to prospect a plethora of microbial metagenomes to identify bioactive compounds producing biosynthetic gene clusters. In the phylogeny-guided mining approach, marker biosynthetic genes based phylogenetic trees provide a road map to prioritize biosynthetic gene clusters and subsequent molecule expression studies (Kang 2017). Two of the most common enzyme families associated with natural product biosynthesis are PKSs and NRPSs. These genes are accountable for the biological synthesis of the several bioactive metabolites of microbial origin. Polyketide and nonribosomal peptide biosynthetic pathways are multienzyme complexes that sequentially construct natural products in an assembly line process from carboxylic acid and amino acid building blocks (Miyanaga et al. 2018). The types of bacteriocins and their distribution among various phyla viz. *Actinobacteria, BV4, Firmicutes, Glidobacteria*, and *Proteobacteria* are analyzed using phylogenetic genome mining. Phylogenetic trees from these alignments are used to study evolutionary relationships between bacteriocins and producer species (Joynt and Seipke 2018).

Ribosomal RNAs and mitochondrial cytochrome oxidase genes have been exploited for establishing phylogenetic relationship between leaf beetles, longhorn beetles, and weevils (Pistone et al. 2016, Aoki et al. 2018). 16s rRNA sequences are commonly used to represent the evolution of entire microbial genomes and its speciation (Clarridge 2004). Indolocarbazoles are a family of natural precursor to multiple anticancer drugs. Its biosynthesis involves the dimerization of two oxygenated tryptophans. Due to their clinical significance, the phylogeny-guided approach was employed to discover novel indolocarbazoles from soil metagenomes (Chang et al. 2015). An improved understanding of these phylogenetic patterns can direct bioprospecting efforts in combination with the metagenomic sequencing. A web tool called Natural Product Domain Seeker (NaPDoS) provides an automated solution to measure the microbial diversity and secondary metabolite biosynthetic gene diversity. NaPDoS analysis is based on the phylogenetic relationships of sequence tags derived from polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) genes, respectively (Ziemert et al. 2012). Other softwares include MEGA

and Seaview which produce efficient sequence alignments and phylogenetic trees (Hall 2013; Gouy et al. 2010).

14.5 Genome Mining as Tool of Novel Chemistries

Advances in genomics have resulted in the accumulation of large quantity of DNA sequence data in public databases. This availability of enormous publicly accessible data has made it possible for bioinformatics intervention to discover the novel chemistries of natural origin. This has greatly facilitated the identification and analysis of gene clusters likely to encode natural product biosynthetic pathways in sequenced genomes. However, the advancement in genomics has stimulated the development of multiple approaches and tools which were not available earlier to discover the natural bioactive chemistries (Zerikly and Challis 2009).

Biosynthetic gene cluster (BGC) analysis tools have been developed to efficiently predict the structure based on functional and architectural domains (Fig. 14.2). These tools have been used for broad profiling for modular assembly-line complexes, such as polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) (Tietz et al. 2017).

Biosynthetic gene cluster (BGC) analysis tools:

1. **anti-SMASH (antibiotics and secondary metabolite analysis shell):** Identification and annotation of secondary metabolite gene clusters. It combines automated identification of gene clusters with compound-specific analysis algorithms. It functions by analyzing the single genome (and integrates the

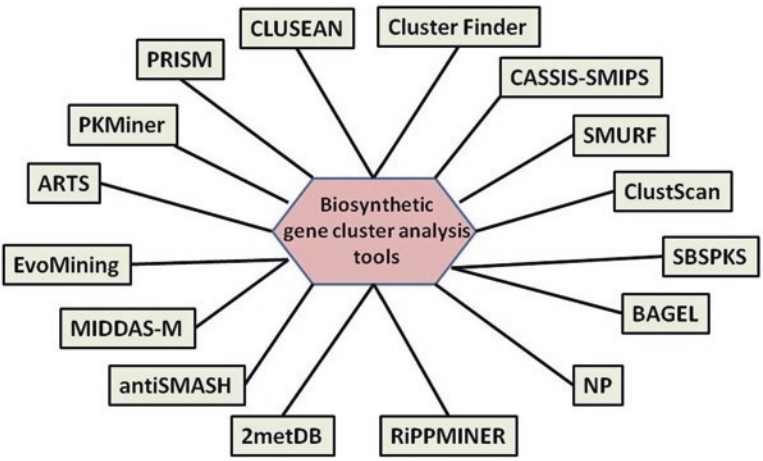

Fig. 14.2 A schematic representation of the web-based tools of biosynthetic gene cluster analysis available for identifying the secondary metabolite producing gene clusters in microorganisms based on the detection of signature gene clusters

RiPP output with analysis of other BGC classes). Along with bacterial genome mining, anti-SMASH is also used for fungal (fungiSMASH) and plant (plantiSMASH) genomes. It works by comparing encoded gene products with a manually curated library of hidden Markov models, describing a range of natural products producing biosynthetic genes (Blin et al. 2013).

2. **PRISM** (PRediction Informatics for Secondary Metabolism): Identification of nonribosomal peptides, type I and II polyketides, and RiPPs (Skinnider et al. 2017). It is used for structure prediction of nonribosomal peptides and polyketides. It executes structure prediction by recognizing biosynthetic genes attained by searching the query sequence with hidden Markov models library of secondary metabolism. It depends on the homology between experimentally characterized biosynthetic enzyme and gene clusters. PRISM is used for the analysis of prokaryotic genome and has limitation in eukaryotic setting
3. **BAGEL** (BActeriocin GEnome mining tool): It is one of the earliest tools developed for the identification of bacteriocin BGCs. BAGEL searches for RiPPs (also defined as class I bacteriocins by BAGEL), class II and III bacteriocins. BAGEL recognizes potent RiPP and bacteriocin open reading frames by employing the combination of genetic context and knowledge-based motif databases. It takes into account the processing, modification, transport, and regulation of RiPPs and bacteriocins. BAGEL2 focused on using profile hidden Markov models for bacteriocin identification led to updating of manually curated databases of known bacteriocins. BAGEL3 was aimed at discovering cyanobactins, sactipeptides, and linaridins producing biosynthetic genes (Jong et al. 2006).
4. **CLUSEAN** (CLUster SEquence ANalyzer): It is a BioPerl-based software pipeline for the annotation of gene clusters responsible for biosynthesis of antibiotics or compounds with novel bioactivities. CLUSEAN contains components for automated homology search, protein domain identification, and substrate prediction for the biosynthetic enzymes. CLUSEAN integrates standard analysis tools, like BLAST and HMMer, with specific tools for the identification of the functional domains and motifs in NRPS/PKS (Weber et al. 2009; Weber and Blin 2013).
5. **Cluster finder**: It is a probability algorithm based on hidden Markow model for predicting signature enzyme independent biosynthetic metabolic gene clusters in bacterial genomes. It is used in the recognition of gene clusters including NRPS clusters, PKS clusters, and NRPS-PKS hybrid clusters from different bacterial genomes (Cimermancic et al. 2014; Naughton et al. 2017).
6. **NP** searcher (Natural Product searcher) is an open-source software program used for prospecting genomes of microorganisms to recognize NRPS, PKS, or hybrid NRPS-PKS gene clusters with their putative bioactive product (Ren et al. 2020).
7. **ClustScan**: It is similar to NP searcher and appropriate for DNA sequences. Cluster Scanner is a commercial software package for gene cluster identification intended for NRPS, PKS, and hybrid NRPS-PKS enzymes. It also guides

the prediction of protein structure attained by the identified gene clusters (Cullum et al. 2011).

8. **SMURF** (Secondary Metabolite Unknown Regions Finder) is dedicated for the analyses of fungal genomes. It dictates cluster boundaries by employing combination of physical genomic distance and enzymes (Khaldi et al. 2010).
9. **CASSIS-SMIPS** (Cluster Assignment by Island of Sites) is also dedicated for fungal genome mining. It exploits the principle of high-density promoter regions in clustered genes compared to non-clustered genes. SMIPS (Secondary Metabolites by InterProScan) is used to predict anchor genes based on domains encoding biosynthetic enzymes in eukaryotic genomic sequences (Wolf et al. 2016).
10. **EvoMining**: It is based on the analyses of evolutionary theory and phylogenomics for predicting secondary metabolite-producing gene clusters in bacterial genomes (Sélem-Mojica et al. 2019).
11. **MIDDAS-M** (Motif-Independent De novo Detection Algorithm for Secondary Metabolite) gene clusters is another platform aimed for the detection of metabolite gene clusters in fungal genome in absence of signature enzymes (Chavali and Rhee 2018).
12. **ARTS** (Antibiotic Resistant Target Seeker) provides a platform for swift recognition of potent biosynthetic gene clusters. It is based on the cumulated analysis of target-directed genome mining and antibiotic gene cluster predictions. It depicts housekeeping genes shoeing resistance owing to gene duplication, colocalization with gene clusters, and horizontal gene transfer. Further advanced versions of ARTS allow the analysis of bacterial kingdom and its comparison with other genomes (Direnc et al. 2020; Alanjary et al. 2017).
13. **2metDB**: Genome mining for polyketides and nonribosomal peptides. 2metDB is a unique tool that offers the mining of PKS and NRPS gene clusters in whole-genome protein FASTA files. It combines the biochemical pattern and gene sequence knowledge to produce translated gene sequence data. Thus, it can be readily used to decipher the structural metabolites coded by the gene clusters (Bachmann and Ravel 2019).
14. **PKMiner**: Genome mining for type II polyketide synthases. PKMiner identifies novel BCGs of PKSs and polyketides based on aromatase and cyclise domains. It is used extensively in exploring novel polyketides that are synthesized by type II polyketide synthases and exhibits high pharmacological significance (Kim and Yi 2012).
15. **SBSPKS**: Sequence analysis of polyketide synthases. SBSPKS is used for multiple chemical analyses of experimentally characterized BGCs encoding PKS/NRPS. Its updated versions provide a platform for structural comparison of a secondary metabolite with intermediate and final products of BGCs. It employs profile-based searches based on HMM. It provides tools of identifying condensation, epimerization, and cyclization domains of NRPS (Khater et al. 2017).
16. **RiPPMINER**: It is used for genome mining and deciphering chemical structures of RiPPs, and BGCs. RiPPMiner predicts chemical structures based on predecessor peptides of lanthipeptides, lasso peptides, cyanobactins, and thio-

peptides. It contains two major modules based on peptides and genomes. RiPPMiner-peptide and genome takes sequence data as input and identifies class, structure, crosslinks, and cleavage sites for RiPP families and BGCs respectively (Agrawal et al. 2017).

14.6 Some Important Compound Analyzed and Identified Using Genome Mining Tools

14.6.1 Lasso Peptides

Lasso peptides are a lariat knot-shaped class of natural products present in bacteria that undergoes post-translational modification. Lasso peptides have a spectrum of bioactivities. It acts as receptor antagonist, inhibits enzyme and bacterial growth. The BGC of lasso peptides includes a total of three genes out of which one codes for a precursor protein and two for enzymes. Various genome mining approaches have been used to identify diverse lasso peptides in bacterial and archaeal phylum. Expression of gene cluster in *Escherichia coli* led to the production of an astexin-1 lasso peptide (Maksimov et al. 2012). The discovery of capistruin produced by *Burkholderia thailandensis* in 2008 marks the initiation point of genome mining era of lasso peptides. The precursor-centric approach and genome mining based on mass spectrometry have been the successful strategies adopted for lasso peptide prospecting (Maksimov and Link 2014). Ortholog neighborhood-based prospecting of mcjB-like genes predicted hypothetical lasso peptides producing multiple gene clusters in *Bacillus, Burkholderia, Caulobacter*, and *Sphingopyxis* (Duquesne et al. 2007).

14.6.2 Bacteriocin

Bacteriocins are group of antimicrobial peptides produced by bacteria, these are ribosomally synthesized peptides. Bacteriocin is a non-growth-associated product. However, it is widely abundant in bacteria owing to its genetic determinants. Phylogeny-based genome mining methods have been used to analyze and identify bacteriocins in various microbial biomes (Kjos et al. 2011). Microbial genome mining resulted in the discovery of 145 bacteriocin gene clusters coding for 290 potent bacteriocin precursors. Seven groups have been identified in the mentioned gene clusters based on comparative genomics (Wang et al. 2011). BAGEL3 uses an identification approach that combines direct mining and context genes-based indirect mining for lanthipeptides, sactipeptides, and glycocins (Jong et al. 2010; van Heel et al. 2013; Javan et al. 2018; Egan et al. 2018).

14.6.3 Cyanobactins

Cyanobactins are a cyclic ribosomal peptides produced by cyanobacteria. Genome mining predicted a horizontal transfer and precursor gene diversification dictated production of complex cyanobactins. Cyanobactin gene clusters are present in the *Oscillatoria, Arthrospira*, and *Microcystis* genera whereas absent from other generas including *Prochlorococcus* and *Synechococcus*. Phylogeny-based genome mining elucidated that cyanobactin biosynthetic gene clusters are grouped together into a single well-resolved clade, i.e., 31 cyanobactin gene clusters in a set of 126 cyanobacterial genomes (Leikoski et al. 2013).

14.6.4 Methanobactins

Methanobactins (Mbns) are a family of copper-binding bioactive compound in methanotrophic bacteria. Mbns are secreted under copper starvation conditions and then re-internalized as a copper source for the enzyme particulate methane monooxygenase. Genome mining studies have led to the identification and classification of operons with a number of putative transports, regulatory and biosynthetic proteins (Kenney and Rosenzweig 2013). Genome mining revealed the presence of Mbn-like precursor peptides encoding operons in 16 new species of both methanotrophs and non-methanotrophs. Aminotransferases, sulfotransferases, and flavin adenine dinucleotide (FAD)-dependent oxidoreductases were some other BGCs identified in these families (Laura et al. 2017).

14.6.5 Polyketide

Polyketides are produced by frequent decarboxylation and condensation cycles on polyketide synthases enzymes. Polyketides have been distributed across multiple and diverse bacterial genomes. Polyketides are diverse pharmacologically relevant secondary metabolites with exciting ecological functions. Polyketides include macrolides, enediynes, polyphenols, polyenes, and numerous other structural scaffolds. Many clinically relevant bioactive agents, such as antibiotics, immunosuppressants, cytotoxins, and cholesterol-lowering substances, are derived from polyketides. Bacillus and related genera in the Bacillales are remarkable producers of highly diverse polyketides (PKs). Bacillaene, Difficidin, Macrolactin, Paenimacrolidin, and Paenilamicin are some PKS isolated and identified from bacterial genomes (Boddy 2014; Helfrich et al. 2014; Aleti et al. 2015; Crawford et al. 2008; Chooi et al. 2013).

14.7 Genome Mining in Biosynthetic Gene Cluster-Based Families to Map the Pathway

Biosynthetic gene clusters (BGCs) are prearranged groups of genes concerned with the production of secondary specialized metabolites. A single BGC can produce one or multiple (up to over 100) comparable bioactive compounds with varying strength and/or specificity. Genome mining is the often prime move toward examining the potency of a microorganism to produce novel bioactive metabolites. BGCs can be associated with "superclusters" that function to produce two or more similar molecules. Access to whole-genome sequence data has highlighted the general occurrence of the cryptic BGCs, thereby renewing their attention for natural product discovery (Martinet et al. 2019). Different structural classes of BGCs exist including non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), terpenes, and bacteriocins. NRPS and PKS are popular targets for natural product discovery as they are known to synthesize a diversity of antibiotics and immunosuppressants with enormous pharmaceutical potential (Chen et al. 2020).

Transcriptomics in combination with metabolite analysis of different plant tissues, developmental stages, and/or elicitor-treated material is also being used to great effect to identify candidate genes for the synthesis of natural products. To identify groups of BGCs that are functionally closely related and encode the production of the same or very similar molecules, approaches have been developed to group BGCs into gene cluster families (GCFs). By examining shared absence/presence patterns of GCFs and compound families (derived via molecular networking of MS/MS spectra across different microbial strains), one can connect BGCs to their expressed products (Kautsar et al. 2021). BiG-SLiCE is a tool that makes it possible to perform GCF reconstruction at very large scales in a computationally feasible way. BiG-FAM can be used to explore the biosynthetic repertoires of specific taxa, investigate the taxonomic and architectural diversity of BGCs of known function, and obtain insights into the novelty of newly sequenced BGCs. Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository provides rich reference data to connect these GCFs to known products based on gene cluster homology, even with clusters from different fungal genera (Satria et al. 2021).

Many effective in silico-guided strategies have been designed using genetics, substrate labeling, and screening for predicted physicochemical properties to characterize novel medicinal chemistries from cryptic gene clusters (Kerstena et al. 2013). One of the comprehensively studied biosynthetic gene clusters from the microbiome is the colibactin pathway. The colibactin pathway has been implicated in colorectal tumorigenesis in mice models. The colibactin gene cluster is a ~55 kb BGC cluster that produces a family of polyketide-non-ribosomal peptide. This gene cluster is found among the *Enterobacteriaceae*, including *Escherichia coli, Citrobacter koseri, Klebsiella pneumoniae*, and *Enterobacter aerogenes*. The "pathway-targeted" molecular networking is used to more finely map expressed secondary metabolic pathways within complex metabolomes to

aid in secondary metabolite identification and characterization (Vizcaino and Crawford 2016).

14.8 Conclusions

Resistance to antibiotic is ever evolving and escalating problem due to population burden, overuse of antibiotics in clinical settings, genetic selection pressure, and poor solid waste. Researchers across the globe are diligently working to find the solution or alternate strategy to curb the mounted resistance. Among the prime strategies adopted is to isolate a novel natural compound from the diverse chemical signatures of plants and microbes. Among this, the relevance of fungi as a repository of novel medicinal chemistries has been persistently substantiated in the literature. Multiple compounds have been isolated and characterized from fungi showcasing the promising potency to curb the antimicrobial resistance. The presented chapter provides an in-depth insight on the myriad of compounds put forth from fungal repository along with its potency to mitigate AMR. Another aspect of the chapter highlights the importance of the advanced strategy, i.e. genome mining adopted to prospect the microbial flora for the ability to produce antibiotics based on the detection of signature secondary metabolite genes. Along with the identification of the gene clusters, multiple comparisons with the curated cluster and compound database are required by using web-based tools. Plethora of advanced tools are available that are used for gene cluster identification, screening for patterns in gene regulation or content. A biosynthetic gene cluster analysis tool that provides an analysis of structure based on functional and architectural domain has been used for broad-scale profiling in deciphering the secondary metabolite gene clusters. The extensive work in the field of genome mining has led to the deposition of a large quantity of DNA sequence data in databases. This has led to the foundation of building tools that helped in potentiating the discovery of natural products.

Antimicrobial resistance in the microbial pathogens is a major challenge for scientific community that poses a threat of morbidity and mortality. Antimicrobial resistance is a major challenge in developing countries compared to developed countries owing to the inappropriate use of easily available antibiotics. Sincere efforts are direly needed to mount a mitigating response against this microbial resistome. The importance of novel chemistries in the present chapter is a promising feat in highlighting genome mining as one of the strategies of prospecting microorganisms for developing novel therapeutic chemistries.

Conflict of Interest There is no actual or potential conflict of interest among authors.

Author's Contribution LG conceived and designed the context of the chapter. RB and NK contributed in summarizing the genome mining and fungal chemical diversity respectively. NK, RB, and LG wrote the manuscript chapter.

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Chapter 15
Phage Therapy as an Alternative Treatment in the Fight Against AMR: Real-World Problems and Possible Futures

Rajni Kaur and Nidhi Sethi

15.1 Introduction

The emergence of resistance amongst antimicrobials has raised grave concerns within the scientific community to further develop antibiotics to fight pathogens. The worldwide rise and spread of new resistant strains of microbes have undermined our capacity to treat common infections, which has resulted in delayed recovery, disability and death (https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance). The development of antimicrobial resistance (AMR) is a continuous process that evolves over time. After a certain period of time, the antibiotic drug loses its effectiveness towards the same pathogenic microbe (such as bacteria, fungi and parasites) it was previously effective. Antimicrobial resistance has led to a lack of availability of efficacious antibiotics and consequently increasing the risk of infectious spreads, severe illnesses and deaths. The year 2019 report released by the United Nations Ad hoc Interagency Coordinating Group (UN IACG) predicts that antimicrobial resistance could cause ten million deaths every year by 2050. Economically, antimicrobial resistance could constrain up to 24 million individuals to extreme poverty by 2030. Currently, at least 700,000 deaths occur every year globally due to drug-resistant diseases. Data show that only in the United States (US), 223,900 cases of antibiotic resistance-driven *Clostridioides difficile* had been reported in 2017, of which there were 12,800 casualties (https://www.cdc.gov/drugresistance/biggest-threats). Antibiotic resistance also adds to various complications in vulnerable patients going through chemotherapy, surgery, joint replacement

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and dialysis, and is considered a huge threat to the public health sector of developing countries and throughout the world. Global data from the year 2017 reflect that around ten million cases of tuberculosis (TB), an infectious disease caused by *Mycobacterium tuberculosis*, had been reported which included 558,000 cases of rifampicin-resistant patients, of which 82% were multi-drug resistant (MDR). There has always been greater number of TB cases (100 per 100,000 or higher) in underdeveloped and developing countries like sub-Saharan Africa, India, and Southeast Asia due to poor understanding of prescription or treatment, inappropriate choices and insufficient dosing of antibiotics, which attributes to their misuse and further adds to the increase in antibiotic resistance (Park et al. 2019). Extreme use owes to easy accessibility of antimicrobial medications as the same can easily be purchased without the prescription of a qualified health professional. It has been clinically proven that prolonged and unscrupulous use of antimicrobials is probably the main reason behind the development and spread of antimicrobial-resistant infections. Other factors include exceptionally vulnerable immunosuppressed patients (cancer patients, transplant receipt and AIDS patients), delicate elderly patients, or long stay in hospitals (Prestinaci et al. 2015). The inability of antibiotics to treat infectious diseases showcases the uncertainty towards the future of the healthcare system. Hence, there lies a constant need to design and develop new antibiotics that could guard us against widespread microbes causing infections and find alternative strategies to reduce the burden on the current overexploited antibiotic therapy. Several therapies have been developed in the fight against antimicrobial resistance. These include probiotics, prebiotics, synbiotics, bacteriocins, SMAMPs (synthetic mimics of antimicrobial peptides), antimicrobial peptides, IDR (innate defence regulator) peptides, peptidomimetics, bacteriophages, vaccines and immunoglobulins, antibacterial oligonucleotides foldamers, antibacterial nucleic acids and immune stimulation by P4 peptide, etc., each one offering its own benefits and limitations. Amongst the mentioned alternatives, this chapter focuses on bacteriophages and how they can be efficiently used clinically to cure bacterial diseases in the form of phage therapy, covering all the aspects of this alternative treatment.

15.2 Antimicrobial Resistance (AMR): The Crisis

Antimicrobial resistance is a natural phenomenon that occurs when microbes are constantly exposed to antibiotic drugs. Whenever an antibiotic is used against disease-causing microbes, the most susceptible microbes are either inhibited or killed, while some are resilient and have a natural resistance to the attack, as expressed in the species. Significant factors contributing to AMR include the unscrupulous use of antimicrobials, patient incompetence to follow the prescribed therapy properly, and unavailability of new drugs within a particular class of antimicrobials that could replace the ineffective ones. Broadly, resistance can be of two types: intrinsic resistance and acquired resistance. In the case of intrinsic resistance, microbes do not possess the target for the antibiotic, while in the case of acquired

resistance, bacteria device a mechanism that allows them to escape the action of the antibacterial drug molecule. Acquired resistance arises through the acquisition of host genetic material or mutations. These two forms of resistance are the most prominent ones and impart greater chances for the survival and multiplication of pathogen (Annunziato 2019; Prestinaci et al. 2015). Antibiotics have consistently been considered the most significant discovery over the last 70 years. Antibiotics are agents used to prevent and treat microbial infections in humans. Between 1950 and 1970, the use of antibiotics formally began with the so-called "golden age" of antimicrobial drugs. Unfortunately, the beginning of the antibiotics era tragically corresponds to the rise of the phenomenon of antimicrobial resistance (Fig. 15.1).

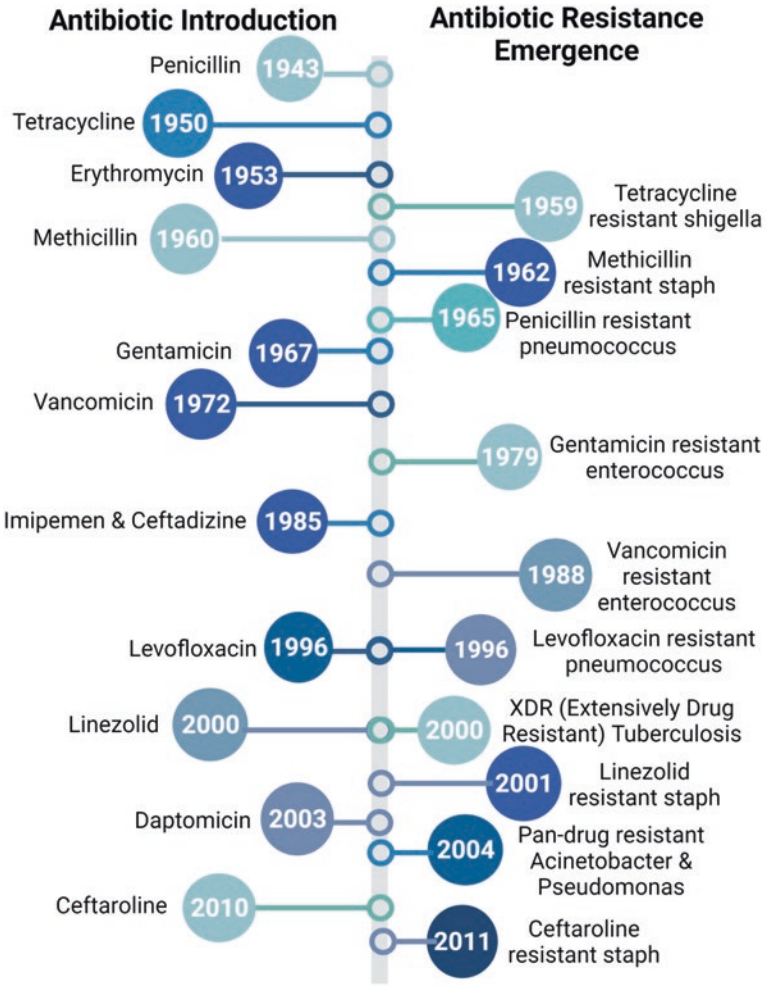

Fig. 15.1 Diagram showing major milestones in the journey of antibiotics and evolution of antimicrobial resistance (Annunziato 2019)

Since the discovery of penicillin, the first antibiotic, Alexander Flemming had expressed concerns and cautioned the scientific community for the higher demand of antibiotics which later could lead to their abuse. This has been widely acknowledged now that, while the utilization of antimicrobials has prompted the control and even elimination of infectious disease; their abuse and/or overuse has led to the development of resistant strains. After a couple of years of the golden age of antimicrobials, an alarming sign of developing resistance has been observed (Annunziato 2019).

15.3 Phage Biology Basics

Bacteriophages or simply phages are viruses that infect bacteria instead of humans, small in size (20–200 nm), diverse and the most abundant biological entities estimated to be around 10^{31}, amounting to ten folds the bacterial population. These are composed of protein or proteolipidic polyhedral capsid containing nucleic acid (mainly DNA and RNA) fragments and complex appendages. The capsid is generally joined to a tail made up of a helical protein structure necessary for the adsorption of the virion (viral particle) to the bacterial cell (Brives and Pourraz 2020). Characterized as natural intracellular bacterial parasites, phages lack the ability to reproduce independently and completely depend on the host bacterial cell for its survival. Phages bind to specific receptors on the bacterium's surface and inject the genetic material into the host cell. After entry into the host, phages may follow a lysogenic or temperate and lytic or virulent path. In the first case, these may amalgamate their genetic material into the host's genome and reproduce to form daughter cells. In the latter case, phages take control over the bacterial replication machinery and produce the next generation of phages followed by consequent lysis of the host cell (Lin et al. 2017). Figure 15.2 demonstrates the lytic and lysogenic life cycle of a bacteriophage. As illustrated, phage attaches to the host cell and rapidly kills the infected cell at the end of the growth cycle. However, in the lysogenic phase, the phage introduces its genetic material into the host genome or stays undetected as plasmids inside their host cells by embedding into the bacterial chromosome (the prophage state) (Inal 2003).

Despite the advances in molecular biology techniques, it still is a cumbersome task to classify the vast varieties of phages. The International Committee on Taxonomy of Viruses (ICTV), an international organization, emphasizes the classification of phages based upon taxonomical properties such as the nature of genetic material, morphology of the virion, physico-chemical characteristics and genome sequencing data. The largest order of bacteriophages, i.e. *Caudovirales*, constitutes more than 96% population of all the bacteriophages, includes caudate viruses having double-stranded DNA and virions having an icosahedral capsid with the DNA and a tail (enabling connection between host and phage to transport its genetic material into the bacterial cell). The order *Caudovirales* further constitute three families: *Myoviridae* (capsid size ~150 nm and a contractile tail); *Siphoviridae*

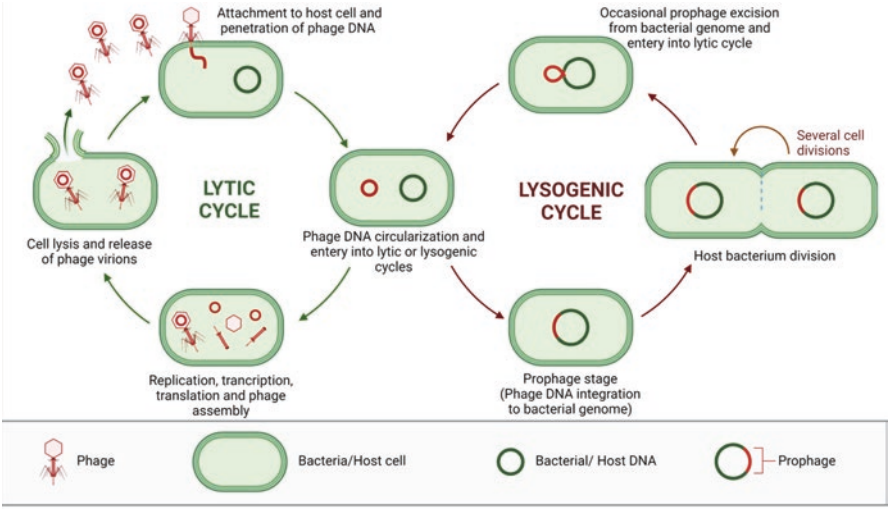

Fig. 15.2 Diagram showing schematic representation of lytic and lysogenic replication cycles of bacteriophage (Brives and Pourraz 2020)

(capsid size ~50–60 nm, with a long, flexible and non-contractile tail) and *Podoviridae* (capsid size ~50–60 nm and a short tail). The remaining 4% of the phage population demonstrate greater morphological diversity (representing as filamentous, cubic or pleomorphic viruses) and hence are studied on an individual basis (Royer et al. 2021).

15.4 Discovery and Early History of Bacteriophages

Earliest reports for bacteriophages date back to 1896 by Ernst Hankin who reported the antibacterial activity of waters from the rivers Ganges and Yamuna in India against *Vibrio cholera* infection (Hankin 1896). Two years later, a Russian bacteriologist also reported similar phenomenon while working with *Bacillus subtilis* bacteria (Samsygina and Boni 1984). Later it was in the early twentieth century that phages were concurrently discovered by British microbiologist Frederick Twort (1915) and French-Canadian microbiologist Felix d'Herelle (1917). It was Frederick Twort who first hypothesized the possibility of a virus to be exhibiting activity against bacteria. However, Twort did not pursue phage research further but it was d'Herelle who methodically explored the nature of phages and investigated their capacity to work as therapeutic agents (Summers 2016). While working at the Pasteur Institute in Paris, d'Herelle utilized the newly discovered phage therapy during World War I, being in-charge of the bacteriological examination of the cases. During this period, he investigated extreme and atypical cases of bacillary dysentery amongst French soldiers stationed at Maisons-Laffitte commune on the

north-western suburbs of Paris. While working on the faecal samples, d'Herelle found out that the not so familiar bacterial culture tend to lyse and clear in some time. When small portions of the cleared cultures were transferred to other samples of bacterial cultures, these would clear them as well. The mysterious antimicrobial activity is retained even after the removal of bacteria from the culture solutions. Following these observations, he went on to coin the term "Bacteriophage"with reference to assumed microbes that bring about the lysis and the lysis phenomenon itself (Ackermann 1917). d'Herelle expanded his research to United States, France, and Soviet Georgia by setting up phage therapy centres at several places (Carlton 1999; Myelnikov 2018).

Kharkov Mechnikov Institute was established in Kharkov, Soviet Ukraine in the year 1886 to carry out bacteriological studies investigated the Donbass region affected by frequent outbreaks of typhoid, scarlet fever and dysentery between years 1929 and 1935. Several therapies such as scarlet fever serum, typhoid vaccine and bacteriophage against dysentery were tried. The scientists and technicians were successful in making phage preparations against Shiga strain of dysentery bacillus (*Shigella dysenteriae*). Later Mel'nyk also isolated bacteriophages from areas near those water sources and used phage preparations against Shiga sub-strains (cultured from the faecal matter of patients). Similar to d'Herelle's findings, the phages were found to lyse the infective microbes, as depicted by clear cultures. The consequent clear cultures were first mixed and then filtered through the bacterial filter to trap bacteria and debris; permitting the passage of phage for further use. This area-specific use of phages to fight localized strains helped deducing the specificity of bacteriophages (Myelnikov 2018). However, with the advent of antibiotics, a decline in further development and use of phage therapy was observed. Initially, two audits on phage therapy were published in the Journal of the American Medical Association (JAMA) in 1934 and 1941, explaining challenges in the utilization of phages and issues concerning their efficacy. During the same period, several derivatives of sulphonamides or sulfa drugs (potent antibacterial compounds) were introduced in Germany and there was enormous production and utilization of antibiotics in the United States in the 1940s. Hence, these sulfa drugs were considered one of the noteworthy accomplishments in the history of therapeutic agents also contributing to the sharp decline of phage therapy in the western world (Brives and Pourraz 2020). However, to overcome the earnest problem of antibiotic resistance arising due to multi-drug-resistant bacteria, interest in phage therapy has revived.

In the year 2017, WHO (World Health Organization) released a list of some disease-causing microbes consisting of antibiotic-resistant bacteria that pose the greatest risk to global well-being. Some susceptible microbes included carbapenem-resistant *Acinetobacter*, *Candida auris*, *Clostridioides difficile*, carbapenem-resistant *Enterobacteriaceae*, drug-resistant *Neisseria gonorrhoeae*, drug-resistant *Campylobacter*, drug-resistant *Candida* vancomycin-resistant *Enterococci* (VRE), erythromycin-resistant Group A *Streptococcus* and clindamycin-resistant group B *Streptococcus*. Therefore, the need to develop phage therapy for the treatment of common bacterial infections like sepsis, some forms of diarrhoea and sexually transmitted infections increases substantially as the world is running out of effective

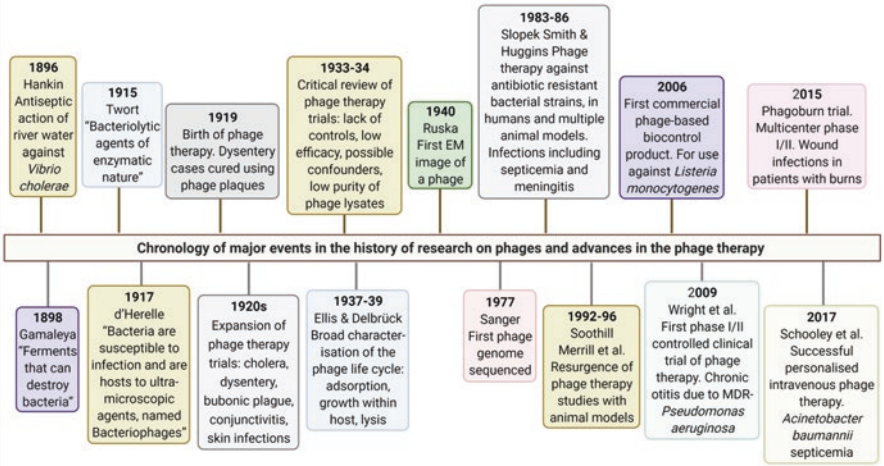

Fig. 15.3 Chronology of major events in the history of research on phages and advances in the phage therapy (Gordillo Altamirano and Barr 2019)

antibiotics (https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis). A brief timeline of events occurring in the development of bacteriophages and phage therapy has been summarized in Fig. 15.3.

15.5 Mechanism of Action of Bacteriophages

Based upon replication cycle, bacteriophages can proceed through lytic and lysogenic mechanisms (Fig. 15.2). Bacterial lysis occurs when the infected cells tend to release the lytic phage progeny. Scientific investigations on lytic mechanism of phages provide insights into the phage therapy. Some bacteriophages utilize amurins (single proteins) which inhibit the synthesis of bacterial peptidoglycan whereas others use the holin-lysin systems. In the year 1992, a model was hypothesized which stated that lysis by phage involved two proteins: holins and endolysins. The model was principally based on molecular and genetic studies carried out on phage lambda. During the morphogenesis of the infectious cycle, the holin encoded by lambda S gets accumulated in the membrane, forming large pores in the cytoplasmic membrane and assisting the endolysins to escape and attack the peptidoglycan and consequently affecting the physical integrity of cell wall of bacteria (Moghadam et al. 2020; Young 2014). Holins are an extremely different class of small phage-encoded membrane proteins. There are three classes of holins based on the differences in amino acids sequence. Class I incorporates proteins that have more than 95 amino acids residues and are represented by *Escherichia coli* phage λ S105 protein and *Staphylococcus aureus* bacteriophage p68 hol15 protein. Class II holins have 65–95

amino acid residues and are characterized by *Clostridium perfringens* bacteriophage Φ3626 hol3626 protein and Lambdoid phage 21 S protein. Class III holins are represented by phage ΦCP26F holin (Dewey et al. 2010). Genetic and biochemical methodologies on S105 suggest that it is the most extensively studied holin. It is a 105 amino acid residue polypeptide with three transmembrane domains (TMDs) encoded by the S gene of phage. S105 produces lethal lesions (~340 nm in diameter) in the bacterial lipid bilayer.

Endolysins, also known as phage lysins, are enzymatic proteins acting through hydrolysing peptidoglycan and subsequent cell wall degradation. They possess muralytic action on the bacterial cell wall mediated through amidase, endopeptidase or glycosidase action leading to bacterial cell destruction. At the end of their phage replication cycle, penetration into the peptidoglycan layer leads to osmotic lysis which eventually causes bacterial cell death and promotes virion progeny release (Cisek et al. 2017). The structural differences in the cell wall of gram-positive and gram-negative bacteria reflect upon the differences between their enzyme targets (Schmelcher et al. 2012). The impact of endolysins on bacterial cell walls is scheduled by certain holin genes which are specified in the dual start model. In this model, the holin gene is an open reading frame that encodes two proteins, holins and antiholins. The proportion of holin to antiholin (holin antagonist) decides the time of release of endolysins. For instance, Class I holin gene, the S gene of bacteriophage lambda not only encodes the effector holin, S105 but also an inhibitor, S107 with a Met1-Lys2-Met3 extension at the terminus (Shi et al. 2012).

15.6 Benefits and Limitations of Phage Therapy

Phage therapy has numerous advantages that make it an attractive option in contrast to antibiotics. Bacteriophages have high specificity for bacterium, unlike antibiotics, which are more active against a wide spectrum of microorganisms and probably cause secondary infections, e.g. with yeast, dysbiosis and other side effects (Romero-Calle et al. 2019). In addition, phages are dominant in nature and producing new phages takes fewer time rather than production of antibiotics. Thus, the development of phage therapy is inexpensive as compared to the production of antibiotics. One of the reasons why antibiotics are rendered ineffective is their metabolism and excretion by the body without reaching the site of infection. However, phages on administration replicate at the site of infection where the host organisms are located and then spread throughout the body. A comparative between phage therapy with conventional antibiotics has been summed up in Table 15.1 (El-Shibiny and El-Sahhar 2017; Romero-Calle et al. 2019).

Generally, phages target the surface receptors of the bacterial cell through virulence factors which later kill the bacteria by rupturing cell wall and cell membrane and eventually leading to the death of bacteria. Also, it is difficult for bacteria to develop resistance against phages but there are a few reports citing incidents of resistance within bacterial species attributed mainly to alteration and attenuation of

Table 15.1 Comparison between antibiotic and phage therapy

Characteristic feature	Antibiotic therapy	Phage therapy
Specificity	Low	High
Side effects	Moderate to high	Usually low
Spread of bacterial resistance	Broad spectrum	Narrow spectrum with some exceptions, phages tend not to cross-species limit
Delivery target	Moderate	Moderate to good. Can enter the blood-brain barrier
Formulation	Fixed	Mostly fixed, sometimes variable
Regulation	Well established	Under process
Kinetics	Single hit	Single hit or self-replicating
Development costs	High	Low to moderate
Immunogenicity	Not fixed	Likely low, but not well established
Clinical validation	Numerous trial studies	Relatively few trials studies
Fate of the drug	Metabolic degradation of the molecule, as it functions	Exponential growth in numbers, so that the drug replicates at the site of diseases, where it is required

phage virulence (El-Shibiny and El-Sahhar 2017). On comparison of phage therapy to traditional antibiotic treatments for use in the cure of bacterial infections, phage therapy offers extraordinary advantages over them. However, the phage therapy like other therapies is not devoid of its limitations. Despite being the largest group of organic entities on earth and offering numerous advantages, application of phage therapy is accompanied by various limitations as outlined in Table 15.2 (Moghadam et al. 2020).

15.7 Phage Therapy against Bacterial Infections in Humans

Since their discovery in early 1900s, bacteriophages have been widely used clinically to treat numerous bacterial infections accompanied by cases of conflicting outcomes in phage trials in the 1930s. Issues concerning the safety and efficacy of phage therapy were also raised in this period because of lack and inappropriate characterization, problems in production and purification of phage preparation (Gordillo Altamirano and Barr 2019). To overcome these obstacles, several clinical trials have been carried out and a few have been completed in recent years (Furfaro et al. 2018). Studies have shown that humans are constantly exposed to bacteriophages everyday and they help in their well-being. However, a number of factors still need to be explored while performing the clinical trials (Parracho et al. 2012). Bacteriophages have been utilized to treat bacterial diseases occurring in diverse body locales with different arrangements. Eastern Europe has a long history of using bacteriophages against bacterial infections (Summers 1999). Various mixed phage cocktails are sold in Russian drug stores as registered items. The

Table 15.2 Advantages and disadvantages of phage therapy in the treatment of bacterial infections

Advantages of phage therapy	Disadvantages of phage therapy
Active against both gram-positive and -negative bacteria	Bacteria capable of developing resistance against phages
Faster isolation and lower cost of development	Do not replicate in absence of target organism
Relatively lower side effects	Phages may act as carriers of bacterial virulence factors or antibiotic-resistance genes
Wider application in food preservation	Immune system perceives phages as invaders and hence, can be removed rapidly
Disruption of bacterial biofilms, XDR and MDR	Lack of regulatory guidelines
Can affect immune system by diminishing C-reactive protein mean values and leukocyte count	Release of endotoxins, superantigens and induction of inflammatory cascade after phage lysis of bacteria that may lead to multiple organ failure
Help reducing damage to normal microbiome	Insufficient data on the function of genes obtained from known phage genomes
Prevent overgrowth of secondary pathogen	Extrapolation of *in vitro* phage growth data to *in vivo* response is difficult
Rapid distribution throughout the body	Needs to identify phage specificity for exact host bacterium
No cross-resistance development to antibiotics	Exclusivity while using lytic phages is a concern
Cocktail of phages, more advantageous, has greater impact on target bacteria	Time-consuming task to diagnose a pathogen in clinical microbiology laboratory and then use a specific bacteriophage for treatment
Recognize different receptors on the surface of cell	Not yet recognized as pharmaceutical drugs
	No health insurance cover provided for phage treatment

XDR: extensively drug-resistant, *MDR:* multidrug-resistant

bacteriophage therapy has been best examined for topical application on bacterial infections occurring in human skin (Sulakvelidze et al. 2001). The first human phage therapy has been accounted for the treatment of skin infections caused by *Staphylococcus aureus*. During the 1980s, phage preparations at the Eliava Institute, Georgia were given to human volunteers with no reported adverse effects. They were mostly effective on immunocompromised patients, new born children and in cases of pelvic inflammatory illnesses. However, it has also been observed that phage preparations have high efficacy in early phases of diseases (Abedon et al. 2011). It is a known fact that burn surfaces quickly get colonized by microorganisms which are fit for producing biofilms and are regularly impervious to multiple antibiotics. Phage treatment might have been utilized to treat burns and prevent sepsis during and after world war II in Eastern Europe (Morozova et al. 2018).

The largest clinical trial on phage therapy carried out in Europe was the Phago Burn trial, in the year 2013. This clinical trial fulfilled the criteria for both Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP). In this randomized but controlled phase I/II clinical trial, 27 patients experiencing burn wounds

infections were enrolled from hospitals situated in France and Belgium. Patients were randomly treated with phage therapy with a cocktail of 12 lytic phages and a standard preparation (1% sulfadiazine silver emulsion cream) to compare efficacy and tolerability of both therapies in patients infected by *Pseudomonas aeruginosa*. Both the formulations were applied topically for 7 days with a 14-day check. In general, bacterial load in burn wounds was less in treated groups but the process was slower than in the control group. No adverse effects were observed in the phage treated group. The restricted adequacy of the phage cocktail was accounted for an essential drop of phage titre. This resulted in lower concentration of phages in the patients than the ones assessed first. Also, in these participants, phage treatment failed as bacteria were found to be resistant to low doses of phage (Jault et al. 2019).

A combination of eight bacteriophages targeted against *P. aeruginosa*, *S. aureus* and *Escherichia coli* were applied on venous leg ulcers in around 39 patients and no side effects were observed relating to the treatment (Rhoads et al. 2009). Another randomized clinical trial was conducted on a group of 24 patients with a persistent otitis externa condition using a topical phage preparation against *P. aeruginosa* and the patients were successfully treated without any adverse effects. Besides this, a combination of *P. aeruginosa* and *S. aureus* phages was developed for application on the skin of infected patients (Wright et al. 2009). In Eastern Europe, oral phage delivery has also been utilized against intestinal diseases nowadays. Nonetheless, before studying the efficacy of phage therapy on numerous people in clinical trials, it is fundamental to first evaluate their safety in humans to guarantee that they do not cause adverse reactions when given orally (Sulakvelidze et al. 2001). While most clinical trials have failed to provide promising evidence of the efficacy of the phage therapy, some of the few case analyses show phage therapy as a successful method to treat life-threatening infections (Pires et al. 2020). For example, a novel method has been used to prepare personalized therapeutic bacteriophages cocktails to protect patients from life-threatening multidrug resistance caused by *Acinetobacter baumanni* infection. This case further consolidated the utilization of phage therapy in treating patients experiencing MDR bacterial infections with limited therapeutic options (Schooley et al. 2017). It is important that the western world should acknowledge the phage therapy and ramp up the clinical trials for their commercial use. There are numerous observational studies that have been performed but have encountered restrictions such as sample sizes and inadequacy in control groups. Every clinical case requires robust clinical trial data to be submitted to the regulators for the improvement of existing clinical guidelines (Payne and Jansen 2003).

15.8 Phage Therapy: Problems and Alternative Solutions

As an antibacterial therapeutic, phage therapy has not been successful in completely replacing antibiotics as alternative. In order to establish phages as therapeutics, lot more investigations need to be done to uncover every aspect of bacteriophages. Due to reluctance of using phages, they have not been tested for their effects

systemically in animal models and hence, they have a rather simplified pharmacokinetic profile. Merrils and coworkers in 1973 first reported infusion of high titre of lambda phage into non-immune germ-free mice. They concluded that phages were quickly cleared by the spleen, other filtering organs and liver (Geier et al. 1973). It has been observed that selected phage strains can remain in circulation for over a period of many days and continue to be accessible for interaction with possible systemic bacterial infectious agents by genetic selection method (Vitiello et al. 2005). This capacity of phages to stay in the circulation for a longer period and clear the toxins and antibiotic resistance genes may be helpful in the administration of phages. This property can further help design and develop phages which are effective with improved activity for specific bacterial strain (Adhya et al. 2014). Another limitation of phages is their moderately narrow spectrum of activity. Apparently, it was thought that the fundamental restricting factor for their clinical use is the number of phages accessible to doctors, which appears to be irrational after recognition of phages to be the most abundant organic entity on earth. The uniqueness of phages for bacterial strains infers the need to have a wide variety of phages with strong lytic action for fighting pathogenic bacteria in the given patient.

After the First International Congress on Microbes and Viruses in 2010 at the Pasteur Institute in Paris, an article on bacteriophages was published in 2011 in which the authors proposed two approaches known as 'prêt-à porter' and 'sur-mesure'. In the 'prêt-à-porter' approach, fixed cocktails of several phages (around 10) are brought together to ensure that at least one the phage will be effective on the bacterial strain carried by the patient. This approach helps standardization of phage cocktail for commercial use whereas in the 'sur-mesure' approach, the patients will be administered only few phages that are specifically active on the infecting strain (Brives and Pourraz 2020). Phage preparations are often contaminated by bacterial debris, especially, macromolecules derived from the host bacteria and culture media with significant amount of endotoxins (Raetz et al. 2007). Injection of even modest quantities can result in cell injury, toxic shock and can be fatal to patients. However, various endotoxin removal methodologies and commercial kits have been developed which help in purification of phage preparations (Merabishvili et al. 2009).

Other key problem associated with phages is the inability to establish scientific proof of phage efficacy. Several articles in defence of phage therapy have been published in Europe in the recent couple of years, stating that the deficiency of clinical preliminaries exhibits the efficacy of phage due to their specificity whereas in infectious diseases, the antibiotics developed in the course of recent years are themselves quite specific, which has not kept them from demonstrating their adequacy (Morrison and Ulevitch 1978).

15.9 Future Challenges and Role of Bacteriophages

Unlike other antimicrobials such as antibiotics, phages show greater diversity in mechanism of action and can be much safer in many instances (Chan et al. 2013). The challenges for phage therapy include: how to put forward the positive attributes

of phage therapy in presence of existing regulatory practices and how phages would find a way into the current economic models that support the distribution and utilization of antibacterial agents (Fig. 15.4) (Międzybrodzki et al. 2012). Phage therapy appears to flourish especially in regions where the regulatory authorities are moderate or with lesser restrictions (for example, in Poland and some countries of undivided Soviet Union). Scientists and researchers believe that phage therapy shall show expansion in years to come as promising candidates for use as antibacterial agents to overcome infections in western medicine system. Thus, it is anticipated that in the following 5–10 years, phage therapy would ascertain its way into clinical practice to cure life-threatening, ongoing chronic bacterial infections that cannot be treated using available antibacterial drugs (Sommer and Dantas 2011). To scale up the production of phages, there are significant issues that have not been satisfactorily addressed. These include removal of endotoxins and pyrogens which are delivered from the ruptured cells during phage lysis after administration of phage preparation. However, it is quite possible to optimize such outcomes. Recently, an endotoxin removal kit has been introduced which purifies the phage preparation before use in clinical trials (Merabishvili et al. 2009). Literature reports show that preliminary clinical studies have been attempted on cocktail of phages and have cleared phase I, showing no adverse reactions related to the utilization of phage cocktails, thus paving way to phase II clinical trials (e.g. APS Biocontrol Limited, Dundee, United Kingdom). Clearance of phage therapy in phase I and II encourages positive model for subsequent investigation in phase III (Rhoads et al. 2009). Another major barrier in bacteriophage delivery is the removal of phages by the immune system. Kim et al. studied the immune response by human body after phage administration by conjugating phage with polyethylene glycol. Results revealed that there was a decrease in the levels of helper T cells and an increase in the blood circulation time in contrast to unmodified phages (Kim et al. 2008).

Currently, phage therapy has been investigated only on fast dividing bacterial species and not on slow dividing bacterial species, where it needs to be developed

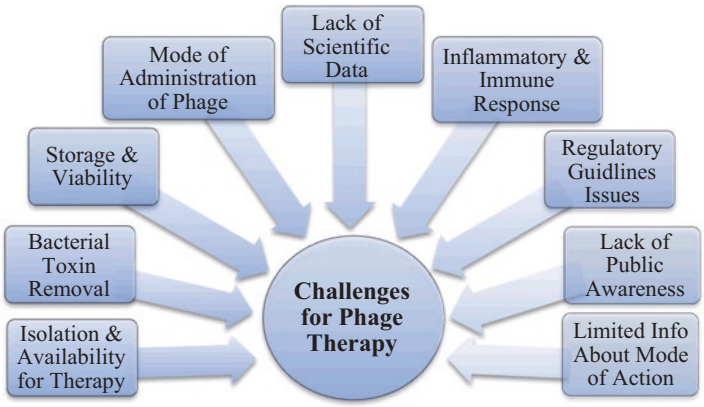

Fig. 15.4 Major challenges faced by phage therapy

and explored. Phage therapy can provide a promising solution for extremely slow dividing *Mycobacterium africanum* and *Mycobacterium leprae* (Dąbrowska 2019; Van Belleghem et al. 2019). Many challenges lie ahead for the therapeutic application of phages such as elimination of endotoxins in the final product, delivery system for the host, inadequacy in identification of quick and immediate therapy, activity potential of phages against specific host bacteria, public awareness and the absence of regulatory guidelines (Manohar et al. 2019). A huge amount of effort is required from government, organizations and academic researchers to translate phage therapy from clinical trials to markets.

15.10 Emerging Approaches in Phage Therapy

Phage therapy has been an area of interest that offers endless advantages over other antimicrobial therapies already threatened by antibiotic-resistant pathogens. Combining antibiotic and phage therapy, or the use of different phages as cocktails might be the most reassuring strategies for the treatment of bacterial infections (Manohar et al. 2019). For example, effectiveness of bacteriophages alone or in combination of amoxicillin, for eradication of biofilm formed by *Klebsiella pneumoniae* B5055 and planktonic cells has been assessed (Bedi et al. 2009). It has also been observed that individual treatments had restricted success rates. However, repeated phage treatments have shown to increase the biovolume of biofilms, as reported in *P. aeruginosa* PA01 (Henriksen et al. 2019). In contrast, the combination of phage and antibiotics leads to biofilm eradication (Díaz-Pascual et al. 2019). Consequently, when an antibiotic is administered in combination with specific bacteriophage, significant destruction of biofilm has been observed. Hence, the phages could be used effectively in conjugation to antibiotic therapy (Bedi et al. 2009). Mechanistically, antibiotics have substantial effects on biofilm structures and enhance the intrusion of biofilm by bacteriophages. An additional benefit of this combination therapy would be its ability to control emergence of resistant mutants that, in any case, progress effectively after utilizing antibiotic and phage therapies separately. It has also been observed that while using antibiotics and phages simultaneously, synergism does not occur for all the phage-antibiotic combinations. It has been reported that higher concentration of antibiotics to phages in combination therapy can also antagonize the proliferation of bacteriophages (Jansen et al. 2018).

Increase in the antimicrobial activity of phages has also been reported when administered along with enzymes. The use of enzyme depolymerase alongside phages has lead to prevention and dispersion of *Staphylococcus* biofilms (Gutiérrez et al. 2015). Other agents that can be effective in combination therapy with phages include hydrogen peroxide, honey, probiotics, xylitol, chlorine and cobalt (II) sulphate (Pires et al. 2020).

Advancement in synthetic biology and engineering methods has enabled improvement in phages by producing novel genomes. Engineered phages have enhanced antimicrobial activity along with the limitations of phage therapies

(narrow host range and host immunity) (Kim et al. 2019). Engineered phages based upon lytic mechanism of phages have been developed. One or more temperate phages are genetically engineered with a lytic phage for use as therapeutic. The most common approach comprises of turning the phage into lytic one by genetic engineering. An illustration of this approach is the utilization of a cocktail made from one natural lytic phage and two engineered temperate phages to effectively treat a 15-year-old patient with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection (Dorscht et al. 2009).

A well-known approach to overcome the problem of narrow range of activity is to use a combination of phages with different spectra in a single cocktail. However, the use of cocktails requires greater optimization to improve their performance. In addition, mixing up a diverse group of phages can lead to more challenges for approval and manufacturing. Subsequently, it would be ideal to create engineered phages with broad-spectrum and enhanced antimicrobial activity instead of simply combining multiple phages. For example, use of a recombinant phage (T3/7) produced by combination of a T3-based hybrid phage whose tail fibre gene I7 is a recombinant between those of T3 and T7. The hybrid phage T3/7 has been found to show better adsorption, effectiveness and a wider host range than both T3 and T7 phages used independently. To enhance the phage antimicrobial activity against biofilms, some enzymes such as lactonase and dispersin B have also been engineered into phage T7 (Lin et al. 2012). Researchers have proposed the use of a restriction enzyme on a non-specific target of a host genome that can be fundamentally extended to 'CRISPR antibacterial' technique which focuses on a specific gene vital for bacterial growth and survival and even more significantly, antibacterial species with their accessible phages (Kim et al. 2019). Hence, engineering methodologies can potentially improve phages' antimicrobial properties, and such phages should be considered therapeutic choices. Additionally, phages that are engineered have simpler patentability and hence are of more commercial interest.

15.11 Conclusions

Undoubtedly, bacteriophages offer vast diversity and have huge potential for development as antimicrobial therapy in a 'post-antibiotic era' world which is short of medications essential for our fight against surging microbial infections. To ensure maximum clinical efficacy of phage therapy, fast-track research needs to be carried out on pathogenic bacteria affecting patients, followed by isolation, identification and screening against individual phage strains or available approved phage cocktails. So far, antimicrobial resistance continues to be one of the preeminent threats to global health, which requires significant intervention at different strata of the society. Considering the limited armamentarium of antibiotics available and an insufficiency in production of newer ones, and despite all its limitations, phages are a nature's gift to humanity which is safe and effective strategy

for combating bacterial infections, with the added advantage of controlling and preventing multidrug-resistant organisms.

To overcome individual limitations of a therapy, a combination therapy approach, wherein two or more therapies can be concurrently used, holds potential in extending the effectiveness of the currently available antimicrobials. The prospects of phage preparations to be used in combination with antibiotics, probiotics and vaccines against chronic infections and resistant pathogenic bacteria can help reduce human illnesses significantly. Measures can be taken in this direction to encourage pharmaceutical giants to invest in development of phage therapy and promote international collaborations amongst academia, doctors and pharma sector, resulting in ensuring advancements in phage therapy. Hence, comprehensive research is the requisite to achieve approval of the regulatory agencies for their inclusion to our repertoire of treatments as well as commercial use.

In conclusion, the looming crisis of antimicrobial resistance has left us with no other choice but to device multidimensional strategies to deal with widespread bacterial infections. The phage therapy offers enormous possibilities and can be instrumental in redefining modern biology by assisting in understanding the biological processes at the molecular level. Therefore, it is time to deliberate the benefits of phage therapy in its entirety with its targeted compassionate use on patients left with no other alternative treatment.

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Part III
Novel Strategies to Combat AMR

Chapter 16
Omics and In Silico Approaches in the Surveillance and Monitoring of Antimicrobial Resistance

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16.1 Introduction

The discovery and industrial production of antibiotics in the early twentieth century were one of the most significant accomplishments in medical history. These drugs increased the quality and expectancy of life (Mohr 2016). Antibiotic abuse has unfortunately resulted in creating multidrug-resistant pathogens. Deaths and infections linked with multidrug-resistant pathogens have surged in recent years (Chan 2016). Drug-resistant diseases cause at least 700,000 deaths globally a year, including 230,000 deaths from multidrug-resistant tuberculosis, and these numbers could increase to ten million deaths globally per year by 2050 under the most alarming scenario if no action is taken. Moreover, it is predicted that around 2.4 million people can lose their lives in high-income countries between 2015 and 2050 without continued effort to manage AMR (Interagency Coordination Group on Antimicrobial Resistance 2019). The diversity of resistant species, the regions affected by drug resistance, and single-organism resistance are enormous and growing (Levy and Marshall 2004). Microbe gains antimicrobial resistance via a variety of mechanisms (Alekshun and Levy 2007) like producing enzymes that catabolize the antibiotic, expressing efflux pumps that remove the antimicrobials, changing the antibiotic's target to prevent binding, triggering an alternate pathway that impedes the action of

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the drugs, and eliminating the antibiotic by downregulating transmembrane porins through which drugs enter the cell (Vila et al. 2007). Drug resistance is mobile, and resistance genes can be passed from one taxonomic community to another through bacteriophages, plasmids, transposons, and other means. Many genes carrying a drug resistance trait can amass in the same organism, and these genes are typically directed toward a family of antibiotics. In the absence of transposons and plasmids (which typically mediate high-level resistance), bacteria undergo development from low-level to high-level resistance through chromosome mutations (Levy and Marshall 2004).

The word "antibiotic resistome" denotes the set of all antibiotic resistance genes found in microorganisms. This term refers to the idea of an antibiotic resistance gene reservoir in environmental samples. Pathogen-derived ARGs make up a small portion of the resistome. Antibiotic resistance genes from antibiotic producers and cryptic resistance genes are examples of resistance genes from nonpathogenic bacteria. The resistome also includes several genes that code proteins with reasonable resistance or antibiotic binding functions that may develop into resistance factors because of selection pressure during evolution. The ultimate external cause of antibiotic resistance is resistance precursor genes (Wright 2007). The widespread use of high-throughput sequencing (HTS) technology has made it easier to identify microbial communities from numerous samples. The growing scale of datasets has created new challenges for exploring and analyzing the information. The identification and classification of antimicrobial resistance genes (ARGs) in large datasets are aided by state-of-the-art databases and analysis pipelines (Yin et al. 2018). However, since bioinformatic platforms can vary significantly, it is critical to choose the resources that are best suited to the user's requirements. This chapter aims to provide detailed information on omic approaches and the most important bioinformatic platforms for identifying and characterizing ARGs.

16.2 Omics of Antimicrobial Resistance

The advancement of many "omic" technologies now allows the high-throughput tracking of the relative abundance of several biomolecules. Genomics aids in the understanding of the whole genome during various biological states. Transcriptomics, which analyzes the whole transcriptome; proteomics, which includes whole proteome studies; metabolomics, which determines various parameters of metabolomes; interactomics, which deals with molecular interactions; and fluxomics, which deals with dynamic alterations of molecules inside a cell, are some common "omic" platforms that have aided microbiology. Yet, no single "omic" analysis can entirely decipher the challenges of microbiology; hence, a unification of many omic approaches, i.e., a "multi-omic" approach, is necessary to evaluate antimicrobial resistance (Zhang et al. 2010). This multi-omic approach in microbes has revealed the involvement of several pathways in determining AMR (Fig. 16.1).

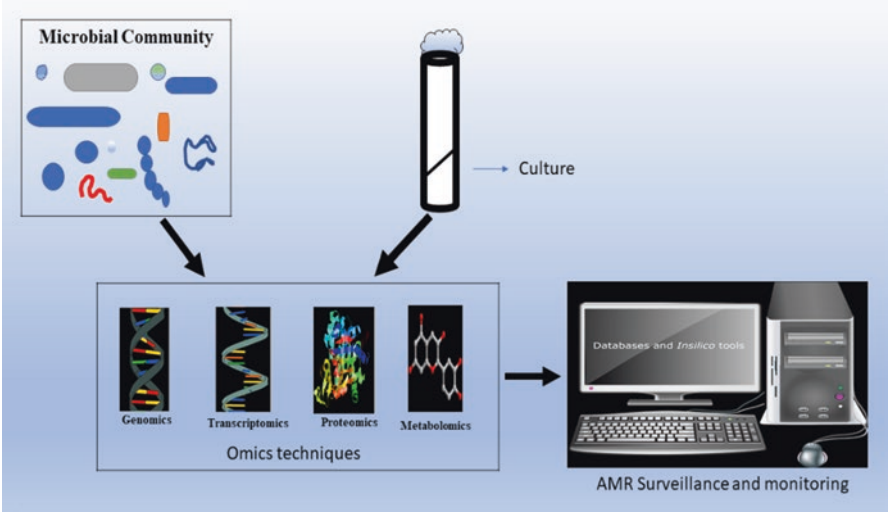

Fig. 16.1 Multi-omic approaches in antimicrobial resistance

16.3 Genomics in Antimicrobial Resistance

Genome sequencing has shown that all bacteria have a significant number of resistance genes. Bacteria have many genes that code for efflux pumps. Microbes like *P. aeruginosa*, an opportunistic pathogen found in a variety of habitats including freshwater and soil, have a remarkable array of efflux pumps (Amsalu et al. 2020). These pumps give microbes the most versatility possible, allowing them to thrive in a variety of environments and increase pathogenicity and biofilm formation (Piddock 2006). Enzymes may contribute to microbial tolerance, one of which is β-lactamases. β-Lactamases have a long history dating back millions of years (Bush 2018). β-Lactamases are the most common enzyme in Gram-negative bacteria that confer resistance to β-lactam antibiotics. Bacteria are thought to be protected from naturally occurring β-lactams by a group of enzymes called β-lactamases, which currently number about 2800 unique proteins (Bush 2018). These β-lactamases are tightly regulated and can be induced shortly by antibiotic exposure. β-Lactamases encoded by genes in resistance plasmids and transposons found in clinical isolates are constitutively expressed because the host organism prefers to live in conditions where antibiotics are abundant: In such cases, regulated expression offers a few disadvantages (Wright 2007). Antibiotic resistance mutations have a negative impact on microorganisms. If the antibiotic is withdrawn, species that are sensitive to the antibiotic outcompete antibiotic-resistant mutants with lower health, weakening antibiotic tolerance in the microbes. As a result, antibiotic cycling has been proposed as a strategy for combating antibiotic resistance. Cycling or rotation consists of the sequential or periodic use of two or more antibiotics not sharing a common mechanism of resistance. Cycling has been proposed as a putative tool for

decreasing current resistance rates or deterring the emergence of resistance (Martínez and Rojo 2011). In most cases, however, it has been demonstrated that, first, compensatory mutations may reinstate fit condition while preserving antibiotic resistance; second, a few mutations that confer resistance have no fitness costs; and third, a few mutations conferring resistance may lead to enhanced fitness (Andersson 2003, 2006). As a result, removing the antibiotic could have little impact on lowering resistance levels. Studies suggest that the ability of microbes to produce compensatory mutations may result in the development of resistance sooner than previously thought (Handel et al. 2006). During the process of drug discovery, another important factor of the effect of mutations on resistance is to determine whether these mutants have less fitness in the host organism and decide whether the compensatory mutations are easily identified (Wright 2007).

Antimicrobial resistance is often caused by mutation, but many mutations are needed for robust resistance to a specific drug. Many genes that affect resistance phenotype indirectly also influence antimicrobial resistance (Chan 2016). Genetic mutations can prompt antimicrobial resistance by giving rise to a protein that impairs the antimicrobial agent, producing a different enzyme that is not suppressed by the antimicrobials, resulting in target alterations, posttranscriptional and post-translational changes in the target (Levy and Marshall 2004), lessened uptake of the antimicrobial agent, and altering actions of efflux pumps and overproduction of the target. It is perplexing how antimicrobial resistance and genetic events are connected in such a complex way (Chan 2016). Antimicrobial resistance is associated with mutations not only in target protein-coding genes but also in a wide range of other genes whose products participate in cellular functions such as DNA repair, replication, transmembrane transport, energy production, and virulence. Comparative genomic study of strains with varying antimicrobial sensitivity is critical for understanding complex antimicrobial resistance mechanisms. The original and antimicrobial genomes can be compared after selecting the corresponding resistant mutants for locating possible target molecules for the drugs. Mutations occur in genes that code for proteins with unknown functions. Antimicrobial resistance phenotype may be significantly influenced by concomitant mutations (Chernov et al. 2019). Today, scientists can disclose the metabolic and genetic potential of new antimicrobials and antimicrobial resistance genes using genomes or genomic fragments assembled from metagenomic studies (Medvedeva et al. 2016). The role of membrane vesicles in antimicrobial resistance has been probed through advanced genomic research. Extracellular vesicles, which channel molecules such as proteins, DNA, RNA, and lipids, can promote intercellular interactions and microorganism adaptation to their surroundings. Antimicrobial resistance determinants are horizontally transferred across these structures (Caruana and Walper 2020). Extracellular vesicles, which help in secretion and intercellular interactions, are uniformly conserved. Although the exact mechanisms of their creation remain unknown, efforts to find vesicular mutants have failed, suggesting that these structures may play an important role in cell biology. Extracellular vesicles may thus be an intriguing target for antimicrobial drug production, both to suppress pathogens and to prevent antimicrobial resistance from spreading through extracellular vesicles (Yang et al. 2018).

16.4 Transcriptomics in Antimicrobial Resistance

Transcriptomics, also known as global gene expression analysis or genome-wide expression profiling, is a technique used for analyzing the entire collection of mRNA molecules (transcripts). Low-throughput methods for calculating single transcripts, such as northern blots and quantitative polymerase chain reaction (qPCR), were employed in the early studies of gene expression. Methods for quantification of gene expression via transcriptomics have advanced over the last few decades (Schena et al. 1995). Moreover, transcriptomics has made it possible to quantitate the expression of mRNA between different states at the genome scale, while sequencing and comparative genomics, which focus on DNA, are undynamic information and normally do not change significantly in response to short-term environmental changes (Zhang et al. 2010). The initial transcriptomic studies were carried out with hybridization-based microarrays, which offer an economical high-throughput choice (Schena et al. 1995). These approaches, however, have many drawbacks, like the need for a prior understanding of the sequences being investigated, cross-hybridization artifacts in the study of highly related sequences, and the inability to reliably measure low and high gene expression (Casneuf et al. 2007). Sequence-based approaches, in comparison with hybridization-based methods, have been designed to elucidate the transcriptome by specifically identifying the transcript sequence.

Next-generation sequencing (NGS) has transformed transcriptomics by allowing RNA analysis via cDNA sequencing. This method, known as RNA sequencing (RNA-Seq), outperforms earlier methods and has redefined the study of the transcriptome complexity and dynamism. Alternative splicing, gene expression, and allele-specific expression can all be studied more thoroughly and quantitatively with RNA-Seq. Deep profiling of the transcriptome and recent developments in the RNA-Seq workflow have facilitated the ability to explain various physiological and pathological conditions and have lightened the labor involved from sample preparation to sequencing platforms to bioinformatic data analysis (Kukurba and Montgomery 2015). Small RNAs (sRNAs) regulate resistance by base-pairing with mRNAs that are necessary for conferring resistance. Antibiotics cause bacteria to drastically alter their sRNA pool. An antibiotic produces a distinct sRNA profile, which may have effects that aid in overcoming the antibiotic challenge. As a result, regulatory RNAs, such as sRNAs, and their protein interaction partners, such as Hfq, may function as antimicrobial chemotherapy targets (Dersch et al. 2017). A more reliable RT-PCR technique can be used to verify transcriptome analysis through microarray-dependent hybridization. Transposon insertion sequencing (TIS) techniques like *transposon sequencing (Tn-Seq)* (van Opijnen and Levin 2020), *transposon-directed insertion site sequencing* (TraDIS) (Langridge et al. 2009), *insertion sequencing* (INseq) (Goodman et al. 2009), or *high-throughput insertion tracking by deep sequencing* (HITS) (Gawronski et al. 2009) may be used to further validate candidate genes or gene products known as possible targets through genomic and transcriptomic techniques, with the expected suppressive

impacts of such mutations. TIS can provide details of the relative impact that disrupts each genomic feature on antibiotic sensitivity and can provide a better perception of how resistance appears, besides guiding the development of new strategies to target resistant bacteria. These studies have shown that antibiotics may have specific targets (e.g., in cell wall synthesis, DNA replication, or protein synthesis) and the genome encodes the bacterial response to antibiotics. Drugs like fluoroquinolones target topoisomerase IV and DNA gyrase, which are enzymes involved in DNA replication. Many TIS experiments cannot assay these targets directly owing to their inherent essentiality. TIS profiles generated under fluoroquinolone exposure implicate the involvement of other genes in DNA replication and repair, like *recN* and *xseA (*van Opijnen and Camilli 2012*;* Geisinger et al. 2018*)*. Antibiotic TIS profiles for each antibiotic tested, and each organism screened, show a role for genes beyond those related to the primary target, indicating the significance of genes with varied functions, like amino acid and carbohydrate metabolism, energy generation, and transport. This shows that although we have some knowledge of the mechanism involved in the inhibition of bacteria by an antibiotic, TIS can unravel this multifactorial process. Yet, conclusions can only be reached after a protein-level validation of antimicrobial targets (Chernov et al. 2019).

16.5 Proteomics in Antimicrobial Resistance

Proteomics is an important method for the study of the emergence and spread of antimicrobial resistance. They play an important role in understanding the molecular mechanisms of bacterial pathogenesis and determinants of diseases. Proteomics' physical associations contribute to methods for developing pathogen-specific treatment strategies that reduce antibiotic resistance. Proteomics is now commonly used to study microbial gene expression because of the recent rapid advances in whole-genome and protein sequencing techniques. Therefore, proteomics has become a dependable tool to study microbes (Khodadadi et al. 2020). Proteomics was first used to footprint the proteomes or sub-proteomes of antibiotic-sensitive and antibiotic-resistant microbes, as well as to classify the differences in protein abundance, a process known as "discovery proteomics." This form of study was later questioned because not all variations in abundant proteins lead to antibiotic resistance. To validate the proposed functions of the proteins of interest, functional validations such as gene deletion and overexpression, as well as enzyme activity, must be performed (Peng et al. 2019). The proteome is a complex system that reacts quickly to changes in the environment. Modification in the pattern of gene expression because of antimicrobials can be used to identify components of the cell that are impaired besides identifying their functions. The unique proteomic responses that appear against antimicrobials allow for the fast identification of mechanisms, compounds involved, and their cellular targets (Wenzel and Bandow 2011). In

general, proteomics of AMR can be divided into two groups: the one that involves comparing resistant and susceptible bacteria, and responses of microbes to antibiotics. Generally, in the first method the least abundant proteins are related to secretion and metabolism, specifically *OmpW* (bacteriocin receptor). The commonly translated proteins are those involved in cell wall biogenesis, known resistance mechanisms, polysaccharide metabolism, and transport. The most cited of these is *Tolc*, an outer membrane channel. In the second case, studies on bacterial response to antibiotic challenge showed that proteins commonly involved are chaperones and those associated with stress response, amino acid, and energy metabolism. Interestingly, a few proteins involved in amino acid and energy metabolism are upregulated, while others are downregulated, indicating the complexity of antibiotic response. Researchers have also reported that some unique mechanisms or groups of proteins are produced at low or indiscernible levels in the unchallenged resistant organisms, and are regulated on antibiotic exposure pointing to them acting as potential targets in novel therapies (Pérez-Llarena and Bou 2016). The design of molecular networks and pathways is for classifying broad datasets obtained using omic technologies. Genes, mRNAs, proteins, metabolites, epigenetic factors, and posttranslational regulators found in the network's "nodes" could be used to produce new antimicrobials. Studying metabolites (the final substrates of the biochemical pathways) can help with the construction of such connections based on data from "omic" approach (Chernov et al. 2019).

16.6 Metabolomics in Antimicrobial Resistance

Metabolomics is rapidly evolving as one of the platforms of "omics." Metabolomics is the systematic identification and quantitation of all metabolites in a given organism or biological sample (Idle and Gonzalez 2007). Metabolomics offers high-throughput metabolite analysis and a forum for comparing biological samples at the same time, revealing their critical roles in metabolism, interorgan, and intraorgan communication. Because of the diverse chemical structure of metabolites, metabolome analysis is particularly difficult (Chandra Mohana et al. 2018). Modification in microbial metabolism, like those linked to biofilm formation or dormancy, regulates antibiotic susceptibility, hinting that bacterial metabolism and antibiotic resistance are correlated. Bacterial pathogen resistome analysis also shows that the production of intrinsic resistance necessitates the coordinated activity of several components, many of them may function in bacterial metabolism (Martínez and Rojo 2011). There is an exometabolome besides the cellular metabolome, which contains metabolites released into the exterior of the cell and is extremely important in the formation of AMR (Birkenstock et al. 2012). Profiling more than 500 intracellular and extracellular putative metabolites in 190 evolved populations revealed that

carbon and energy metabolism strongly constrained the evolutionary trajectories, in terms of both speed and mode of resistance acquisition. This analysis, together with genome sequencing of resistant populations, identified condition-dependent compensatory mechanisms of antibiotic resistance, such as the shift from respiratory to fermentative metabolism of glucose upon overexpression of efflux pumps (Zampieri et al. 2017). Changes in the levels of metabolites that occur during the advent of AMR can aid in the identification of previously unknown antimicrobial therapy targets. Inhibiting these targets with other pharmaceuticals may act as a selection pressure, reducing the rate with which AMR mutations are fixed. As a result, this method may be employed in the development of integrated and multitargeted antimicrobial therapies, as well as new treatments that develop selection pressure on resistant phenotypes to return them to a susceptible state (Chernov et al. 2019).

16.7 Meta-omic Approaches in Antimicrobial Resistance

Meta-omic approaches have allowed working on uncultured species found in the environment. The widespread existence of ARGs in several presumably sterile habitats, despite no documented exposure to synthetic antimicrobials, is one of the most intriguing findings from functional metagenomic studies (Chernov et al. 2019). Random sequencing or PCR amplification of target genes is commonly used to analyze metagenomic clones. Alternatively, functional metagenomics can be used to find genes whose function is not known based on their sequence (Torres-Cortés et al. 2011). State-of-the-art high-throughput functional metagenomics helps scientists to multiplex up to 400 functional metagenomic selections on a single Illumina sequencing lane. A dedicated customized tool like PARFuMS (parallel annotation and reassembly of functional metagenomic selections) aids scientists to perform demultiplexing, quality filtering, trimming, assembly of the reads into full-length metagenomic fragments, and annotation in a single automated step, significantly lowering the cost of the experiment (Boolchandani et al. 2017). Metagenomic sequencing has aided the finding of new antimicrobial-resistant genes and their interactions by providing a potent way of obtaining information about ARGs, or "resistomes," that are the hallmark of different ecosystems. Current metagenomic approaches rely heavily on sequence similarity computation to predict antibiotic resistance attributes, which have substantial drawbacks. To allow consistent and accurate ARG recognition, such computations of similarity require a quality and cutting-edge antimicrobial resistance gene reference and annotation database. Furthermore, the absence of a systematic target gene for alignment limits the reach of such studies to previously identified ARGs. However, through using structural (3D) analogs, computational attempts have been made to annotate new antimicrobial-resistant genes in order to extend the sequence repertoire (Arango-Argoty et al. 2020).

16.8 Bioinformatics in Antimicrobial Resistance

Antimicrobial resistance gene prediction necessitates assurance of the data quality of genome, data trimming, genome assembly, and annotation. All of these procedures can now be performed in a short period using bioinformatic analysis, depending on factors such as computer processing capacity, database efficiency, and Internet speed (Chan 2016). The fast amassing of data on ARG necessitates the development of sophisticated databases for organizing and effortless accessibility to data. These databases not only store data, but also include bioinformatic resources for analyzing data for genes encoding possible antimicrobial biosynthetic pathways and AMR in microorganisms isolated from various sources, including microbes having unknown culture conditions (Chernov et al. 2019). Commercial or open-access platforms may be used to detect ARGs, and both require varying degrees of user skills. Freely available services are typically available online at public genome data centers or as an independent program for installation and use on a computer. Most bioinformatic software can process assembled sequences or raw reads, but to avoid ambiguous/false-positive results, users must check the validity of sequence that is submitted (Lal Gupta et al. 2020). As of now, there are close to 47 publicly available bioinformatic tools for detecting AMR in DNA or amino acid sequence data (Hendriksen et al. 2019). In this chapter, we discuss a few AMR detection methods and databases that are currently available.

16.9 Comprehensive Antibiotic Resistance Database (CARD)

CARD is a curated database offering reference sequences of protein and DNA, detection models, and the molecular biology of bacterial AMR. The antibiotic resistance ontology (ARO), developed to merge with software design for resistome analysis, such as CARD's Resistance Gene Identifier (RGI), aims at offering reference data of high quality (Alcock et al. 2020). The RGI uses BLAST to compare genome or assemblies to CARD and makes an all-around report with projected genes and targeted drug groups concerned with antibiotic resistance. This encompasses antibiotic resistance caused by mutations in their targets or by antibiotic resistance gene products. The information about resistance genes in CARD is connected to entries in Web resources like GenBank, PubChem, PubMed, and Protein Data Bank, allowing a connection between literature and databases (McArthur and Wright 2015). CARD has evolved significantly since 2017, with comprehensive reference sequence curation, updating the ontological framework, curation of over 500 novel antimicrobial resistance detection models, and development of a new classification approach and analytical methods. As of now, a new resistome and variant module, for example, offers statistical analysis and description of computer-assisted predicted resistance variants from 82 pathogens and over 100,000 genomes. A researcher can summarize predicted resistance using CARD, key out patterns in AMR mobility, and identify novel resistance variants (Alcock et al. 2020).

16.10 ResFinder

ResFinder is a widely cited tool that recognizes both short reads and assembled genome or contigs for tracking and locating gained ARGs in complete or partial sequences of bacterial isolates using KMA- and BLAST-based approaches. This tool includes a built-in reference ARG database that users can download and use for custom pipelines (Zankari et al. 2012). ResFinder is centered on a database of over 2000 ARGs that is searched using BLAST. A 50% identity over at least 3/5 of the length of the gene was laid as the criterion for notifying a match between a gene in the ResFinder and the input genome (Kleinheinz et al. 2014). ResFinder is a freely available Web-based tool. To make a prediction, the following four steps need to be taken: (1) Select the file containing the genome sequence applying the "Browse" button. (2) Under "Pick Antimicrobial configuration," the researcher can select the antimicrobial agents against which resistance genes are to be identified. ResFinder looks for resistance genes for all 12 forms of antimicrobial agents by default. (3) The "Select threshold for percent ID" dropdown menu is used to set the percent identity threshold among genes in the ResFinder database and genes in the input sequence. By default, the ResFinder database reports only genes in the input genome that are 98% similar to genes in the ResFinder. (4) The input genome's format has to be defined using the "Select sort of your reads" dropdown menu. The input sequence is assumed to be a draft or full genome in FASTA format by design. The time taken to study a genome is decided by a variety of factors. It is usually less than 10 min (Kleinheinz et al. 2014). ResFinder includes only acquired ARGs, making it ineffective for resistance detection caused by chromosomal mutations (Lal Gupta et al. 2020). The latest version of ResFinder was improved by rewriting the program and developing and broadening the database of PointFinder and ResFinder. It uses the same Web interface as old ResFinder versions. It also produces *in silico* antibiograms other than detecting AMR genes and chromosomal mutations. Antimicrobial resistance genes (ResFinder), gene mutations specific to chromosomes facilitating antimicrobial resistance (PointFinder), genotype-to-phenotype translation, and species-specific modules for *in silico* antibiograms are all included in the new version of ResFinder (Bortolaia et al. 2020).

16.11 PointFinder

A new Internet-aided tool for whole-genome sequence-based tracking of AMR in bacterial pathogens is linked to chromosomal point mutations. PointFinder is made up of a chromosomal gene database with reference sequences in fasta format and a database for chromosomal mutation with positions of codons and substitutions. Only hits with an identity of less than 80% are further examined by PointFinder, which uses BLASTN to determine the similarity for each gene in the chromosomal gene database. The software compares each position in the query with the

corresponding position in the database sequence for each alignment. The chromosomal mutation database is used to save and compare all mismatches (Zankari et al. 2017). In their databases, both CARD and ARG-ANNOT have attempted to include chromosomal point mutations. Since both ARG-ANNOT and CARD do not take into account the microbial species, both methods produce mutations and sequences linked to mutational resistance, which are unrelated to the bacteria in question. To use these tools, the user must have prior knowledge of the mutational genes and mutations that they are searching. To fix some of these concerns, PointFinder was built to make the identification of chromosomal point mutations linked to resistance more user-friendly. The Web tool's functionality is user-friendly and easy to understand, describing observed nucleotide and amino acid codon shifts, mutations, and expected resistance (Zankari et al. 2017).

16.12 MEGARes

MEGARes is designed for high-throughput sequencing of already reported antimicrobial resistance genes in metagenomic results. It aims to offer an extensive database that includes all available reference sequences for genetic determinants of antimicrobial resistance. It also employs an acyclic hierarchical annotation system that aids high-throughput statistical and classification analysis. MEGARes is not an alternative to source databases like ResFinder and CARD, which offer comprehensive accession details, but instead provides an easier, hierarchical ontological framework that is specifically modeled for high-throughput analysis of metagenomic resistome data. MEGARes also emphasizes reported sequences than concentrating on discovering new variants. Metagenomic data are a source for discovering new AMR genes, which necessitate the use of specialized bioinformatic tools (e.g., fARGene) (Berglund et al. 2019), Meta-MARC (Lakin et al. 2019) and PCM (Ruppé et al. 2019). MEGARes, on the other hand, aims to make bioinformatic detection of already reported sequences related to resistance to antimicrobial drugs, metals, and biocides that are more reproducible and statistically relevant (Doster et al. 2020).

16.13 PRAP (Pan Resistome Analysis Pipeline)

PRAP (Pan Resistome Analysis Pipeline) is a freely available pipeline for detecting antibiotic resistance genes (ARGs), characterizing pan-resistomes using annotations, and predicting the impact of ARG on resistance phenotypes using machine learning. PRAP helps to predict AMR phenotypes straight from whole-genome sequences, allowing researchers to dig deeper into possible ARG features. PRAP's workflow is divided into three sections: input file preprocessing, ARG recognition, and pan-resistome characterization. PRAP supports a number of sequence file formats for input data preprocessing, including raw reads files (fastq), GenBank

annotation files (gb), fasta nucleic acid files (fna), and fasta amino acid files (faa). PRAP retrieves protein-coding sequences (CDSs) from GenBank annotation files and generates both fna and faa files. In a nutshell, PRAP allows the rapid recognition of ARGs from multiple genome files besides helping in the discovery of possible ARG transmission and distribution "laws" within the population (He et al. 2020).

16.14 Other Databases

Antibiotic Resistance Genes Database (ARDB) was an effort to make preliminary ontology and gene catalog for ARG. ARDB is a database that integrates virtually all the freely accessible ARG and offers an authentic annotation tool to scientists looking into the molecular biology of bacterial resistance (Liu and Pop 2009). Since ARDB was last updated in 2009, it does not have any newly explored ARGs (Arango-Argoty et al. 2020). ARG-ANNOT was the maiden database to locate point mutations in target genes in chromosomes linked to AMR. ARG-ANNOT maintains a repertoire containing sequences of genes linked to mutational resistance and particulars on the gene's location and mutation. Since ARG-ANNOT does not trace these mutations automatically, the user must manually search along the alignment to find them (Zankari et al. 2017). AMRFinderPlus is a tool developed by NCBI that uses protein annotations and assembled nucleotide sequences to identify AMR genes and resistance-associated point mutations. The pathogen detection pipeline utilizes AMRFinderPlus, and the results are shown in NCBI's Isolate Browser. AMRFinderPlus relies on curated collections of hidden Markov models and NCBI's curated Reference Gene Database (Feldgarden et al. 2019). Another Web-based tool, the Resistome Tracker, was created to make resistance data more available. It was created for the National Antimicrobial Resistance Monitoring System to help researchers study the contagious property of resistance by providing vast volumes of resistome data available to a wide range of users. Resistome Tracker helps in the discovery of new resistance factors, variations in the occurrence of resistance genes, and the spread of resistance genes over time. Resistome Tracker is also updated on a regular basis, allowing users to identify early resistance risks. Here, data dashboards are solely dedicated to the study and visualization of AMR genes derived from the NCBI's full genomes. User orchestrated searches for the data are possible with Resistome Tracker. It allows users to start a search with a particular resistance allele and finish with an evolutionary study of similar strains since it is directly linked to the NCBI pathogen database. It is currently based on foodborne bacteria, but it can be tweaked to look for AMR genes in any genome (Hendriksen et al. 2019). The structured ARG database (SARG) was developed by incorporating sequence information from ARDB and CARD in a hierarchical structure. SARG allows ARG-like sequences found through similarity searches to be automatically classified into various types and subtypes, saving time and effort. Using SARG, an online research pipeline (ARGs-OAP) is also open. ARGs-OAP

was created to make analyzing metagenomic sequences for ARG data easier using the Galaxy Web server and SARG, which has a user-friendly interface. Users may use this pipeline to assess ARG profiles from many samples with one step. The pipeline aims to better understand the distribution and propagation of ARGs in various environments by using the power of metagenomic research (Yang et al. 2016). PathoFact is a pipeline for identifying virulence factors, toxins from bacteria, and ARGs in metagenomic data that is simple to use, modular, and reproducible. In addition, our tool blends pathogenicity factor estimation with the detection of mobile genetic elements. This adds complexity to the study by taking into account the genetic background of the relevant genes. PathoFact's modules for toxins, virulence factors, and ARGs may also be used separately, making it a versatile and portable method (de Nies et al. 2021).

AMR reference databases are divided into those for detecting resistance to specific antimicrobials in specific bacteria and solutions for detecting any AMR influencing sequences in any nucleotide or amino acid sequence. AMR reference databases have significant variations that users must know in order to choose the best database for their needs. First, the criteria for inclusion in AMR reference databases vary. For example, for CARD, submissions have to be published in peer-reviewed journals, but publication is not a prerequisite in ResFinder. A GenBank number is required for each gene, and GenBank entries need to be analyzed by an expert. In addition, the kinds of submissions vary between databases, with most containing antimicrobial resistance genes and some concerned with mutations in chromosomal genes facilitating antimicrobial resistance. Ultimately, the databases for AMR vary in terms of entry format (fasta, json, etc.), download capability, and curation capacity and frequency. To lessen ill-suited outputs due to differences in algorithms, synchronization of the databases is needed (Hendriksen et al. 2019). In theory, *in silico* AMR detection works by querying input data like nucleotide or protein sequence for the existence of a predetermined set of AMR determinants stored in reference databases using a search algorithm. Sequence data can be processed as reads or assemblies by various bioinformatic applications (Boolchandani et al. 2019). When utilizing assembly-based approaches, differences between assemblers can compromise the outcome's comparability. Following assembly, BLAST and hidden Markov model searches and so on are used for the comparison of the input data with the antimicrobial resistance reference databases. BLAST-based tools can produce a variety of results depending on the default settings for gene length and similarity percentage. If the settings are too high or too low, this can have a negative impact on specificity. Furthermore, approaches based on assembly are very intensive computationally. Despite these drawbacks, assembly-based tools can have more worth in AMR monitoring by allowing genetic analysis of the causative AMR genes, such as their mobilizing potential. To match reads to AMR databases, read-based methods may use a variety of tools, such as Bowtie2, BWA, and KMA (Boolchandani et al. 2019). The KMA (k-mer alignment) was recently developed to directly map raw reads against redundant AMR databases. In comparison with other methods of mapping such as BWA, which were designed for broad reference genomes such as the human genome and then applied scientifically to

microbiology, the KMA tool was designed particularly for speedy and precise bacterial genome analyses. KMA uses the Needleman–Wunsch algorithm to correctly align extensions from k-mer seeds, as well as k-mer seeding to speed up mapping. To ensure a precise choice of templates, multi-mapping reads are handled by adopting a new sorting strategy (ConClave scheme) (Clausen et al. 2018). Read-based techniques enable the detection of antimicrobial resistance genes that are present in low abundance and genes that might miss out during incomplete assemblies (Boolchandani et al. 2019). The complexity of the analyzed dataset, the study's objectives, and the explicit questions posed influence the choice of suitable bioinformatic methods and ARG tools.

16.15 Global Collaborations for the Surveillance of AMR

AMR is a global problem in developing countries, where the burden is high and the process of replacing older antibiotics with new ones is daunting due to budget constraints (Ganguly et al. 2011). For addressing the factors influencing AMR, one must consider the healthcare system, the efficiency of regulatory authorities, the socioeconomic status, and geographical properties of a particular country (Winters and Gelband 2011). Many regulatory authorities, collaborations, and bodies are set up internationally for the management of AMR. In this session, we will briefly describe a few important ones. The Global Antibiotic Resistance Partnership (GARP) was started in 2009 for developing action plans and proposals relevant to low- and middle-income countries (Ganguly et al. 2011). GARP aims to define policy solutions by investigating the particular contexts of target countries. National working groups, with support from the Center for Disease Dynamics, Economics, and Policy (CDDEP), developed strategies tailored to local conditions in the GARP countries, based on the data collected and analyzed in this study. The strategies include reducing the antibiotic demand by cramping the occurrence of infections in hospitals and by controlling the use of antibiotics in humans and livestock (Winters and Gelband 2011). In India, a network of microbiology laboratories (Indian Network for Surveillance of Antimicrobial Resistance—INSAR) at reputed hospitals was formed with support from WHO. The network monitors AMR and assesses the extent of complications associated with it (Joshi et al. 2013). The FDA Centre for Veterinary Medicine (CVM) launched the National Antimicrobial Resistance Monitoring System (NARMS) in 1996 as a means to monitor the influence of antimicrobial use in agriculture moreover studying the evolution of AMR in clinical isolates (Gilbert et al. 2007). NARMS monitors AMR in microbes like *Campylobacter*, *Escherichia coli* O157:H7, non-Typhi *Salmonella*, *Enterococcus*, *Salmonella* Typhi, and *Shigella (*Holmes and Chiller 2004*)*.

A monitoring system concentrating on European countries is the European Surveillance of Antimicrobial Consumption (ESAC-EU) project that was launched in 2001. ESAC is a network of monitoring systems that collect reliable

data on the use of antibiotics in European countries (Coenen et al. 2007). ESAC-Net collects and analyzes data on antimicrobial consumption from both communities and hospitals. The data are used to give timely information on indicators of antimicrobial consumption (European Centre for Disease Prevention and Control 2021a). Another globally accepted initiative is the South African Society for Clinical Microbiology (SASCM). The objectives of SASCM include the monitoring of AMR patterns in the public and private medical sector in South Africa like the publication of AMR patterns in selected publications. SASCM handles the oversight of antimicrobial surveillance activities, and it is incumbent upon the society to educate and raise awareness around antimicrobial susceptibility testing issues that have implications for treatment and surveillance data (Singh-Moodley et al. 2018). Following the announcement of the World Health Organization's (WHO) Global Action Plan on Antimicrobial Resistance in 2015, AMR has begun to receive international political publicity. The member states of the Pan American Health Organization (PAHO) supported this in a resolution adopted in September 2015, emphasizing the political allegiance of all associated countries to develop AMR action plans. The Global Action Plan lays out five strategies for containing AMR: education and awareness, surveillance and study, infection prevention and control, effective antimicrobial use and research, and the establishment of the economic case for long-term investment. A multisectoral One Health response is key to implementing these action plans. Countries have made progress in policies, measures, and strategies since the adoption of the Global Action Plan to ensure the successful design and execution of their action plans in line with the regional plan of action and the One Health strategy for containing AMR (Pan American Health Organization 2021).

The European Antimicrobial Resistance Surveillance Network (EARS-Net) is Europe's largest publicly funded surveillance system for AMR. Data from EARS-Net are crucial in raising awareness among public health officials, scientists, and the general public. The European Antimicrobial Resistance Surveillance System (EARSS), established in 1998, is the predecessor of EARS-Net. The objectives of EARS-Net are to collect comparable and accurate AMR data, analyze spatiotemporal tendencies of AMR in Europe, and encourage the implementation and improvement of national AMR surveillance programs (European Centre for Disease Prevention and Control 2021b). GLASS (National Antimicrobial Resistance Surveillance System) is a WHO-supported system that was introduced in October 2015 to promote a systematic approach for the collection, review, and sharing of data related to AMR at the global and regional levels. GLASS aims to promote national surveillance frameworks and harmonized global standards, estimate the global scope and burden of AMR using selected metrics, analyze and monitor global AMR data regularly, identify emerging resistance and its international spread, inform the implementation of targeted prevention and control initiatives, and evaluate the impact of interventions (Basheera 2020). All these agencies and collaborations result in an effective exchange of information, and this can certainly help in the effective monitoring of AMR.

16.16 Conclusions

The NGS platform, combined with bioinformatic tools, is nowadays becoming the tool for the study and prediction of AMR. This is primarily due to reducing expense and reduced overall time spent on the experiment. Today, for typical bacteria, the entire process of analyzing AMR from raw sequence reads needs only 3 min on a laptop using Mykrobe predictor software (Bradley et al. 2015). The co-carriage of particular genes having various multidrug-resistant patterns, their genetic background including the possibility for horizontal transfer, allelic trends over time, and their distribution can be revealed using bioinformatic analysis. Furthermore, the presence of co-resistances not identified by typical drug workflows, such as those conferred by disinfectant and heavy metals, is revealed. This degree of "deep surveillance" will reveal additional potential modulators of AMR perseverance and phylogeny as well as the possibility of a more polished microbial risk assessment based on traits that confer resistance. The kind of approved input data, the existence of tools for searching within an AMR determinant database that can be unique to a tool or cloned from other resources, and the search method used (based on mapping or alignment) are some factors that differentiate bioinformatic resources. As a result, each method has potency and constraints in terms of sensitivity and specificity of AMR determinant detection, as well as in terms of implementation, which have been highlighted in benchmarking exercises and research papers for some methods. Today, the number of tools and DNA sequence data available is increasing, allowing for worldwide pathogen and AMR monitoring based on genomic data. Calibration of databases and pipelines, and prediction of phenotype based on data are, however, needed for extensive workflows. A dependable genomic workflow to assay AMR gene necessitates well-curated, harmonized, and constantly updated reference databases to ensure comparable results. Even though there are challenges, the bioinformatic approach is certainly a high-throughput method to do monitoring and surveillance of AMR globally.

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Chapter 17
Antimicrobial Activity of Bioactive Compounds (Thymoquinone and Eugenol) and Its Nanoformulation Therapeutic Potential

Jasmeet Kaur, Mohamad Taleuzzaman, and Iqra Rahat

17.1 Introduction

Essential oils, generally representing the essence of the all-natural plant and common feature of all plant extracts, have been widely used since ancient times due to their wide range of pharmacological effects. In fact, essential oils are important bioactive constituents of medicines from herbal plants for the development of good health with very few side effects. Further, based on recent scientific publications, eugenol and thymoquinone claim to be interesting bioactive compounds exerting beneficial effects on humans and possessing broad-spectrum antimicrobial activity.

17.1.1 Chemistry and Sources

These compounds comprise isoprene units (C_6H_8) and are being used to control, cure, or prevent microbial infections. Eugenol, belonging to the class of phenylpropanoids, is amphipathic hydroxyphenyl propene with the chemical formula $C_{10}H_{12}O_2$. This aromatic yellowish oily liquid has a pleasant odor and taste. It is chemically expressed as 4-allyl-2-methoxy phenol or 1-allyl-4-hydroxy-3-methoxybenzene (Mishra et al. 2013).

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17.1.1.1 Eugenol

Eugenol was first isolated as a volatile compound in the year 1929 from *Eugenia caryophyllata* and was thus named after this genus. Eugenol is present in a variety of herbal plants including *Melissa officinalis* L. (lemon balm) (Jaganathan and Supriyanto 2012), *Abutilon indicum* L. (country mallow) (Kumar et al. 2013), *Aegle marmelos* (Indian bael), *Illicium anisatum* (Japanese star anise) (Jaganathan and Supriyanto 2012), *Myristica fragrans* (nutmeg), *Pogostemon cablin* (patchouli) (Chen et al. 2013), *Piper betel* (betel) (Gundala and Aneja 2014), *Acorus calamus* (sweet flag) (Mythili Avadhani et al. 2013), and *Aphanamixis polystachya* (pithraj) (Shaikh et al. 2012). Also, an important source of eugenol includes *Syzygium aromaticum* (clove buds and leaves), various *Cinnamon* species such as *Cinnamomum zeylanicum*, *C. verum*, and *C. cassia*, and *Ocimum* species such as *Ocimum basilicum* L, *O. sanctum* L, *O. americanum* L, *O. gratissimum* L, *O. micranthum* Wild, *O. minimum* L., and *O. adscendens* Wild (Marchese et al. 2017). Furthermore, eugenol can be commercially produced via biotransformation method involving various microorganisms such as *Corynebacterium* sp., *Bacillus cereus*, *Pseudomonas* sp., and *Streptomyces* sp. and chemically synthesized by using allyl chloride for allylation of guaiacol (Mishra et al. 2013; Molina et al. 2013).

Eugenol is a highly volatile compound with limited solubility and low chemical stability. Due to volatility and limited stability, it is highly sensitive to oxidation and chemical interactions (Majeed et al. 2016). Upon administration, eugenol is rapidly absorbed and metabolized in the liver with an excretion rate of 95% within 24 h. Thus, it is necessary to encapsulate eugenol to improve its efficacy and solubility while preventing its early absorption (Majeed et al. 2016; Joris et al. 2008). A wide range of biological activities has been reported to be associated with eugenol such as analgesic, antiviral, antispasmodic, anticancer, anti-inflammatory, antiseptic, and antioxidant. It has been reported to be used for the treatment of many diseases like acne, arthritis, warts, asthma, diarrhea, skin diseases, and various allergies (Arana-Sánchez et al. 2010; Shah et al. 2013).

17.1.1.2 Thymoquinone

On the other hand, thymoquinone is a naturally occurring phytochemical compound that belongs to the chemical class of benzoquinones. It is a main bioactive constituent present in the seeds of the plant *Nigella sativa* (black seed), belonging to the Ranunculaceae family. The chemical formula of thymoquinone is $C_{10}H_{12}O_2$ with a molecular weight of 162.4 g/mol and is chemically expressed as 2-isopropyl-5-methylbenzo-1, 4-quinone. The substance was first isolated in the year 1963 and appears to be a potential candidate against many diseases (Abu Khader and Khan 2017). Besides, *Nigella sativa* being the main source of thymoquinone, the substance is present in a large number of plants such as *Nigella sativa* L. seeds (black bread weed) (Havlik et al. 2006), *Satureja* (winter savory) (Kubátová et al. 2001), *Origanum* (oregano) (Lukas et al. 2009) (Economakis et al. 2002; Ipek et al. 2005),

Agastache (giant hyssops), *Monarda* (bee balm), *Cupressus*, *Coridothymus* (wild thyme), *Thymus* (garden thyme) (Fleisher and Fleisher 2000), and *Juniperus* (Mastelić et al. 2000). The solubility of the substance ranges from 0.549 to 0.740 mg/ml for a period of 24–72 h and is thus categorized as a fat-soluble molecule. Therefore, the drug is highly unstable in an aqueous solution. Upon oral administration, the drug undergoes chemical and enzymatic alterations in the gastrointestinal tract and follows hepatic first-pass metabolism (Abu Khader and Khan 2017). The use of nanocarriers for the enhancement of bioavailability of thymoquinone seems to be a plausible alternative. Interestingly, *Nigella sativa* seeds have been traditionally reported to be used for the treatment of various ailments such as asthma, cough, headache, bronchitis, fever, influenza, and rheumatism. Moreover, many kinds of literature have reported the beneficial effects of *Nigella sativa* seeds as antidiabetic, immunomodulatory, anxiolytic, antioxidative, anti-inflammatory, antidepressants, antipsychotic, anticonvulsant, gastroprotective, antibacterial, hepatoprotective, cardioprotective, anticancer, and memory impairments (El-Far 2015; Darakhshan et al. 2015; El-Far et al. 2014).

17.1.1.3 Significance of Natural Products

Herbal drugs being natural products are valued for their use in various health ailments since the prehistoric period. Moreover, as these drugs are plant extracts, the side effects and other complications associated with these drugs are very few. Herbs are a rich source of ingredients that plays a critical role in drug synthesis and development. Many herbs are used for a wide range of biomedical applications such as gene therapy, tissue engineering, the medicinal industry, and skin regeneration. Many herbs are used as blood cleansers, which are responsible for eliminating and altering metabolic toxins. Hence, these drugs can be used to improve immunity and are used in fevers, as Chirata. Herbs such as aloe, ginseng, cinnamon, black pepper, and sandalwood are used in case of boils, wounds, and sores. Many herbs also possess antibiotic properties such as turmeric. It works by inhibiting the growth of harmful microbes, germs, and bacteria. Moreover, it is widely used since ancient times for healing cuts and wounds. Some herbs such as marshmallow roots act as antacids by neutralizing the gastric acid. Traditional Indian medicine practitioners recommend the use of Indian sage for snake bites. Many herbs such as ginger, eucalyptus, cardamom, and cloves possess the expectorant property and are used in cough syrups. These herbs are responsible for the elimination of mucus from the lungs. Apart from these, herbs are widely used as an ingredient in the food, dye industry, pest control, and perfume industry. Despite these advantages, herbal drugs possess low solubility, an increased absorption, higher instability, and rapid metabolism and excretion. Therefore, the administration of herbal drugs requires modification for achieving enhanced efficacy and to achieve sustained release. To achieve a rational dosage regime of herbal drugs, it is important to understand the bioavailability and pharmacokinetic profile of these drugs. These parameters require the usage of new technologies such as nanotechnology for the formulation of herbal

drugs to achieve site-specific delivery at a predetermined rate. Nanoformulation of herbal drugs includes many benefits such as targeted drug delivery, enhanced component solubility, reduced dose, increased absorption, reduced metabolism and elimination of bioactive ingredients, and enhanced bioavailability (Huang and Chang 2009). It helps in improving stability and achieving precision targeting, thereby improving the effectiveness of phytoconstituent. This can be achieved by incorporation or encapsulation of herbal drugs in a suitable carrier system such as liposomes, nanoparticles, and nanoemulsions, which can convert a poorly available herbal drug into a favorably bioavailable drug candidate.

17.2 Pharmacology

17.2.1 Eugenol

The antimicrobial activity of eugenol is due to the presence of free radical scavengers in the molecules. These free radicals are responsible for blocking the production of reactive oxygen species (ROS). Several pieces of kinds of literature have been reported for antimicrobial activity of eugenol. In the year 2004, Burt along with his coworkers hypothesized that eugenol prevents enzyme action by binding to the proteins (Burt 2004). Later in 2006, Gill and Holley demonstrated that eugenol affects membrane nonspecific permeability and alters ion and ATP transport by disrupting the cytoplasmic membrane (Gill and Holley 2006). This result was further validated by Filgueiras and Vanetti and concluded that eugenol increases phosphate ions permeability upon addition of bacterial concentration (Filgueiras et al. 2006). In particular, eugenol has been tested against a broad spectrum of microorganisms that are responsible for causing infectious diseases in humans. For example, microorganisms responsible for causing respiratory infections such as *Stenotrophomonas maltophilia* and *S. pneumonia* were found to be most susceptible to eugenol with inhibition zone (IZ): 28 mm, followed by an inhibition zone of 27 mm for *Haemophilus influenzae*. The researchers reported *S. aureus* and *Klebsiella pneumonia* to be the most resistant strains with IZ of 18 and 10 mm (Fabio et al. 2007). In the same year, other researchers reported the antibacterial activity of eugenol against various Gram-negative bacteria including *E. coli*, *S. typhimurium*, *S. typhi*, *Shigella flexneri*, and *P. aeruginosa* (Dabur et al. 2007). In 2009, Pei et al. demonstrated the antibacterial activity of eugenol against *E. coli* and reported its minimum bacterial concentration of 1600 mg/l using broth macrodilution assay (Pei et al. 2009). Devi along with his coworkers demonstrated that eugenol alters membrane permeability leading to extensive loss of ions and cellular content followed by cell death. The authors evaluated this mechanism of action for antibacterial activity against *Salmonella typhi* (Devi et al. 2010). In the later years, Das along with his coworkers demonstrated the antibacterial activity of eugenol against *S. aureus*. The authors evaluated that eugenol was able to produce intracellular reactive oxygen

species, which results in inhibition of cell growth, cell membrane disruption, and DNA damage followed by decomposition of cell and ultimately leading to cell death (Das et al. 2016). Another investigation shows the activity of eugenol against bacterial enzymes such as amylase, protease, ATPase, and histidine carboxylase (Hyldgaard et al. 2012). The major mechanism of action of eugenol is described in Fig. 17.1.

Besides being highly effective against Gram-negative and Gram-positive bacteria, some researchers have evaluated its activity against fungi. Eugenol being lipophilic in nature, the authors evaluated that this may enter in the lipid bilayer membrane resulting in altering its fatty acyl chains, which ultimately disturbs the membrane fluidity and permeability. Ahmad along with his coworkers evaluated the activity of eugenol against *Candida* spp. The researchers found that eugenol inhibited the H^+-ATPase and glucose-stimulated H^+-extrusion of *Candida* spp. at a minimum inhibitory concentration of 500 μg/ml (Ahmad et al. 2010). Moreover, other researchers showed that the treatment of *Candida albicans* with eugenol and methyl eugenol resulted in induction of oxidative stress by increasing superoxide dismutase levels and elevating ROS, which causes peroxidation of lipids present in cytoplasmic membrane leading to cell death (Khan et al. 2011). In fact, the first study for antifungal activity of eugenol was conducted in 1982 against 31 strains of *Candida albicans* and 33 strains of *Cryptococcus* reforms (Boonchird and Flegel 1982). Moreover, the alteration in the structure of eugenol results in more potent derivatives of eugenol. For example, the addition of morpholinyl group in the eugenol results in much higher antibacterial activity as compared to eugenol (Abrão et al.

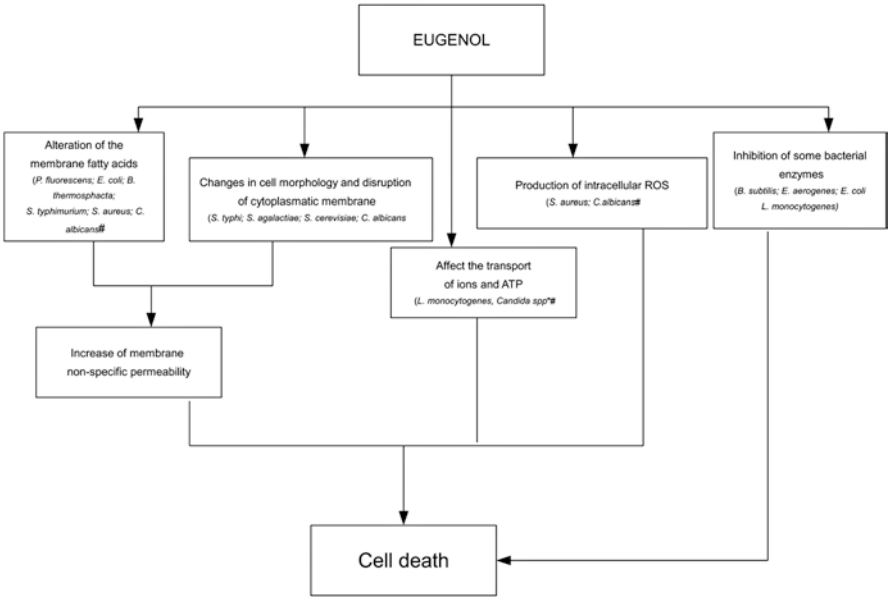

Fig. 17.1 Eugenol mechanism of action against bacteria and fungi

2015). The activity of per acetyl glucoside derivative of eugenol showed better potency against *Candida glabrata* and was less cytotoxic than eugenol (Hipólito et al. 2018) (Fig. 17.1).

To exhibit the synergistic effect of eugenol, it is necessary to combine the essential oil or standard antibiotics with the eugenol. This synergism results in various interactions such as interfering of substance with the cell wall, which results in an increased sensitivity toward the other substance and inhibitory effects of both substances in the same biochemical pathways. For example, eugenol and thymol result in synergism where thymol allows eugenol to reach the cytoplasm and interact with enzymes by disintegrating and disrupting the outer cell membrane of Gram-negative bacteria (Bassolé and Juliani 2012). Another example involves the additive and synergistic effect of Streptomycin and eugenol against *L. monocytogenes* and *S. typhimurium* (Liu et al. 2015).

17.2.2 Thymoquinone

Interestingly, the tiny seeds of *Nigella sativa* are a rich source of thymoquinone. Many scientific articles have been published, which refer to the antimicrobial activity of thymoquinone and black seed. This bioactive constituent shows potent activity against a broad spectrum of Gram-negative and Gram-positive bacteria such as *Listeria monocytogenes, Micrococcus luteus, Bacillus aureus, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa, Typhimurium, Vibrio* spp., and Salmonella enteric by preventing the formation of bacterial biofilm (Chaieb et al. 2011). The diverse therapeutic action of thymoquinone is demonstrated in Fig. 17.2.

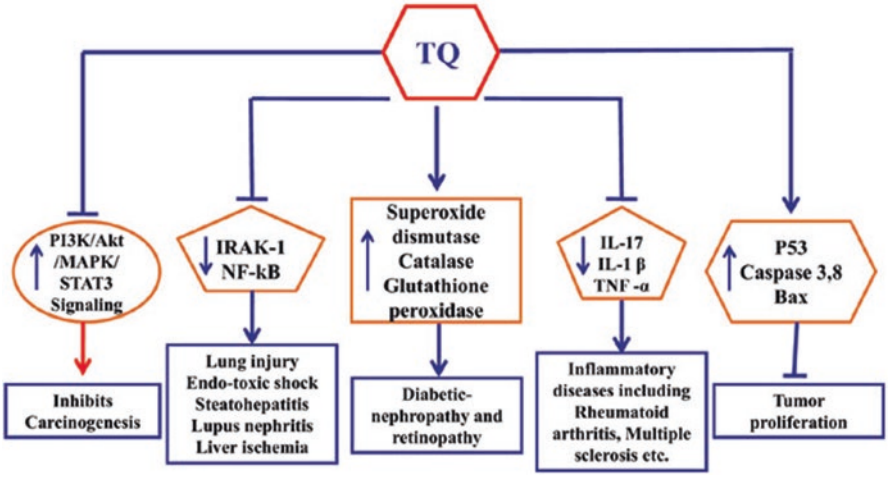

Fig. 17.2 Mechanism of action of thymoquinone

Interestingly, the antibacterial effect of oil of *Nigella sativa* has been reported to be effective against multidrug-resistant bacteria such as *Staphylococcus aureus* (Ugur et al. 2016). Moreover, the food industry uses black seed oil as an antimicrobial agent during the production of food to prevent food spoilage. Arici along with his coworkers reported the ability of black seed oil to inhibit the growth of spoilage, and pathogenic and lactic acid bacteria (Arici et al. 2005). Hasan et al. reported the antibacterial effect of methanolic and water extracts of black seed against Gram-positive bacteria such as *Streptococcus pyrogen, Proteus vulgaris, Klebsiella pneumonia*, and *Pseudomonas aeruginosa* (Hasan et al. 2013). The literature revealed the antifungal property of thymoquinone against *Fusarium solani* and *Aspergillus niger*. Moreover, the researchers were able to compare the antifungal property of thymoquinone with amphotericin B, an antifungal drug (Aljabre et al. 2015). Interestingly, the in vivo antifungal property of aqueous extract of black seed against candidiasis resulted in a high inhibitory effect with many-fold decrease in kidney, liver, and spleen (Khan et al. 2003). Other researchers evaluated the methanolic and ethanolic extracts of black seed and reported higher potency of black seed extract than that of standard amphotericin B against *Candida albicans, C. tropicalis, Cryptococcus laurentii, Cryptococcus albidus, Aspergillus fumigates*, and *Aspergillus flavus*. Also, literature revealed the antiviral activity of *Nigella sativa* oil. For example, Salem and Hussain reported the protective effect of black seed oil against murine cytomegalovirus. They found that the oil was able to completely inhibit the virus in the liver and spleen (Hussain and Hussain 2016; Salem et al. 2011). Despite these, the black seed oil was found to be effective against the influenza virus, laryngotracheitis virus, and hepatitis C virus. Further, the administration of black seed leads to improvement in clinical conditions and oxidative stress and results in decreasing viral load in the patients (Barakat et al. 2013). Therefore, while referring to the amount of literature present, eugenol and thymoquinone prove to be effective antimicrobial agents against various bacteria, fungi, and viruses. Moreover, the drugs are relatively safe with long-term traditional use. Thus, it is important to develop novel antimicrobial drugs using natural products and this can be achieved by applying nanotechnology and by completely understanding the mechanism of action of both drugs on the microbial cell.

17.3 Nanoformulation of Active Compounds

As discussed above, herbal medicine is an ideal candidate for antimicrobial activity against various Gram-negative and Gram-positive bacteria. They act by disrupting bacterial cell membranes or by damaging intracellular biomolecules as well as DNA and proteins. Moreover, chemical antimicrobial agents possess high toxicity and side effects. Therefore, the focus has been shifted toward formulating an antimicrobial drug incorporating natural sources. Since natural products are poorly soluble, highly unstable, and undergo chemical and enzymatic alterations, it is necessary to incorporate nanotechnology for the development and designing of new drugs for the

treatment of microbial infection. The introduction of nanotechnology plays a promising role in natural medicine by improving the pharmacokinetic profile of the drug candidate. Nanotechnology involves encapsulation or coating of a drug candidate in an appropriate polymer, which results in increasing the surface area, thereby increasing the solubility, permeability, and delivery of the drug to the target cells. The enhancement of drug delivery can be achieved by using chitosan, carbon, and ceramic nanoparticles. Various types of nanoformulations include nanoemulsions, nanocrystals, self-nanoemulsifying drug delivery systems, polymeric nanoparticles, polymeric micelles, lipid nanoparticles, and carbon nanotubes (Fig. 17.3). Among these, nanoemulsions, nanocrystals, and polymeric nanoparticles are widely used approaches. These methods are effective in reducing the particle size to the nanoscale. For example, nanoparticles of paclitaxel have been reported to cause a many-fold increase in bioavailability causing higher therapeutic activity. Nanocrystal and nanosuspension formation results in increased dissolution, saturation solubility, and relatively higher bioavailability (Zhang et al. 2011; Thadkala et al. 2014). Besides these, dendrimers, polymeric micelles, and self-nanoemulsifying drug delivery systems are gaining much attention. Nanomicelles have been reported to improve the stability of formulation and inducing a strong inhibitory effect in tumors. Dendrimers have a high affinity for both hydrophobic and hydrophilic drugs and have the ability to increase the solubility, transfect the cells, sustain the release of drugs, and enhance penetration and bioavailability of drugs (Pathak and Raghuvanshi 2015). Despite various advantages, the toxic effects of nanomedicines are related to their small sizes and intrinsic toxicity to the surface. This can be overcome by the development of herbal nanomedicines. Various literatures have been reported regarding different nanoformulations of thymoquinone and eugenol, which demonstrate enhanced physicochemical properties of the drugs (Fig. 17.3).

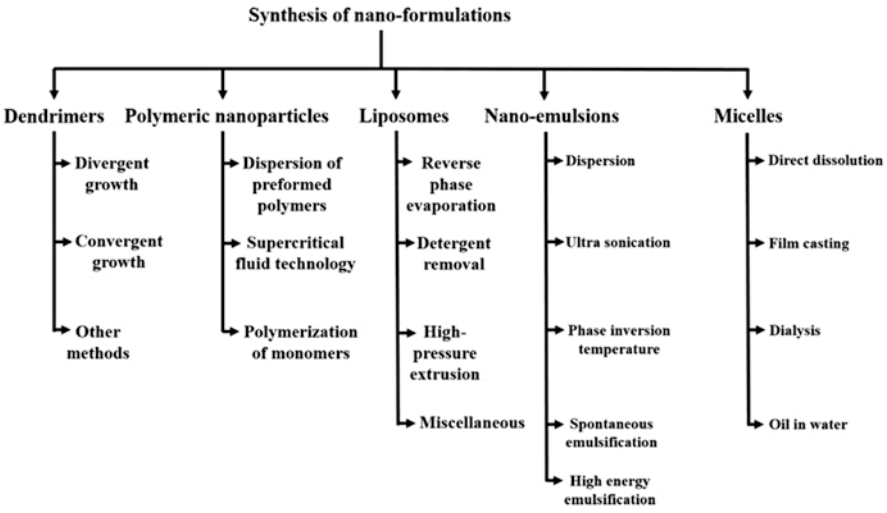

Fig. 17.3 Types of nanoformulations

17.3.1 Eugenol

Eugenol exhibits limited solubility and chemical stability, and due to its rapid absorption and metabolism, the rate of elimination is high. It is highly sensitive to chemical interaction and undergoes oxidation. This factor leads to poor bioavailability of eugenol. Therefore, encapsulation is necessary to improve the aqueous solubility and to prevent early absorption of eugenol resulting in improved efficiency and bioavailability (Jahangir et al. 2020). Nanoemulsion of eugenol with other carrier oil results in enhancement of transdermal permeation and stability of the drug. Encapsulation of eugenol with β-cyclodextrin shows promising antimicrobial activity of eugenol alone; similarly, the same formulation was developed by the molecular inclusion method. After freeze-drying, the developed formulation was smaller in size and showed negative zeta potential along with higher efficiency of encapsulated eugenol. Chitosan-based nanoparticles result in increased thermal stability of eugenol (Taleuzzaman et al. 2020). The synergistic effect of eugenol and carvacrol using chitosan for encapsulation shows effective antimicrobial activity against *S. aureus* and *E. coli*. Zhao along with his coworkers evaluated the ulcerogenic activity of oral microemulsion of ibuprofen and eugenol ester (Zhao et al. 2005). The solubility of optimized formulation was many folds higher than that in water. Further, according to Garg and his coworkers, solid lipid nanoparticles resulted in a sixfold increase in the activity of eugenol as compared to the free form of eugenol (Garg and Singh 2014). In another study, eugenol nanoemulsion gel was prepared. The optimized nanoemulsion was converted into Carbopol gel for the treatment of periodontitis. The formulation showed antibacterial activity and was effective in the treatment of periodontitis by acting as an anesthetic and analgesic. Another similar study involves the preparation and evaluation of controlled release eugenol mucoadhesive tablet for periodontitis. The composition includes Carbopol and hydroxyl methylcellulose in the ratio of 1:1, 1:2, and 2:1 along with 10 mg eugenol. Controlled drug release of the eugenol formulation was observed for a period of 8 hours. The Carbopol results in prolonged release of eugenol. The eugenol showed promising results as antibacterial, anesthetic, and analgesic (Razzaq et al. 2018).

Said and his coworkers formulated a water-based microemulsion of eugenol. The authors used the oil titration method for the incorporation of oil and eugenol in the Tween 20 solution. The formulation was further evaluated for antioxidant and antimicrobial activity. The antioxidant activity was measured by DPPH-free radical scavenging assay, while antimicrobial activity was measured by the agar disk diffusion method. The results showed promising antimicrobial activity of eugenol microemulsion in comparison with the crude eugenol (Hamed et al. 2012). In another study, novel eugenol–chitosan nanoemulsion was prepared by ultrasonication method. The nanoemulsion was prepared using chitosan and eugenol at a ratio of 1: 1 and Tween 20 at 1% w/v at 450 W for 6 min. The resulting formulation was spherical in shape and showed promising antioxidant and antimicrobial activity. Moreover, the formulation was stable at a greater extent and

showed prolong storage (Shao et al. 2018). The prepared eugenol-loaded solid lipid nanoparticles were evaluated for antifungal activity. The formulation was used in immunosuppressed rats for the treatment of oral candidiasis. The formulation showed faster drug release, higher efficiency, and greater antifungal activity. Poly(DL-lactide-co-glycolide) (PLGA) nanoparticles of eugenol and trans-cinnamaldehyde were developed for the evaluation of an antimicrobial activity. The formulation had greater efficiency in inhibition of Gram-positive bacteria (*Listeria* spp.) and Gram-negative bacteria (*Salmonella* spp.). In another study, eugenol-loaded nanoemulsion was prepared for the antiproliferative action of eugenol against liver and cancer cells. The apoptosis was measured by flow cytometry as a result of reactive oxygen species generation, and cell viability was measured by MTT assay. Several other works of literature have also been reported for the development and evaluation of eugenol-loaded nanoformulations for anti-inflammatory, anticancer, antibacterial, antifungal, and analgesic activity with greater therapeutic efficiency of eugenol after encapsulation. Gaysinsky et al. formulated eugenol microemulsion using carvacrol as micelle for encapsulation. The authors evaluated that an efficient antimicrobial activity could be the result of surfactant. They concluded that the surfactant was able to increase the permeability of the bacterial cell wall. Further, the addition of a hydrophilic surfactant may result in increasing the solubility of the drug. Also, other scientists concluded that improvement in the efficiency of a drug could be due to the adsorption of carvacrol to the bacterial cell surface, which further increases the concentration of eugenol in the target organ. According to the literature, antimicrobial microemulsion interacts with bacterial cell walls by the addition of surfactant. This interaction results in loss of cellular components leading to loss of viability of bacteria. Literature reveals that microemulsion formation results in distortion of a phospholipid bilayer, which further disrupts the plasma membrane leading to cell death (Gaysinsky et al. 2008) (Table 17.1).

17.3.2 Thymoquinone

Literature review reveals the use of thymoquinone nanoparticles as anticancer, anti-inflammatory, antimicrobial, antioxidant, and immunomodulatory. Bakal along with his coworkers evaluated the antibiotic property of thymoquinone against multidrug-resistant bacteria (Bakal et al. 2017). Also, other researchers compared the antifungal activity of different nanoparticles such as ketoconazole, amphotericin B, and thymoquinone against *Candida albicans* yeast and biofilm. They evaluated that antifungal activity of thymoquinone nanoparticles (160 μg/ml) was two- to fourfold higher than that of amphotericin B nanoparticles (0.31 μg/ml) and ketoconazole nanoparticles (0.62 μg/ml) (Randhawa et al. 2015). Nihei et al. developed a thymoquinone nanoparticle using a cold wet milling system and evaluated its pharmacokinetic profile. The researchers found that the synthesized

Table 17.1 Eugenol Nanoformulations

S. No.	Nanoformulation	Facts	References
1.	Microemulsion	Prepared microemulsion of eugenol and clove bud essential oil (CEO) investigates the antimicrobial activity by DPPH-free radical scavenging assay and agar disk dilution method. Outcome of these methods had shown that the microemulsion of eugenol was more effective than CEO	Hamed et al. (2012)
2.	Eugenol oil nanoemulsion.	Seed cotton is infected by fusarium wilt, and it can be controlled by eugenol oil nanoemulsion. The developed formulation-optimized result indicated that the *Z*-average diameter was 80 nm, and its shape is spherical. Three different concentrations of this formulation were evaluated by experiments in vitro and in vivo. Outcome of this study reveals antimicrobial effect of nanoemulsion	Abd-Elsalam and Khokhlov (2015)
3.	Eugenol–chitosan nanoparticles	Antibacterial and antioxidant activity of eugenol–chitosan nanoparticles is examined. Antimicrobial activity was assayed with gram-positive bacterium (*Staphylococcus aureus*) and gram-negative bacterium (*Escherichia coli*). A better or equivalent activity is that of the unmodified chitosan nanoparticles	Chen et al. (2009)
4.	Eugenol-entrapped ethosome nanoparticles	The formulation released drug in sustained manner and it used against fruit anthracnose, antibacterial activity (>93%) against fruit pathogens had founded greater efficacy that free eugenol. The advantage of sustained drug release was prolonged antibacterial action	Jin et al. (2019)
5.	Clove oil nanoemulsion	Nanoemulsions (NEs) of clove oil prepared for the treatment of infection are caused by microorganisms *Candida albicans* and *Candida glabrata*. It is suitable for topical application, use co and pluronic F-127 in different concentrations for the preparation of NEs by ultrasonic processor. Benefits of this formulation are less irritation on skin, and low dose had shown therapeutic effects	de Oliveira de Siqueira et al. (2019)
6.	Eugenol gel for periodontal delivery.	Prepared a eugenol gel formulation incorporated in NLC for sustained delivery. Many advantages of this formulation such as high bioavailability, low-dose high therapeutic value, and effectively delivered	Pokharkar et al. (2011)

(continued)

Table 17.1 (continued)

S. No.	Nanoformulation	Facts	References
7.	Nonionic nanoemulsion.	Antifungal activity investigated of oil-in-water nanoemulsion, prepared through low-energy method using nonionic surfactants. Optimized this formulation against *Fusarium oxysporum* f.sp. lycopersici (FOL)	Sharma et al. (2018)
8.	Nanoformulation of eugenol	Formulation contains eugenol (oil phase), tween 80 (surfactant), and PEG (co-surfactant), a novel nanoemulsion—Carbopol®934 gel. Evaluate this formulation on the basis of their pH, drug content, syringe ability, and mucoadhesion on the animal goat buccal mucosa. This formulation shows significant antibacterial effects in the treatment of periodontal disease	Ahmad et al. (2019)
9.	Eugenol with nanoparticles.	Periodontal diseases cured by the eugenol, but lipophilic nature of the molecule create challenge for the formulation development. With the application of nanotechnology, the therapeutic efficacy of the drug enhanced. Nanoparticles used for the delivery of drug have potential for the treatment of periodontal disease. Nanoparticles help to modulate the drug kinetics and help to deliver the drug in the right site at right time	Garg et al. (2018)
10.	Quantification of eugenol in *Myristica fragrans* seed extract by HPTLC	Developed a HPTLC method for the estimation of eugenol in *Myristica fragrans* seed, this method was robust and can be used for the optimization of nanoformulation of eugenol	Taleuzzamana et al. (2017)

thymoquinone nanoparticle was more photostable than that of free thymoquinone. Moreover, there was a sixfold increase in the oral bioavailability of nanoformulation thymoquinone as compared to the free form of thymoquinone solution (Nihei et al. 2016). Further, it is necessary to develop a proper strategy to overcome the problems that occurred during the nanoformulation of thymoquinone. As a large amount of excipient is required in the preparation of nanothymoquinone, therefore excipient should be stable, biocompatible, and biodegradable and should be free from side effects. Also, a prodrug can be designed using in silico modeling where the ADME profile of thymoquinone can be acquired for developing drug-likeness of thymoquinone by modifying its structure during the encapsulation of thymoquinone within nanoformulation (van de Waterbeemd and Gifford 2003). Literature review reveals that the first thymoquinone-loaded nanoparticle was developed in the year 2010 by Shah et al. where preparation, characterization, and in vitro analysis of nanothymoquinone were evaluated. Nanoformulation is relatively advantageous to other methods such as facilitate drug release by modulation of physicochemical properties, thereby enhancing its biological behavior. Moreover, the drug can be protected from degradation in the gastrointestinal tract

by encapsulation technique, which on the other hand enhances the stability of the drug and lower the dose. Besides these advantages, nanoformulations can be easily taken up lymphatic tissues resulting in effective and direct delivery of a drug in the blood and target organs. Various studies have been conducted where polymeric nanoparticles are used for intranasal delivery of thymoquinone to the brain. They concluded that upon encapsulation, nanoparticles were able to protect the core drug molecule from biological and chemical degradation. Further, being small in size, the nanoparticles were able to transport across the blood–brain barrier for efficient treatment of Parkinson's disease. Therefore, an increased bioavailability of drugs can be achieved in the brain. Thus, polymeric nanoparticles prove to be an attractive approach for in situ nasal gel formulation (El-Far et al. 2018).

Ganea et al. evaluated copolymeric nanoparticles of thymoquinone against the MDA-MB 231 cancer cell line and reported it to be much effective than that of free thymoquinone (Ganea et al. 2010). In the year 2016, Abdel mottled reported the anticolorectal properties of polycaprolactone-encapsulated thymoquinone nanoparticles using 6-week-old male BALB/c mice (Abdel-Mottaleb 2016). Sustained effects and enhanced proliferative activity of thymoquinone nanoparticles have been demonstrated against MCF 7 cell line by Shaarani et al. (2017). Anti-inflammatory effects of thymoquinone lipospheres have been reported by Jain et al. for psoriasis. The authors conducted in vitro and in vivo analyses. The in vitro study was conducted against RAW 264.7, a murine macrophage cell line, and decreased levels of interleukin 2, interleukin 6, and tumor necrosis factor-alpha were reported. On the other hand, the drug was able to reduce interleukin-17 and tumor necrosis factor-alpha in psoriatic skin of BALB/c mice (Jain et al. 2017). However, studies regarding safety concern for long-term use of thymoquinone are still lacking and need immediate attention (Table 17.2).

From the above findings, it can be concluded that nanotechnology is a rapidly emerging field for efficient drug delivery. Various efforts are being done to enhance bioavailability and patient compliance. The nanotechnology-based system appears to be a promising, effective, and safe drug delivery system for achieving site-specific drug targeting and overcoming the limitations of a potential drug candidate with a narrow therapeutic window and poor bioavailability. Therefore, nanotechnology has been widely used for the formulation of phytoconstituents. Despite the above advantages, it is important to understand detailed concepts of phytoconstituents and modern scientific methods to overcome the toxicity of bioactive constituents.

17.4 Toxicity of Bioactive Constituents

Since phytoconstituents are considered safe for administration, but due to broad range of application in the treatment of diseases, concern regarding the toxicological profile of natural products has also increased in recent years. Several

Table 17.2 Thymoquinone Nanoformulation

S. No.	Nanoformulation	Facts	References
1.	Thymoquinone-loaded PLGA nanoparticles	Formulation of TQ prepared, it encapsulated in nanoparticle PLGA (poly(DL-lactide-co-glycolide)) and examined its antimicrobial properties against *E. coli, Staphylococcus aureus*, and *Salmonella typhi* strains. DPPH radical scavenging activity was founded	Nallamuthu et al. (2013)
2.	Biofilm formation inhibited by TQ	Gram-positive bacteria and Gram-negative bacteria growth inhibited and also anti-biofilm activity. Studied with minimum inhibitory concentration in the range of 1.56–100 μg/ml. To understand the mechanism studied DiSC3, NPN, and ROS assay	Goel and Mishra (2018)
3.	Formulation of L-ascorbic acid and thymoquinone encapsulated in chitosan nanoparticles	Use nanoparticles to solve the challenge of combination hydrophilic and hydrophobic molecule delivery. Due to high surface area chitosan, NPs (CNPs), the L-ascorbic acid and thymoquinone, are more efficiently delivered and uptake by cells, prepared CNPs-LAA-TQ via ionic gelation routes because the preparation is nontoxic. Encapsulated together hydrophilic (LAA) and hydrophobic (TQ) in CNPs increase their therapeutic efficacy	Othman et al. (2018)
4.	Assessment of antibacterial activity of synthesized silver nanoparticles using *Nigella sativa*	Biosynthesized silver nanoparticles (Ag-NPs) from *Nigella sativa* (NS) seed extract using ethanol and chloroform. Studied antibacterial activity against some drug-resistant bacteria and it has been used for the treatment and prevention of drug-resistant bacteria. Microtiter broth dilution process using ciprofloxacin as standard for assessment of antibacterial activity of Ag-NPs–NS seed extract	Shehensha and Jyothi (2020)
5.	Nanoformulation of amphotericin-B, ketoconazole, and TQ	Amphotericin-B, ketoconazole, and TQ drugs encapsulated in nanoparticle for its better efficacy using the ball milling technique. Comparative study of nanoformulation and its conventional forms against *Candida albicans*, yeasts, and *Candida* biofilm have been done. Founded two to four times more effective disinfectant efficacy	Randhawa et al. (2015)
6.	Thymoquinone liposomes	Diabetic mice with infection of *C. albicans* treated with Liposomal formulation of TQ (Lip-TQ). Used dose of 10 mg/kg of Lip-TQ result founded that in blood glucose level reduced and systemic infection of *C. albicans* also. The survival rate of mice was 70% when treated with Lip-TQ, and in the case of free TQ, the survival rate was 20%	Khan et al. (2018)

(continued)

Table 17.2 (continued)

S. No.	Nanoformulation	Facts	References
7.	Thymoquinone niosomes	Quantification of TQ has been done by HPLC and the formulated TMQ-loaded niosomes (TMQNIOS). Studied various parameters such as permeation study, in vitro drug release, surface morphology and confocal laser scanning microscopic (CLSM), enhancement of permeation founded in TMQNIOS. Efficacy of this formulation is high	Gilani et al. (2019)
8.	Nanocarrier PEGylated LMW chitosan used for TQ	This formulation was stable and had better antimicrobial activity. Optimize the formulation-founded stable and constant particle size along with polydispersity index. Performed in vitro studied, results indicate that the percentage of micellization of TQ compared to mixed micelles	Vignesh Kumar et al. (2020)
9.	Thymoquinone-loaded coconut oil nanostructured lipid carriers (NLCs)	NLC was used for the development of TQ formulation, using also Compritol® 888 and coconut oil. Melt emulsification and sonification method used for the preparation of formulation using Poloxamer 188 as a surfactant. In in vitro study, a biphasic release pattern was observed, and burst release of drug in first 2 h followed by a gradual release	Fahmy et al. (2020)
10.	Encapsulation of thymoquinone using solid lipid nanoparticle	Formulated, thymoquinone-encapsulated solid lipid nanoparticle (TQ-SLNs) studied the thermal behavior result found that a melting enthalpy value of $\Delta H = 131.7$ J/g and attained peak at 69.5 °C indicate greater stability and reduced lipid crystal matrices; additionally, stability analysis of TQ-SLNs showed a stable encapsulation enhancement effect and almost increased five times bioavailability after oral administration	Surekha and Sumathi (2016)

pieces of literature have been reported for toxicological effects of thymoquinone and eugenol.

Though no chronic toxicity of thymoquinone was reported in the literature, the authors revealed various side effects along with acute and subacute toxicity in vitro and in vivo studies. In the year 2012, Harzallah et al. evaluated the oxidative damage effect of thymoquinone in the liver and kidney. The study involves the administration of intraperitoneal injection of thymoquinone at various doses (10, 20, 40, and 80 mg/kg) to BALB/c mice. An induction in oxidative damage activity was reported in higher doses of thymoquinone, i.e., 40 mg/kg and 80 mg/kg. The oxidative effect was recorded due to an increase in malondialdehyde levels and catalase activity. The authors further evaluated the genotoxic activity of thymoquinone at 80 mg/kg and found a significant increase in chromosomal aberration with a high DNA damage index in the organs. In another study, thymoquinone was administered intraperitoneally to albino rats for a different time period (1, 4, 7, 10, and

14 days) at different doses (0.5, 1, 2, 4, 6, and 8 mg/kg). The authors evaluated thymoquinone effects on blood lipids in the rat. The drug was able to reduce triglyceride levels, high-density lipoproteins (HDLs), and low-density lipoproteins (LDLs). The animals were safe at all doses except at 8 mg where animals died within 1 week of administration and showed signs of peritonitis (Mashayekhi-Sardoo et al. 2020).

Badary et al. evaluated the cytoprotective activity of thymoquinone in Swiss albino mice. Thymoquinone was added to drinking water at 100, 200, and 300 ppm concentration. The water was given to male Swiss albino mice for 90 days. Microscopic examination of tissues such as kidneys, heart, and liver showed no damage. Other serum concentrations such as urea, triglycerides, creatinine, and LDH were normal. Since no mortality occurred, therefore, no toxicity was recorded. However, a significant decrease in fasting blood sugar levels was observed. The results concluded that it was safe and possess cytoprotective activity (Badary et al. 1998). Ong et al. formulated thymoquinone nanoparticles by encapsulation of crude drug in lipid carrier. The resulting thymoquinone nanostructured lipid carrier (TQNLC) possesses high drug loading capacity and high encapsulation efficiency. The authors further conducted in vivo studies using female BALB/c mice. Oral administration of thymoquinone and TQNLC was done at three different doses such as 5, 50, and 300 mg/kg body weight. Death was seen in mice receiving 300 mg/kg within 24 h of treatment. Delayed death was seen after 48 h of treatment in the case of mice receiving TQNLC. No mortality was observed in mice receiving 5 and 50 mg/kg dose of thymoquinone and TQNLC. The LD50 of both the formulation was found to be 50–300 mg/kg and 300–2000 mg/kg. The authors concluded that encapsulation of thymoquinone results in reduced toxicity of thymoquinone. Therefore, encapsulation could be a promising approach for the delivery of thymoquinone. Later, the authors administered 1, 10, and 100 mg/kg of both formulations in both sexes of BALB/c mice for a period of 28 days. During the experimental period, no significant changes in biochemical and hematological profiles were seen. In the liver of both sexes, cell degeneration was observed following oral administration of 100 mg/kg dose of both formulations. However, no mortality was observed in either sex (Haron et al. 2018).

The results of literature research were consistent, and it can be concluded that it is completely safe to use thymoquinone for clinical applications as no chronic toxicity was observed in animal models following thymoquinone administration.

Though eugenol is considered safe in the food industry, toxicity studies of eugenol are of greater concern due to its wide range of applications in the pharmaceutical industry. Various adverse effects have been reported after the use of dental products containing eugenol including ulcers, tissue necrosis, irritation to the skin, and allergic dermatitis. Researchers found a similar result between eugenol and paracetamol poisoning. The authors reported a case study involving ingestion of 5–10 ml of clove oil by a 2-year-old boy. The patient was in a deep coma after 3 h of administration. The patient suffered from generalized seizures for 8 h, and within

24 h of administration of clove oil, the patient suffered from liver failure along with disseminated intravascular coagulopathy. The patient recovered and gained consciousness after 1 week of symptomatic treatment (Hartnoll et al. 1993). Gaulet and his coworkers studied the toxic effects of eugenol at anesthetic doses in clawed frogs. The authors demonstrated renal tubular apoptosis where distal tubules were affected in the medulla. They reported some morphological changes such as hepatic necrosis, adipose tissue hemorrhages, and the presence of hyaline membranes in the lung. In another study, the authors evaluated the cytotoxicity of eugenol using human osteoblastic cells. The toxic effects were evaluated by performing cytotoxicity and cell proliferation assay. They demonstrated significant periapical toxicity of eugenol. Eugenol inhibited cell proliferation by decreasing the activity of dehydrogenase enzymes. The IC50 was reported to be 0.75 mmol/l. However, the addition of *N*-acetyl-L-cysteine was able to protect the cells from the inhibitory action of eugenol (Ho et al. 2006).

The cytotoxicity of eugenol was determined in HL-60 human promyelocytic leukemia cells. Eugenol-treated HL-60 cells were evaluated for cytotoxicity and apoptosis. The results demonstrated apoptosis by a significant increase in fragmented DNA. Moreover, 50% of cell growth was inhibited by eugenol at a concentration of 23.7 μM. Further, the authors evaluated the putative pathway for the action of eugenol. They concluded that apoptosis occurred as a result of ROS generation, which induces mitochondrial permeability, the release of cytochrome c in the cytosol, and reduced levels of anti-apoptotic proteins (bcl-2) levels ultimately leading to cell death (Yoo et al. 2005). In another study, the authors demonstrated the effects of eugenol and cinnamaldehyde in isolated rat liver mitochondria. Due to the lipophilic character of both components, they were able to penetrate in the intracellular organs. Both compounds were able to decrease the membrane potential, inhibit Na^+/K^+ ATPase activity in the kidney and intestine, and stimulate ATPase at a concentration equal to or greater than 0.3 μM, ultimately disturbing mitochondrial functions. Toxicity study of aspirin eugenol ester was evaluated for a period of 15 days at a dose of 50, 1000, and 2000 mg/kg. The authors evaluated a significant increase in total bilirubin levels, alanine aminotransferase, and aspartate aminotransferase. However, no adverse effects were observed at a daily dose of 50 mg/kg. Another case study reported unexpected hypersensitivity reactions of zinc oxide eugenol in an 8-year-old boy during dental treatment. It was found that eugenol causes dermatitis with evident redness in the body parts including the face, neck, lower and upper limbs, and behind the ears. The composition consisted of 10% zinc oxide, 1% aqueous formaldehyde solution, and sodium chloride along with eugenol. The skin prick test revealed a negative response for all the compounds except for eugenol (Tammannavar et al. 2013).

Since extensive research has been done on the biological activities of eugenol, research related to the toxicity of eugenol is still limited. A long-term in vitro and in vivo human research is still undiscovered related to the chronic toxicity of eugenol.

17.5 Challenges for the Development of Herbal Nanoformulation

Herbal drugs have achieved great concern in today's era as people seek natural remedies. They have been used since ancient times for the treatment of diseases and maintenance of health. They serve as a common ingredient in homeopathic, ayurvedic, Unani, and another system of medicines and are considered safe. They are widely used in the treatment of cirrhosis, gall stones, menopause, asthma, Alzheimer's, acne, migraine, and impotence and as an antimicrobial, anti-anxiety, anti-inflammatory, antidiabetic, antispasmodic, hepatoprotective, and analgesic. Herbal drugs possess many advantages including fewer side effects, enhanced tolerance, and low cost, and are efficient. The main disadvantage related to herbal medicine involves complexity in the standardization of drugs, stability, and solubility of drugs. Due to poor solubility, phytochemicals suffer from poor absorption and poor bioavailability. Moreover, rapid clearance and high protein binding of phytochemicals pose a major problem in the design and development of the drug. Further challenges in the development of herbal medicine include toxicity issues of the plants, low yield of a bioactive constituent, limited literature for possible side effects, shortage of medicinal plants, and limited documentation for drug–phytochemical interactions. This can be overcome by using nanotechnology for the enhancement of efficacy, solubility, and stability of drugs.

Taxol being a potent anticancer agent isolated from the barks of *Taxus brevifolia* is capable of inhibiting tumor cells during mitosis. In the year 1992, the drug got the approval of the FDA against ovarian cancer, and in the year 1994, FDA approved its use for metastatic breast cancer. Due to low yield and difficulty in isolation of taxol, it accounts for a high cost. To improve the solubility of taxol, it was encapsulated in cremophor, but later researchers found that camphor was toxic for endothelial, epithelial, and living cells. However, further research for the treatment of cancer lead to the development of nanoformulation of paclitaxel bounded to albumin. The protein-bound paclitaxel reported higher solubility and higher tissue distribution. The drug was effective with other cytotoxic agents and prolonged the survival of patients. The formulation was further approved by FDA (Wani and Horwitz 2014). The targeted therapy has been widely applied in cancer to further enhance the efficacy of herbal medicines. This involves passive targeting, active targeting, and target-activated system. The passive targeting includes the incorporation of the drug in a nanoparticle that can passively reach the tumors. The active targeting involves the delivery of the herbal drug by attachment of ligands to the polymer. Target-activated systems are activated by pH or certain enzymes that respond to the target tissue. The major challenge in the development of nanoformulation includes its stability. Some literature reveals that nanoparticles are not able to hold the drug due to leakage of contents upon interaction with blood components. Another challenge is the residence time of nanoparticles in the metabolism system of the body. Stealth coating of nanoparticles with polyethylene glycol (PEG) was able to overcome

this issue. However, the disadvantages of PEG are of great concern. Another issue related to nanoformulation involves changes in the structure of drug components, which can adversely affect the components of the formulation with negative effects. This can further alter the safety, efficacy, and stability of the herbal product. The acceptance of nanoformulation for clinical use has to undergo various standards and desirable pharmaceutical and physicochemical characterization including surface morphology, size, distribution, surface charge, chemistry, and pharmacokinetic profile of the nanoparticles. While these components seem to be similar to that are faced by any drug, the multicomponent nature, complexity, and a large number of additional variables related to nanoformulations make it difficult to control the processes and predict the biological behavior of the formulation in the body (Pathak and Raghuvanshi 2015).

Aseptic manufacturing of nanoparticles is another major challenge. Nanoformulation of the sterile product might get damaged by the techniques of sterilization such as autoclaving and gamma irradiation. In the case of flexible particles such as liposomal preparation, sterile filters can be used as an alternative. However, on the other hand, rigid particles such as polymers, silica particles, and metals may possess difficult infiltration due to the pore size of the particles and filtration membranes. The nanoformulation of dry materials is another challenge as they are distributed as aerosols. Such particles get deposited in the lung resulting in pulmonary toxicities such as pulmonary fibrosis, pleural effusion, and granuloma. Therefore, current challenges involve the design and development of methods to control the problems associated with interactions of nanoformulations with biological systems (Desai 2012).

New challenges related to nanoformulation involve a meeting of international standards for targeting efficiency, biocompatibility, and toxicity profile of the drug. Also, the ease of scale-up processes and the requirement of a multifunctional system for fulfilling biological and therapeutic demands are other challenges that need much concern in the future. The smaller size of nanoparticles captures more surface area per unit mass, making it more reactive to the cellular environment. This leads to the enhancement of the intrinsic toxicity of the particles. The major component of these particles is carbon, which may cause cardiovascular effects. Moreover, particles that are fine and ultrafine in nature can easily be inhaled resulting in an assessment of vasculature leading to cardiovascular dysfunction. Clearly, the design and development of herbal nanoformulation come up with a lot of obstacles.

The future of nanoformulation involves the design and development of personalized medicine for the clinical application of a particular drug in a subgroup getting benefit from the therapy. This approach can further reduce the size of clinical trials leading to immediate approval. Additionally, tissues such as tumors act as a natural biological barrier for the delivery of the drug to the organ. This can be controlled by a better understanding of the active transport mechanism and delivery of nanomedicines. The health authorities need to respond to such techniques that could provide steps for the development of quality, safe, and efficient drugs in the market and can lead to advances in drug development.

17.6 Conclusions

The literature reveals that the use of nanotechnology for the delivery of herbal medicines has been the most promising way to achieve the efficient biological activity. Thymoquinone and eugenol, active natural constituents derived from natural sources, are widely used in the treatment of a wide range of diseases as antimicrobial, antidiabetic, analgesic, anti-inflammatory, anesthetic, immunomodulatory, hepatoprotective, and antioxidant. These natural sources come up with several side effects and a lack of information regarding potential toxicity. Therefore, the incorporation of herbal drugs in novel drug delivery systems leads to enhanced bioavailability, sustained release action, and reduced toxicity. Extensive research has been done for the antimicrobial activity of thymoquinone and eugenol against a broad spectrum of Gram-positive and Gram-negative bacteria. The attempt to use bioactive constituent-loaded nanoparticles has its own advantages and could come up with an effective treatment strategy to be seen shortly.

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Chapter 18
Nanoparticles as a Future Alternative Against Multiple Drug Resistance

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18.1 Introduction

Microbial infections are a leading cause of chronic infections and mortality (Gargano and Hughes 2014). Antibiotics have been used for treatment against microbial infections due to their cost-effectiveness and potent outcome. However, several studies have reported that the widespread use of antibiotics has become a major cause of the development of multiple drug-resistant bacterial strains. Bacterial multiple drug resistance against antibiotics has become a major challenge for human health for the treatment of bacterial pathogenic disease (Desselberger 2000). The statistical data of research reports against infection caused by multiple drug-resistant bacteria to antibiotics have increased many folds day by day. These reports have shown that resistance developed by bacteria against multiple antimicrobial agents becomes a major threat to human medication in Europe. In Europe, the mortality rate from multiple drug-resistant bacterial infections is enhanced and the amount of money spent by the government becomes €1.5 billion per year of the economy (Rémy et al. 2015). Nowadays, about two million people in the USA are suffering from antibiotic-resistant microorganisms per year with a rate of mortality of about

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23,000 as a direct result. The development of new antibiotics or antimicrobial agent is not an easy task, and this gap in new antibiotics or antimicrobial agent has further become worse in the circumstances of antimicrobial resistance (Li and Webster 2018).

Nanotechnology is a multidisciplinary promising area of biological science in the advancement of nanoscale research (Wong et al. 2013). Interacting with biological molecules at the nanoscale opens up a broad field of research and application. There are different methods for the preparation of nanoparticles and classified into conventional and biological methods. A conventional method deals with the fabrication of nanoparticles by using chemicals by adopting the top-down approach and a bottom-up approach. However, by biological methods, nanoparticles are fabricated by using living things such as plants leaves, stems, seeds, flowers, latex, and microbes (Shamaila et al. 2016a). The main constituents of plant extracts are bioactive polyphenols, sugar, alkaloids, terpenoids, phenolic acids, etc., which may have a very important responsibility in reducing the metallic ions and their stabilization (Castro et al. 2011).

Nanotechnology is providing a platform to modify important properties of metals and develop nanoparticles with potential applications in drug delivery, diagnostics, cell labeling, biomarkers, and novel antimicrobial for the treatment of various diseases (Singh and Nalwa 2011). Hence, researchers are shifting toward future alternatives of multiple drug resistance and silver nanoparticles as well as gold nanoparticles may be alternative. Silver is precious heavy metal that has been used as antibacterial agent since ancient times. In the current scenario, due to the beginning of antibiotics progress, the uses of silver in medical applications against bacterial infections were day by day declined (Chen and Schluesener 2008). The manipulation in size of silver by using nanotechnology technique resulted in their enhanced antimicrobial effects. Due to changes in physicochemical properties, AgNPs have served as antibacterial agents because of their high ratio of surface area to volume and their exclusive chemical and physical characteristics (Kim et al. 2007). It has been reported that the AgNPs with size range of 10–100 nm have potent antimicrobial properties against Gram-negative and Gram-positive microorganisms (Morones et al. 2005). The antibacterial or antimicrobial activity of AgNPs are extensively studied by many researchers against the multiple drug-resistant strains as well as pathogenic bacteria. It was found that AgNPs act as potent weapons against the multiple drug resistant organisms such as vancomycin-resistant *Staphylococcus aureus,* ampicillin-resistant *Escherichia coli*, and erythromycin-resistant *Streptococcus pyogenes*.

Gold is another precious heavy metal. Gold nanoparticles (AuNPs) are commonly used in many biological applications such as medical, gene therapy, and biosensor for disease diagnosis. AuNPs are easily prepared by co-precipitation methods and have lower toxicity as compared to other metallic NPs such as AgNPs (Shamaila et al. 2016a, b). Gold nanoparticles and ionic form have been studied for antibacterial activities. Recent research reports showed that AuNPs have antibacterial properties (Zhou et al. 2012).

Bacteria became resistant to multiple antibiotics via various pathways; thus, the therapeutic efficacy of antibiotics against infectious microorganisms declines. Most

of the bacterial antibiotic-resistant mechanisms are not relevant for nanoparticles (NPs) of AgNPs and AuNPs because these nanoparticles directly act on cell wall of bacteria after contract. AgNPs and AuNPs exert their antibacterial properties through differing metabolic and biochemical pathways, thus acts effectively against a variety of multiple drug-resistant microorganism stains. It is very difficult for bacteria to develop resistance against these NPs. This chapter covers a general layout of nanoparticle fabrication, the mechanism involved in the development of antibiotic resistance, and the mechanism of action of silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs) in bacterial cells by which we may consider these NPs as a future alternative against MDR.

18.2 Antibiotic Resistance Mechanisms

Generally, the current running antibiotics or antimicrobials that are most frequently being used against harmful bacteria or microorganism during infection have their mainly three action sites such as the bacterial cell wall, replication of bacterial DNA, and translational of proteins. Unfortunately, bacteria become resistant against antimicrobial agent by induction of mutation in bacterial chromosomes or by intrinsic factor contribution. After the development of resistance against antimicrobial agent, the normal type of microorganism may be terminated by using of drug. However, the microorganism that became mutant can survive and spread the resistance via vertical or horizontal transfer of genetic material (Arzanlou et al. 2017). There are the following mechanisms by which bacterial cell may be able to develop resistance against antibiotics or antimicrobial agent and nonantibiotics or nonantimicrobial agent (Fig. 18.1).

1. **Drug influx**: The porin channel is responsible for drug influx in bacterial cell. It has been found that very poor drug influx occurs via porin channel due to modification in bacterial cell wall permeability by mutation (Gupta and Birdi 2017). For example, the resistant development in *Pseudomonas aeruginosa* against imipenem.
2. **Drug efflux**: It has been observed that majority of the antimicrobial drugs that enter the bacterial cells undergo rapid drug efflux from the target bacterial cell. For instance, conversion of *Pseudomonas aeruginosa* into a multiple drug-resistant pathogen due to mutation in the major regulatory protein responsible for rapid and active drug efflux. The rapid and active drug efflux and poor influx alter drug effective concentration at action site that may cause sublethal drug concentration at the site of drug action and leading cause of target-based drug resistance. Some of the microbial strains have developed antimicrobial resistance against some drugs such as sulfonamides, aminoglycosides, streptogramins, tetracyclines, and chloramphenicol (Munita and Arias 2016).
3. **Inactivation of drug molecules**: For the development of drug resistance, molecule inactivation via covalent binding is also a common mechanism. For exam-

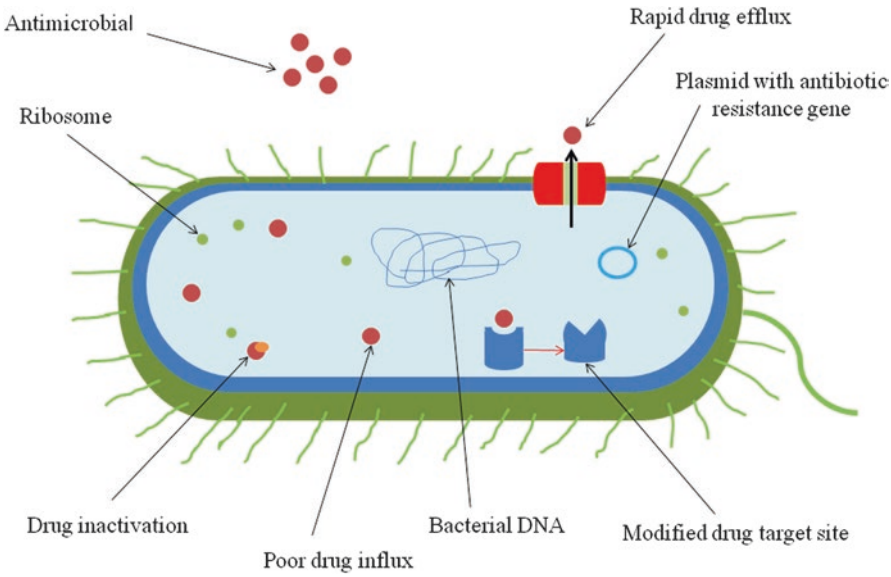

Fig. 18.1 General mechanism of bacterial resistance against antibacterial resistance

ple, β-lactam antibiotics (amoxicillin, ceftazidime, imipenem, penicillin, ampicillin, piperacillin, etc.) are inactivated by the cleavage of β-lactam ring in *H. influenza, S. aureus*, and *N. gonorrhea* (Munita and Arias 2016).

4. **Resistance plasmids**: Resistance plasmid is also called R plasmid, which has multiple resistance genes and is also responsible for transmission resistance. Bacteria have mobile genetic elements known as transposons, and these transposons facilitate horizontal genetic material transfer in condition of bulk metal ions and antibiotics (Baker-Austin et al. 2006).
5. **Modification/protection of target site**: Here the resistance occurs due to modification or alteration of microbial genes which induces conformational changes in the drug's target site making it inactive or therapeutically less effective. As for example, the rpoB gene of *Mycobacterium tuberculosis* after mutation become resistance to rifampin (Ao et al. 2012), and penicillin resistance in *Streptococcus pneumoniae* by the modification in penicillin-binding proteins (Kocaoglu et al. 2015).
6. **Biofilm formation**: Surface-associated microorganism generates three-dimensional biofilm made up of extracellular polysaccharide matrix that acts as protective cover for microorganisms against antibiotics or antimicrobial agents. The biofilm-forming bacterial strains are susceptible for gene mutation. Quorum sensing is another mechanism by which microbial gene expression may be responsible for secretion and synthesis of extracellular polymeric matrix. These extracellular polymeric matrixes make a protective cover around bacterial cell and prevent from high concentration of antimicrobial or antibiotics that is leading cause of some chronic bacterial infections like endocarditis, periodontitis,

cystic fibrosis, and prostatitis. Bioavailability of antibiotics reduces biofilm matrix due to slowing down diffusion of drugs. There are many scientific reports based on 3D bacterial biofilms as an inducer of resistance in several microorganisms (Liaqat et al. 2019).

These resistance development mechanisms of microorganism are leading cause of hindering the antibacterial drugs/antibiotic effectiveness against harmful microorganism.

18.3 Nanoparticle Fabrication Approaches

Nanotechnology is the branch of science, which deals with the fabrication of nanoparticles and their application in biological systems. There are different methods for the fabrication of nanoparticles; they are classified into conventional and biological methods (Fig. 18.2). By conventional methods, nanoparticles are fabricated by using chemicals by two basic approaches, the top-down and bottom-up approaches. The metallic substances are generally available in bulk materials, and the preparation of metallic nanoparticles from bulk materials into fine nanoparticle top-down approach will be applicable. But this approach has its limitations such as high energy consumption, formation of heterogeneous nanoparticles, very complex process of synthesis, and not eco-friendly, whereas bottom-up is a process in which we can easily prepare clusters of atoms and molecules by its self-assembly.

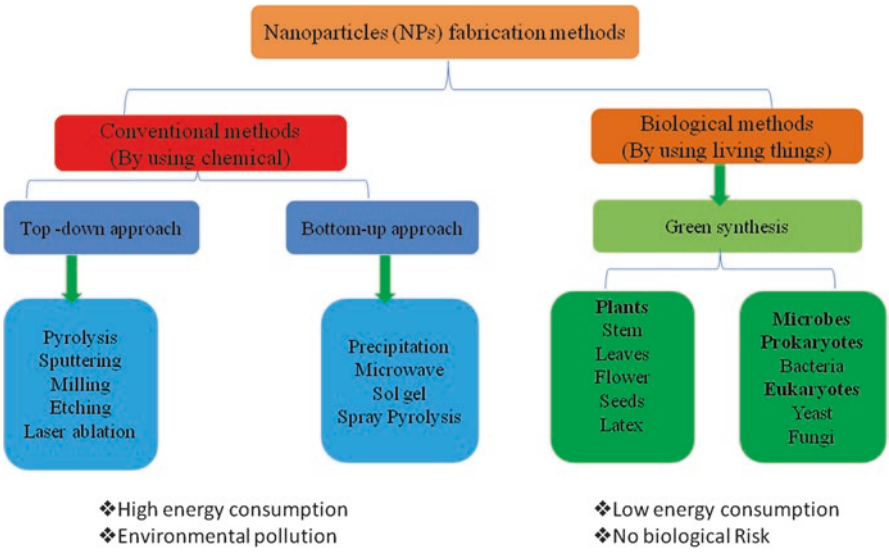

Fig. 18.2 Schematic representation of different approaches for nanoparticle fabrication

The bottom-up approach produces desired homogeneous metallic nanoparticles. By biological methods, we fabricate nanoparticles by using living things such as plants leaves, stems, seeds, flowers, latex, and microbes. Biological methods are less energy-consuming and eco-friendly.

18.3.1 Silver Nanoparticles (AgNPs)

Silver is a precious metal having antibacterial efficacy and has been recognized from ancient times (Reidy et al. 2013). Recently, various researchers have been reported that AgNPs as promising antimicrobial efficacy can be used as antimicrobial agent for the treatment against microbial infections (Natan and Banin 2017). The method of chemical reduction or green chemistry is a very traditional method for AgNP synthesis by using plant extract/microbial (Ribeiro et al. 2018). Many researchers have reported that the antibacterial activity of AgNP can be attributed to the cell death caused due to disruption of bacterial cell wall (Bondarenko et al. 2013), inactivation of respiratory chain enzyme, ROS production, and cellular components decomposition (Dakal et al. 2016). The permeability of bacterial cell membrane increased after the incorporation and adsorption of AgNPs due to depolarization of the bacterial cell wall. This ultimately makes the bacterial cell wall more permeable.

When AgNPs enter into the bacterial cell they induce ROS generation that is responsible for the inhibition of ATP production and DNA replication (Ramalingam et al. 2016). At the same time, AgNPs can release Ag^+ that is responsible for antibacterial activity by interacting with thiol-containing proteins (Durán et al. 2010). Another study has been reported that displays AgNPs as potent antimicrobial agent as compared to other antibiotics (Saeb et al. 2014) such as vancomycin or gentamicin against bacteria *P. aeruginosa*. One more study has reported that AgNPs are capable to inhibit *E. coli* and *S. aureus* growth through upregulation of ATPase pumps and expression of an antioxidant gene (Nagy et al. 2011). Recently, some researchers have been reported that AgNPs from P. amarus extract have potent antimicrobial agent against *P. aeruginosa* MDR bacteria (Singh et al. 2014). Another recent study has reported that rod-shaped and spherical AgNPs have a potent antibacterial agent for Gram-positive and Gram-negative bacteria (Acharya et al. 2018). AgNPs with conjugate have excellent antibacterial activity. Recently, Pal et al. have reported that AgNPs with a cysteine-containing AMP conjugate exhibit increased stability of Ag-S bond and enhanced antibacterial activity (Pal et al. 2016). On the basis of various previous reports, we have summarized the role of silver nanoparticles (AgNPs) against antimicrobial resistance and their mechanism of action in Table 18.1. At the end of this section, we have a clear view about AgNPs as possible candidature as antimicrobial agent against drug-resistant bacteria and still more extensive research work is required to explore more possibilities.

Table 18.1 Silver nanoparticles (AgNPs) against antimicrobial resistance and their mechanism of action

Targeted microorganism	Antibiotic resistance type	Mechanism of action	References
S. pneumoniae	Teicoplanin-resistant type	By generation of reactive oxygen species, silver ion uptake by cell membrane, intracellular cascades	Thapa et al. (2017)
P. aeruginosa	Ofloxacin-resistant type	By evading efflux pumps of multiple drugs	Ding et al. (2018)
S. mutans, Enterobacter cloacae	Biofilm formation	By reactive oxygen species-mediated disruption of cell membrane	Kulshrestha et al. (2017)
E. coli	MDR	By generation of reactive oxygen species	Siddiqi et al. (2018)
B. subtilis, S. aureus, K. pneumoniae	MDR	By the penetration of bacterial cell	Acharya et al. (2018)
A. baumannii	MDR	By structural change in cell wall permeability	Chang et al. (2017)
S. epidermidis, Mycobacterium smegmatis Vibrio fluvialis, P. aeruginosa	Formation biofilm/MDR	By adenosine monophosphate conjugation	Lambadi et al. (2015), Jaiswal et al. (2015)

18.3.2 Gold Nanoparticles (AuNPs)

Gold is precious heavy metal since ancient times, and it is inert and nontoxic in nature. From bulk gold materials, gold nanoparticles (AuNPs) can be prepared by gold salt through traditional chemical reduction method (Shah et al. 2014). The AuNPs can be synthesized most commonly by chloroauric acid reduction by citrate through chemical synthesis method (Fernandes and Baptista 2017).

According to some research reports, AuNPs have potential antimicrobial agents, but based on some researchers, controversy still remains (Shamaila et al. 2016a, b). AuNP concentrations have also played important role in bactericidal properties. According to some research reports, AuNPs generally do not serve as antimicrobial agent at low concentration and serve as weakly antibacterial agent at very high concentration (Shareena Dasari et al. 2015; Zhang et al. 2015). However, some research reports have described that the antimicrobial effect of AuNPs is associated with hindering ATPase activity, collapse in membrane potential, hindering the binding to tRNA of ribosomal subunit (Cui et al. 2012), and attachment to the respiratory chain of microorganism (Shamaila et al. 2016a, b).

Gold NPs in cationic and hydrophobic functionalized form have been reported as antibacterial agent for the Gram-positive and Gram-negative uropathogen. According to the research report, it has been reported that AuNPs are very less toxic in nature for mammalian cell and its chance to become resistant is very low (Li et al. 2014). Also, gold nanoparticles with ultra-thin graphite or graphite-like carbon

nitride have served as potent antibacterial displaying potential against MDR Gram-positive as well as Gram-negative bacteria and high effectiveness against biofilms (Wang et al. 2017). Other studies have described that AuNPs to the conjugate of antibiotics enhance their inherent antibacterial potential against strain of multiple drug resistance (Payne et al. 2016). On the basis of previous reports, we have summarized the role of AuNPs against antimicrobial resistance and their mechanism of action in Table 18.2. In the end, keeping all research reports and researchers' views in our mind we can say that AuNPs have proved their possible candidature as antimicrobial agents against drug-resistant bacteria but still need more research work to further explore more possibilities in this field.

Table 18.2 Gold nanoparticles (AuNPs) against antimicrobial resistance and their mechanism of action

Targeted microorganism	Antibiotic resistance type	Mechanism of action	References
K. pneumoniae, *E. coli*	Cefotaxime-resistant type	By damage of bacterial genetic material, through bacterial cell wall disruption	Shaikh et al. (2017)
A. baumannii, *Proteus mirabilis*	Carbapenem-resistant type	By osmotic balance disturbance and disruption of bacterial cell wall integrity	Shaker and Shaaban (2017)
Streptococcus bovis, *E. Aerogenes*	Kanamycin-resistant type	By disruption of cell wall	Payne et al. (2016)
S. aureus	Methicillin-resistant type	By use of photothermal therapy with reactive oxygen species generation	Millenbaugh et al. (2015), Mocan et al. (2016), Hu et al. (2017), Ocsoy et al. (2017)
P. aeruginosa, *S. aureus*	Formation biofilm	By laser excitation for efficient photothermal response and efficient killing of biofilm	Yu et al. (2016), Pallavicini et al. (2014)
E. coli, *P. aeruginosa*, *B. Subtilis*	Formation of biofilm	By generation of reactive oxygen species	Wang et al. (2017)
S. epidermidis, *S. haemolyticus*	Formation of biofilm	By combination with antimicrobial agent	Roshmi et al. (2015)
S. aureus	MDR	By photoacoustic detection and photothermal therapy	Galanzha et al. (2012)
K. pneumoniae, *E. cloacae*	MDR	By use of photodynamic therapy	Khan et al. (2017)
E. coli	MDR	By generation of ROS	Zhang et al. (2013)
P. aeruginosa	Formation of biofilm/MDR	By conjugation with adenosine monophosphate.	Casciaro et al. (2017)
Salmonella typhimurium	Formation of biofilm/MDR	By adenosine monophosphate conjugates.	Yeom et al. (2016)

18.4 Mechanism of Action of Nanoparticles in Bacterial Cell

The antimicrobial or antibacterial potential of metallic nanoparticles against drug-resistant microbe and three-dimensional polymeric biofilms depend on a variety of factors. These factors are large surface area of nanoparticles in the contact with microorganism through intermolecular forces as van der Waals forces for polarization of particles, electrostatic attraction for long-range interaction, the particle size, and their stability collectively with drug concentration (Li et al. 2015). For the possible mechanism of nanoparticles, candidature as antimicrobial agent is inhibition of enzyme action, generation of reactive oxygen species for oxidative damage of microbes, change in gene expression, and protein deactivation.

Nowadays, metallic particles have been extensively used as potential antimicrobial agent and possible mechanism for action of nanoparticles to serve as antimicrobial agent is release of metal ion in bacterial cell, generation of reactive oxygen species for oxidative stress-mediated damage of microbes, and nonoxidative-mediated damage of microbes (Zaidi et al. 2017). The most common two metallic nanoparticles such as AgNPs and AuNPs have been included in this chapter, and their mechanism of action against microbes as antibacterial candidature is mentioned in Fig. 18.3.

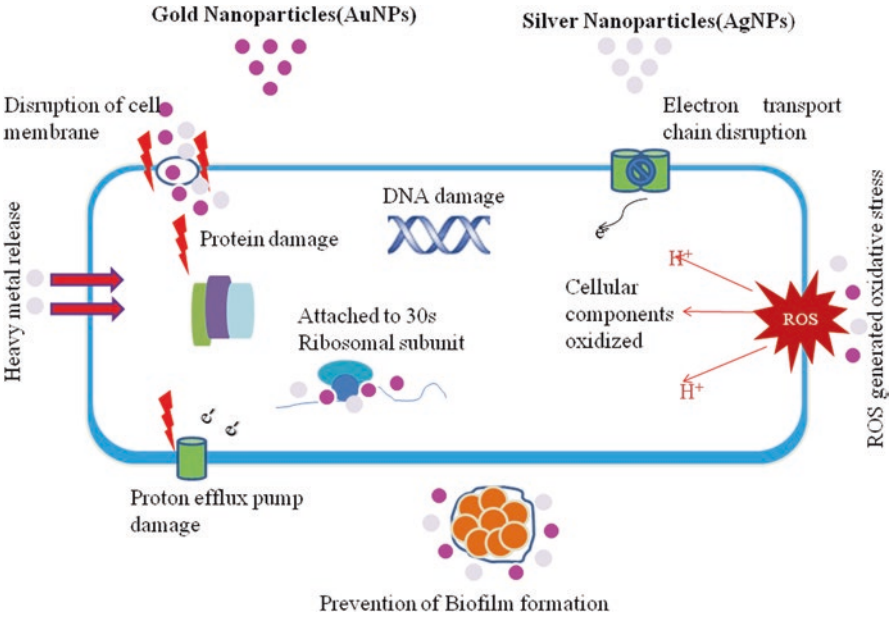

Fig. 18.3 Mechanism of action of AgNPs and AuNPs in bacterial cell

18.4.1 Mechanism of Action of AgNPs

In this section, we will explore the mechanism of action of AgNPs to prove their antibacterial potential against microbes. The mechanism of action has been classified into mainly two types, oxidative and nonoxidative. In oxidative damage, AgNPs generate reactive oxygen species that are responsible for oxidative-mediated death of microorganism. Therefore, AgNPs induced oxidative stress play important role in their antibacterial activity (Rudramurthy et al. 2016). Cellular oxidative metabolism generates abundant reactive oxygen species as by-product, and these reactive oxygen species are involved in cell survival and death, and meantime, these reactive oxygen species molecules play a very significant role in cell signaling and cell differentiation. During aerobic respiration in bacterial cell, reactive oxygen species are produced. These reactive oxygen species are neutralized by cellular intrinsic defense mechanism, but due to massive insult reactive oxygen species may cause oxidative damage of biomolecules and cell components (Li et al. 2012). The generation of massive reactive oxygen species causes cellular redox potential that is responsible for oxidative stress-mediated damage, alteration or modification in protein structure, deoxyribonucleic acid structure, and membrane lipid damage (Dwivedi et al. 2014). Production reactive oxygen species depends upon the chemical nature of nanoparticles. Silver had antimicrobial activity from ancient times, and metallic nanoparticle application against bacterial infection has become more popular in recent. A recent research report showed that AgNPs were generated superoxide radicals that lead to cellular oxidative damage and served as antibacterial efficacy (Zhang et al. 2013). Researchers have reported antibacterial effect of AgNPs against drug-resistant bacteria through silver ion (Ag^+) uptake across cell membrane. Further, the AgNPs can impart antibacterial effect through inhibition of drug efflux pump, intracellular cascades, production of reactive oxygen species, cell modifications affecting the membrane permeability, membrane disruption, and conjugation with adenosine monophosphate. These all are well summarized in Table 18.1.

The nonoxidative way includes the penetration of the AgNPs into the cell and disruption of the membrane permeability, alteration into the cellular components, and inhibition of the protein synthesis by incorporating into the purines and pyrimidines of the DNA and RNA. The AgNPs have a strong positive charge and the cell wall has a negative charge, and the AgNPs attach to the cell wall via electrochemical interaction. The active Ag^+ ions are generated via dissociation after attachment to the cell wall; furthermore, Ag^+ ions are generated by the Trojan horse mechanism (Singh et al. 2016). The Ag^+ ion interaction causes disruption of the membrane integrity of lipid bilayer, hence the cellular leakage due to increased permeability (Agnihotri et al. 2014). This leads to cell death of bacteria due to loss of primary barrier for the defense.

18.4.2 Mechanism of Action of AuNPs

The AuNPs have been being extensively used as antibacterial agent. There are major concerns of researchers to explore the mechanism of action of AuNPs as antimicrobial agent. According to a research report, researchers have reported that reactive oxygen species generation and release of metal ion potentiate the antimicrobial properties of AuNPs via without coating with gold nanoparticles under ultraviolet electromagnetic radiation at the wavelength of 365 nm (Zhang et al. 2013). Another research report showed that the antimicrobial activity of AuNPs against *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli*, and *Salmonella Typhi* was due to intracellular ROS-mediated oxidative stress (Umamaheswari et al. 2014). There are so many studies that have reported antimicrobial properties of AuNPs against drug-resistant microbes through reactive oxygen species-generated oxidative damage and well summarized in Table 18.2.

Another mechanism of action of NPs is a nonoxidative. In this mechanism, nanoparticles interact closely with bacterial cell wall and hamper them. As we all know that cell wall of microorganism is an outer protective layer and served as a defensive barrier that protects from environmental insults, hampering this protective layer definitely hampers the growth of microorganisms. The peptidoglycan polymer is the main constituent of Gram-negative bacteria. The bacterial outer membrane is made up of lipopolysaccharide and protein that are highly specialized in selective entry of macromolecules (Zaidi et al. 2017). Gram-positive bacteria cell wall is also composed of peptidoglycans and has several pores that allow the penetration by external or foreign particles that may able to interfere with bacterial cell proper functioning (Sarwar et al. 2015). So, these are possible ways by which gold nanoparticles serve as potent antibacterial agents and maybe probable candidates against microbial infection.

18.5 Mechanism of Action of NPs on Biofilm Formation

Biofilms are the three-dimensional film of polysaccharide matrix generated by microorganism. The biofilm has a great impact on public health. The microorganisms associated with generation of polymeric matrix are very less susceptible to antimicrobial action. Recently, researchers have reported that the formation of polymeric matrix with surface in the form of biofilm has become another way by which microorganisms protect themselves from antibiotics (Khameneh et al. 2016). By the formation of biofilm, cells adhere to the extracellular polymeric substance that acts as a protective barrier from antibiotics that is responsible for antibiotic resistance, inhibition of the most important process of phagocytosis during innate immune response by phagocytes, and the

leading cause of serious health threat globally (Bjarnsholt 2013). However, the formation of polymeric three-dimensional matrix by microorganism has become a challenge for antimicrobial agent and meantime researchers have reported that nanoparticles can disrupt cell wall of microorganisms and generation of polymeric matrix that are the major causes to reduce microbial survival (Peulen and Wilkinson 2011; Chen et al. 2014; Miao et al. 2016). Therefore, the properties of AgNPs and AuNPs against biofilm formation give an extra advantage of these NPs to fight against microbial infection and a better optional candidate as antimicrobial agents.

18.6 Conclusions

Antimicrobial resistance has become global health threat. It has a great impact on global socioeconomic condition. Due to the development of drug resistance in bacteria, it becomes very crucial in medication against infectious disease in health sector. Microorganism becomes resistance to antimicrobial agents or antibiotics through alteration in their cell wall to cheek drug permeability from cell membrane, modification in target protein, inactivation of antimicrobial agent to bind bacterial ribosome, and development of active efflux of drugs. Nanomedicine is a budding branch of medicine that uses advancement in nanotechnology for the prevention and treatment of infectious disease. The advancement in nanotechnology has become an effective approach for the fabrication of metallic bulk materials in nanosized particles for the treatment against drug-resistant microbes. There are many metallic nanoparticles that have efficient antibacterial properties. Nowadays, there is no doubt on the potentiality of silver and gold nanoparticles against drug-resistant bacteria. AgNPs and AuNPs are well-established antimicrobial agents against both non-MDR and MDR bacterial strains. These NPs have developed antibacterial potential against multiple drug-resistant microorganisms via adhesion to the bacterial cell membrane and cell penetration, causing various structural disruptions and dysfunction through ROS-generated oxidative stress and inhibition of the formation of polymeric matrix from substrate in the form of three-dimensional biofilm by microorganism. The antibacterial activity of AgNPs and AuNPs may be the new alternative against multiple drug-resistant microbes. Furthermore, extensive studies are required on these nanoparticles to explore their potential as antibacterial agents. Also, there is a need for extensive research on these nanoparticles with an impact on gene level for wider application of these particles against MDR in healthcare system.

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Chapter 19
Nano-Cargo Boarded Defensins to Combat Multidrug Resistance

Saurabh Bhorkade, Pawan Kumar Pandey, Md. Meraj Ansari, and Sanyog Jain

19.1 Introduction

Antibiotics are compounds that inhibit the growth of microorganisms through their cytostatic or cytotoxic action (Pizzolato-Cezar et al. 2019). The discovery of antibiotics was a providential event in the history of medicine as they have been used exhaustively over the decades to save numerous lives and decrease overall mortality vis-à-vis infectious disease (Davies and Davies 2010). Several classes of antibiotics have been developed over the past years and used for the set of infectious diseases. But lately, these antibiotics have started failing to provide the expected results and have become ineffective in eradicating infections due to the development of resistance in pathogenic microorganisms. An era of antibiotic resistance followed after an interminable failed attempt by the researchers to discover novel antibiotics, immoderate use by clinicians, and antibiotic abuse by humankind in medicine and agriculture (Wright and Sutherland 2007). The resistance gene can transfer vertically or horizontally to the microbes present in their surroundings. With exposure to other antibiotics for a more extended period, they might develop resistance toward more than one antibiotic. The condition is known as multidrug resistance, and this has been a significant challenge in medicine. The emergence of multidrug-resistant strains has led to serious concerns for clinicians as it creates a burden on existing resources. With many Gram-negative bacteria acquiring pan-resistant, it now appears as a severe threat to humankind. Novel approaches to combat multidrug

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resistance of microbes are being investigated, and in this regard, the beacon of hope can be seen in novel microbicidal compounds like antimicrobial peptides (Magiorakos et al. 2012).

Scientists have indefatigable efforts to decipher new targets to intervene in the different mechanisms of resistance development and find novel compounds to combat antibiotic resistance (Lima et al. 2019). In recent studies, many approaches have been explored to combat the problems mentioned above. Preventing SOS response, blocking intracellular communication in bacteria to reduce the horizontal transfer of resistant genes, and inhibiting efflux transporters have been targeted to combat multidrug resistance in microorganisms (Wright and Sutherland 2007). Besides these, an appreciable amount of work has been done to discover novel antimicrobial compounds. Recent studies' most exciting outcome is the emergence of natural peptides as antimicrobial agents known as antimicrobial peptides (AMPs). Antimicrobial peptides are oligopeptides having a chain length of 5–100 amino acids equipped with antimicrobial activity. They have broad-spectrum and potent activity against numerous pathogenic microorganisms. First discovered in the 1980s, they have shown appealing results in conquering multidrug resistance. Moreover, they are ubiquitous in nature and found in plants, animals, bacteria, fungi, and viruses (Lei et al. 2019). AMPs are synthesized by almost all living organisms and form an indispensable component of the host defense mechanism. They may have specific or nonspecific interaction with the cell membrane of invading pathogen. Their mechanism of action has been found to be different based on their structure. Due to their cationic or amphiphilic nature, they interact with negatively charged components of the cell membrane to interfere with the cell wall synthesis. While conventional antibiotics intervene in cell membrane synthesis, the AMPs interact with precursor molecules essential for cell wall synthesis. These antimicrobial peptides can also evoke the immune system, resulting in increased cytotoxic and cytostatic activity against pathogenic microorganisms.

These peptides have been isolated from different organisms and have shown similar activity against microbial infection. The first-ever AMP was isolated from moths, i.e., cecropins A and B, which showed selective toxicity against bacteria, whereas magainin was the first AMP to be extracted from vertebrates. Later, Lehere et al. identified a class of AMP isolated from humans known as defensins. Defensins were first isolated from human neutrophils and were known as human neutrophilic peptides (Yamaguchi and Ouchi 2012). Other human antimicrobial peptides include cathelicidins and histatins. Defensins are nonglycosylated cationic AMPs with a molecular weight of below 5 kDa with six cysteine residues cross-linked via disulfide linkage. They have a vital role in the innate and adaptive immunity of the host organism. Recently, several studies were conducted on defensins' antimicrobial activity, but due to poor pharmacokinetics and stability-related problems, they have not been commercialized. Lately, the development of nanoformulation loaded with antimicrobial peptides has been explored to bypass the stability and pharmacokinetic-related limitations.

Nanotechnology and the development of nanoparticle-based drug delivery system are a well-established and promising field in therapeutics and diagnostics,

which have been extensively studied to overcome the limitations of poor pharmacokinetic profile, limited solubility, poor absorption, probable adverse effects, and poor stability of numerous drug molecules. Different nanomaterials with unique properties have been designed to improve the efficacy and overall performance of the drug molecule with respect to pharmacokinetic profile (Teixeira et al. 2020). Nanoparticles with sizes in the range of 10–1000 nm have been helpful in various clinical setups due to their size and flexibility by fabricating them with different materials. Their surface properties allow them to act as cargos to deliver compounds ranging from simple molecules to complex ones like peptides and proteins. Several nanocarriers like metallic, inorganic, organic, and polymeric nanocarriers including dendrimers, micelles, and liposomes have played a pivotal role in delivering the perverse molecules to the targeted site (Mirza and Siddiqui 2014). These nanocargos can act as Trojan horse for drugs, which cannot be given through conventional formulation (Gulyaev et al. 1999). In the same line, defensins can also be loaded into these nanocarriers and thus overcoming the limitations of stability and poor pharmacokinetics. Defensins have shown promise in combating multidrug resistance, whereas some nanomaterials have their own antimicrobial activity; by combining these, there is possibility of getting an improved efficacy and overall clinical outcome.

19.2 Defensins

These are cationic (+2 to +11 net charge) in nature with molecular weight of 3.5–4.5 kDa. The term defensin was coined by Lehrer to describe the cationic peptides stabilized by disulfide linkage and having broad antimicrobial activities against fungi, bacteria, and viruses (Ganz et al. 1985; Selsted et al. 1985). The defensins represent a class of antimicrobial peptides that play important role in innate and adaptive immunity. Defensins have antimicrobial activity through innate immunity against bacteria, virus, and fungi (Fig. 19.1).

19.2.1 Classification of Defensins

Defensins, based on the structure and disulfide topology, are classified into three major subfamilies: α-defensins, β-defensins, and θ-defensins. Only α- and β-defensins are found in humans, whereas unique, circular, ladder-shaped θ defensins stabilized by parallel disulfide bonds are present in rhesus macaques leukocytes (Gabay et al. 1989). Apparently, humans contain RNA transcript of rhesus θ-defensins, but its subsequent translation is abolished by the presence of stop codon in upstream signal sequence (Lehrer et al. 2012). Both α- and β-defensins have triple straned β sheets with different lengths of protein segments between six cysteine residues.

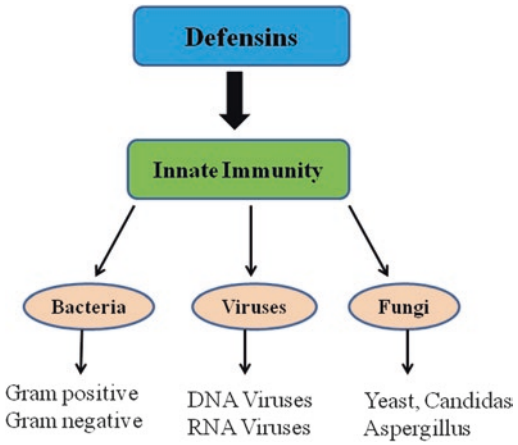

Fig. 19.1 Defensins as antimicrobial through innate immunity

19.2.1.1 α-Defensins

α-Defensins are present in neutrophils of humans, rats, guinea pigs, and rabbit macrophages. α-Defensins, based on expression and gene structure, are further classified into two groups, four human neutrophil peptides (HNP 1–4), also known as myeloid defensins, present in the azurophilic granules of neutrophils, and two human defensins (HD 5–6), also known as enteric defensins, secreted by colon and small intestine (Lehrer and Lu 2012; Nikitina et al. 2012). The three most abundant defensins present in the human neutrophils are HNP 1, HNP 2, and HNP 3. HNP 4 is also present in human neutrophils at concentration of <2% of HNP 3 levels. HNP 2 contains an extra amino acid (30 amino acids) compared to HNP 1 and HNP 3 (29 amino acids), whereas HNP 1 and HNP 3 differ in their amino acid residue at N-terminal. HNP 1 contains alanine, and HNP 3 has aspartate at its N-terminal. This replacement of single nucleotide residue at N-terminal by acidic aspartate amino acid makes HNP 3 less effective against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. HNP 4 contains 33 amino acids and has shown antimicrobial activity against *E. coli, Streptococcus faecalis*, and *Candida albicans* (Wang 2014). Azurophilic granules containing HNPs undergo restricted secretion and are fused with phagolysosomes releasing the content inside at high concentration and killing phagocytosed microbes.

Enteric defensins (HD 5 and HD 6) were isolated for the first time from the Paneth cells of the small intestinal crypt. These defensins play a crucial role in regulating intestinal microbes and protecting intestinal stem cells (Bevins et al. 1999; Bevins and Salzman 2011). HD 5 is also found in vaginal fluids (1–50 μg/ml) and is induced in the reproductive organs on infection of sexually transmitted diseases (Klotman et al. 2008; Porter et al. 2005). HD 5 have shown concentration-dependent antimicrobial activity against *Candida albicans, E. coli, Salmonella typhimurium,* and *Listeria monocytogenes*. Moreover, the antimicrobial efficacy of HD 5 against multidrug-resistant *Acinetobacter baumannii* is also confirmed by Wang and coworkers (Wang et al. 2018).

19.2.1.2 β-Defensins

β defensins differ from α-defensins in their disulfide linkage and have a short N-terminal α-helix. These were first time isolated from the bovine tracheal epithelium, where they were induced as a result of infection. Unlike α-defensins, the expression of human β-defensins (hBD) is transcriptionally regulated by keratinocytes of epithelial cells of the respiratory, gastrointestinal, and genitourinary tract. Scientists have reported more than 30 hBDs out of which only four (hBD 1 to hBD 4) have been studied in detail to date (Zasloff 2007). hBD 1 is expressed constitutively by the epithelial cells, whereas hBD 2 and hBD 3 are expressed in response to inflammation and infection (Ghosh et al. 2007). These hBDs also differ in their expression among the different tissues leading to tissue-specific upregulation. hBD 2 is abundantly expressed in the lungs, and hBD 3 in skin and tonsils, whereas hBD 4 is expressed highly in testicles and stomach (Tecle et al. 2010). β-defensins are also expressed in the genital tracts of males and females highlighting their protective roles in sexually transmitted diseases.

hBD 1 has shown strong microbicidal action against *P. aeruginosa* and *E. coli* and has proven effective in cystic fibrosis patients. On reduction of disulfide bond, hBD 1 acquires potent antimicrobial activity against *C. albicans*. On the other hand, hBD 2 has proven effective against Gram-negative bacteria like *E. coli* and *P. aeruginosa* along with *C. albicans, A. baumannii,* and S. *marcescens.* However, only bacteriostatic activity is observed against Gram-positive bacteria like *S. aureus*. Thus, hBD 2 can be employed in the treatment of gastric infection by *E. coli* and has also shown antitubercular properties.

The bactericidal activity of hBD 3 against Gram-negative bacteria like *P. aeruginosa, E. coli*, and *C. albicans* and Gram-positive bacteria like *S. aureus and Streptococcus pyogenes* is well established. Further, hBD 3 is also found effective against multidrug-resistant *S. aureus, P. aeruginosa, Enterococcus faecium, Acinetobacter baumannii, Stenotrophomonas maltophilia,* and vancomycin-resistant *Enterococcus faecium* (Dhople et al. 2006; Wang 2014). hBD 2 and hBD 3 have also shown anti-HIV activity by blocking viral replication (Pace et al. 2017). hBD 4 is found effective against bacterial infection *with P. aeruginosa.* Newly discovered β-defensins, hBD 5 and hBD 6, have shown antibacterial activity against *E. coli* and are widely expressed in the epididymis. Out of the most recently discovered defensins, hBD 19, hBD 23, hBD 27, and hBD 29, hBD 23 is found most effective against Gram-negative and Gram-positive bacteria like *S. aureus* and *E. coli* (Chow et al. 2012).

19.2.1.3 θ-Defensins

θ-Defensins are cyclic peptides with 18 amino acids connected with three disulfide linkages between cysteines. These were first time discovered in rhesus macaque leukocytes. Genomic analysis has shown the presence of θ defensins genes in

genomes of macaques, colobus monkeys, siamangs, and orangutans. These defensins are resistant to proteases, have higher antibacterial efficacy, and are more stable due to their cyclic nature. Unlike acyclic peptides, the antimicrobial activity of these defensins is not dependent upon disulfide bonds and is more stable at physiological salt concentration giving them three times more activity against *E. coli* and *S. aureus* compared to acyclic defensins at osmotic conditions of body (Tang et al. 1999). One of the θ-defensins, rhesus θ-defensin 1 (RTD), has shown antimicrobial activity against multidrug-resistant *S. aureus* (methicillin-resistant *S. aureus*) and *P. aeruginosa* (Tai et al. 2015). The absence of θ-defensins increases the susceptibility of HIV infection highlighting its anti-HIV activity where the probable mechanism is blockade of viral entry (Conibear and Craik 2014; Wang 2014). However, anti-herpes and anti-influenza activities of these defensins are also observed (Doss et al. 2009; Yasin et al. 2004). θ-Defensins bind and neutralize bacterial toxins as well. For example, retrocyclin 1 and analogs of retrocyclin 1 have activity against anthrax and neutralize anthrax toxin (Wang et al. 2006). However, acyclic analogs of retrocyclin were unable to bind and neutralize bacterial toxins highlighting the role of sulfide bind and the cyclic backbone. Several attempts have been made to synthesize the cyclic analogs of these defensins having greater stability and broad antimicrobial activity.

19.3 Mechanism of Action of Defensins

AMPs like defensins are cationic in nature due to positively charged amino acid, which electrostatically interacts with the negatively charged cell membranes of bacteria. This interaction between defensins and cell surface leads to disruption of cell membrane, ultimately death of organism (Guilhelmelli et al. 2013). In contrast to human cell membrane, enriched with neutral phospholipids like phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylcholine (PC), bacterial cell wall is enriched with negatively charged phospholipids like phosphatidylserine (PS), phosphatidylglycerol (PG), and cardiolipin (CL). Additionally, Gram-positive bacteria have lipopolysaccharide-rich outer layer that imparts negative charge to the membrane. This contrasting nature of cell membranes is responsible for the selectivity of defensins toward bacteria. Further, the presence of cholesterol in human cell membrane imparts additional stability to human cell membrane making the interaction with AMPs less likely (Jin and Weinberg 2019).

The formation of pores inside cellular membrane is widely believed as the mechanism for antibacterial activity of AMPs resulting in the uncontrolled flow of ions across the membrane. Initially, interaction between the cationic amino acids of defensins and negatively charged phospholipids of cell membrane takes place leading to the accumulation of defensins on cell membrane (Epand et al. 2016). After a certain threshold, these AMPs self-assemble forming a pore inside the membrane. This process of pore formation is explained by three models: (1) barrel stave model, according to which there is perpendicular insertion of these AMPs

inside the cellular membrane leading to the formation of pore, (2) carpet mechanism, which proposes parallel absorption of peptides on the membrane reaching a particular threshold, and this results in detergent-like effect causing membrane disintegration, and (3) toroidal pore mechanism, which also proposes perpendicular insertion of peptides but forming a curvature in the membrane with pores lined partly by phospholipid heads and partly by peptides (Yeaman and Yount 2003; Nguyen et al. 2011) (Fig. 19.2).

Variability among these mechanisms of action is also observed depending upon the type of defensin and its target. For example, antibacterial activity of HD 5 is by increasing the permeability of cellular membrane and by binding to DNA, thereby inhibiting cell replication. Similarly, HNP 1 acts against *E. coli* by destroying cell membrane, whereas against *S. aureus* by restricting wall precursor lipid II. In case of β-defensins, reduction in disulfide bridges is reported to increase the antibacterial activity. According to recently discovered mechanism, hBD 1 forms a Web-like structure with neutrophils and traps the bacteria. hBD 2 acts by binding to the membrane phospholipid leading to cell death, whereas hBd 3 is reported to act by two mechanisms, by binding with cellular lipid II precursor and by membrane destruction (Pachón-Ibáñez et al. 2017). Along with this generalized mechanism of action, i.e., pore formation and membrane permeabilization, other mechanisms are also

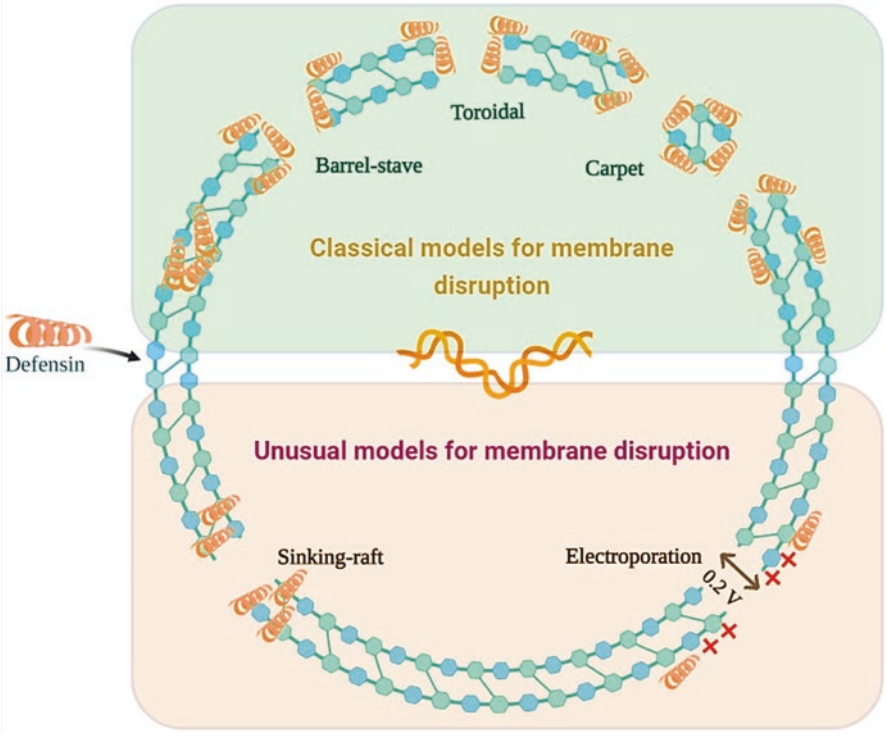

Fig. 19.2 Mechanism of action of defensin

depicted by the researchers, for example, inhibiting intracellular functions and bipolar synthesis (Yeaman and Yount 2003).

19.4 Defensins as Therapeutic Candidates

Multiple activities of the human defensins have drawn considerable attention of the scientist over the period, making them highly potential therapeutic candidates. Several companies have started investing or have future programs for engineering these peptide molecules into consumable therapeutic entity. The rapid development of antimicrobial resistance has further boosted the need for these novel antimicrobial agents as these defensins are less susceptible to resistance development when compared to other antimicrobial agents, and scientists across the globe have responded to it. Human defensins have ability to kill bacterial strains and enveloped and non-enveloped viruses. Defensins can also neutralize the bacterial toxin thus are ideally suited as broad anti-infective therapeutic agents. For example, recent study by Teles et al. has shown that the induction of endogenous gene regulation is effective against treatment of leprosy and other diseases (Teles et al. 2013).

19.4.1 In Vitro Synthesis of Defensins

19.4.1.1 Recombinant Synthesis

Several protocols have been developed and tested over time for the production of recombinant human defensins (Piers et al. 1993; Valore and Ganz 1997; Zhang et al. 1998; Xu et al. 2005a, b; Chen et al. 2006; Zhong et al. 2006). However, the smaller size of these defensin peptides (30–35 amino acids) often hampers the successful production by PCR technique as it is below the limit of protein size required for stable expression. This problem is further worsened by the presence of basic and cysteine residues resulting in incorrect folding and very low levels of expression. To overcome the issue related to smaller size of defensins, these are often expressed as fusion proteins with higher molecular weight partner proteins. Properly selected partner fusion protein also allows desired alteration of solubility suitable to particular protocol. In most of the protocols, various strains of *E. coli* were used for the production of recombinant defensin peptides. Low cost, ease in scaling up, ease in availability of suitable plasmids, and quick generation of large number of peptides in short time are some of benefits of using *E. coli* strains. Mainly, two main strategies are described for the production of recombinant defensins, (1) expression of insoluble inclusion bodies and (2) generation of soluble proteins. Both of these strategies involve the production of fusion proteins using the suitable carrier protein. Several

other expression systems like yeast cells, tobacco plant cells, and infected insect cells were also employed for the production of recombinant defensins.

19.4.1.2 Chemical Synthesis

Due to smaller size of these peptides, chemical synthesis is often used as an alternative to recombinant technology for their production. Introduction of various non-natural modifications into these peptides is much easy using chemical synthesis when compared to recombinant technology. Further, ease in the purification of these peptides in the environment, which is simple and better defined compared to one in recombinant expression technology, is add-on benefit of this technology. However, synthetic peptides are generated in the nonfolded form and incorporation of folding, which involves multiple disulfide bonds, may be difficult in chemical synthesis. Low solubility of these peptides in basic solution due to the presence of cationic charge causes their precipitation during the process of synthesis. One of the protocols developed by Lu and coworkers involves amino acid sequence of mature defensins subjected to oxidative folding in the presence of 2 M urea and 25% DMF to minimize the aggregation and to avoid denaturation of proteins. Up to 80% of yield was achieved using these protocols, and four neutrophil defensins were folded and oxidized successfully (Wu et al. 2004).

19.4.2 Limitations of Defensins as Therapeutic Candidates

Although the defensins can be potential therapeutic agents, their progress as pharmaceuticals is majorly delayed due to some limitations. Very few of these novel peptides have made it up to the clinics, and most of them are natural cyclic peptides. One of the major factors limiting their development is high cost involved in the extraction of purified form of these AMPs, which largely affect the clinical trials. Until recently, biological studies of these defensins were limited by the lack of sufficient quantities of purified peptides. Efficient and economical production of pure form of these peptides is key for the clinical development of these therapeutic agents. Defensins are also reported to have nonspecific hemolytic activity and osmotic sensitivity to human cells. Low stability of these peptides and rapid elimination from the body is also a major hurdle as most of the gastrointestinal enzymes like carboxypeptidase, exopeptidases, and aminopeptidases rapidly digest these amino acid backbones. Thus, improvement is needed in terms of stability, targeted delivery, controlled release, transportation, and immunogenicity of these peptides before they can be used as therapeutic agents. The major challenge lies in the development of suitable formulation for these defensins to overcome above limitations.

19.4.3 Approaches to Overcome Limitations of Defensins

Notorious molecules like peptides have always been difficult to deliver, and there has been a continuous effort to improve delivery aspects of such components. Several researchers have applied different approaches to modify the properties of peptides like defensins. These include chemical modifications to increase the stability and protect it from enzymatic degradation, increase its intestinal absorption and half-life in plasma in human body, and increase its solubility and bioavailability. Many such modifications like cyclization, lipidization, and PEGylation are some of the methods that can be considered for modifying the properties of antimicrobial peptides. Fabrication of hybrid peptide is another method to improve the possibility of using antimicrobial peptides as therapeutics in near future.

Cyclization process can augment the stability of antimicrobial peptides through head-to-tail, head-to-side chain, tail-to-side chain, or side chain-to-side chain method. Nowadays, numerous methods exist to achieve cyclization of peptide molecules. Bacitracin is an example of cyclic peptide; after its discovery, the concept of cyclization has been a lucrative option for delivering peptides with improved stability. In a study conducted by Rozek et al., a linear peptide indolicidin was cyclized to achieve cell selectivity and at the same time stability in the presence of proteases up to 4.5-fold. Moreover, cyclization reduced hemolytic toxicity of the indolicidin, hence encouraging the possibility of oral delivery of the antimicrobial peptide (Rozek et al. 2003). However, this approach is not universal and some of the peptides have shown increased toxicity and decreased stability (Unger et al. 2001).

19.5 Nano-Cargos for Delivery of Antimicrobial Peptides

Nanomaterials have existed since the development of material science, but in last couple of decades they have gained significant attention. They were used in engineering and material sciences, but in last couple of decades they have attracted significant attention in therapeutic and diagnostic field (Pitkethly 2004). Many studies explored the potential of nanomaterials in drug delivery, which showed promising outcomes. Numbers of nanocarriers have been approved in last two decades with improved therapeutic efficacy and reduced toxicity. Many of these materials when reduced to nano-size have shown to have their own antimicrobial activity. They accentuate intracellular delivery of cargos, decrease frequency of administration, and attenuate the development of drug resistance strains by providing alternate mechanism for antimicrobial activity (Sánchez-López et al. 2020). Thus, loading of these antimicrobial peptides with nanocarriers can have ameliorating effect on microbicidal activity and it can further circumscribe the chances of development of resistance. Further are discussed some antimicrobial peptide-loaded nano-cargos for combating multidrug resistance.

19.5.1 Inorganic/Metallic Nanoparticles

In recent past, metallic nanoparticles fabricated with silver, gold, nickel, and silicon have been studied for their antimicrobial activity (Zhang et al. 2013). Metal nanoparticles have been shown to improve antimicrobial activity of drugs against resistant bacteria. They are easy to fabricate and provide uniform shape and size. Generation of reactive oxygen species is a principal mechanism of these metallic nanoparticles with respect to their antimicrobial activity. Studies have been conducted extensively with silver and gold NPs to explore their potential in providing better efficacy against resistant microbes (Abdeen et al. 2013) .

Tachyplesin-1, an antimicrobial peptide, has β-hairpin-like structure and has shown exemplary membranolytic activity, but it suffers from limitations in terms of stability and poor permeability. Shangshang et al. fabricated a multifunctional peptide-coated AgNPs to overcome the limitations of tachyplesin-1. The multifunctional peptide was equipped with a matrix metalloproteinase (MMP) cleavable sequence, a target peptide, and tachypresin-1 as an antimicrobial peptide, wherein the target peptide was pegylated in order to increase the circulation time in vivo, which was further assisted by MMP cleavable sequence. When this MFP reached the site of infection, the components get separated and by MMP which digests the MMP cleavable sequence releasing the tachyplesin-1 and restraining the spread of infection. It was observed that amalgamation of cationic MFP and anionic AgNPs led to improved bioactivity against MDR-AB, which opens new realm in curbing multidrug resistance (Li et al. 2020).

Gold nanoparticles have been investigated for their antimicrobial activity on both Gram-positive and Gram-negative bacteria. AuNPs have also shown ability to inhibit the growth of infection in MDR strains with lesser toxicity compared to other metallic NPs. Loading these AuNPs with antimicrobial peptides can further improve the antimicrobial therapy and is a preferential candidate for the development of nano-based delivery system of peptides for circumventing MDR (Li et al. 2014). Casciaro et al. demonstrated that covalently conjugated AMP esculentin-1a on AuNPs using poly(ethylene glycol) linker produced AuNP@Esc(1–21), which increased the microbicidal activity of esculentin-1a up to 15 times (Table 19.1). AuNPs formed were found to be less toxic to keratinocytes. Moreover, the nanoparticle system provided extended protection against proteolytic degradation and require lower concentration to break down membrane structure (Casciaro et al. 2017). Rai et al. illustrated the increased stability and antimicrobial activity of cecropin–melittin (CM)-conjugated gold nanoparticles. The CM-conjugated AuNPs with controlled size and polydispersity demonstrated higher stability in presence of proteolytic enzymes with significant increase in bactericidal activity. Moreover, the CM-AuNPs showed considerable reduction in side effects against human cells. The improved profile of the AMP indicated that the use of nanoparticle-based system for delivery of antimicrobial peptide can have multifarious merits in terms of efficacy and shows better therapeutic result against multidrug-resistant bacteria (Rai et al. 2016).

19.5.2 Polymeric Nanoparticles

Polymeric nanoparticles have gained significant attention in drug delivery with wide range of polymers to select from. Extensive numbers of publications are available on polymeric NPs. Most popular among polymeric nanoparticles are PLGA (poly-lactic-co-glycolic acid) owing to its biodegradability, biocompatibility, and multifaceted degradation kinetics (Sandreschi et al. 2016) (Table 19.1).

Chereddy et al. studied the wound-healing property of PLGA nanoparticle-loaded LL37 peptide, which is a component of host defense mechanism to counter the pathogenic microbes. PLGA nanoparticles have the merit in wound-healing formulation as they can supply lactate, which is required for faster recovery of epithelial membrane. The LL37-loaded PLGA nanoparticles fastened up the process of wound healing and increased the rate of angiogenesis. Furthermore, it demonstrated antimicrobial effect against *E. coli.* The authors concluded that LL37-PLGA NPs moderated inflammatory immune response and effectively restrained the infection owing to enhanced antimicrobial activity of LL37 peptide (Chereddy et al. 2014). Similarly, Chunhua et al. fabricated nisin/γ-PGA/chitosan nanoparticle, which demonstrated better MIC value compared to free drug. It was observed that drug release from Nisin/γ-PGA/chitosan NPs was pH-dependent manner. The Nisin-loaded NPs were efficiently able to restrain the growth of *E. coli* and *Listeria monocytogenes* at

Table 19.1 Nanoparticle-based delivery systems for AMPs

Nanocarrier	AMP	System	Reference
AuNPs	Magainin-1	Development of a one-step synthetic route form functionalization of AuNPs with AMPs	Rai et al. (2016)
AgNPs	MBP-1	Study of the synergistic antibacterial effect of plant peptide MBP-1 and AgNPs on infected wounds caused by *S. aureus*	Salouti et al. (2016)
Anionic mesoporous SiNPs	LL-37	Study of membrane interaction with AMPs in different types of mesoporous particles	Braun et al. (2016)
PLGA nanoparticles	Colistin	Development of a system of nano-embedded microparticles (NEM) for sustained delivery of CAMPs in the lung	d'Angelo et al. (2015)
PLGA nanoparticles	Plectasin	Intracellular antibacterial activity against *S. aureus* in epithelial cells	Jorrit Jeroen Water et al. (2015)
Hyaluronic acid nanogel	Novadin	Novel nanogel-based novadin delivery system	Jorrit Jeroen Water et al. (2015)
Pectin nanoparticles	Nisin	Study of a safe suitable antimicrobial system to be used in food industry. Influence of pectin degree of acetylation on NP properties	Krivorotova et al. (2016)
Chitosan/PGA nanoparticles	Nisin	Influence of chitosan coating on colloidal stability, loading capacity, and encapsulation efficiency	Wu et al. (2016)

lower concentrations compared to free drug and chitosan boarded Nisin nanoparticles. Hence, it implies that nanocarrier boarded antimicrobial peptides exhibits lower MIC values and thus are less susceptible to the development of resistance (Wu et al. 2016).

19.6 Nano-Cargo Boarded Defensins in Combating Multidrug Resistance

Defensins are a class of antimicrobial peptides found in humans, also known as human defensins primarily located in Paneth cells in intestinal crypts. Their discovery is a boon to combat infectious diseases and an alternative to combat MDR. Their commercialization is limited due to their poor stability and pharmacokinetics. Hence, some researchers have modified human defensins to make it suitable for in vitro conditions. The modified defensins have shown comparable antimicrobial activity with more circulation time. Furthermore, attaching these modified human defensins to nanoparticles significantly improves the therapeutic efficacy and overall outcome of treatment.

Zhao et al. modified human defensin by introducing couple of Arg moiety near C-terminal region of human defensin-5 (HD-5). The modified HD, i.e., T7E21R-HD5, was having an atypical dimer with stability against enzymatic hydrolysis. In addition, it showed potent microbicidal activity in saline solution making it an ideal candidate for in vitro bactericidal activity eliminating resistant bacteria and thus turns out to be a next-generation antibiotic. Mesoporous silica nanoparticles (MSNs), which provide controlled size, surface area, and biocompatibility, have been explored widely for better drug absorption, controlled release, and targeted delivery of drugs, and were prepared using CTAB as precursor for silica and TEOS as pore-forming agent, which was then loaded with T7E21R-HD5. Degradation of peptide in the stomach is a major limitation of oral peptide delivery. Hence, the MSN formed was further coated with succinylated–casein (SCN) layer, which prevents the release of peptide in acidic environment and helps in delivering and releases the peptide at the site of infection, i.e., intestine. The controlled release was observed with MSNs@T7E21R-HD5@SCN in the presence of trypsin indicating an improved drug release profile and successfully halted MDR *E. coli* in vivo. Further, it demonstrated subsided adverse effects in the host and inhibited the production of inflammatory factors, thereby reducing the intestinal distress. Loading of T7E21R-HD5 in SCN-coated MSNs enhanced the potency of modified defensins by 2.1-fold against MDR *E. coli* (Li and Shi 2014; Zhao et al. 2019).

The results obtained from the abovementioned study unveil the role of nanocargos in delivering peptide to overcome the limitation and improve their activity against multidrug-resistant bacteria.

PLGA nanoparticles form excellent polymeric nanocarriers for various drug molecules with great biocompatibility and easy manufacturing. They have been explored widely in drug delivery system; hence, they are always a suitable option

for construction of nano-cargos. In another study by Liang et al., PLGA (polylactide-co-glycolic-acid) nanoparticles were loaded with recombinant bovine neutrophil defensin-5 (B5) and its activity was investigated against *Mycobacterium bovis,* which belongs to *Mycobacterium tuberculosis* family. Anti-TB medicines are now being less effective due to the development of resistance in *M. tuberculosis,* and it necessitates the development of novel strategies to counter the minacious disease. The authors developed PLGA nanoparticle encapsulating B5 peptide using solvent evaporation method. The surface of resulting NPs was negatively charged, and sustained release was observed under physiological environment. B5-PLGA NPs demonstrated significant reduction in microbial burden in lungs and spleen within 1 month. Loading of B5 in PLGA NPs was associated with improved immune response in terms of lung infection where secretion of inflammatory mediators was elevated (Liang et al. 2020).

Hangxiang et al. developed a noncovalently self-assembling human α-defensin-5 nanobiotic via formation of amide bond, which demonstrated broad spectrum of antimicrobial activity. The C-terminal myristoylated HD5 revealed intensified activity against *E. coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) owing to its membranolytic activity. Moreover, it exhibited protection from MRSA and *E. coli*-induced sepsis, which can be lethal and limited the systemic infection and organ damage in rescued mice. The self-assembling human α-defensin-5 nanobiotic also revealed insignificant hemolytic activity and reduced toxicity in animals. The findings in abovementioned study revealed that modification of defensin to make self-assembling nanobiotics can be a crucial step to combat multidrug resistance. The formation of myristoylated-human α-defensin-5 was simple procedure, which not only improved the stability but also provided broad-spectrum activity to the antimicrobial peptide (Lei et al. 2018).

Defensin-loaded NPs have improved the therapeutic outcome in several cases and have established the role of nanocarriers in various fields including multidrug resistance. The encapsulation of naive or modified form of defensin has demonstrated improved toxicity and pharmacokinetic profile along with reduced ill effects related to host tissues. Nano-cargo boarded defensins were observed to have improved potency against MDR strains of bacteria.

19.7 Conclusions

Defensins, a major family of AMPs, play vital role in human innate immunity by protecting it from various strains of Gram-positive and Gram-negative bacteria, viruses, and fungi. Growing recent evidences have highlighted their role in the treatment of multidrug-resistant strains. Thus, defensins have great potential to be used as therapeutic agent for antimicrobial treatment. However, their applications are limited due to certain difficulties like low stability, higher toxicities, and high production cost. Some corrections are needed to be done for successful clinical translation of such peptides. Novel nanotechnological approaches offer a way out of such

problems. Formulating these peptides into novel drug delivery systems like polymeric micelles, metallic nanoparticles, and lipidic nanoparticles has shown great promises by improving stability, half-life, selectivity, and even therapeutic potential. Herein, we envisage and optimize further development of novel nano-cargos loaded with defensins and other AMPs for the combat against multidrug resistance.

Conflict of Interest There is no conflict of interest among the authors.

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Chapter 20
Implementation of Diverse Nano-cargos to Disguise and Fight Multidrug Resistance

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20.1 Introduction to Nano-vehicles

Antimicrobial resistance call for efficient ways to combat adamant pathogenic micro-organisms, which gradually develop resistance towards modern drugs that can no longer be effective with the same dose regimen and may even lead to fatal complications if untreated (World Health Organization (WHO) 2020). Nanotechnology is emerging as one of the promising tools to curb widespread infections caused by several multidrug-resistant microbes, especially metallic nanoparticles, as they possess intrinsic antibacterial properties (Suresh 2015). We shall discuss various nano-vehicles, which are being introduced as efficient antimicrobial adjuncts in combating multidrug resistance.

Nano-vehicles are supposedly pharmacologically inert and biocompatible materials chemically synthesized using inorganic and organic sources. Physically, these nanostructures are available in different dimensions of solid core and/or hollow structures ranging from sub-nanometre to few micrometres. Being devoid of any chemical reactivity and driven by their high surface-to-volume ratio, surface functionalization, charge-based drug and cellular binding, and high potential for targeted drug delivery, various nanostructures are employed as carriers of potent pharmaceutical drugs (Panyam and Labhasetwar 2003). Nanocarriers have been revolutionizing healthcare sectors, especially in clinical prognosis. These nanostructure paraphernalia allowed researchers and clinicians to defend prevailing pathogenic infections in the inertia of antimicrobial resistance (Baptista et al. 2018). In the interest of current chapter, the applicability of nano-vehicles is limited to antimicrobial resistance; however, several other applications of nano-vehicles in

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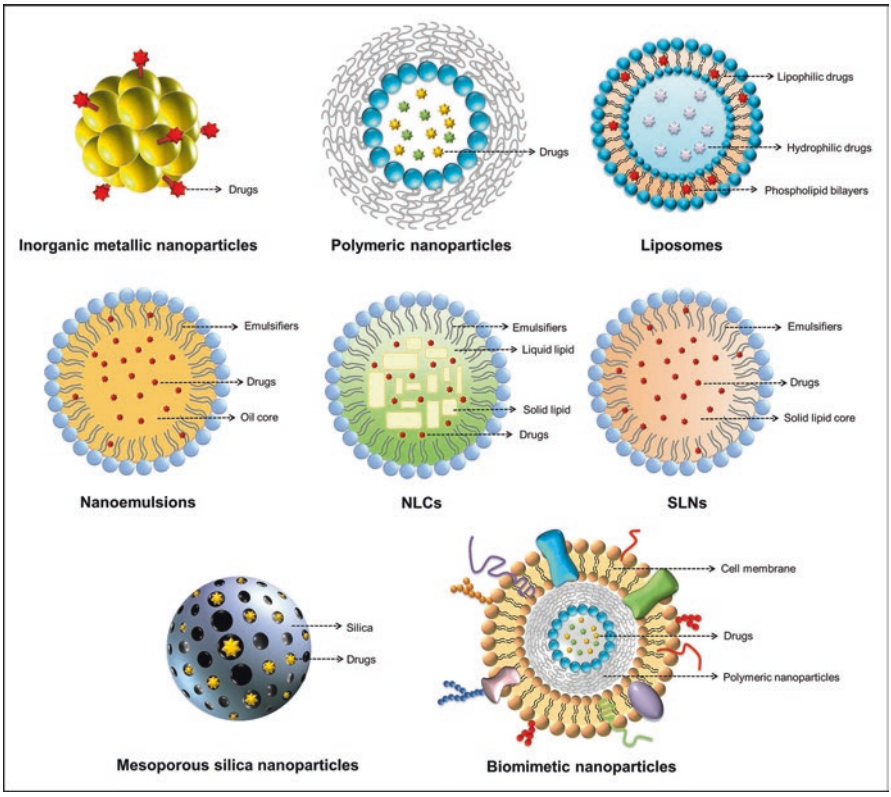

Fig. 20.1 Different types of nano-vehicles used in drug delivery. Reproduced from (Yeh et al. 2020) © 2020

biomedical fields are vastly reported. List of commonly used nanostructures to disguise and fight multidrug resistance is discussed in the below sections with relevant examples, and few selected nano-vectors are illustrated in Figs. 20.1 and 20.2.

20.1.1 Metallic Nanoparticle-Based Nano-cargos

Metallic nanoparticle is metal-based nanoparticles that are fabricated by reducing metal precursor salts that yield different core materials (gold, silver, platinum, palladium, zinc, iron), which fall in the size range from 1 to 100 nm. Such nano-scaled matter possesses exceptional intrinsic properties like high surface-to-volume ratio, chemical reactivity, catalytic activity, and physiochemical, optoelectronic and biological properties. These unique functionalities of nanoparticles driven by specific core, size and shape are leveraged in the fields of bio-medicine, food and agricultural, electronics, health care, industrial and textile (Anil Kumar and Khan 2010).

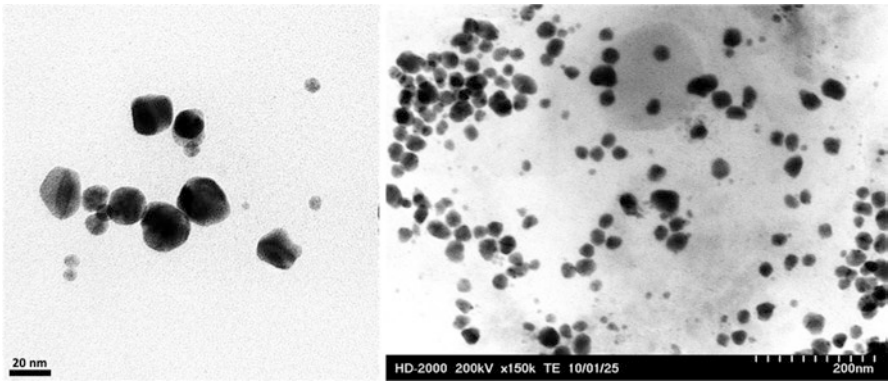

Fig. 20.2 Selected TEM images of metal nano-vehicles used in drug delivery. Transmission electron microscopy of metallic gold (**a**), silver (**b**) and silver sulphide (**c**) nanoparticles

Various metallic nanomaterials that are physiologically inert are employed as carrier platforms in accommodating antimicrobial peptides and antimicrobial entities onto their surface, which potentiates the bactericidal activity.

One among those is gold nanoparticles, which are well known for their biocompatible nature. Amenable surface functionalization property of gold nanoparticles explored handful of possibilities in tuning the nanoparticle surface by multiple functionalization intended to target desired application. For instance, regulating functional groups on gold nanoparticles surface with cationic monolayers could interfere with Gram-positive and Gram-negative bacteria. Xiaoning Li et.al synthesized gold nanoparticles by incorporating different chain lengths and aromatic groups to impart and understand the cationic properties and observed that synthesis using n-decane end linkage provided greater hydrophobicity and is effective against 11 clinical isolates of uropathogenic *E. coli* strains. Growth of all the selected *E. coli* strains was suppressed, which are otherwise resistant against seventeen different drugs depending on the strain used. These functionalized nanoparticles acted by disrupting the integrity of bacterial cell membrane to induce toxicity and were effective against superbugs like *Pseudomonas aeruginosa* and *Staphylococcus aureus* including the methicillin-resistant *S. aureus* (Li et al. 2014). In addition to surface functionalizations, gold nanoparticles were also used to conjugate antibiotic drugs like vancomycin and methicillin to potentiate the effect of antimicrobial efficacy. Mohammed Fayaz et al. compared the antibacterial activity of vancomycin alone and vancomycin-bound gold nanoparticles (VBGNPs) against *S. aureus* and *E. coli*. Compared to free vancomycin, VBGNPs non-specifically bound to transpeptidase of the glycopeptidyl precursors prominent on the vancomycin-resistant *S. aureus* to lyse the cell wall inducing toxicity (Fayaz et al. 2011). In another study, gold nanodots prepared by depositing antimicrobial peptide surfactin showed greater antimicrobial activity towards both non-multidrug and multidrug-resistant bacteria due to the synergistic effect of disrupting bacterial cell membrane. Using these antimicrobial peptide-loaded

gold nanodots as dressing material for methicillin-resistant *S. aureus* wound healing showed rapid healing in rats (Chen et al. 2015).

Significantly, several other bimetallic nanoparticles such as Au/Ag, Au/Pt and Au/Fe_3O_4 and metal oxide nanoparticles such as graphene oxide, TiO_2 and CuO are reported to deploy antibacterial activities against multidrug-resistant bacteria with their intrinsic mechanisms (Baptista et al. 2018). Different ways by which different metallic nanoparticles interact with bacterial cell and pathogenic biofilms are illustrated in Fig. 20.3.

20.1.2 Carbon-Based Nano-vehicles

Nanostructures are made of pure carbon atoms arranged in different allotropic fashions, characterized by sp^2 bonding in the core (Bianco et al. 2005). Carbon dots, fullerenes, carbon nanotubes (single-walled/multiwalled), graphite, graphene and graphene oxide are different classifications of carbon nanoparticles based upon their dimension. Desired carbon nanostructures can be easily synthesized by microwave

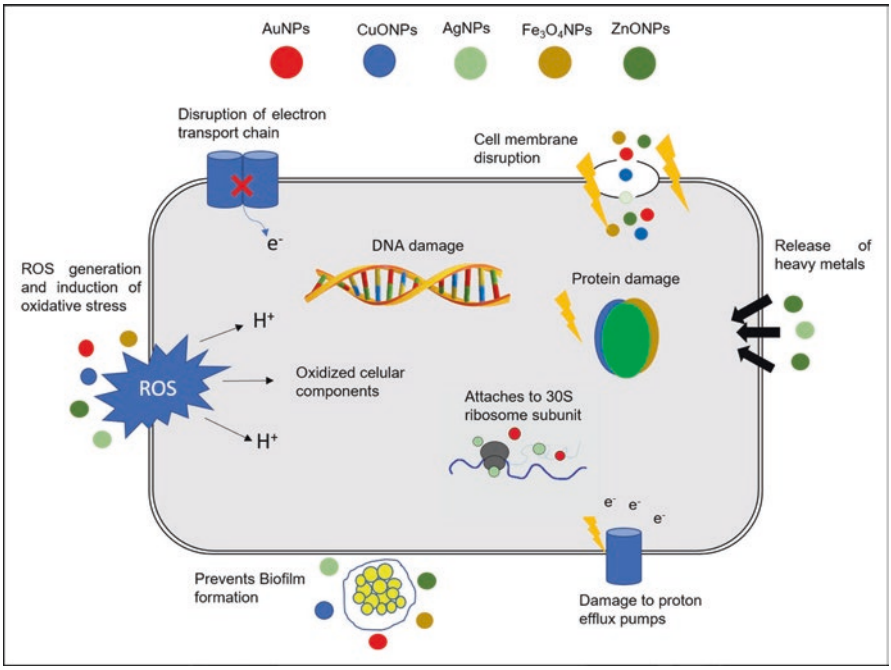

Fig. 20.3 Illustration of different mechanisms by which nanoparticles can elucidate toxic responses to bacterial cells. DNA—deoxyribonucleic acid; ROS—reactive oxygen species; AuNPs—gold NPs; CuONPs—copper oxide NPs; AgNPs—silver NPs; Fe_3O_4NPs—iron oxide NPs; ZnONPs—zinc oxide NPs. Reproduced from (Baptista et al. 2018) Copyright © 2018

irradiation, pyrolysis, carbonization and chemical vapour deposition. Due to the arrangement of carbon atoms in different dimensions, each structure exhibits unique electrical, thermal conductivities, high stability, and mechanical and optical properties (Baddour and Briens 2005). Therefore, carbon-based nanoparticles find applications in varied fields like biomedical engineering, drug delivery and cancer therapies.

Carbon being biocompatible and greater surface areas provided by multiple nanostructures opened up scope to utilize them as nano-vehicles in drug delivery. Very recently, Tak H. Kim reported that carbon nanoparticles conjugated with antibiotic tetracycline improved its efficacy on antimicrobial-resistant *Klebsiella pneumoniae* by tenfold. In contrary to the earlier methods, tetracycline-CNP conjugates are functional in inhibiting the process of drug extrusion, an inherent efflux mechanism of drug-resistant bacterial strains to protect itself and further generations from the antibiotic threat. Tetracycline when used alone showed less inhibitory action due to active drug efflux mechanism by bacteria, thereby escaping from the clutches of free antibiotic moieties. Whereas covalent bonding of tetracycline with CNPs disguises bacterial cell to misinterpret the tetracycline-CNP complex to tetracycline drug moieties and instantly initiate drug efflux mechanism, the tetracycline-CNP complex thus appeared in the vicinity of cytoplasmic membrane, thereby impairing the efflux system subsequently resulting in cell toxicity mediated by tetracycline-bound complex. These disguised mechanisms decipher the novel possibilities of implementing nanostructure for efficient drug deliveries (Kim et al. 2020).

Carbon nanotubes are made from either single-walled or multi-walled carbon atoms forming long hollow tubes by rolling of single or multiple graphene sheets. The aspect ratios of carbon nanotubes are as high as 132,000,000:1; i.e., length of carbon tube is usually greater than its diameter (Wang et al. 2009). These single lattice carbon nanotube structures have great scope to accommodate drug moieties because of their large surface areas and hollow channels. Afeefah Khazi-syed et al. demonstrated that suspending single-walled carbon nanotubes (SWCNTs) in doxycycline and methicillin water suspensions led to the non-covalent binding of drug onto the nanotube surface, which promoted the antibacterial effect in methicillin-resistant *Staphylococcus epidermidis*. SWCNT-methicillin complex was reported to exhibit 40-fold enhancement in inhibition of bacterial colony development. SWCNT internalization was likely due to their cell membrane permeability, thereby releasing methicillin to induce toxicity. On the other hand, SWCNTs also protect drug moieties from degradation (Khazi-Syed et al. 2019). Similarly, Mohyeddin Assali et al. reported the functionalization of carbon nanotubes with high payloads of ciprofloxacin, the most effective antibiotic of fluoroquinolones. Antibacterial activity of these functionalized SWCNTs was intensified by 16-fold against *Staphylococcus aureus* and *Pseudomonas aeruginosa* and by eightfold against *E. coli* when compared to free ciprofloxacin activity. High surface areas of SWCNTs favour abundant ciprofloxacin charging, and multiple nanotubes are exposed with bacteria thereupon augmenting the amount of ciprofloxacin interaction with the bacteria. On the other hand, these functionalized SWCNTs cripple the efflux pump defensive mechanism of bacteria used to gain resistance to antibiotics (Assali et al. 2017).

20.1.3 Liposomes-Based Nano-assemblies

Liposomes are small vesicular structures composed of lipid bilayer assemblages, enclosing aqueous cavities (Cevc 2012). The amphiphilic property of phospholipids gives an arrangement of hydrophilic heads facing the surface and hydrophobic tails towards the core-forming unilayer oligo and/or multi-lamellar bilayers. Liposomes can be prepared using natural or synthetic lipid molecules in situ, by techniques like rehydration of dried lipid films, sonication, micro-emulsification, membrane extrusion and solvent dispersion. Liposomes are widely used for incorporating nutrients and pharmaceutical drugs either in the aqueous core or in the phospholipid bilayers (Karmali and Chaudhuri 2007). Nanotechnology-driven liposomes are fabricated within 100–200 diameters that can efficiently penetrate infectious biofilms and reach the bacteria. The fusogenic property of liposomes, i.e., the ability of liposomal bilayer to directly fuse with bacterial cell membrane and expel highly potent antimicrobial drugs, provides great advantage in liposomal antimicrobial carriers over free antimicrobials (Scriboni et al. 2019). Either strains of bacteria that are resistant to selected antibiotics showed susceptibility to such drug encapsulation strategies. Liposomal encapsulation of drug also merits for conserving antibiotics from bacterial enzyme degradation. In the interest of infection control, and liposomal properties, these can be classified into natural, anionic, cationic, zwitterionic and fusogenic liposomes, which are capable of delivering selective agonistic interactions on pathogenic bacteria. Anne-Sophie et.al demonstrated the encapsulation of antibiotic tobramycin in anionic liposomes (negatively charged), which affirmed selective delivery of antibiotic to *Burkholderia cepacia* complex, a causative organism of lung infections that has intrinsic resistance to several antibiotics. Moreover, electrostatic interactions arising between negatively charged lipids and tobramycin anionic liposomes depicted increased loading of tobramycin by eight times, when compared to neutral liposomes, implying the abundance of antibiotic concentrations available at the proximity of pathogenic biofilms (Messiaen et al. 2013). Similarly, combinations of antibiotics such as incorporating both hydrophobic and hydrophilic drugs in the lipid bilayers were also employed for efficient antibacterial activity and drug delivery. Readers may further refer Wang et al. (2009) who compiled different antibiotics encapsulate using liposomal carriers.

20.1.4 Liquid Crystalline Particle-Based Nano-carriers

These are also made up of lipid bilayers but are curled to develop two- and three-dimensional structures bridged by water molecules; therefore, they are intermediate forms between solid and liquid phases. Thermotropics and lyotropics are the two types of liquid crystals classified based on the crystallinity exhibited as a function of temperature and concentration, respectively (Mo et al. 2017). Thermotropics are employed in monitor display screens, whereas lyotropics find applications in drug delivery owing to their good solubility, withstanding enzyme degradation and good

absorption by human tissues. Liquid crystals are formed by suspending any amphiphilic groups in suitable solvents, where the amphiphilic molecules aggregate to form a monolayer exposing hydrophilic heads outward by protecting hydrophobic tails in the core rounded up to form micelles. Upon availability of more such amphiphilic solute groups, the liquid crystal pattern extends to form phases of hexagonal, cubic and lamellar arrangements (Mo et al. 2017). Desired liquid crystal forms are used to dissolve the drugs in liquid matrix so that they are steered towards the selective pathogenic sites for targeted drug delivery. Chelsea R. Thorn et al. recently performed an interesting research availing drug delivery from monoolein liquid crystal nanoparticle triggered by bacterial lipase. Addressing the limitation of using ciprofloxacin, these nanoparticles showed 82-fold and sevenfold higher release of rifampicin and alginate lyase, respectively, by diffusing monoolein liquid crystal nanoparticle through bacterial lipase. Rifampicin is a drug of choice to treat predominant MRSA infections, while alginate lyase actively degrades bacterial secretions that form biofilm. The non-regiopecific lipase activity actively digested the cubic structure to lamellar, thereby provoking the antibiotic release. Thus, such MO-LCNPs find efficient applications in infections caused by *Pseudomonas aeruginosa* and *Staphylococcus aureus* that secrete lipase as a part of natural living process (Thorn et al. 2020). Another recent study performed by Xiangfeng Lai et al. illustrates the use of phytantriol-based cubosomes against polymyxin-resistant Gram-negative bacteria (*Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa)* for improved efficacy. Two model systems of Gram-negative bacteria, one with lipopolysaccharide membrane and the other lacking lipopolysaccharide membrane, were interacted with cubosomes. Good number of cubosomes are adsorbed on the surface of lipopolysaccharide-deficient model, which leads to rearrangement of bacterial cell membrane and penetration of cubosomes into bilayer systems, thereby exchanging lipids and subsequently disrupting the membrane (Lai et al. 2020).

20.1.5 Dendritic System Nano-assemblages

Dendrimers are highly branched polymeric molecules, which are nanometre in size obtained by controlled synthesis pathways. Dendrimers are usually monodispersed, symmetric and spherical in nature having multiple end groups. Polyamidoamine (PAMAM), polyglycerol and carbosilane are some of the common dendrimers (Martin-Serrano et al. 2019; Abedi-Gaballu et al. 2018; Yang and Zimmerman 2015; Jiménez et al. 2012). The dendritic end groups can be functionalized with selective functional groups to impart desired physiological/pharmacological activity that can have clinical significance. Reinaldo G. Bellini et al. studied the association of antituberculosis drug rifampicin with a fourth-generation poly(amidoamine) (G4-PAMAM) dendrimer and evaluated the activity in neutral and acidic pH conditions. The complex was stable in neutral pH but instantly expelled the contents in acidic pH. This controlled behaviour of the complex demonstrated the selective

release of the drug in acidic conditions, which is efficient against mycobacterium that usually resides in the acidic region of macrophage. The stability of complex in physiological pH and drug triggering behaviour by the change in pH credits the successful fabrications of dendrimers to deliver antibiotics without suffering the bacterial resistance (Bellini et al. 2015).

20.1.6 Polymer-Based Nano-matrices

Polymers are formed by long repetitive chains of smaller molecules (monomer). DNA, cellulose, chitosan and proteins are the examples of natural polymers, whereas polystyrene and PEG are synthetic in origin formed by the process of polymerization. Length of polymers ranges from sub-nanometre to few micrometres and can be microstructured into linear, branched, unbranched and cross-linked structures by the physical arrangement of monomer molecules (Martin-Serrano et al. 2019). Depending upon the structure, polymers possess unique physiochemical properties like tensile strength, diffusability, crystallization and melting transitions, ionic bond formations and electrical insulations. Therefore, polymers are tremendously used in packaging, manufacturing industries, synthetic fibres and medical applications. Polymer nanoparticles are being used extensively in biomedicine due to their biocompatibility/biodegradable nature. Antibacterial polymeric nanosystems have become phenomenal in tackling multidrug-resistant bacteria and their infectious biofilms through effective detection and binding abilities (Faya et al. 2018). Antibiotic encapsulations in these polymeric systems such as nanogels, vesicles and polymeric micelles serve as effective drug vehicles (Ding et al. 2019). Such encapsulations when selectively designed are capable in delivering multimodal therapies by responding to bacterial-infected environments such as alteration in pH and overexpression of enzymes. Abilities of certain MDR bacteria to produce hydrolytic enzymes such as penicillin G, amidase and B-lactamase, which console antibiotic activity of potent antibiotics such as penicillin, cephalosporins, cephamycins and carbapenams, are rate limiting the efficiencies of antibiotics. Yamin et al. synthesized bacterial enzyme-responsive polymer vesicles with amphiphilic diblock copolymer containing enzyme cleavable linkages (hydrophobic) and PEG blocks (hydrophilic), which can allow encapsulation of desired hydrophobic antibiotics, viz. deprotonated quinupristin/dalfopristin in hydrophobic bilayers and hydrophilic antibiotics, like gentamicin, vancomycin and parasin I in aqueous layers. Such antibiotic-loaded polymer vesicles experience structural rearrangement to expel the antibiotics to curb the growth of MRSA (Li et al. 2016). Similarly, antibody-loaded polymeric nanogels and antibody-conjugated polymeric nanosystems with series of hyperbranched antibiotics like gentamicin, tobramycin and neomycin exhibited potent antibacterial efficiency. Amjed et al. successfully sorbed antibiotic penicillin G onto fluorescent polystyrene nanoparticles. Even at lowest concentrations used the antibacterial activity was enhanced against Gram-positive and Gram-negative

strains of bacteria tested which also includes MDR strains that are specifically resistant to penicillin G (Alabresm et al. 2020).

20.1.7 Hydrogel-Based Nano-networks

Hydrogels are basically cross-linked polymer chain networks and are hydrophilic in nature. They are highly absorbent containing about 90% of water (Narayanaswamy and Torchilin 2019; Hu et al. 2019). Hydrogels are chemically composed of polyvinyl alcohols, polyethylene glycol, sodium polyacrylate and acrylate polymers. Modifying the polymer concentration and cross-linking concentration provides great mechanical properties to hydrogels, thus making that available for various applications. Hydrogels containing antibiotics are promising technology in dealing with drug resistance by designing combination of synthetic antimicrobial polymers with specific antibiotics. Hydrogels containing poly(N-hydroxyethyl acrylamide)/salicylate were developed by Zhao et al., which possess antimicrobial activities against *E. coli* and *S. epidermidis*. Salicylate is a natural antibacterial compound extracted from plants, and abundant loading of salicylate (up to 80%) hindered the growth of bacteria by 98%. The release of salicylate from hydrogel complex was attributed through diffusion process as the function of concentration gradient of salicylate from hydrogels to biological fluids (Zhao et al. 2013). Similarly, Tan et al. also introduced seaweed-containing PVA/PVP hydrogels, which are highly active against clinically relevant pathogenic bacteria like *S. aureus*, *E. cloacae* and *C. perfringens*. Hydrogel containing antibiotics, antimicrobial metal nanoparticles, antimicrobial polymers and peptides are few other strategies being developed in this area (Ng et al. 2014).

20.1.8 Cyclodextrin-Based Nano-vesicles

Dextrins are cyclic oligosaccharides composed of macrocyclic rings of glucose subunits linked by alpha-1,4 glycosidic bonds giving a hollow structure. Cyclodextrins are usually produced from starch through enzyme conversion. Based on the number of glucose subunits involved in the ring creating a cone shape, cyclodextrins are classified as α-alpha (6), β-beta (7) and γ-gamma (8) cyclodextrins and easily form complexes with selective compounds owing to their hydrophilic interior and hydrophobic exterior surfaces (Crini 2014). Therefore, cyclodextrins are widely chosen as vehicles in delivering variety of drugs due to the high solubility and stabilities conferred by cyclodextrins to the selected drugs. Incorporating antibiotics in the cavities of cyclodextrins enhances their solubility, efficiency and reduced dosage regimen, thus counteracting antibiotic resistance. Yucai Wei et al. recently synthesized enrofloxacin/florfenicol-loaded cyclodextrin metal-organic framework, which showed increased bactericidal activity on

S. aureus and *E. coli* compared to free antibiotics. Antibiotics were encapsulated in the porous structures of the framework, thus increasing the solubility and bioavailability of antibiotics. Moreover, the antibiotic release was sustained for longer times, continually up to 4 hours demonstrating higher and longer inhibition capabilities of bacteria (Wei et al. 2021). Overcoming the limitation of reversible/virustatic activity of sulfonated cyclodextrins, an antiviral for HIV, Samuel T Jones et al. further modified the cyclodextrins with mercaptoundecane sulfonic acids that conferred efficient virucidal molecules effective against different viruses like herpes simplex virus (HSV), respiratory syncytial virus (RSV), dengue virus and Zika virus (Jones et al. 2020).

20.1.9 Aptamer-Based Nano-templates

Aptamers are monomers of nucleic acids such as RNA, single strands of DNA or peptides forming short chains, which are capable of binding to target moieties. Aptamers are formed in vitro by the process called systemic evolution of ligands by exponential enrichment (SELEX) (Mallikaratchy 2017). Through this process, a number of aptamers can be developed that selectively bind to the target, therefore supporting both the clinical and research needs. Their smaller size, ease of modification and in vitro synthesis afford great merits to encourage their use in biomedicine, biosensing, imaging and target drug delivery. Nanoparticle-conjugated aptamers are majorly used in drug delivery because their high selectivity and nanoparticles protect nucleic acid from enzymatic digestion (Ding et al. 2017; Poolsup and Kim 2017). The stability of antimicrobial peptides can be increased in association with nanoparticle conjugates. Boeun Lee et al. demonstrated such study by loading an antimicrobial peptide $HPA3P^{His}$ onto gold nanoparticle-DNA aptamer selective against *Vibrio vulnificus* that is resistant to antibiotic regimens. The developed conjugate was functional in intracellular delivery of $HPA3P^{His}$, thereby interfering with the pathogenic bacteria besides protecting the antimicrobial peptide from proteolytic degradation. Intravenous injection of the conjugate in *V. vulnificus*-infected mice showed 100% antibacterial activity with selective targeting and 0% toxicity to the mammalian cells. Thus, such efficient intracellular uptake mechanism also minimizes the antibiotic dose regimen besides being highly effective (Lee et al. 2017). Fan Chen et al. identified a single aptamer (NK2) from the SELEX process, which has high affinity towards highly virulent strain Mycobacterium tuberculosis (H37Rv). The aptamer NK2 upon selectively binding to H37Rv provokes the CD4+T cells to produce IFN-γ, thereby significantly lowering the bacterial abundance (Chen et al. 2007). Similarly, aptamers can thus be used further upon drug conjugations and chemical modifications to treat several bacterial and viral infections.

The list of such nano-vehicles may become exhaustive in the near future due to extended research progress simultaneous evident by efficient treatment modalities in combating the pathogenic infections caused by antimicrobial-resistant bacteria.

As the nano-vehicle-based drug delivery is still in infancy, efforts are needed to fasten various nano-vehicles for their optimal therapeutic use.

20.2 Limitations on the Use of Nano-carriers

From the pool of metallic nanoparticles available for varied clinical applications, selection of highly biocompatible core metals is imperative to ensure treatment compliance. Majority of metal nanoparticles (except gold) used as either carriers alone or incorporated in suitable encapsulations tend to dissolute into respective ions in aqueous atmospheres, which interferes with the healthy host cells and may hamper the active cell metabolisms. Physiochemical properties of nanoparticles like size, shape and surface charge influence the stability of nanoparticles when exposed to different physiological conditions like pH, enzymes and secretions, which may prone to aggregate nanoparticles if they are instable. Peptide molecules such as antimicrobial peptides are also limited to short life span and poor permeability (Lee et al. 2017). Therefore, peptides may get denature before they could reach the target. Hydrogel-based nano-carriers through bio-inert suffer the limitations of oxidative degradation such as in PEG-based hydrogels and poly-HEMA-based hydrogels known to cause local tissue dehydration as these hydrogels have low hydration levels, thus driving the water diffusion through concentration gradient. Nonetheless, bacterial-specific and bacterial-targeted deliverable capability of several nano-carries addressing subtle limitations as evidenced from careful monitoring is highly needed to ensure host cell compliance and optimal nano-carrier utilization.

20.3 Conclusions

In summary, while introducing the readers on the emerging of nano-based carriers ‘nano-vectors’ for targeting microbial killing. The emergence of various forms of nano-vectors that have evolved to address the needs of current therapeutics to decrease the after-effects of drugs by attaining high payloads is discussed one by one. Choice of nano-cargos to dictate a selective biomedical implication is discussed as a crucial factor, as not all nanoparticles can be used for all applications. Desired nanoparticle has to be designed depending on the objective and nanoparticle properties to adopt the same. Interaction of these nano-cargos with various bacterial systems to combat multidrug resistance and the various mechanisms by which the bactericidal action is induced is discussed. Several mean by which nanoparticles can pose threat to a microorganism by inducing cell membrane, cell organelle and DNA damage are discussed. Along with cellular response to nanoparticle, stress in terms of ROD-induced oxidative stress is highlighted. Finally, limitations with regard to the exiting nano-carriers are discussed with a scope to design novel nano-carriers with unique properties.

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Chapter 21
Silver Nanoparticles as Potent Multidrug-Resistant Incorporants in Biomedicine

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21.1 Introduction

Currently, there is inadequacy in the development of new antibiotics to tackle the emergent antimicrobial resistance. Continuous rise in the emergence of resistant strains has not outweighed by the readiness of novel therapeutic agents for several reasons (Rawson et al. 2020). Primarily, policymakers should practice to elude the recommendation of new antibiotics until they are obligatory for resistance development. Conversely, society wishes that pharmaceutical companies should design and develop innovative drugs with no negative impacts on health. Furthermore, the durability of antibiotics being short-term; companies cannot make an unrelenting revenue, and high screening and development cost of the design, monitoring onus for novel drugs makes it difficult to meet the demand of affordable antibiotics (Wilson et al. 2020).

Since earlier decades, noble metal nanoparticles exhibiting unique physical, chemical, and biological properties due to their size in the nano regime have elicited tremendous interest. Metal nanoparticles are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biomedical and pharmaceutical applications. Among the various inorganic nanoparticles, silver nanoparticles are employed extensively since ancient times, to fight bacterial infections and control spoilage of food.

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21.2 Silver Nanoparticles as an Alternative Therapeutic to Disguise Multidrug-Resistant Microbes

Resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections when becomes nonfunctional due to continuous exposure is termed as antimicrobial resistance. Resistant microorganisms (including bacteria, fungi, viruses, and parasites) can eventually withstand attack by antimicrobial drugs over time, and so the standard treatment becomes inefficient and the infections persist increasing the risk of patient threat and further spread (Kingwell 2016). The evolution of resistant strains is a natural phenomenon that occurs when microorganisms replicate themselves erroneously or when resistant traits are exchanged. The use and misuse of antimicrobial drugs accelerate the emergence of drug-resistant strains. With continuous use, antibiotics became usual which on onehand act as a curable agent against disease but also have a profound impact on the life of bacteria, which results in the development of resistive nature against antibiotics and chemotherapeutic agents. Poor infection control practices, inadequate sanitary conditions, and inappropriate food handling encourage the further spread of antimicrobial resistance (Laws et al. 2019).

Multidrug resistance is a natural phenomenon of insensitivity of microbes like bacteria, fungi, viruses, and parasites to the administered antimicrobial drug which are structurally different and have different molecular targets, which leads to ineffective and spreading of infection. As per the survey performed across various regions of the globe, it has been found that a number of antibiotic-resistant species have been evolved showing inhibitory effects against antibiotics with increased mortality and hence referred as “superbugs”. These superbugs obstruct the drug action by intensifying the spreading of resistant microbes. Also, the difference in resistance profiles of bacterial and fungal pathogens is responsible for the impact on the efficacy of antimicrobial agents. Therefore, multidrug resistance is considered a threat to human health in the modern era (Gray and Wenzel 2020).

The action of antimicrobial agents on the microbe could hinder the metabolic pathway of nucleotide amalgamation, where a number of alleles (allele count) are inflated, relative to the expected mutation which inhibits the DNA/RNA synthesis and hence disrupts the cell membrane. With the development of multidrug resistance nature in microorganisms, the effectiveness of drug decreases as the organisms gain the tendency of chromosomal mutations or exchange of extrachromosomal DNA elements through conjugation which causes alteration in the composition of cell membrane resulting in decreased permeability and uptake of drugs by the microbial cell (Klemm et al. 2018). Another mode of multidrug resistance is the artificial expression of drug-targeted enzymes which generate alternate target molecules and cause interference in protein synthesis which affects the drug approach to the targeted site. Additional causatives of multidrug resistance are enzymatic degradation, conformational changes, certain point mutations, and chemical transformation of antimicrobials. Chemo resistance during the course of cancer treatment is also a major problem, which has emerged as multidrug resistance. When antibiotics

were misused for the cure of bacterial infection, it caused a genetic modification in the bacteria, which made bacteria antibiotic-resistant (Cole 2014).

As far as the clinical resistance is concerned, it arises when the concentration of antimicrobial drugs such that it poses higher probability of therapeutic failure. Some diseases which cause human death because of multidrug resistance are tuberculosis (Srinivasan et al. 2020), pneumonia (Cillóniz et al. 2019), HIV (Günthard et al. 2019), influenza (Guk et al. 2020), malaria (Ross and Fidock 2019), and several yeast infections. Immunocompromised conditions like HIV, diabetes, severe burns are key factors responsible for the spread of multidrug resistance. This is because the old conventional drugs have been replaced by novel drugs with more treatment time resulting in the increment of treatment cost and economic burden for the patient. With the introduction of nanotechnology, it becomes possible to utilize unique physicochemical properties of metallic nanoparticles which arise due to higher surface to volume ratio, in various fields of medical science (Zhao et al. 2020). Considering the antimicrobial potential of silver nanoparticles (AgNPs) against broad spectrum of microorganisms, they have been employed as curable agents against multidrug resistance and nonmultidrug resistance strains, because of their microbicidal activity which leads to dysfunctions and structural disruptions of cells. Also, a number of functionalization/conjugation of AgNPs with commercial antibiotic drugs is performed that is greatly going on to improve the bactericidal activity of AgNPs leading to the development of a new class of nano-based nanobiotics against multidrug resistance.

21.3 Impact of AgNPs in Biomedicine

Although modern medicine has advanced at an astonishing pace and helped increase life expectancy, a remedy for incurable/untreatable diseases, epidemics and pandemics suffer from disadvantages, such as side effects, drug administered deaths, deal with health side effects and added sickness than promoting health and can be overcome by nanomedicines. A wide range of NPs, including metal NPs, silica NPs, lipid-based NMs, dendrimers, polymers, nanogels, carbon-based NMs, graphene oxides are developed to enhance the pharmacological and therapeutic properties of conventional drugs (Giner-Casares et al. 2016). Metal NPs are considered as promising candidates for biomedical research due to their exceptional physiochemical properties, large surface area, photothermal potential, and electrostatic charge. In future, medicines based on nanotechnology have significant potential to increase the pharmaceutical market, targeted drug delivery systems, and bioimaging. AgNPs are used in different biomedical applications, viz. drug delivery systems, catheters, dental applications, bone healing, wound healing, and other medical applications (Li et al. 2016).

AgNPs used in biomaterials like endodontics, periodontics, and implant dentistry prevent biofilm formation, micro leakage, and secondary caries. Further, they enhance the mechanical properties of restorative material and improve the overall

bond strength between dentin and biomaterial. Also during the root canal therapy, complete elimination of microorganisms is not possible; hence AgNPs are used as irritants in place of sodium hypochlorite, ethylene diamine tetraacetic acid (EDTA) for having a complete clean sterile canal. Lotfi et al. showed that at lower concentrations, AgNPs show effective irrigation due to its deeper penetrating antimicrobial effect. Although the application of AgNPs in orthodontics is still at its primitive states, they have been incorporated in cement for orthodontic cases to prevent white spot lesions (Corrêa et al. 2015). AgNPs find application in different fields of dentistry, like implantology dental prostheses, restorative and endodontic dentistry. The profound characteristics of AgNPs hold a conspicuous place in nanomaterial-related restorative, regenerative, and multifunctional biomedicine (Nguyen and Hiorth 2015). Silver has shown promising results in caries prophylaxis in the form of nanosilver diamine fluoride (SDF), the disadvantage associated is tooth staining. As the particle size reduces, antibacterial effect enhanced due to increased reacting surface area. Similarly, in nano regime AgNPs have large contact surface area which further enhances the antimicrobial property (Santos et al. 2013).

AgNP-based nanosystems in particular have been assessed as appropriate transporters of several therapeutic molecules comprising antioxidant (Jiang et al. 2018), anti-inflammatory (Muhammad et al. 2016), anticancer (Al-Obaidi et al. 2018), and antimicrobial (Venugopal et al. 2017) substances. Due to their inherent bactericidal potential, AgNPs have grabbed distinctive consideration, and are well appraise as efficacious antibacterial drug-delivery systems, functioning both passive as well as active carriers for nano drugs in antimicrobial drugs delivery.

For the procurement of innovative and improved efficiency of drug delivery systems in response to different factors (optical, thermal, and pH) intonations to spot infectious, inflammatory, and malignant disorders, hybrid molecular units comprising of AgNPs were efficaciously chosen, owing to their incomparable biocompatibility, sturdy absorption characteristics, and its low toxicity. In biomedical devices like catheters, AgNPs play pivotal role to prevent device like infection (Thomas et al. 2015). Further, AgNPs-modified CVCs have shown very well inhibition against gram-positive as well as gram-negative bacterial biofilm. The attachment of AgNPs with bacterial cells is dependent on surface area to volume ratio hence bactericidal effects are size-dependent. Silver-treated catheters epitomize a possible strategy for decreasing dialysis-associated infections in patients enduring peritoneal catheters; though antimicrobial efficacy and method of procuring Ag^{+} may vary (Aflori 2014). It has been observed that hydrophilic surface properties of polymer matrix occur due to infused AgNPs which lead to retardation of biofilm formation and protein and electrolyte deposition accountable for coating and attachment of microorganisms onto the surface (Samuel and Guggenbichler 2004).

Some biocomposites comprising of silver-like AquacelTM (ConvaTec), PolyMemSilverTM (Aspen), ActicoatTM and BactigrasTM (Smith & Nephew), and TegadermTM (3 M) have been approved by Food and Drug Administration (FDA). Along with these commercial products, encouraging results have been witnessed in regard to the amalgamation of AgNPs with natural biomaterials for

improved wound-healing treatment, like modified cotton fabrics, chitosan, bacterial cellulose, and sodium alginate (Emam et al. 2015). The AgNPs and Ag^{+} carriers also find applications in treating the delinquent wound healing process of diabetic patients as wounds in these patients are escorted by various secondary infections thereby helping diabetic patients in wound healing at early stages. The enhanced antibacterial effects shown by AgNPs have gained special interest as their application in treatment of wounds, coating of medical devices, though their safety and biocompatibility need to be thoroughly studied (Rigo et al. 2013).

On comparing the effects of different nanoparticles, it has been observed that AgNPs increase the process of differentiation in preosteoblast cells (MC3T3-1) by following tissue mineralization like bone (Qing et al. 2018). Today, silver-coated prostheses present an alternative in the prophylaxis of tumor-related infections along with general trauma-related infections. However, no clinical studies associating with the lasting clinical effect of nano silver-coated implants used for revision arthroplasty are not reported (Brennan et al. 2015). The self-repairing ability of bones becomes limited during bacterial infection when occurs in bone defects. When compared to typical antibiotics, silver in nano dimension exhibits greater antibacterial effects of broad spectrum. Moreover, the resistance in bacteria against AgNPs is an infrequent phenomenon, therefore highlighting antibacterial mechanisms of nanosilver is essential. In antibiotic-resistant bacteria (methicillin-resistant *Staphylococcus aureus*), AgNPs have shown inhibiting potential in development of biofilm (Lu et al. 2016). In techniques like bone-replacement, doping materials are used as AgNPs in artificial as well as bioinspired bone scaffolds. The inimitable physiochemical and biofunctional characteristics like antiplatelet, antiangiogenesis, anti-inflammatory, antiviral, antifungal, and antibacterial activities, have made AgNPs a promising tool for implementing in biomedical applications. In fact, AgNPs were explicitly studied for their encouraging anticancer property. It was observed in different human cancerous cell lines (U251 glioblastoma cells, MDA-MB-231 breast cells, and IMR-90 lung fibroblasts) (Thapa et al. 2017). AgNPs are readily taken up by the mammalian cells, penetrate and cellularly localize *via* energy-dependent internalization pathways. Another enticing characteristic of AgNPs is precise fluorescence behavior which helps in detection and diagnosis applications (Mattea et al. 2017; Burdus et al. 2018).

Cardiovascular diseases (CVDs) are one of the reasons for global human death, with an history of greater than 17.7 million deaths in the year 2015. Prosthetic silicone heart valve having elemental silver was first silver-grafted cardiovascular medical device to evade bacterial infection and to reduce inflammation (Jiang et al. 2017).

The human eye is a multifarious organ, with extensive vascularization and innervation which are generally subjected to microbial infections. Compounds which have nanosilver have shown advancement of therapies even for the sensitive portions of our system the “eye infection”. Coating of AgNPs with calcium indicators has reduced the damage in cells of the retina and may perhaps be experimentally suitable for retinal imaging in animal mouse models (Weng et al. 2017). The antibacterial effects of AgNPs are important facets for ocular applications. The

broad-spectrum properties of AgNPs make them promising candidates not merely for antimicrobial effects, but also in multidrug resistance confronting tactics (Pind'akova et al. 2017).

21.4 Silver NPs as a Potent Antimicrobial Therapeutic Against Multidrug Resistance

Metal-based nanoparticles are effective against pathogens because of their nonspecific bacterial poisonousness nature along with their small size and hence exhibit a range of antibacterial action toward gram-positive and gram-negative bacteria. It has been used since ancient time not only for jewellery but also because of its antiseptic property specifically for open wounds and burns. AgNPs have an effective bactericidal nature against a broad spectrum of bacteria and can inhibit biofilm during wound healing. It is used as a substitute for conventional antibiotics like antifungal, antiviral, and anti-inflammatory. Considering the antibacterial effect of AgNPs that it exhibits by various modes of action in which silver ions interrupt DNA replication in bacterial cells, thus causing denaturation and rupture of cell organelles *via* oxidation (Marambio-Jones and Hoek 2010). In this type of cell lysis, AgNPs having a size ranging from 1 to 10 nm with minimum inhibitory concentration ranging from 0.003 to 0.5 mg/mL cause cell inhibition for microbes *Fusobacterium nucleatum*, *Streptococcus mutans,* and *Actinomycesoris* (Lara et al. 2010). Sondi et al., in 2004, showed that AgNPs cause 100% inhibition of *Escherichia coli* when MIC is 50–60 μg cm^{-3} (Sondi and Salopek-Sondi 2004). A mechanistic approach of interaction of AgNPs with antibiotics where amikacin-functionalized AgNPs revealed enhanced antibacterial activity against *E. coli* than normal antibiotics. Interaction of AgNPs possibly induces conformational changes and folding characteristics of amikacin molecules (Uddin 2018).

The size, shape, and surface properties of AgNPs and its interaction with cells can be either a physical or chemical transformation (Fig. 21.1). The physical interactions include disruption of membranes, membrane activity, transport processes, protein conformation/folding, and protein aggregation/fibrillation. Simultaneously, chemical interactions include the production of ROS, oxidative damage through catalysis, lipid peroxidation, and disturbance of cell membrane transport activity. Both physical and chemical processes cause cell death. The production of ROS is considered a critical chemical factor of causing cell cytotoxicity. However, the physicochemical properties of AgNPs are strongly responsible for the change in membrane morphology and stability. AgNPs are also potent enough to affect mitochondrial function, leading to apoptosis. Generally, the movement of NPs ends up in lysosomes where the cell could either digest or excrete out, based on its physicochemical properties. NPs can interact with protein corona because both are of the same size magnitude and compatible electron transfer. It is believed that ROS is responsible for DNA damage.

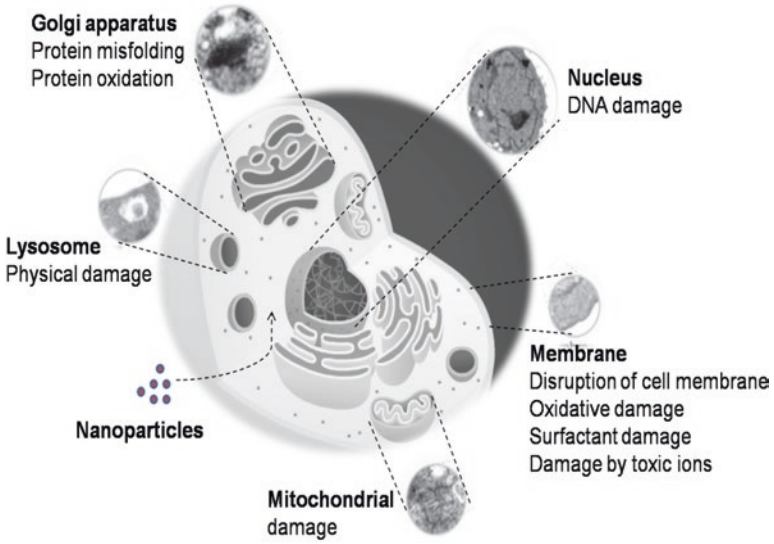

Fig. 21.1 Various modes of interaction of nanoparticles with cells

21.5 Mechanistics of AgNPs on Multidrug-Resistant Bacteria in Biomedicine

The mode of action of AgNPs in case of multidrug-resistant bacteria (MDRB) differs greatly by which outmoded antibiotics act, and therefore resistance with regards to antibiotics can be overcome by replacing conventional antibiotics with nanoparticles. Though the antimicrobial effects of AgNPs have been comprehensively investigated, their bactericidal mode is still ambiguous. Some studies indicated that AgNPs attack to the cell wall; penetrate through it, causing structural damage in cell membrane, increasing permeability, uncontrolled transport *via* cytoplasmic membrane leading to cell death.

Kim and coworkers reported that generation of free radicals might be attributed to antibacterial mechanisms leading to membrane damage (Kim et al. 2007). Morones and coworkers anticipated the idea that silver ions have affinity for thiol groups in most of the phosphorus-containing bases and enzymes, asserting the impairment caused by interactions of AgNPs with DNA (Morones et al. 2005). This contact can inhibit DNA replication and cell division, eventually causing cell death. Shrivastava and coworkers suggested that AgNPs inhibit bacterial growth by modifying the phosphotyrosine residue of putative bacterial peptides which further can affect cellular signaling (Shrivastava et al. 2007). Hwang et al. studied mechanistic aspect of toxicity of AgNPs and the silver ions on stress-specific bioluminescent bacteria. It was observed that ROS produce due to movement of silver ions in the cells and lead to membrane damage by AgNPs (Hwang et al. 2008). Klueh and coworkers proposed inhibition of bacterial growth occurs through silver atoms

which restrict enzyme activity by binding to thiol groups of the plasma membrane components and alter the function of components of cell membrane, which is essential energy generation and ion transport. Silver induces the compilation of disulfide bonds in between oxygen and thiol groups (R-S-S-R) within the cell. The disulphide alters the shape and structure of enzymes thus affecting the functioning of enzymes. He also put forward that bactericidal effects of the AgNPs could be attributed to the Ag^+ ions, which make way into the cell and intercalate with pyrimidine and purine bases of DNA thereby disturbing the hydrogen bonding between the two antiparallel strands ultimately leading to denaturation of DNA (Klueh et al. 2000).

It has been observed that Ag^+ at the concentration of 900 ppb, reacts with cells alters the functioning of enzymes (Succinyl Co-A synthetase, fructose bisphosphate aldolase, maltose transporter (MalK)). The translation of protein was restricted through binding of Ag^+ ions with the 30S ribosomal subunit which disables the ribosome complex formation.

The TCA cycle enzyme, succinyl coenzyme A synthetase was down-regulated on exposure of Ag^+ ions. Therefore, the proteins, key players of cells, are altered by the application of AgNPs, resulting in bacterial cell death (Yamanaka et al. 2005).

21.6 Factors Influencing the Bactericidal Effect of AgNPs

Microbes are unlikely to develop resistance against silver, as they do against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms, which means that they would have to develop a host of mutations simultaneously to protect themselves. There are following factors that can significantly influence the bactericidal effect.

21.6.1 Nanoparticle Species

While the properties of core particle species can be a great role in dictating the potential causative in microbial toxicity, relative toxicity of one species to another is not simple as factors such as particle size, shape, stabilizers, and syntheses can influence toxicity. Suresh et al. suggested that, as nanoparticles size decreases or change in its curvature occurs which further leads to the reactivity and directly influences the enhancement of toxicity (Suresh et al. 2013). Surface coating of the nanomaterials can also have enhanced the microbial toxicity. Rigid controlling of physical characteristics is highly challenging, and various syntheses may pertain to differences in surface coating and contaminant toxic after reaction leftovers. Moreover, diverse encapping processes may involve additives, surfactants, and chemicals that are not fully eliminated from the reaction. For example, C60 that was initially toxic was later demonstrated to be safe, and it is the remnant of tetrahydrofuran that is used in the synthesis of C60 was the toxicity culprit. Likewise, despite

of their use of using Ag-resistant *E. coli* in the studies, presence of formaldehyde was demonstrated for the killing of Ag-resistant bacterium (Marambio-Jones and Hoek 2010). Therefore, the properties of nanoparticles may also rely in par on other leftovers present in the reaction formulation and the coatings associated with the nanoparticles.

Evidence supporting the role of silver ions upon its dissolution from parent AgNPs as the reason toxicity is unfolding. For example, the antibacterial potential of 9 nm spheres of AgNPs against silver-resistant bacterium has been attributed to chemisorbed Ag^+ ions that are formed under extreme oxygen-sensitive conditions (Levard et al. 2012).

21.6.2 Particle Appearance and Morphology

Differences in the iterated antimicrobial potential of AgNPs also correlate to particle's size and/or shape distribution. Decrease in the size of the particle leads to increase in the ratio of surface area to mass thereby altering to the physicochemical properties such as surface atom reactivity, electronic and optical properties. Such a phenomena result in clumping of particles and can significantly influence the particle reactivity and cell-binding characteristics (Rai et al. 2012). The change in properties of nanoparticles is largely governed by their small size rather than their bulk counterparts. The properties of materials change as their size approaches the nanoscale and as the percentage of atoms at the surface of a material becomes significant. The smaller the size, greater the surface area to volume ratio, hence greater is the bactericidal effect of AgNPs. Since the particles in nano dimensions have greater surface reactivity, thus the antibacterial effects of silver are size-dependent. AgNPs undergo a size-dependent interaction with human immunodeficiency virus type 1, preferably *via* binding to gp120 glycoprotein knobs (Raimondi et al. 2005). The morphology of nanoparticles plays a key role on antibacterial effect, Pal and coworkers investigated the effect of different morphology (spherical, rod, and triangular) nanoparticles synthesized by chemical route against *E. coli* at various concentrations. It has been observed that triangular shape nanoparticles were shown more inhibition than spherical nanoparticles followed by rod shaped against *E. coli* (Pal et al. 2007). In Fig. 21.2, UV-Visible absorption spectra of silver nitrate ($AgNO_3$) and after synthesizing AgNPs are shown. Further, crystalline nature AgNPs explored through XRD pattern and surface morphology are also shown in TEM micrograph.

21.6.3 Concentration

Morones and coworkers studied interactive effects of AgNPs of various concentrations on bacterial cells (*E. coli*) at mid-log period of bacterial growth. Further dose-dependent activity of AgNPs is also measured on both the *Gram* + as well as

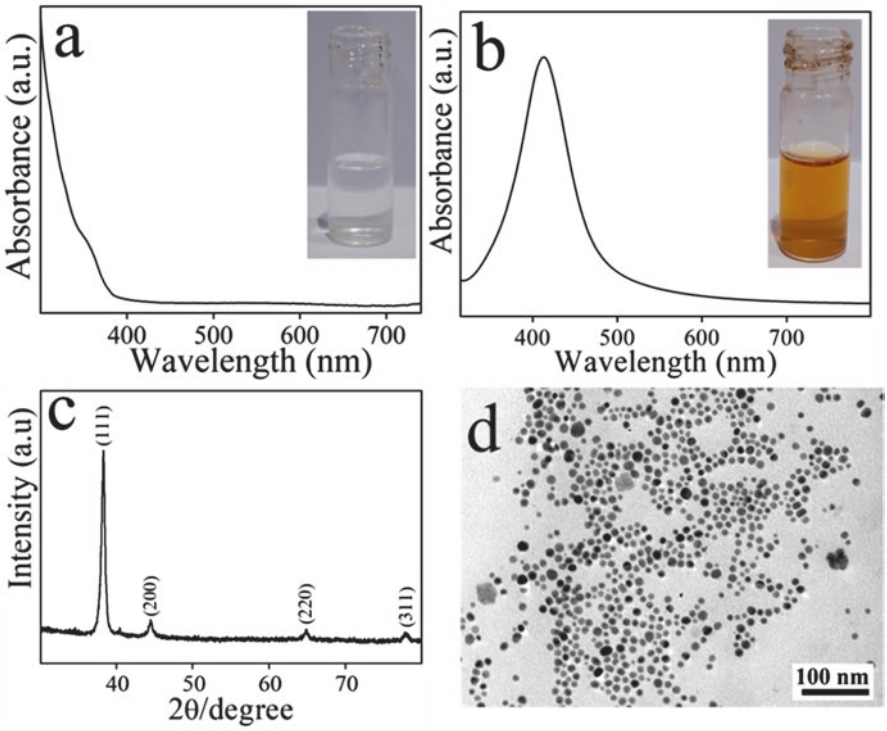

Fig. 21.2 UV-Visible spectra of (**a**) precursor ($AgNO_3$) as-synthesized AgNPs, (**b**) AgNPs showing their morphological distribution, (**c**) powder XRD-pattern of AgNPs demonstrating their crystallinity, (**d**) TEM image

Gram-bacteria. It has been observed that the action of AgNPs is dose-dependent and AgNPs depict conspicuous antibacterial effects against gram-negative bacteria in comparison to the gram-positive ones.

21.6.4 Stabilizing Agents

Silver nanoparticles are encamped through stabilizing agent and its determinant in dictating mechanistic approach and environmental as well as biological destiny of nanoparticles, as the surface is often primary approach of interaction. Charge on nanoparticles influenced by surface capping, which in turn can affect the binding of the nanoparticle to the cellular surface. Figure 21.3 shows the transmission electron microscopy image analysis of mode of interaction of AgNPs with bacteria. The binding of the nanoparticles to the surface can further induce stress,

and localize potential toxic impacts. Additionally, the surface coating can influence the release of metallic ions from the nanoparticles in reaction medium

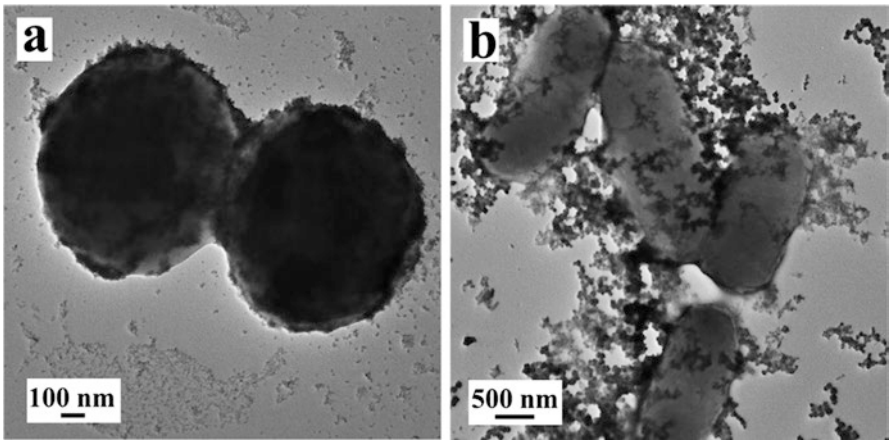

Fig. 21.3 Transmission electron microscopy images of interaction of AgNPs with bacteria

or aqueous environments. Suresh et al. while demonstrating the role of various surface coatings on AgNP use three types of encapping agents; peptide-coated biogenic, colloidal, and oleate (Suresh et al. 2010). The surface features are also strongly influenced by environmental parameters such as pH, ionic strength, presence of organic molecules, and temperature. Consequently, the surface coatings can be difficult to prescribe and can strongly influence the apparent toxicity of the nanoparticle. For example, the sensitivity of *N. europaea* to Ag^+ ions in comparison with 20 and 80 nm diameter AgNPs under varied physiological parameters showed that seemingly minor factors like the sequence of addition of particles and the presence or absence of medium constituents can have huge influence on the stability, reactivity, and toxicity of the nanoparticles including the subject microorganism.

21.7 Conclusions

Silver nanoparticles have been proven as potent therapeutic alternative to disguise multidrug- resistant microorganisms, which effectively inhibit various pathogenic bacteria, fungi, and viruses either by membrane destruction, ROS generation, DNA damage, enzyme inactivation or protein denaturation. AgNPs had shown unique properties and therapeutic action, exhibit different antibacterial effects against gram-positive and gram-negative bacteria which depend upon particle appearance and morphology, dose and nanoparticle species. This chapter summarizes AgNPs and cell interaction in bacteria through which they inhibit multidrug resistance in bacteria.

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Chapter 22
Role of Gold Nanoparticles Against Multidrug Resistance (MDR) Bacteria: An Emerging Therapeutic Revolution

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22.1 Introduction

Infectious diseases are increasing continuously in our day to day life and this reason has now become a serious threat globally. This has led to concern of increase in the development of multidrug resistance bacteria which has now become a serious nationwide burning topic in the public health sector. Large numbers of antibiotics are used against enormous number of both gram-positive and gram-negative bacteria in humans, animals, and fish resulting in the selection of pathogenic bacteria resistant to antibiotics and antimicrobials. Multidrug resistance bacteria may arise due to, first, accumulation of multiple genes each coding for resistance to a single drug inside a cell/R plasmids. Second, due to increased gene expression that codes for multidrug efflux pumps. Third, due to altered physiological state, which is the occurrence of "persister" cells, i.e., it was observed that high concentration of antibiotics does not kill a bacterial population, leaving behind a persister population which is genetically identical to the susceptible cells (Nikaido 2009). Hence, the use of nanostructure material or nanoparticle has been developed as an emerging technique to overcome the threat of multidrug resistance bacteria. Nanoparticles have a size varying within the range of 1-100 nm. Nanomaterials are used as potential candidates in disease diagnostics, drug delivery system, and medical imaging due to their improvised

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characteristic such as high surface area to volume ratio and small size effect (Okkeh et al. 2021). Different types of nanoparticles can be used which may be metallic, organic or carbon nanotubes. They can be used to assist the delivery of drugs, inhibit the formation of biofilm and activity of bio efflux pumps, and thus circumvent antimicrobial activity by themselves. Thus, nanoparticles furnish us a promising solution by acting as a carrier for antibiotic and antimicrobial compound (Baptista et al. 2018). In context to this, gold nanoparticles can be used as tool against multidrug resistance bacteria. However, gold nanoparticles offer excellent bactericidal activity against MDR gram-positive and gram-negative bacteria unlike other free drugs. One of the most extraordinary properties of AuNPs is their capacity to transform light into heat under laser irradiation. Thus, this property can be exploited to develop photothermal nanovectors to destroy MDR bacteria at a molecular level. Gold nanoparticles are considered as inert and nontoxic and can be synthesized by chemical synthesis based on the reduction of chloroauric acid by citrate (Baptista et al. 2018). These AuNPs enhance the inhibitory effects of antibiotics when conjugated with ampicillin, kanamycin, and streptomycin. They lower the minimum inhibitory concentrations of the antibiotic counterparts against the bacteria. Since they act at nanoscale range; hence, they can interfere with the molecular pathway, interact with bacterial cells, and regulate cell membrane penetration. Fluoroquinolone antibiotics and AuNPs can be used for the treatment of multidrug-resistant *E. coli* bacterial strains (Lee et al. 2019). Gold nanoparticles exhibit optical properties which are determined through Plasmon resonance, associated with the excitation of electrons and localized from the visible to the infrared (IR) region, depending on the particle size, shape, and structure (Dykman and Khlebtsov 2011). They act as a metal precursor, reducing agent, and a stabilizer. Certain in vitro researches have condemned that AuNPs once incorporated within a cell may give rise to endogenous reactive oxygen species (ROS) which may later result to DNA damage, cell death, and eventually cell cycle arrest. Furthermore, it decreases the viability of human cells about 100% at a concentration of 0.1 ppm to less than 40% at a concentration of 10 ppm resulting in dose-related toxicity. But due to the natural characteristic of gold nanoparticles such as biocompatible nature, ease of surface functionalization, optical properties, and antibacterial efficacies, they are widely used to fight against both multidrug resistance gram-positive and gram-negative bacteria (Okkeh et al. 2021). Antimicrobial actions of Cephalosporin type antibiotic (cefaclor) conjugated with AuNPs interact with the outer peptidoglycan layer of the bacteria where it has been observed that cefaclor enhance the membrane porosity and AuNPs create holes in cell walls. This interaction resulted into hindered DNA unwinding and transcription and resulted into high cefaclor concentration in the bacteria. Antibiotic named Cefotaxime when conjugated with AuNPs generates extended spectrum beta-lactamase which resulted into the deposition of high cefotaxime concentration in the bacterial cell. Furthermore, AuNPs when conjugated with methylene blue reduce the bacterial survival capacity enhancing the generations of ROS other than singlet oxygen (Shaikh et al. 2019).

22.2 Antimicrobial Mechanism of Gold Nanoparticles

Of late, the bacterial drug resistance has been greatly curtailing the effective antibiotic options and is consequently posing perils globally. Therefore, novel methodologies are highly sought-after and are straightaway required to significantly enhance the antimicrobial activity. However, associating antibiotics to nanoparticles is bringing about a promising scope by increasing overall drug avidity, bioavailability, and internalization by mammalian cells (Singh et al. 2017). Ag, Cu, and Zn ions along with zinc and copper oxides are some of the nanoscale compounds that have proven exalted antibacterial efficacy. The common consensus with regard to antibacterial activity is that the gold nanoparticle, AuNP-drug conjugates demonstrate stronger antibacterial activity than specific nanoparticles and drugs when administered separately.

Gold particles at the nanoscale (AuNPs) have demonstrated uniquely useful antimicrobial capabilities. Being the metal with bare minimum activity, gold exhibits steady chemical properties, nontoxicity, and excellent biological compatibility (Hammer and Norskov 1995). AuNPs are multivalent, and their substantial surface area provides AuNPs with a plethora of specific binding sites for the targeted bacterial cell (Dreaden et al. 2012). AuNPs have been shown to have a wide range of antibacterial action against gram-negative and gram-positive bacteria (Slavin et al. 2017). In contrast to traditional antibiotics, AuNPs do not easily acquire drug resistance (Yang et al. 2018) as they are shown to target a variety of components (DNA and protein) in bacteria, thereby posing perils for the pathogen to evolve and evade all the damage. The bactericidal characteristics of AuNPs are directly proportional to their size, dispersibility, and surface modification. By the means of electrostatic adsorption on the bacterial surface, reactive oxygen species (ROS) damage, and membrane damage to proteins and DNA, the gold nanoparticles induce death and bring about cell lysis. AuNPs are cytotoxicant-free and have a high degree of biocompatibility (Hammer and Norskov 1995). Currently, the majority of antibacterial investigations using gold nanoparticles have been conducted on traditional bacteria, with just a fraction on multidrug-resistant bacteria. This is therefore a critical step in paving the path for future study. Antibacterial activity is quantified in terms of the minimum inhibitory concentration (MIC), the number of bacteria reduced, the zone of inhibition, the rate of bacteria inactivation, and the proportion of bacterial survival (Amini et al. 2019). Due to the disparate units, it is tedious to conduct impartial comparisons across research. As a result, this becomes one of the many elements of nanoscience that needs standardization. Additionally, coalescing theoretical research and practical application is a tremendous task. Numerous problems remain unresolved. The selectivity and location of action of nanoparticles on the cytomembrane, as well as their transmembrane activity, are of primary importance (Gu et al. 2021). In general, when nanoparticles come into contact with bacteria, they induce oxidative stress mechanisms, enzymatic inhibition, protein deactivation, and gene expression alterations. The antibacterial effect of natural products (NPs) is mediated by oxidative stress caused by reactive oxygen species (ROS) (Rudramurthy et al. 2016;

Dwivedi et al. 2014). ROS are naturally occurring byproducts of cellular oxidative metabolism and play a significant role in the regulation of cell survival and death, as well as cell signaling and differentiation. ROS are produced during aerobic respiration in bacteria, and their formation is precisely controlled by the cell's antioxidant machinery (Li et al. 2012). Excessive generation of ROS disrupts redox equilibrium, resulting in oxidative stress that damages membrane lipids and substantially changes DNA and protein structure (Dwivedi et al. 2014). While endogenous antioxidants may neutralize O_2- and H_2O_2, singlet oxygen (1[O2]) and •OH have been found to cause acute microbial mortality (Zaidi et al. 2017). Gold NPs may generate distinctive ROS, such as hydroxyl radical (·OH) and hydrogen peroxide (H_2O_2) (Wang et al. 2017). In this way, the amount of ROS produced by NPs is determined by the chemical composition of the nanoparticles. Due to their high surface-to-volume ratio, metallic NPs have demonstrated significant antibacterial effectiveness and are presently being explored for the treatment of bacterial infections. An increased generation of ROS, including free radicals, is generally associated with an elevated ratio. It was shown that under UV irradiation, ROS production and metal ion release greatly increased the antibacterial activity of uncoated AuNPs in aqueous solution (365 nm). Antibacterial activity of AuNP against *E. coli, Salmonella typhi, Pseudomonas aeruginosa,* and *Klebsiella pneumoniae* was ascribed to oxidative stress caused by increased intracellular ROS generation (Umamaheswari et al. 2014). Studies evaluating the gold nanoparticles and laser-AuNP combination therapy against *C. pseudotuberculosis* suggested that the mode of action is directly linked to ROS production, which explains why an increase in oxidative stress in microbial cells in the form of vacuole formation is a sign of potent activity. This effect was enhanced by AuNPs-laser, resulting in a dramatic degradation of the integrity of bacterial cell membrane owing to laser light. As a corollary, AuNPs' antibacterial activity is increased by at least onefold. Metal ions produced by metal oxides are absorbed by cells and reach the intracellular compartment, where they interact with functional groups on proteins and nucleic acids, such as carboxyl (–COOH) groups, amino (–NH), and mercapto (–SH) groups (Wang et al. 2017). This kind of interaction changes the cell structure, inhibits enzyme activity, and disrupts bacteria's regular physiological functions. Copper oxide (CuO) nanoparticles have been demonstrated to substantially affect protein expression and to prevent denitrification. Additionally, it has been shown that CuONPs alter the proteins that are involved in nitrogen metabolism, and electron transport. The interaction of gold–superparamagnetic iron oxide nanoparticles with bacterial proteins influences the metabolism and redox system of bacteria (Niemirowicz et al. 2014). Nanoparticles may also enter the bacterial cell through absorption, and thereby releasing metal ions into the extracellular environment. It may also bind to the bacterial cell membrane's negatively charged functional groups. Silver ions (produced from silver NPs) deposited on the cell membrane, for example, have been shown to cause protein coagulation (Jung et al. 2008).

The bactericidal action of graphene oxide/Cu/Ag NPs against *E. coli, P. aeruginosa, K. pneumoniae, S. aureus*, and Methicillin-resistant *S. aureus* (MRSA) was recognized by Jankauskaite and colleagues as a potential synergy between various

damaging routes (Jankauskaitė et al. 2018). The NPs' interaction with the cell wall is nonoxidative.

Bacterial cell walls and membranes serve as protective barriers against deterioration in the environment. Different adsorption pathways for NPs are provided by cell membrane components (Lesniak et al. 2013). The cell wall of the gram-negative bacteria is made up of lipoproteins, phospholipids, and lipid polysaccharides (LPS) which form a barrier that enables only specific macromolecules to get through (Zaidi et al. 2017). On the surface of their cell walls, gram-positive bacteria have a strong negative charge. Because teichoic acid is only produced in gram-positive bacteria, LPS binds NPs by providing the negatively charged sections of gram-negative bacteria's cell walls; NPs are distributed throughout the phosphate chain. Gram-positive bacteria have a stronger antibacterial effect as a result (Wang et al. 2017). Yu and colleagues produced hydroxyapatite whisker (HAPw)/zinc oxide (ZnO) NPs and tested them for antibacterial activity against *S. aureus, E. coli,* and *Streptococcus mutans*. According to the researchers, the components and structure of the bacterial cell wall affect antibiotic effectiveness.

The antibiotic action of these NPs may be increased in gram-positive bacteria, and certain components may inhibit NPs from sticking to the bacterial cell barrier. A plethora of excellent research on the antimicrobial activity and toxicity of gold nanoparticles have been reported in recent decades. AuNPs are thus reckoned as composite innovations with attributes that are distinctive from those of conventional materials, and they have enormous potential for using antimicrobial agent against MDR bacteria.

22.3 Combination/Conjugation Strategies of Gold Nanoparticles

Vancomycin when conjugated with AuNPs acts as a reducing and capping agent and thus reduced the antibacterial capacity. Similarly, when conjugated with colistin reduces the minimum inhibitory concentration providing improved efficacy. Conjugation in this case can be achieved by both covalent and noncovalent interaction. It have synergistic effect against *E.coli, S. aureus*, *E. faecium*, *A. baumannii*, and *P. aeruginosa* when conjugated with fluoroquinolone, polymyxin B, ciprofloxacin, ceftazidime, ampicillin, clindamycin, or erythromycin. They also induce reversal of antimicrobial resistance and boost antimicrobial effects when conjugated with antibiotics against vancomycin-resistant *Enterococcus* and methicillin-resistant *S. aureus* (Okkeh et al. 2021; Baptista et al. 2018). AuNPs when conjugated with antimicrobial peptides increase the stability of the peptide and are protected from protease degradation and have the property of water solubility. They can functionally fold itself into amphiphilic α-helical structure and thus reduce its antimicrobial activity. One such example of conjugation is antimicrobial peptide (AMP), $HPA3P^{His}$, when loaded onto a gold nanoparticle-DNA aptamer

(AuNP-Apt) conjugate (AuNP-Apt-HPA3P^{His}) is an effective therapeutic tool against *V. vulnificus* infection. Photoluminescent gold nanodots (AuNDs) when conjugated with surfactin, AuNP when conjugated with Cecropin melittin Magainin-1 Tet-20, and AuNPs when conjugated with antiapoptotic peptide show several healing features in the human body. Antibiotics such as ciprofloxacin when conjugated with NPs result into antimicrobial effect against MDR microorganisms. Peptide named 1018 K6 when conjugated to AuNPs can maintain its antimicrobial activity by folding into a functionally relevant α-helix structure when in the presence of a membranous environment. Cecropin-melittin (CM-SH) has antibacterial properties and thus when conjugated in the surface of AuNPs through Au-S bond, resulted in higher antimicrobial activity and higher stability (Lee et al. 2017; Teixeira et al. 2020). Drugs such as kanamycin are known to be broad spectrum antibiotic. This drug when conjugated with AuNP develops antibacterial activity against multidrug bacterial strains. It provides improved efficacy against both resistant and susceptible gram-positive –negative bacterial strains. kan-AuNP reduces minimum inhibitory concentration in comparison to free kanamycin when tested. They result into bacterial cell lysis. Prior to this AuNPs when conjugated with ampicillin, streptomycin also reduces the minimum inhibitory concentration compared to the free drug counterparts. In addition to this, we also have vancomycin-resistant based on mutation or cell membrane when conjugated with gold NPs. Another example, AuNPs and AuNPs-laser combined therapy against *C. pseudotuberculosis* and the mechanism of action are related with ROS production that results in vacuole formation as a potent activity. Conjugation AuNPs with Carbapenems (Imipenem (Ipm) and Meropenem (Mem)) as a delivering strategy against carbapenem-resistant gram-negative bacteria resulted in significant increase in antibacterial activity against all the tested isolates which were previously isolated from an infected individual. Cefotaxime-conjugated AuNPs are used to target drug-resistant CTX-M-producing bacteria. Concanavalin-A (ConA) directed dextran capped AuNPs (GNPDEX-ConA) when conjugated with methylene blue (MB) (MB@GNPDEX-ConA) and photodynamic therapy (PDT) enhanced the efficacy and selectivity of MB induced killing of MDR microorganisms. Glucosamine-gold nanoparticle-graphene oxide and UV-irradiated GlcN-AuNP-GO were evaluated against *E. coli* and *E. faecalis* (Payne et al. 2016). AuNPs when cross-linked with collagen, gelatin or chitosan provide great efficacy and easy binding with macromolecules. AuNPs inhibit the ATP synthase activity and the multidrug-resistant bacteria are killed by non-ROS-dependent pathway. Flavonoid when coated with gold nanoparticles on colonization of *Enterococcus faecalis* resulted in higher reduction of bacterial counts both in in vitro and in vivo. Trisodium citrate (TSC) and chlorogenic acid (CGA) can synthesize gold nanoparticles (CGA-AuNPs) with different particle sizes indicating antibacterial activity. Green synthesis of dispersed cuboid gold nanoparticles using algae indicated that the synthesized gold nanoparticles had certain potential for certain pathogens like *E.coli* and *Staphylococcus aureus*. Stems of *Tinospora cordifolia* can synthesize gold nanoparticles (AuNPs) and showed greater effect on the biofilm of *Pseudomonas aeruginosa* PAO1. The aqueous extract of C. sativa stem can be used

to synthesize gold nanoparticles without adding other reducing, stabilizing or capping agents and upon conjugation it showed bactericidal effects against *Pseudomonas aeruginosa* and *Escherichia coli*. Flavonoid glycerin can be used as a reducing capping agent and upon conjugation with synthetic gold nanoparticles showed antibacterial efficacy against opportunistic MDR-resistant bacterial pathogens. Green synthesis of gold-chitosan hybrid nanoparticles (Au-CS hNPs) showed antibacterial activity (Singh et al. 2020; Riaz et al. 2020; Su et al. 2020). This is an advancing technology to combat against multidrug resistance bacteria where RNA is used to target DNA recognition and the cas enzymes thus destroy the nucleic acids. They enable specific removal of microorganisms and enhance the antimicrobial property by targeting specific resistance gen, undesirable polymorphism, biofilm formation, and virulence. Thus, they can enable the selective and specific removal of microorganisms. Carbapenem-resistant *K. pneumonia* resulted in efficiency of genome editing which facilitates resistance to carbapenems (Lima et al. 2019). These conjugates have shown increased efficacy and enhanced synergistic action by increasing the concentration of drugs at the target site. They reduce the toxicity at cellular level by reducing reactive oxygen species generation. Gold nanoparticles conjugated with aminoglycosides showed efficient antibacterial agents against *Staphylococcus aureus*, *Micrococcus luteus*, *E. coli,* and *Pseudomonas aeruginosa*. A hydrophobic cationic-conjugated gold nanoparticle reduces the minimum inhibitory concentration (MIC) of fluoroquinolone against multidrug-resistant bacteria by 8–16 times. Carbapenem when conjugated with gold nanoparticles resulted in antibacterial activity against MDR bacteria such as Klebsiella *pneumonia*, *Proteus mirabilis,* and *Acinetobacter baumannii.* Imipenem-conjugated gold nanoparticles indicated fourfold decrease in the MIC. Meropenem upon conjugation decreased the MIC by threefold. Mannose-substituted gold metal nanoparticles resulted in high efficiency of antibiotics capped AuNPs compared to free antibiotics against MDR bacteria. Gold NPs conjugated with cephalosporin showed high efficacy of AuNPs-cefaclor against *E. coli* and *S. aureus* (Masri et al. 2019). These conjugates inhibit the biofilm formation, trigger both innate and adaptive host immune response, generate reactive oxygen species, and induce intracellular effects, thus can be greatly used against multidrug-resistant bacteria. They are often used as a vector to carry antimicrobial moiety, hence improving biocidal property. Green synthetic AuNPs can be used as effective nanoantibiotics to combat biofilm-related infections caused by *Pseudomonas aeruginosa*. Combination of synthetic thiol ligands with p-mercaptobenzoic acid adapted Au- nanoparticles, and this conjugate showed antibacterial effects against MDR bacteria. They delayed the development of resistance compared to the antibiotic alone by affecting the transcription process for a wide number of genes. An amino-substituted pyrimidines-conjugated Au nanoparticle had antibacterial activities against MDR bacteria through chelating agents such as Mg^{2+} or Ca^{2+} and thus suppresses the ATPase activities. Combination of gold nanoparticles with 4-Dimethyl aminopyridinium propylthioacetate showed antibacterial effects against *E. coli* (Baptista et al. 2018).

22.4 Different Variants of Gold Nanostructures in Combating MDR

Gold Nanoparticles (AuNPs) Gold nanoparticles are small structures having size of about 0.1–100 nm. Among nanomaterials of other metals, gold nanoparticles have garnered much attention as they are more stable in comparison to the others (Hu et al. 2020). The methods for their synthesis include chemical, physical, and photochemical reduction of gold salts, microwave irradiation, etc. (Chen et al. 2008). Their application ranges from electronics to therapeutic drug deliveries and biosensors. The growing threat of microbial antibiotic resistance calls for novel therapeutic techniques and the use of gold nanoparticles in this scenario is a promising field (Tao 2018). Li et al. (2014) showed that the surface modification of gold nanoparticles displayed high antimicrobial effect against multidrug-resistant gram-positive and gram-negative uropathogens, with very low toxicity to mammalian cells. Khan et al. (2017) developed a complex of Methylene blue and Concanavalin-A (ConA) directed dextran-capped gold nanoparticles (MB@GNPDEX-ConA) that showed efficient light-activated antibacterial potential against a wide range of MDR bacteria like *Escherichia coli* and *Enterobacter cloacae,* with low toxicity to mammalian cells, thus giving a prospective for photodynamic therapy against such MDR bacteria.

Gold Nanorods (AuNR) Gold nanorods are gold nanoparticles that are given an elongated rod-like shape rather than the conventional spherical one. They have been preferred more than the other gold nanoparticles owing to their exceptional optical properties (Pérez-Juste et al. 2005). Due to their elongated shape, the plasmon frequency position increases longitudinally which gives them the advantage of having absorptions along wavelengths ranging from visible to infrared and also gives high quantum yield of fluorescent molecules (Stone et al. 2011). They have been synthesized by methods like seed-mediated chemical growth, electrochemical method, photochemical reduction, lithographic methods, catalytic methods, template methods, etc. (Huang et al. 2009). Because of their optical properties, they have been used in areas like biological sensing and imaging, tracking, drug delivery, photothermal therapies of cancer cells, etc. (Stone et al. 2011). Studies have also shown the potential use of these gold nanorods as antimicrobial agents. Prepared AuNRs with surface charge transformable properties showed compatibility with mammalian cells while displaying net positive charge in blood stream, while they changed to net positive charge in acidic fluids, corresponding to microbial infections. They were able to show that these nanorods had a potent photothermal antibacterial efficiency (Qiao et al. 2020). Shokri et al. (2015) showed in a similar way that when AuNRs were conjugated with antiprotein A antibody and used it as a targeting agent against methicillin-resistant *Staphylococcus aureus* with the application of photothermal therapy. The results showed a significant reduction in the bacterial cell viability both in vitro (82%) and in vivo (73%). Chen et al. (2020) developed a drug delivery system against *S. aureus* by integrating AuNRs, having photothermal potential and BF2b, an

antimicrobial peptide. The results confirmed that AuNRs and BF2b conjugate can be used as a potential tool against drug-resistant bacteria.

Gold Nanoclusters (AuNC) AuNCs contain several 100 gold atoms, having size not more than 2 nm. They are a new form of gold nanoparticles that have attracted the attention of many because of their exceptional properties like high biocompatibility, high catalytic activity, long fluorescence lifetime, etc. (Cui et al. 2020). They differ from other larger AuNPs in the fact that they possess fluorescence emission in the near-infrared to visible region, but no surface plasmon resonance absorption in the visible region (Zheng et al. 2017) and in the catalytic activity. These characteristics make them a potent candidate for various applications in the field of fluorescence sensing, bioimaging, drug delivery system, etc. As such, their potential against multidrug-resistant microbes has also been explored. Li et al. (2020) developed highly water-soluble and positively charged AuNCs by using (11-mercaptoundecyl)-NNN-trimethylammonium bromide (MUTAB) and found that they possess antimicrobial activity against a wide range of microbes including the multidrug-resistant ones, causing bacterial cell membrane and DNA damage, with very low toxicity to mammalian cells and no apparent drug resistance. In another study by Zheng et al. (2019), AuNCs were conjugated with daptomycin, an antimicrobial peptide to obtain an antibacterial hybrid with high potential against *Staphylococcus aureus*. The resulting conjugate showed efficiency in conferring bacterial DNA damage and also reducing the drug resistance ability of the bacteria. Also, the fluorescence of the resulting hybrid structure was found to be enhanced.

Gold Nanostar (AuNS) Gold nanostars are a type of polyhedral gold nanostructures having a central core and several uneven arms of various lengths (Jiang et al. 2013). They have gained popularity due to their unique optical properties, like their localized surface plasmon resonance can be manipulated to the near infrared region. This makes them an exceptional candidate for application in various fields, like bioimaging, sensing, nanomedicine, etc. (Niu et al. 2015). The antimicrobial properties of these gold nanostars have also been observed. Rovati et al. (2019) prepared antimicrobial surfaces using gold nanostars by functionalizing them with a monolayer of thiols, which did not change their antibacterial property, but instead improved their stability. An antimicrobial film was prepared by Rossi et al. (2020) by conjugating gold nanorods and gold nanostars with crystal violet, a photosensitizer dye. The one involving gold nanostars showed efficient antimicrobial activity against gram-negative bacteria within 4 h of exposure of light due to the reactive oxygen species generated by the particles. This study thus showed the possibility of developing antimicrobial surfaces of objects that come in contact frequently.

Gold Nanocages (AuNCG) Gold nanocages are a class of gold nanoparticles with hollow interior and porous walls size less than 100 nm. As with the other nanostructures, the localized surface plasmon resonance of these nanocages can also be altered throughout the visible and IR region. These properties make them suitable for use in various biomedical applications including cancer therapy (Skrabalak 2008). Wu

et al. (2019) developed a conjugate of gold and silver silica-coated nanocages to study their antibacterial effects. Consequently, this conjugate showed high efficiency against a broad range of gram-positive (*Staphylococcus aureus*) and gram-negative bacteria (*Escherechia coli*) by the synergistic action of the released silver ions and the near IR-induced hyperthermia. The study was demonstrated in vivo as well, on a rat model with wound infection.

22.5 Antimicrobial Drug Resistance Mechanisms in Bacteria

Resistance to antimicrobial agents has been turned into a foremost trace of morbidity and mortality worldwide. With the discovery of antibiotics in the 1900s, the healthcare community thought that the conflict with contagious diseases was won. However, it was soon discovered that a lot of bacteria hold befall resilient to many microbial agents. The comprehensive effect of antibiotic resistance is potentially devastating, threatening to regular earlier improvements against certain infectious diseases to the preantibiotic epoch. Even though nearly all antibiotic-resistant bacteria firstly emerged in hospitals, drug-resistant strains are seemly further ordinary in the community. Studies have disclosed that bacteria acquire resistance mechanisms by two routes, either by mutation or by means of directives provided by their genetic material (Munita and Arias 2016). A proper summary of antimicrobial resistance must perceive between the intrinsic antimicrobial resistance that characteristic bacteria may possess during planktonic growth and the recalcitrance to antimicrobial penetration accessible to bacteria in their protective biofilm mode of growth, familiar to the majority of bacterial infections. Over 300 years back, Antoni van Leeuwenhoek described that vinegar, as an obsolete antimicrobial, killed merely bacteria that inhabited at the outside of a bacterial layer affixed to a surface, what known as "biofilm" today (Donlan and Costerton 2002). Thereupon, biofilm formation can be categorized as a focal implement of antibiotic resistance along with other intrinsic mechanisms such as enzymatic inactivation of antimicrobials, target modification, immunity and bypass, and efflux (Yu et al. 2020).

Biofilm Formation Biofilms are complex architectural colonies of microorganisms within a self-composed extracellular polymeric matrix containing polysaccharides, extracellular DNA, structural proteins cell debris, and nucleic acids (Kassinger and van Hoek 2020). The occurrence of a biofilm involves attachment of cells to a surface, multiplication, maturation, and assembly of polymeric matrix, and colonization of new surfaces. To produce a protective extracellular matrix or "slime", the bacteria will afterward start to guise a monolayer. Initially, matrix formation is dominated by eDNA, and assumed over shortly by polysaccharides and structural proteins. During these stages, microcolonies are formed with significant growth and quorum sensing. The biofilm enlarges in a three-dimensional approach and the attachment is now irrevocable. In the last stage, some cells get detached from the mature biofilm and

dispersed into the environment as planktonic cells potentially repeat the cycle of biofilm formation (Kostakioti et al. 2013) (Fig. 22.1).

The biofilm mode of growth confers on the coupled organisms a perceptible decline in antimicrobial vulnerability. The consequence on responsiveness may be intrinsic or acquired (Donlan 2001). Biofilms of selected strains, adhering keenly to hydrophobic silicon rubber, bent an extensive EPS matrix and therewith became not as much of susceptible to gentamycin (Muszanska et al. 2012). However, these strains merely feebly adhered to hydrophilic, polymer brush-coated silicon rubber, parting the bacteria in the notion of being in a planktonic state and suitably had alike responsiveness to gentamycin as planktonic bacteria. In a biofilm, depth-dependent gradients exist, toward the foundation of a biofilm that grounds a stumpy metabolic activity, coupled with an advanced tolerance to antibiotics. These at a low level metabolically dynamic or metabolically sluggish bacterium in a biofilm mode of augmentation are in a so-called "dormant" state. They remain genetically unchanged, but are phenotypic variants. Persisters having 1% of a biofilm population, with their antibiotic tolerance frequently related to chronic infections as they be capable of recoil regrowing when antibiotic pressure is relieved (Cohen et al. 2013). Antibiotic-resistant bacteria have genetically altered to deter antibiotic activity and grow in the poise of antibiotics. Generally, a biofilm has heteroresitance phenomenon, housing subpopulations of bacteria which are liable and tenacious to an antibiotic (El-Halfawy and Valvano 2015). Biofilm research is being paid new awareness due to pervasive allocation of biofilms in diseases and their resistance to plentiful antimicrobial

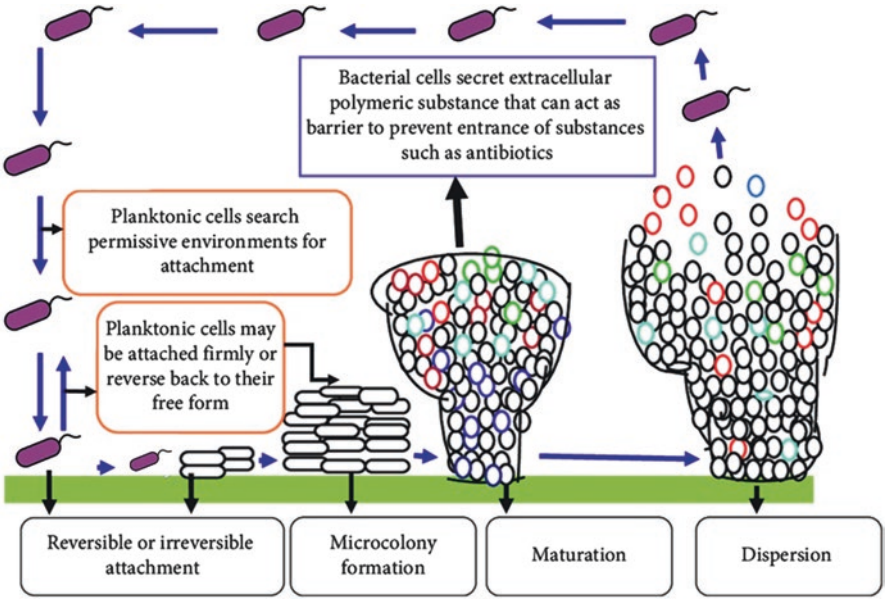

Fig. 22.1 Biofilm formation and structure (Adapted with own modification from Abebe (2020))

treatments. Even after understanding the basic structure and development of this protective shield of bacteria, underlying processes responsible for the transition from planktonic to sessile cells are up in the air.

Enzymatic Inactivation of Antimicrobials Countless bacterial strains own the aptitude to ooze enzymes to protect themselves against antibiotics or other antimicrobials (Egorov et al. 2018). After secretion, these defensive enzymes will add an acetyl or phosphate group maintaining the specificity with the antibiotic which will inactivate it by blocking a specific molecular site as well as induce structural changes to the molecule. These alterations can prevent the antibiotic molecules from binding to specific proteins by steric hindrance, forthwith generating antibiotic resistance. Thousands of enzymes have been identified till date inactivating different classes of antibiotics such as β-lactams, rifampicins, etc. For example, β-lactamase enzyme destroyed the core of β-lactam antibiotic to inactivate serine residues or Zn^{2+}, i.e., the active sites (Tooke et al. 2019).

Target Modification Alteration in the target sites of antibiotics is a frequent means of resistance (Lambert 2005). Different antibiotics have different cellular components including genetic material, cytoplasmic membrane, etc. to kill a bacterium. Antibiotics bind to their targets with lofty resemblance to disapprovingly shape the typical functioning of the target. However, after achieving antibiotic resistance, target sites are capable of being modified to lower the affinity of the antibiotic which makes it unable to interact with its target. The main reason behind changes in target site is spontaneous mutation of a bacterial gene on the chromosome and selection in the manifestation of the antibiotic. For example, mutations in DNA gyrase and RNA polymerase, generating resistance to the quinolones and rifamycins, respectively. In some different cases, attainment of resistance may interest transmission of resistance genes from other organisms through genetic exchange mechanisms including conjugation, transduction, or transformation, e.g., modification of peptidoglycan precursors, etc.

Efflux Pump These are transport proteins that help in the extrusion of substrates which are potentially toxic, such as drugs or compounds or chemicals from the cellular interior to the external environment (Sharma et al. 2019). These proteins are present in both gram-positive and -negative bacteria, and also in the major part of eukaryotic organisms. Effective efflux from prokaryotic as well as eukaryotic cells fanatically modulates the endeavor of a large quantity of antibiotics. In the group of primary active transporters, pumps may be specialized for one substrate or antibiotics of multiple classes, which can be coupled with MDR (multiple drug resistance). Again, the class of secondary active transporters includes five major families; they are- MF (major facilitator), RND (resistance-nodulation-division), SMR (small multidrug resistance), MATE (multidrug and toxic efflux), and ABC (ATP binding cassette). Each of these families consumes the proton motive force as an energy source, except ABC family which hustles the export of substrates through ATP hydrolysis. There are several different efflux pumps which indicate their ancestral origins have

been studied in entire bacterial genome. These systems help to pump out the solids out of the cell. Efflux pumps set aside the bacteria to police their internal environment by removing deadly substances together with metabolites, antimicrobial agents, and quorum sensing signal molecules. Efflux pumps may be produced by a single component or by manifold components, with the later life form brings into being exclusively in gram-negative bacteria. Clinically, germane efflux systems in gram-negative bacteria mainly fit into the RND super family and are typically tranquil of a cytoplasmic membrane pump, an outer membrane protein channel, and a periplasmic protein (Routh et al. 2011). The efflux pump systems found most frequently in gram-positive bacteria such as Bmr and Blt in *Bacillus subtitis* and NorA in Staphylococcus aureus; involve MFS and ABC transporters (Kumar et al. 2013). Efflux pumps are prominent in provisions of high efficiency of drug extrusion as well as broad substrate specificities. It is well known that in quorum sensing regulation which regulates the expression of several virulence factors, and also the biofilm differentiation, efflux pumps have been involved. For example, in *Pseudomonas aeruginosa*, QS-dependent lecA::lux expression is found to be down-regulated by a mutation in a probable RND-like efflux pump transporter. Posterior studies have also demonstrated that QS is partly dependent upon efflux. Later studies comprise in addition demonstrated that QS is fairly dependent upon efflux. Hence, an active transport through efflux pumps is necessary for the signal molecule 3OC12-HSL in case of *P. aeruginosa* to diffuse across the cell membrane. Consequently, an increase in efflux pump activity may increase extrusion or intrusion of QS molecule which might have numerous impacts on biofilm formation.

22.6 Intracellular Uptake of Gold Nanostructures

The entry of the NPs into the cells represents the initial step of drug delivery process. Endocytosis is one of the key pathways for cellular uptake of nanoparticles (Behzadi et al. 2017). Recent studies have revealed that cellular uptake in case of GNPs depends upon the size and surface charge of the NPs. In case of AuNPs uptake, shape also plays a vital role (Manzanares and Ceña 2020). Between the spherical AuNPs and rod- or bar-shaped AuNPs, spherical ones are easily absorbed by the cells and rod- or bar-shaped are further easily extruded from the cells (Chithrani and Chan 2007). Additionally, star- and rod-shaped AuNPs taken up by the cells through CME with a significant involvement of CVME for the later in manifestation of FBS, period in its absence, star- and rod-shaped AuNPs are absorbed by macropinocytosis and following an independent pathway respectively (Ding et al. 2018). Again, 15-45 nm spherical AuNPs go through CME process in presence of FBS, whereas follow macropinocytosis in lack of FBS. According to several other studies, 15-30 nm AuNPs, form a complex with DNA, taken up by CME (Wang et al. 2019a, b). However, due to its larger size, 80 nm AuNPs are endocytosed by macropinocytosis. Again, in agreement with the other studies which indicate mainly for nearly all gold nanostructures, the major uptake pathways considered to be receptor-mediated endocytosis

(RME). It is also called as clathrin-mediated endocytosis which forms vesicles, allowing the adsorption of receptor-specific substances only. Once surface proteins (ligands) adsorb on AuNPs, network with receptors on the cell membrane, RME occurs. The ligand-receptor correlation has been exploited in applications to convalesce targeting of NPs. The plasma membrane possesses several specific receptors which play the major role in helping GNPs to get through the outer surface casing of the cell. Inside the cell plasma, a protein is present known as clathrin protein which guides and plays the leading role, the whole process of endocytosis and vesicle formation, respectively. This cytoplasmic protein has a trimeric structure with three clathrin heavy chains and three light chains (Kirchhausen et al. 2014). After adsorbing by the surface proteins, the gold nanostructures first bind to their specific receptors present on the plasma membrane. Some adaptor proteins are also present in the cytoplasm which helps the clathrin protein to get attached with the receptors. Adaptor proteins are made up of four components- AP-I, AP-II, AP-III, AP-IV. The attachment of the clathrin protein with the receptors via adaptor proteins results in the formation of an invagination. As the process is mediated by clathrin protein, it is known as "clathrin-mediated pit." This pit takes the form of a vesicle with the help of dynamin, a GTPase enzyme which converts GTP into GDP, inorganic phosphate, and energy. The vesicle formed is called as "clathrin-coated vesicle" (Fig. 22.2).

$$GTP \rightarrow GDP + iP + \text{energy}$$

This is now followed by the uncoating process. The bond between the adaptor protein and clathrin protein breaks and both go to their former places to repeat the

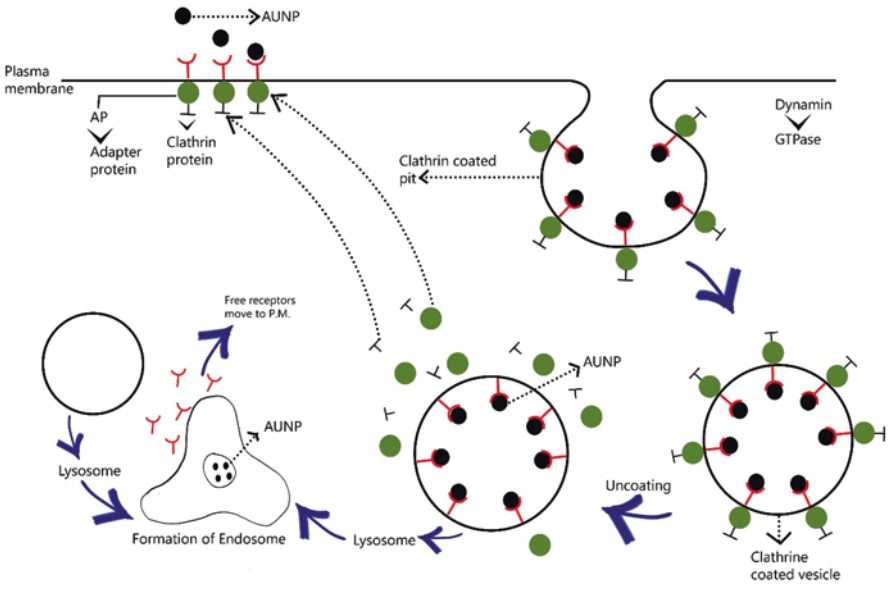

Fig. 22.2 Intracellular uptake of gold nanostructures

cycle. After this, fusion of the vesicle with the lysosome occurs, leading to the formation of secondary lysosome or endosome. This results in the digestion of the gold nanostructures. The receptors get free and take up the initial specific positions (Popova et al. 2013).

22.6.1 Pharmacokinetic and Pharmacodynamic Characteristics of AuNPs

Nanoparticles are found as the most effective tool in diagnosis as well in therapeutics because of their adaptable character (Kang et al. 2015). As a drug delivery system, nanoparticles provide a hopeful deal to achieve a drug formulation with modified pharmacokinetic and pharmacodynamic properties (Marcato 2014).

Half-life: The half-life of gold nanoparticles (AuNPs) is inversely proportional to the particle size, which means half-life increases when size of the particle decreases.

Absorption: It is very much necessary to classify the precise physicochemical characteristics which consent to most uptake and growth in secondary target organs of nanoparticlulate drug delivery system. A very less percentage of AuNPs, i.e., about 0.37–0.01% for large size, are absorbed orally. After the oral administration, absorption of AuNPs across intestinal barriers and accumulation in secondary target organs are regulated by its size and the surface charge. In rats, negatively charged AuNPs have a higher absorption than positive particles (0.37% vs. 0.14% respectively; 2.8 nm). Again, inhalation absorption of gold nanoparticles is 0.06–5.5%.

Tissue distribution: After venous injection, 51.3–96.9% and 2–11.4% of AuNPs are distributed to the liver and spleen, respectively. It crosses the blood-brain barrier (BBB) to a lower degree. Gold nanoparticles travel across the human placental barrier in restricted amounts. The placental conveying of AuNPs depends equally on embryonic/placental maturation and size and/or surface composition and modification. However, distribution of these particles to tissues remains for a long time, i.e., more than 6 months.

Metabolism: AuNPs get degraded within the endosomal compartments in case of mammalian cells.

Elimination: The elimination of metallic NPs through urinary and biliary pathways is normally low leading to their enduring accrual in the liver and spleen. Also, NPs exhibit prolonged tissue retention as they do not go through biodegradation into biologically benign components which lead to amplified toxicity. In case of AuNPs-

1. Renal elimination is low: After parenteral injection in rats, 9% of 1-4 nm AuNPs are eliminated within 24 hours.
2. If the size of the particle is less than the threshold value of 5.5 nm, then renal elimination is more efficient than biliary excretion.
3. Again, if the particle size is 13 nm, then biliary excretion is higher than urinary excretion.

22.7 Novel Therapeutic Approach of Gold Nanostructures for Combating MDR: Phage Therapy, Combined Ablation Photothermal Therapy, Photodynamic Therapy

22.7.1 Phage Therapy

Phage therapy is a controversial against bacterial infections till a decade ago when penicillin was discovered in 1928 by Alexander Flaming (Lin et al. 2017; Gaynes 2017). The term phage indicates the bacteriophage or bacteria-specific viruses in short which were found in use to against pathogens to treat them. The phages have the ability to disrupt bacterial metabolism pushing bacterium to lyse. D'Herelle used phages to treat dysentery in the year 1919 under the supervision of Professor Victor-Henri Hutinel, which is supposed to be first attempt to use phages in therapy (Sulakvelidze et al. 2001). The drug resistance bacteria are currently a global threat. It is seen that number of infections increase which become impossible to treat having high morbidity and mortality. In such situation, the use of bacteriophages which can kill bacteria is studied as strategy to combat antibiotic resistance (Altamirano and Barr 2019). The cause of antibiotic resistance can be considered both as natural as well as man-made. It is observed that in microbial population, genes conferring resistant to modern antibiotics are exist. The clinical and industrial overuse of antibiotic is regarded as one of the major cause that accelerates the increase in number of drug-resistant bacteria. The current use of phage therapy has been improved using the knowledge of phage biology, genetics, immunology, and pharmacology (Altamirano and Barr 2019). The utility of phage therapy is analyzed using in vivo animal models. The *P. aeruginosa* phage therapy is very popular now. The mouse model for administration of *P. aeruginosa* is found successful between 80% and 100% when administered intraperitoneally or intranasally. Phage therapy is also found very successful to treat ulcerative lesions and chronic otitis in catfish and dogs (Pires et al. 2015).

Phage therapy is applied for treatment of infected postoperative wounds in cancer patients too which helped in faster cleaning of wounds and healing without deforming scars in comparison to patients treated with antibiotics. It is also mentioned that highly specific bacteriophages are more effective than poly-specific phage cocktails. On the other hand, bacteria create colony very fast on burned skin. They produce a biofilm which is resistant to antibiotics. Again such patients often suffer from sepsis, intoxication, changes in microbiota, and lymphopenia. Here also phage therapy is potential to prevent sepsis (Morozova et al. 2018). The phage therapy is successful to eliminate multiple drug-resistant *P. aeruginosa* or successful skin graft. The report also says that phage therapy might be alternative to human ulcers where antibiotics are common practice.

22.7.2 Combined Ablation Photothermal Therapy

Photothermal ablation therapy employs agents that can absorb near infrared light (NIR) then convert it to heat to ablate cancer cells which are considered to be a highly efficient as anticancer strategy (Wang et al. 2019a, b). The ideal photothermal ablation is able to eradicate primary tumor as well as induce antitumor immunity by activating the immune system that prevents tumor recurrence. The thermal ablation has advantage over conventional methods like flexibility, minimal invasiveness, and cost-effective (Ashikbayeva et al. 2019). The search of therapeutic agents for photothermal therapy is a challenge. However, the gold nanoparticles (GNPs) high biocompatibility and its ability to be injected intravenously accumulate in cancer cells efficiently because of its high permeability and retention effect. The GNPs that are available in various forms including nanospheres, nanoshells, nanorods, nanocages, nanostars, etc. are found to be useful in photothermal treatment (Li et al. 2014). The functionalized GNPs are also effective against both gram-negative and gram-positive uropathogens as well as multidrug-resistant bacteria (Kang et al. 2020). GNPs have unique ability to convert NIR into heat energy which can induce hyperthermia due to surface plasmon resonance (SPR). Hence it is useful for photothermal ablation (Kang et al. 2020). Hybrid magnetic gold nanoparticles (HNPs) are found effective as catalyst to treat colorectal cancer liver metastases (CRLM) using photothermal sensitization (Yang 2017). The photothermal ablation (PTA) is able to perform in three basic modes such as (a) using only light, (b) light along with molecule for photothermal conversion, and (c) light along with metal nanostructures for photothermal conversion. Due to high efficiency of metal nanostructures in photothermal conversion, it is a good choice as photothermal agents. However, they are not easy to deliver in the cancer sites as well as possess potential toxicity. Noble metals like silver, gold nanoparticles are biocompatible with low toxicity. So they are most discussed as PTA agents. To be suitable PTA agent, metal nanostructure must possess (a) small size, (b) spherical or nearly spherical shape, and (c) strong, narrow NIR absorption. For intracellular uptake, the nanostructure shall be in optimal size range that minimizes friction too. Smaller sizes also help in tunable SPR effects which instead help in strong NIR absorption (Nadejda and Zhong 2009).

22.7.3 Photodynamic Therapy

The photodynamic therapy (PDT) is a technique that requires light, photosensitizer, and tissue oxygen (Jelveh and Chithrani 2011). On irradiation of photosensitizer, it reacts with ground state molecular oxygen ($_3O_2$) to form excited singlet oxygen ($^1O_2$) (Stacey and Pope 2013) and other reactive oxygen species (ROS) that can destroy target tissues. In the process, the photosensitizers are excited with light of suitable wavelength to generate ROS which help in inducing apoptosis or necrosis. To get the

effective results, the photosensitizer is administered in the target sites followed by activation by light. The PDT got its regulatory approval using hematoporphyrin derivative to treat cancer in advanced stage of lung, digestive tract, genitourinary tract in Netherland, Japan, Germany, USA, and Canada in 1993 (Dougherty et al. 1998). Recently gold nanoparticles (GNPs) are found to be preferable delivery agents for PDT drugs. GNPs-based delivery offers enhanced permeability and retention (EPR) effect providing accumulation of drugs in tumor sites. Russell and coworkers first reported the self-assembly of GNPs with photosensitizers (Hone et al. 2002). Various studies have revealed that GNPs possess low toxicity and biocompatibility that make it applicable for biomedical applications. More often GNPs are combined with photosensitizer as has advantages like enhanced production of singlet oxygen as well as other ROS. Unlike PTA agents, the use of GNPs is not limited to spherical-shaped nanoparticles but other nanomaterials like nanorods, nanostars, nanoclusters are also reported (Calavia et al. 2018). It is emphasized that we need to monitor the levels of tissue oxygenation during PDT for clear understanding of therapeutic application. As photochemical consumption and vascular inhibition deplete the oxygen levels, it is believed that PDT efficacy also reduced accordingly.

22.8 Future Perspectives and Conclusions

The nanomedicine era has hardly begun. Nanotechnology has a ton of potential in terms of health and biological science. Due to their physicochemical properties, gold nanoparticles are developed as therapeutic and diagnostic agents both in vitro and in vivo among the various inorganic nanoparticles available today. Because of their possible applications in a variety of fields, including chemical, biomedical, and optical fields, AuNPs have taken a big hit. They are widely acknowledged as biosafety and have been actively used as drug carriers, cosmetics, surgical fillings, and antimicrobial materials (Selvaraj et al. 2010). Although there are many AuNPs antibacterial-based methods, and the majority of them appear to be successful, a plethora of drug researches have investigated AuNPs as drug carriers, while the side effects are under consideration. Until AuNPs may be used in clinical environments, their toxicity to species must be thoroughly understood. AuNPs have been shown to have cytotoxic effects both in vitro and in vivo, with various toxic effects identified and associated with AuNPs size, shape, doses, sampling points, surface coating and functionalization, and cell lines or animal models. AuNPs have demonstrated an array of effectiveness in a number of fields, but they are still in the initial phages of development and are not yet suitable for use in theranostics. The constraint to translational research on these systems in the future will be a dearth of deeper understanding of the interactions between AuNPs, biomacromolecules and the immune system, homogeneity of material preparations, and a lack of information about pertinent biomarkers, along with the biocompatibility concerns. Green AuNPs synthesis, multimodality bioimaging analyses, and theranostics are all pertinent areas of research that are of interest.

Gold nanoparticles interact with bacteria in a variety of ways, allowing them to bypass their resistance mechanisms. Researchers have discovered that these resistant bacteria are often vulnerable against these new therapeutic techniques, ranging from mechanical stress caused by gold nanoparticles acting on bacterial cell walls to hyperthermia and ROS produced by photoactivation. The mechanisms of action of the various antibacterial gold nanoparticles are still being investigated and understood, but they are providing new hope in an era when bacterial resistance continues to rise despite almost any other available treatment choice. Long-term biosafety and experimental observations in vivo and in vitro are critical for transferring this novel therapeutics into clinical practice and guiding their development. Another field, where we believe there is room for progress, is the standardization of experiments in order to obtain comparable studies. We expect to see secure and successful nano-gold-based therapeutics in the foreseeable future, where gold's properties are thoroughly used to eliminate the world's most deadly superbugs forthwith.

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Chapter 23
Carbon Nanoparticles: A Potential Cost-Effective Approach to Counter Antimicrobial Resistance

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23.1 Introduction

Antimicrobial resistance (AMR) is one of the most important problems the world is facing at the moment. The World Health Organization (WHO) has declared AMR as "one of the top 10 global public health threats facing humanity" (WHO n.d.). Infectious diseases are a major cause of deaths worldwide and the emergence of multidrug resistant pathogens results in increased death rates and significantly higher therapeutic costs (Dadgostar 2019). The list of these pathogens is ever-growing and no longer limited to specific geographical locations or healthcare settings (Prestinaci et al. 2015). It has been predicted that by 2050, 300 million additional deaths would occur and an extra US$100 trillion would be incurred as a result of this issue (Dong et al. 2020). To address this problem, there is a global push to search for new antimicrobial agents that are resistance-free, and ideally cost-effective and of natural origin.

Nanoparticles have emerged as one of the effective alternatives to traditional antimicrobials. Apart from being effective, nanoparticles offer several advantages over other antimicrobials. Nanoparticles are relatively safer and less vulnerable to development of resistance due to their multifaceted actions and the inability of microbes to build up multiple gene mutations simultaneously (Plank 2009; Ruddaraju et al. 2020). Some examples of nanoparticles that have been tested for their antimicrobial actions include silver, silver oxide, titanium dioxide, zinc oxide, gold, calcium oxide, silica, copper oxide, magnesium oxide, and carbon-based nanomaterials (Azam et al. 2012; Besinis et al. 2014; Emami-Karvani and Chehrazi 2011; Usman et al. 2013; Chen et al. 2013; Pal et al. 2007; Zarei et al. 2014; Cataldo and Da Ros 2008). Besides being cost-effective and relatively easy to synthesize,

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carbon nanoparticles have a high surface-to-volume ratio and unique chemical and physical properties that make them attractive potential alternatives to traditional antimicrobials (Maleki Dizaj et al. 2015).

23.2 Antibacterial Activity of Carbon Nanoparticles

Carbon nanoparticles have been shown to have broad-spectrum antimicrobial activities (Table 23.1). In contrast to metal nanoparticles that can potentially accumulate in animal tissues and cause cytotoxicity, carbon nanoparticles, especially those derived from natural sources, are safer alternatives to conventional antibiotics. Carbon nanoparticles derived from date pits have been tested for antibacterial activity against *Staphylococcus epidermidis*, *Serratia marcescens*, *Mycobacterium smegmatis*, and *Escherichia coli* (*E. coli*). The nanoparticles inhibited the growth of all four bacteria at a concentration of 0.5 mg/mL (Altaikyzy et al. 2018). Similarly, carbon nanoparticles isolated from kitchen soot (derived from wood and coconut husk) were found to be effective against *Proteus refrigere*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* (*S. aureus*), and *Streptococcus haemolyticus* (*S. haemolyticus*) (Varghese et al. 2013). Another study by the same group demonstrated that CNPs and CNPs encapsulated in functionally modified β-cyclodextrin effectively inhibited the growth of *S. aureus*, *S. haemolyticus*, *Klebsiella pneumoniae* (*K. pneumoniae*), and *E. coli* (Varghese and Kuriakose 2016). In yet another study by a different group, carbon nanoparticles derived from butter and mustard oil were shown to be effective against *K. pneumoniae* (Mohanty et al. 2007).

Another group of carbon nanoparticles, known as carbon dots (CDots; dimensions less than 10 nm) have been shown to have antimicrobial activities (Al Awak et al. 2017; Meziani et al. 2016). It is believed that as the size of carbon particles reduces, their antimicrobial activities increase on account of their larger surface area per unit volume and high extent of atoms in the surface and close to surface layers (Dong et al. 2020). Since the effects of CDots are mediated primarily through photodynamic inactivation (PDI), the use of CDots is a minimally invasive and safe option suited for multiple applications (Maisch 2009; Yin et al. 2015). Carbon dots functionalized with 2,2-(ethylenedioxy)bis(ethylamine) effectively inhibited growth of *E. coli* both in suspensions and on culture plates (Meziani et al. 2016). Other microbes that were shown to be susceptible to the antimicrobial actions of CDots include *S. aureus*, *Bacillus subtilis*, and *methicillin-resistant S. aureus* (MRSA) (Li et al. 2018; Hou et al. 2017). In another study, CDots embedded in polydimethylsiloxane and polyurethane inhibited the growth of *S. aureus*, *E. coli*, and *K. pneumoniae* (Kovacova et al. 2018). Efforts to enhance the antimicrobial activity of CDots by surface modification to increase their affinity to bind with bacteria have met with success. Examples include spermidine-bound CDots (Li et al. 2016) and hybrid CDots with metal and metal oxides (Yan et al. 2019; Liu et al. 2017; Zhang et al. 2018; De et al. 2015). Another approach that has been tested included a combination of CDots and

Table 23.1 A summary of published studies on the antibacterial activity of carbon-based nanoparticles

Type of nanoparticles	Carbon source	Effective against microorganisms	References
Carbon nanoparticles	Date pits	*Staphylococcus epidermidis*, *Serratia marcescens*, *Mycobacterium smegmatis*, and *E. coli*	Altaikyzy et al. (2018)
Carbon nanoparticles	Kitchen soot (wood and coconut husk)	*Proteus refrigere*, *Pseudomonas aeruginosa (P. aeruginosa)*, *S. aureus*, and *S. haemolyticus*	Varghese et al. (2013)
Carbon nanoparticles and carbon nanoparticles encapsulated in β-cyclodextrin with {5-[4-(dimethylamino) benzylidene]-4-oxo-2-thioxo-1, 3-thiazolidin-3-yl} acetic acid	Kitchen soot	*S. aureus*, *S. haemolyticus*, *K. pneumoniae*, and *E. coli*	Varghese and Kuriakose (2016)
Carbon nanoparticles	Butter and mustard oil	*K. pneumoniae*	Mohanty et al. (2007)
Carbon dots functionalized with 2,2-(ethylenedioxy) bis(ethylamine)	Commercially supplied carbon nanopowder	*E. coli*	Meziani et al. (2016)
Carbon dots	Vitamin C	*S. aureus*, *Bacillus subtilis*, *Bacillus sp. WL-6*, *E. coli*, and ampicillin-resistant *E. coli*	Li et al. (2018)
Graphitic carbon dots	Ciprofloxacin hydrochloride	*S. aureus* and *E. coli*	Hou et al. (2017)
Carbon dots capped with polyurethane	Polyoxyethylene-polyoxypropylene-polyoxyethylene, Pluronic 68	*S. aureus* and *E. coli*	Kovacova et al. (2018)
Spermidine-capped carbon quantum dots	Ammonium citrate	*E. coli*, *S. aureus*, *B. subtilis*, *P. aeruginosa*, and methicillin-resistant *S. aureus* (MRSA)	Li et al. (2016)
Carbon dot decorated titanium dioxide	Graphite rods	*S. aureus* and *E. coli*	Yan et al. (2019)

(continued)

Table 23.1 (continued)

Type of nanoparticles	Carbon source	Effective against microorganisms	References
ZnO/graphene quantum dot nanocomposites	Citric acid	*S. aureus* and *E. coli*	Liu et al. (2017)
Carbon dots decorated $Na_2W_4O_{13}$ composite with WO_3	Ethanol	*E. coli*	Zhang et al. (2018)
OMMT-carbon dot reduced Cu_2O nanohybrid	Banana	*S. aureus*, *Bacillus subtilis*, *K. pneumoniae*, and *P. aeruginosa*	De et al. (2015)
2, 2′-(ethylenedioxy) bis(ethylamine) (EDA) capped carbon dots	Carbon nanopowders	*E.coli* and *Bacillus subtilis*	Dong et al. (2017)
Carbon dots functionalized with ampicillin	Citric acid	*E. coli*	Jijie et al. (2018)
Carbon dots as carrier of ciprofloxacin hydrochloride	Gum arabic	*Bacillus subtilis*, *S. aureus*, *E coli*, and *P. aeruginosa*	Thakur et al. (2014)
Carbon dots	Tamarind	*K. pneumoniae*, *E. coli*, *S. aureus*, and *P. aeruginosa*	Jhonsi et al. (2018)
Carbon dots	Vitamin C	*S. aureus* and *E. coli*	Li et al. (2018)

other antiseptic agents. A synergistic effect was observed between CDots and hydrogen peroxide but not between CDots and sodium carbonate or acetic acid (Dong et al. 2017). Carbon dots have also been used as carriers of antibiotics to improve their stability and facilitate sustained release to minimize the development of antibiotic resistance (Jijie et al. 2018; Thakur et al. 2014).

23.3 Antifungal Activity of Carbon Nanoparticles

Fungal infections are, in general, more time-consuming and challenging to treat due to reasons including the difficulties with fungal culture and sensitivity and a limited availability of antifungal agents. This issue is further compounded by the problem of antifungal resistance. Carbon nanoparticles have been demonstrated to have antifungal activity (Table 23.2). Studies have shown CDots to be effective against *Candida albicans* (*C. albicans*) (Jhonsi et al. 2018), and *Pyricularia grisea* and *Rhizoctonia solani* (Li et al. 2018). Apart from their antibacterial activity, some of the hybrid CDots have also been shown to have antifungal activity against *C. albicans* (Priyadarshini et al. 2018; Gao et al. 2019) and yeasts (Thakur et al. 2014). Further studies are required to investigate the activity of various types of carbon nanoparticles against different fungal species.

Table 23.2 A summary of published studies on the antifungal and antiviral activities of carbon-based nanoparticles

Type of nanoparticles	Carbon source	Effective against microorganisms	References
OMMT-carbon dot reduced Cu_2O nanohybrid	Banana	*Candida albicans*	De et al. (2015)
Carbon dots	Tamarind	*Candida albicans*	Jhonsi et al. (2018)
Carbon dots	Vitamin C	*Rhizoctonia solani* and *Pyricularia grisea*	Li et al. (2018)
Carbon dot and gold encapsulated carbon dot	Citric acid and PEG	*Candida albicans*	Priyadarshini et al. (2018)
Nitrogen and chloride co-doped carbon dots		*Candida albicans*	Gao et al. (2019)
Benzoxazine monomer-derived carbon dots	Benzoxazine monomer	Flaviviruses (Japanese encephalitis, Zika, and dengue viruses) and nonenveloped viruses (porcine parvovirus and adenovirus-associated virus)	Huang et al. (2019)
Nitrogen and chloride co-doped carbon dots	Citric acid, Choline chloride, and urea	*Candida albicans*	Khanam et al. (2013)

23.4 Antiviral Activity of Carbon Nanoparticles

Although there are relatively fewer studies on the antiviral effects of carbon nanoparticles, the available evidence suggests that carbon nanoparticles also have some antiviral activity (Table 23.2). It has been reported that CDots can block infection of cells with certain viruses, including porcine parvovirus, Japanese encephalitis, Zika, and dengue viruses (Huang et al. 2019). More research is warranted to evaluate if carbon nanoparticles can block infection of cells with different viruses or inhibit viral replication inside the body.

23.5 Synthesis of Carbon Nanoparticles and their Mechanism of Action

Compared to other nanoparticles, carbon nanoparticles are relatively easy and inexpensive to synthesize (Fig. 23.1). Carbon nanoparticles can be collected from sources such as candle soot in a simple, facile, and low-cost manner (Khanam et al. 2013). Other researchers have used different sources such as date pits (Altaikyzy et al. 2018), wood and coconut husks (Varghese et al. 2013), butter and mustard oil (Mohanty et al.

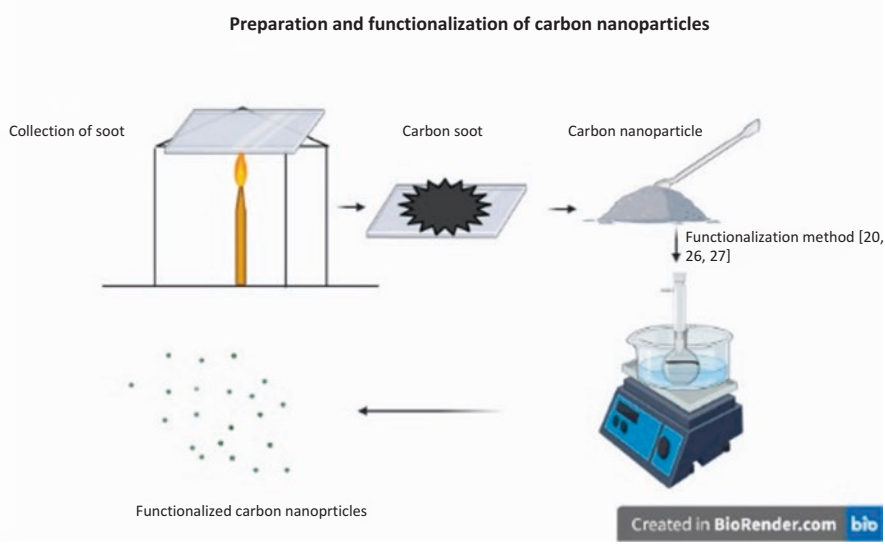

Fig. 23.1 A schematic diagram of the synthesis and functionalization of carbon nanoparticles

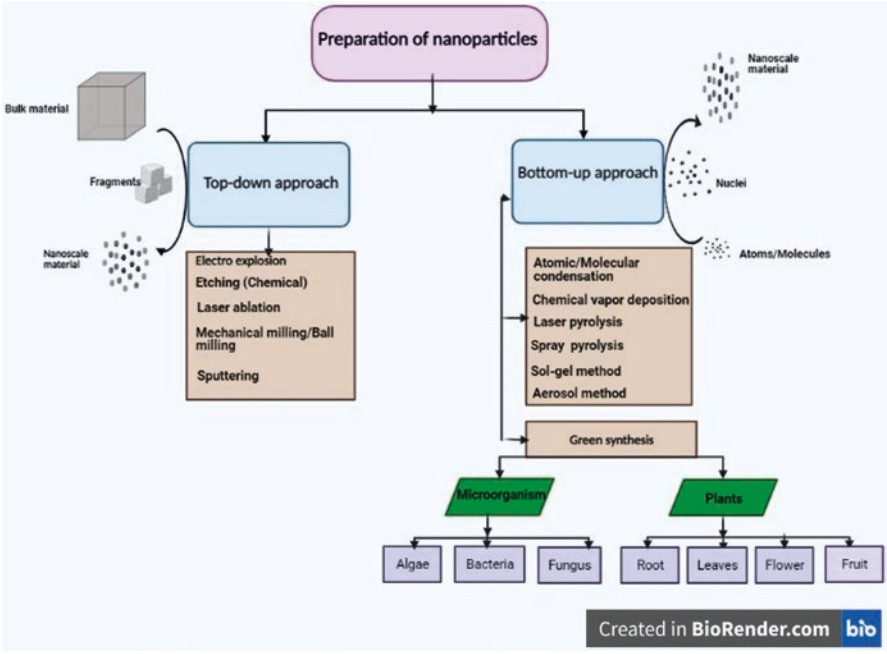

Fig. 23.2 Top-down and bottom-up approaches used for synthesis of carbon dots

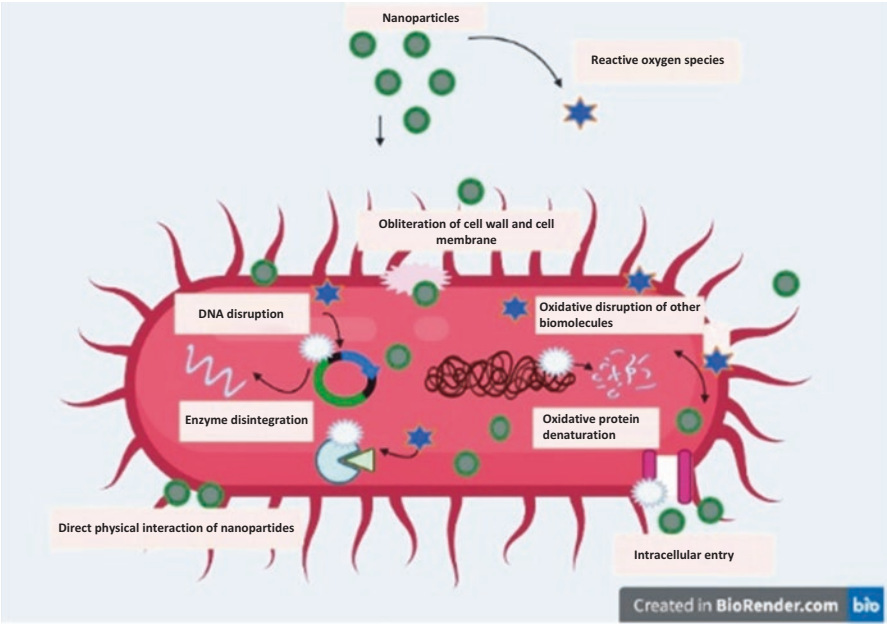

Fig. 23.3 Mechanisms of antimicrobial action of carbon-based nanoparticles

2007). Carbon dots can be prepared using either a top-down approach or a bottom-up approach (Sharma and Das 2019). The top-down approach follows fragmenting the bigger carbon structures into nanoscale material through electrochemical oxidation, discharge, chemical oxidation, and ultrasonic processes (Lim et al. 2015). This approach has some limitations including difficult reaction conditions, prolonged reaction time, and costly reagents (Wang and Hu 2014). The bottom-up approach involves transforming smaller carbon structure into carbon dots using thermal decomposition, ultrasonic treatment, carbonization, microwave synthesis, pyrolysis, solvothermal, and hydrothermal treatment methods (Fig. 23.2). Different mechanisms of action have been proposed for the antimicrobial activity of carbon-based nanoparticles (Fig. 23.3) including oxidative stress and direct interaction with the cell membrane and intracellular structures (Maleki Dizaj et al. 2015; Varghese et al. 2013).

23.6 Conclusions

Owing to the broad-spectrum antimicrobial activity of carbon nanoparticles and the ease with which they can be synthesized and functionalized, this class of nanoparticles offers a potentially viable alternative to traditional antimicrobials against which resitance is observed. Carbon nanoparticles are also a potentially good option

for treatment of multidrug resistant infections in developing countries where the high cost of some traditional antimicrobial agents makes them an unaffordable or impractical option. Clinical studies are warranted in future to confirm the antimicrobial activity and safety of carbon nanoparticles in live animals.

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Chapter 24
Antimicrobial Interfaces as Augmentative Strategy Against Antimicrobial Resistance

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24.1 Introduction

Advancement of lifesaving drugs has effectively decreased the mortality rate in human population, but overuse of such drugs (mainly antibiotics) has increased the threat of antimicrobial resistance globally (McEwen and Collignon 2018). In recent years, we have seen antimicrobial resistance enormously emerges at global scale and is spreading from country to country at faster rate than we thought. Resistant microbes which are also known as superbugs are endemic in many parts of the world (Christaki et al. 2020) and uncontrolled use of antimicrobial drugs is majorly responsible for the consequences of antimicrobial threat (Zaman et al. 2017). The presence of microorganisms like bacteria, fungi, moulds, and viruses in our immediate surrounding causes undesirable and serious health problems. In order to combat such health issues, the patient relies on antibiotics, and excessive consumptions of antibiotics sometimes result in secondary infections and antimicrobial resistance is one of them. Antimicrobial resistance (AMR) is not a recent phenomenon, but it has become a critical health issue in recent times due to the excessive use of antibiotics in lot many sectors like animal farming (Xu et al. 2020a), agriculture (Suriyasathaporn et al. 2012), sewage treatment plants (Tambosi et al. 2010), and

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many more. In year 2017, World Health Organization (WHO) published a list of 12 bacteria stating them as threat of AMR to human health (Duval et al. 2019). The causes and consequences of antimicrobial resistance in different countries are very complex and may develop through the practices of healthcare toward the use of various types of antimicrobials (Ayukekbong et al. 2017). Although the use of drugs and antibiotics in healthcare and clinics is necessary but the research community is falling behind in the process of making new drugs to overcome the threat of newly discovered antimicrobial-resistant species (Moo et al. 2020). Poultry, which is one of the most widespread food industries across the world, has raised a major concern due to the direct/indirect involvement in creating AMR among human population (Xu et al. 2020a; Alam et al. 2019) and in order to minimize these adverse effects, certain rules need to be followed on the usage of antibiotics and antimicrobials in animal livestock (Lhermie et al. 2017). Contrary to this, effluents from hospitals containing high level of antibiotics and various other toxic chemical residues challenge the microbial population and confer the resistance property against them (Rowe et al. 2017). Likewise, faulty agriculture practices especially in irrigation further enhance the problems of AMR for the human population (Hong et al. 2020). Various action plans toward antimicrobial stewardship, improvisation in antibiotics, and techniques for the identification of drug-resistant microbes are promising toward combating the threat of AMR (Jansen et al. 2018). The aim of antimicrobial stewardship programs is to implement antimicrobial-targeted interventions to reduce inappropriate antimicrobial prescribing in order to prevent the development of antimicrobial resistance (Cole et al. 2019). We can use vaccines to control infections over a long time without becoming antiquated because vaccines work prophylactically (Tagliabue and Rappuoli 2018). All these steps are being advised to minimize the use of antibiotics in order to overcome the adverse effects from them.

But still there are significant areas such as animal and food industries where the use of antibiotics could not be reduced to great extent in order to fulfill the needs of overgrowing human population. This is a major concern especially in developing and/or overpopulated countries like India, China, and Bangladesh where agriculture and animal food sector use the antibiotics in large amount thereby causing serious health issues to the people (Van Boeckel et al. 2015). For the prevention and cure of such human diseases, there is unimaginable use of antibiotics and chemicals in daily life. Majorly these kinds of infections can be avoided by taking proper precautions but still the problems exist due to population, pollution, poor facilities of wastewater treatment, and many more. These problems are commonly found associated with the occupation of the individual especially health workers in hospitals and people associated with cleanliness, making them more prone to infections. The presence and growth of microorganisms on their clothes cause serious health problems and also deteriorate the quality of textiles fabrics (Ravindra and Murugesh Babu 2016). A hospital in China has observed methicillin-resistant *Staphylococcus epidermis* in the hand samples of nurses and doctors (Xu et al. 2020b). Likewise, in Saudi Arabia similar thing has been observed among hospital as well as nonhospital personnel for the presence of antibiotic resistance strains of *Staphylococcus aureus.*

Along with this the equipment used in such professions and some other work place is also the source of causing serious health issues to the individual coming in direct or indirect contact with them. As these infections are mostly bacterial, this consequently leads to the consumption of antibiotics and thereby causing concern for AMR. By keeping these things in account, the production of antimicrobial textiles and antimicrobial coating materials seems to be quite promising in reducing the problem of AMR. The use of antimicrobials in textiles such as silver- and zinc-based nanoparticles, silane quaternary ammonium compounds, and chitosan imparts the antibacterial properties to the textiles products for combating the threat of AMR. Antimicrobial's coating of healthcare devices and surfaces of surgical equipment with electrochemical deposition polymer nanoparticles are also found very effective for fighting against bacterial infections. Antimicrobial textiles and coated textiles possess effective potential to reduce the microbial growth and it also enhances the quality and durability of fabrics. This chapter talks about advancements in antimicrobial textiles and coating materials along with their applications and importance in present world.

24.2 Antimicrobial Fabrics in Textiles

Working in a microbial environment such as hospitals, laboratories, and wastewater treatment plants confers higher chances of bacterial infections than any other place. This risk of infection can increase several folds if the textiles used as garments and dressing materials do not offer hindrance to microbial attachment and colonization as they are in direct contact with the body of individuals. Also, microbial growth on fabrics can lead to deterioration of its quality. This necessitates the development of textile fibers which not only offer greater degree of antimicrobial activity but also have more durability. Various approaches to develop such fabric have been considered so far which include treatment of fabrics with reagents as well as naturally derived fibers.

Having a healthy lifestyle is one of the primary requirements for well-being of our body, but high prevalence of pathogenic microbes in our surroundings has created many problems (Emam et al. 2019). The use of antimicrobials in textiles intends to provide protection against microbial invasions (Kramer et al. 2006) as well as such antimicrobial impregnations in textiles have also been found to be quite beneficial for improving the quality of fabrics and reducing potential health risks, e.g., cytotoxicity, bad odor, sensitization, and skin allergies (Kramer et al. 2006). The implementation of antimicrobials in textiles has attracted the world with a conflict of interest aiming to reduce the infective microbial load, unpleasant odor, and enhancing the life span of textile fabrics. Recent advancements include the use of less soluble or nonleaching antimicrobials which have been found to be advantageous in overcoming the toxic effect on aquatic life due to their leaching. Another such advancement is the development of antimicrobial textiles which are effective against a broad-spectrum of pathogenic microorganisms, but do not harm the

nonpathogenic microbes present on the skin of wearer. There is a huge market of antimicrobial textiles in healthcare, food packaging industries, and home furnishing owing to their wide applications. In comparison of traditional textiles, the global markets of present textile approaches are becoming highly competitive. Many technologies have been developed to prevent microbial growth but implementation of these techniques in textiles is not as per current requirements (Hiremath et al. 2018).

Various types of antimicrobials agents are used in textiles depending upon their different biocidal effects and target mechanisms against microorganisms. These different mechanisms can be (Morais et al. 2016):

- Inhibiting cell wall synthesis and targeting monomer polymerization
- Blocking of cross polymer linking beta-lactam by some antimicrobial agents
- Inhibition of protein synthesis and nucleic acid synthesis (DNA/RNA)

The eco-friendly attribute of antimicrobial chitosan has facilitated its applications in textiles. The use of nanomaterials such as silver and zinc nanoparticles as antimicrobials in textiles is advantageous due to their physiological as well as biological properties. Green synthesis and environment friendly approaches in nanomaterials are very reliable due to their antibacterial properties. Recent advances in field of microbicidal polymeric ammonium compounds and their activeness against broad-spectrum range of microorganisms have attracted the concern of textile industries for various applications.

24.3 Imparting Antimicrobial Property by Chemical Treatments

Treating fabrics such as cotton and polyester with certain specific chemicals and use of different scientific techniques for the same have been the prime focus of interest in research involving the development of antimicrobial textiles for varied applications. Such innovations not only increase the microbe fighting ability of textiles, but also contribute to the enhancement of their quality and durability. The finished antimicrobial fabrics can be applied to multiple industries to prevent infections caused by various microbes and improve public health (Chen et al. 2019). In one such research, covalent bonding of novel tert-butoxylcarbonyl (BOC)-protected guanidine which contains an isocyanate group was performed on the surface of cotton fabric. The BOC group was later on deprotected through the process of acidification. The fabric thus developed, showed 88.5% and 99.9% antibacterial activity for *E. coli* and *S. aureus,* respectively (Cao et al. 2020) which is much greater in comparison to the normal untreated cotton fabric. Another finding on cotton fabric was carried out using the process of heterogenous transesterification and the acetoacetyl group was attached directly on the surface of cotton fabric for imparting dual function of hydrophobicity and antibacterial properties. The cotton-acac modified with gentamicin and octadecyl amine (Rong et al. 2019) and the results were very promising which led to improvement in overall bacterial growth inhibition.

Polyester fabrics are also one of the popular choices for textiles. However, polyester does not offer adequate protection against microbial activity by themselves. Therefore, in order to be used as textiles in medical sector, it becomes important that polyesters undergo certain kind of modifications. One such innovation involves the modification of micro denier polyesters with sericin which is used to be extracted from yellow silk cocoons. The novel fabric thus developed shows outstanding effects against both bacteria as well as fungi (Rajalakshmi et al. 2018). Apart from this many a time blending of two fabrics in varying proportions can also increase the antimicrobial activity of the synthesized textile by several folds. Such blended mixtures can even give rise to a large number of knitted fabrics. Polyester silver nanocomposite fibers when blended with normal polyester fibers in varying proportions in terms of yarn count and knitting machine gauge can produce up to 27 types of knitted fabrics which provide good antibacterial activity (about 65–99%) against both *S. aureus* and *E. coli* bacteria (Khude et al. 2020).

Such innovations involving chemical treatment of fabrics and fibers seem to be quite promising in the future for the development of alternative approaches for textiles offering antimicrobial protection to be used in healthcare centers and laboratories and in places where the risk of microbial infection is quite high.

24.4 Developing Antimicrobial Textiles Using Eco-Friendly Approach

Chemical treatment of textiles has been proven beneficial in enhancing their antimicrobial property largely. However, if such enhancements can be made using naturally derived products and using eco-friendly procedures, then such approaches are always the center of attention. The idea to produce antimicrobial fabrics by modification with reagents from natural sources is the most preferred of all and has shifted the focus of new research, as the developed fibers are also biodegradable.

In a study on bamboo-derived fibers, it was found that natural bamboo fibers showed about 8–95% reduction in the growth of *K. pneumoniae* and about 3–50% in the growth of *S. aureus* (Prang Rocky and Thompson 2020). However, when the bamboo fibers are regenerated, it reduces its antibacterial properties significantly (Ali et al. 2021). Many a time substances derived from natural sources can also be utilized due to their natural antagonistic effects toward microbial growth. There are many such substances around us, which can show promising results. Casein, a naturally occurring product can be harnessed for the development of antimicrobial textiles. Casein derived from bovine milk, after being subjected to acid hydrolysis in aqueous media, is blended into PP matrix in the melt phase by extrusion. After processing this blend by the melt spinning process, production of multifilament takes place that can be further woven into textiles. Such textiles show excellent antibacterial activity against both gram-positive as well as gram-negative bacteria (Belkhir et al. 2021). These results are also promising in the perspective for

preventing the wastage of dairy products and even their recycling. Another such product is natural pine/spruce rosin that has also been proven beneficial in improving the antibacterial properties of textiles. Using procedures like intrusion compounding and melt spinning, a mixture of rosin with polyethylene, polypropylene, polylactic acid, polyamide, and corn starch-based polymer can produce fibers for wide applications like filters and medical textiles. Polyethylene with about 10% rosin content had significant antibacterial effects against *E. coli* (Kanerva et al. 2019).

The antimicrobial activity of neem (*Azadirachta indica*) leaves and bark extracts implemented with cotton fabrics was found to be effective against gram-negative bacteria. Neem extract was applied on medical textiles by direct application and microencapsulation. It was found that durability of textiles given by direct method to washing was poor but application by encapsulation method showed good durability after washing (Radhika 2019). These modifications of cotton fabrics with neem extracts enhance the properties of fabric such as elongation, tensile strength, and antimicrobial performance. In recent studies, it was found that eucalyptus oil has different medical application and also property of cleansing agent. It shows antimicrobial and antifungal properties. Eucalyptus shows the antimicrobial properties against *E. coli* by applying on wool and cotton fabrics. But modified fabrics lose its antimicrobial property after every wash cycle. In woolen clothes its durability is high due to low washing period of woolen clothes is limited (Radhika 2019). Polyethylene terephthalate (PET) has been widely used for treatment of fabrics due to properties like easy processability and quick drying. Finishing of fabrics with PET exhibits durable broad-spectrum antimicrobial activities against gram-negative, gram-positive bacteria and fungi (Chen et al. 2019).

Betulin is a natural compound that can be obtained from the outer bark of birch. In an experiment, carboxyl-functionalized cellulose was prepared after oxidation and later on by esterification was attached covalently to betulin which led to the improvement in the antibacterial property by 99% removal and growth inhibition of bacteria (Huang et al. 2019). Similarly, lysozyme is a natural enzyme, which is known to show efficient antimicrobial preventive properties. Harnessing lysozyme from chicken egg white and its immobilization onto wool fibers by cross-linking has produced fibers having better antibacterial properties and even its durability (Yang et al. 2020).

Use of biopolymers and eco-friendly natural antimicrobials for the development of textile materials for medical sectors and food industries maintains health and hygiene of products. Various methods researched and reported the fabrics such as cotton, wool, and linen finish with antimicrobials to be effective against various types of bacteria, viruses, and fungi (Kost et al. 2020).

When a person is injured, the growth of microbes on the dressing materials can slow down the process of healing of wound, which necessitates the development of a biodegradable alternative having antimicrobial properties. In a recent approach, quercetin-loaded polylactide-based fibers have been developed through electrospinning and the obtained woven fabric showed good effects against *S. aureus*, *K. pneumonia,* and *E. coli* (Rigo et al. 2018).

24.5 Surface Modification of Textiles and Appliances with Antimicrobial Coating

The process of formation of biofilms and bacterial colonies on the surface of different kinds of medical devices, food processing equipment, and appliances related to the daily use can cause serious infections as well as unwanted side effects. So, in order to improve the sterility of such surfaces or medical devices like catheters, scissors, and other equipment used in surgery which comes in direct contact with the patient body, there is a huge need of antimicrobial coatings on them. Nanoscience-based solutions used as antimicrobial coating on surfaces are very reliable due to their surface modification properties that provide microbial resistance. Antimicrobial surfaces developed using nanotechnology include hydrogel films with temporal release of active agents (Langowska et al. 2014) and nanoreactors which again are catalytically active compartments to produce reactive agents (Li et al. 2018). The surface coating of materials with QAC is also an effective and promising method for the formation of microbe-free appliances used in food industries, wastewater treatment plants, and health care (Jiao et al. 2017).

In addition to these, contact killing and antimicrobial properties of copper nanoparticles (Maghimaa and Alharbi 2020) are also effective tools which can be used for coating of surfaces to make effective against microbes. Various properties like antifungal and antibacterial properties make copper nanoparticle suitable for use in various healthcare devices as coating agent. All these properties of different elements and compounds have made promising role toward fighting against microbial infections.

24.6 Silver and Zinc Oxide Nanomaterials as Antimicrobial Agent for Natural and Synthetic Textiles

The research in the field of nanomaterials has significantly increased in past few years due to their extensive biological, magnetic, optic, and catalytic properties (Ozin and Arsenault 2015). Developments in nanoscience research have flourished much more in recent times and it is now considered in every technical research for developing new products and similar to various applications, it can also help to tackle problems like AMR and related speculations (Aarthi et al. 2018). Green synthesis of nanoparticles has emerged as an alternative approach, as it is cost-effective and even minimizes the consumption of hazardous chemicals (Ozin and Arsenault 2015). The ongoing researches about the use of antimicrobials in textiles highlight their effectiveness in resisting the growth of microbes. Owing to their antimicrobial applications, the use of silver nanoparticles (AgNPs) has significantly increased in textiles and numerous studies focus on exploring the greenway synthesis of AgNPs. It has been found that after treating with AgNPs, the fabrics like cotton, wool, and

linen show effective activity against bacteria like *E. coli* and *S. aureus.* In addition to this, the use of AgNPs instead of toxic chemicals is also much more cost-efficient and eco-friendlier (Nateghi and Shateri-Khalilabad 2015). Due to these advantages, the demand of antimicrobial fabrics such as cotton, wool, and polyester has significantly increased. Also, by enhancing the durability, nanomaterials used as antimicrobials in textiles seem to be quite promising. Impregnation of textiles with silver nanoparticles shows reduction in growth of microbes like *E. coli* to an extent of about 99% and it is not found to have any harmful and toxic effects on wearer and environment (Fiedot-Toboła et al. 2018). Such modified textiles products are effective against both gram-positive and gram-negative bacteria present on surfaces (Sirelkhatim et al. 2015). Despite all these advantages, incorporation of silver nanoparticles in textiles requires post surface treatments that affect the durability of fabric after several washes. In addition to AgNPs, antimicrobial activity of zinc oxide (ZnO) nanoparticles has also gained significant attention worldwide in developing antimicrobial textiles. Zinc oxide is a biosafe material that possesses photo-oxidizing and photocatalysis activity on biological species (Sharma et al. 2019). Also, ZnO is known to reduce the occurrence of foodborne diseases which has led to an increase use of zinc oxide as antimicrobial in the food packaging industries. However, in some cases, the excessive use of zinc oxide nanoparticles also found to increase the defects on modified surfaces. Incorporation of zinc oxide nanoparticles in textiles and on surface coatings is effective against the broad-spectrum of pathogenic microorganisms. Although, the use of silver nanoparticles is preferred over zinc oxides as in a study on linen fabrics the incorporation of silver nanoparticle resulted 100% reduction in the growth of microorganisms (Hasan et al. 2020) and therefore considered as better approach. Deposition of antimicrobials on nylon fabric surface in terms of excellent coloration and antibacterial properties has been developed by chitosan-mediated silver nanoparticles synthesis (Kloos and Musselwhite 1975). Use of silver nanoparticles as antimicrobials shows promising effect against wide range of microbes with very low toxicity and used in textiles as an antimicrobial agent to prevent infections which in long run helps to reduce the instances of AMR arising from irresponsible and unnecessary consumption of antibiotics (Fig. 24.1).

24.7 Quaternary Ammonium Compounds (QACs) as Antimicrobials

The quaternary ammonium compounds consist of 12–18 carbon atoms, a long chain of alkyl group, and a compatible anion. The mechanism of action of QACs against microbes includes process like inhibition of nucleic acid (DNA and RNA) and damages to the cell membrane of bacteria; along with this it also inhibits the multiplication of bacteria. These compounds possess greater degree of antimicrobial and anticarrier activities, and are very effective against bacteria like *Staphylococcus*

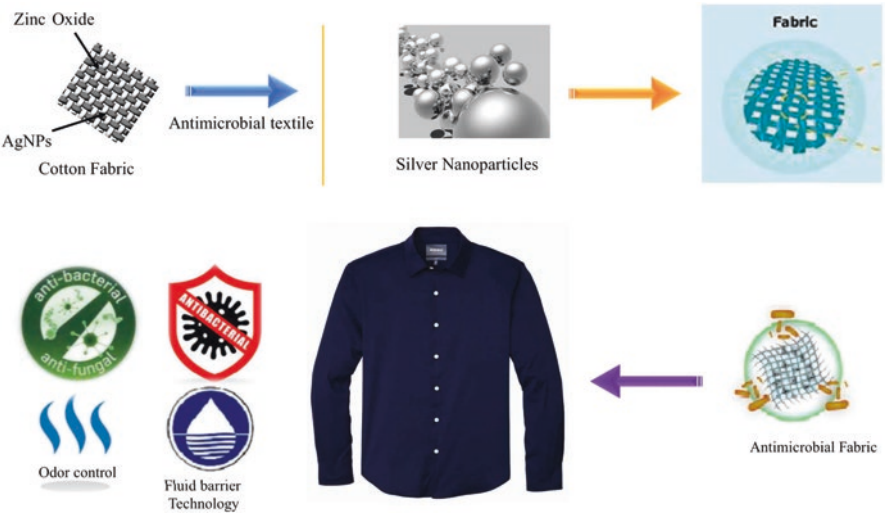

Fig. 24.1 Zinc oxide and silver nanoparticle-based antimicrobial fabrics

aureus, Streptococcus and *Bacillus* species (Kennedy et al. 1995). Newly developed QACs are found to possess virucidal activity and may be used as antimicrobials (Żywicka et al. 2018). Various bacterial membranes have been modified with these QAC compounds and these have been used in wastewater treatment plants because of their effective antimicrobial properties (Żywicka et al. 2018; Elena and Miri 2018). The covalent surface grafting of QACs seems to be quite promising in the formation of effective antimicrobial materials that could be used in medical sector, food and textile industries (Caschera et al. 2019). In textile industries, QACs containing long alkyl chains (12–18 C atoms) are most widely used for the treatment of fabrics to make them resistant to microbes. The antimicrobial action of these compounds depends on the length of alkyl chain, presence of perfluorinated groups, and the cationic ammonium groups. These QACs used as antimicrobial agents are very effective against gram-positive bacteria, gram-negative bacteria, viruses, and also some fungi. The use of QAC in textiles showed 99.99% effectiveness against *E. coli* and *S. aureus* (Khan et al. 2015).

24.8 Natural Polymeric and Graphene Oxide Nanocomposites with Antimicrobial Activities

Graphene, an atomically thin carbonaceous material, has been a popular research topic in the recent past, due to its mechanical, electrical, and antimicrobial properties. Reduction of graphite oxides offers a wide range of possibilities for synthesizing graphene nanocomposites using very easy methodologies (Konwar et al. 2016).

Hence, the production of antimicrobial compounds with impregnations of natural polymeric and graphene oxide nanocomposites exhibits a considerable antimicrobial activities and key tool in combating AMR problems instead of toxic chemicals. It is noted that impregnation of iron oxide-coated graphene nanocomposites on different appliances shows significant antimicrobial activity against bacterial pathogens (gram-positive and gram-negative) such as *E. coli, S. aureus,* and *Candida albicans* (Zhang et al. 2016a). The natural polymeric nanocomposites have received a lot of attention from the past decades due to their various antimicrobial activities and that is why natural polymers such as chitosan, dextran, rubber, and fibrin are enormously used in textiles instead of synthetic ones due to their elastic and antimicrobial properties. Antibacterial activities of natural green polymeric nanocomposites make them useful for having their applications in packaging materials, textiles, and surface coating (Kowalczyk et al. 2017). These polymers maintain the stability of fabrics such as cotton and also impart the treated surface with new functional properties such as UV resistance. But the main problem with respect to implementation of these natural polymers to textile fabrics is the reduction in durability of fabrics. To overcome this issue, use of inorganic nanoparticles with improved finishing is being applied on fabrics. Graphene and its derivatives such as graphene oxides exhibit excellent antimicrobial and mechanical properties. The best antistatic properties can be obtained by coating the material with 1.5% of graphene provided the surface resistance and volume resistance should be of order $10^{\wedge 5}$ and $10^{\wedge 3}$ Ω, respectively and these kinds of properties make the fabric suitable in acquiring antimicrobial properties (Knetsch and Koole 2011).

24.9 Antimicrobial Coatings on Medical Devices

Antimicrobials are commonly added to paint formulation, food processing equipment, medical devices to maintain the coatings against microbial attacks and to provide protection against fungal growth. Applications of nanomaterials implemented as coating agents have been used to prevent adhesion and subsequent biofilm formation (Wei et al. 2019). Recent advances in context to threat of AMR have led to developing new antimicrobial coating materials. Bacterial colonization and biofilm formation on medical devices potentially cause life threatening infections and hazardous impacts on patient's health. The microbes present in biofilm that develops on medical devices are very difficult to control using conventional antimicrobial agents as consequences of intrinsic resistance mechanisms. Several approaches such as hydrophilic coatings, bacteria-repellent coatings, and hydrogels-loaded antibiotics seem to be promising in their ability to prevent microbial infections associated with medical devices (Zhang and Wagner 2017). The considerable way to prevent life threatening infections caused by microorganisms is to modify the surfaces of medical devices in such a way that no microbial attachment on these devices could occur. These approaches of complete surface treatment require modifications with mostly hydrophilic polymeric surface coatings (Wei et al. 2019).

Hydrophilic coatings, when applied to medical equipment confer self-cleaning, antimicrobial, and anticorrosive properties (Mehta et al. 2010). Hydrophilic polymer coating evaluates that this method has significant capabilities linked with devices used in clinical settings (Zakrzewski et al. 2021).

The surface modifications of orthodontic appliances materials have provided an effective antimicrobial and mechanical characteristic during orthodontic treatment. Nanotechnology and its application in the field of orthodontics including antimicrobial properties enhancement is leading to overcome the problems like enamel demineralization and friction during orthodontic movements (Ryu et al. 2012). The use of orthodontic braces without any antimicrobial coatings significantly increases the risk of dental plaque and microbial infections. Nanoparticles such as silver platinum have a larger surface area to volume ratio that helps in providing more surface area for antimicrobial activities (Ryu et al. 2012). Silver-platinum (Ag-Pt) alloys, which have high degree of biocompatibility, excellent resistance to sterilization conditions, and antibacterial properties to different bacteria, are associated with long-term antibacterial efficiency (Yemmireddy and Hung 2017). Due to the biofilm formation and colonization of bacteria, the enamel decalcification occurs and this ends up to orthodontic treatments. Organic acids produced by the adherent bacteria can cause enamel demineralization around a bracket, which can deteriorate the outcomes of treatment (Yemmireddy and Hung 2017). The release of silver ions from the Ag-Pt coating of brackets provides a resistance against *Streptococcus mutans* and reduces the problems of tooth decay. Various bacterial infections can be reduced by use of fluoride and antibiotics but in addition to these, methods such as coating of Ag-Pt alloys has proved to be quite effective against bacterial infections in post dental treatments.

24.10 Antimicrobial Photocatalytic Coating

Improper cleaning and microbial association with surfaces cause life threatening infections and hazardous impacts on society. Several approaches such as cleaning, sanitation, and disinfection of surfaces of more equipment have been used to address current and emerging microbial safety challenges. Advanced oxidation processes involving photocatalytic nanomaterials coatings have shown great significant effects on a wide range of microorganisms and chemical contaminants (Wei et al. 2014). Copper-containing coatings can provide significant protection against wide range of microbial strain but due to the poor corrosion efficacy and high cost, the use of titanium dioxide (TiO_2) is preferably more active against microbes. These photocatalytic coatings show excellent properties against microbes in outdoor environment (Jaksik et al. 2018). Normally, the reduction in number of species of bacteria takes approximately 5 h and same numbers of bacteria get eliminated in 2 h with implementation of TiO_2 coating (Jaksik et al. 2018). In this coating, photocatalyst is used as ingredients to remove the microbes from the surfaces. The active ingredient is titanium dioxide which does not dissolve in water and used for coating of various

surfaces. The surfaces when implemented by this coating have shown antibacterial activity against *E. coli* and *S. aureus* with 99.5% reduction in bacterial population. Photocatalytic coating surfaces happen to be free from biofilm formation and colonization of bacteria for a long time and this decreases the burden of healthcare (Zhang et al. 2016b).

In textiles, TiO_2 is often used in development of antimicrobial-efficient cotton fibers. Spray of TiO_2 emulsion with polymer generative additives, on the cotton fibers, along with heating up to 100 °C generates the TiO_2 -coated fabric surfaces. Coating of fibers with TiO_2 uniformly across the entire surface of the fiber without any cracking enhances the antimicrobial and nondegrading properties of fabrics. The observation and testing of TiO_2-imparting cotton fabrics confirmed with antimicrobial activity, which further invent the application of photocatalyst coating in the field of textiles and antimicrobial coating materials.

24.11 Antimicrobial Durability and Cost Efficiency

For an ideal and effective antimicrobial, the durability and its cost efficiency are the most significant factors among other factors. Durability of antimicrobials used in textiles mainly depends on implementation method and subjected to various treatments such as drying and washing. This challenge is further more complicated by the evaluation of durability that is more complex and time-consuming than that on antimicrobial activity (Xu et al. 2018). A large number of nanoparticles have been used for their antimicrobial properties but implementation of nanoparticles with textiles creates an aspect of durability too. The covalently attachment of L-cysteine to the fibers such as cotton and adhesion of silver nanoparticles to the surface of fibers significantly increase durability of fabrics with great antibacterial activity (Ramya and Subapriya 2012). Cost efficiency is also an influencing factor for the use of antimicrobials agents. Synthesis of nanoparticles such as silver, copper, and their implementation with textiles is a costly process (Aly and Albutti 2014). Due to this problem, antimicrobial textiles and antimicrobial coating materials are not for every consumer of the society. In context of textiles, use of herbal-synthesized nanoparticles approaches the long-term investment toward the public health and maintains the cost efficiency of antimicrobials. New innovative techniques to combating AMR with the use of significant antimicrobials imparting with textiles overcome the problems of durability and cost effectiveness in recent time.

24.12 Environmental Impacts of Antimicrobials

The demand for antimicrobial fabrics, antimicrobial coating materials significantly increases in recent time due to its combating efficiency for AMR and health-related potency. But high use of antimicrobials induces a negative impact on human health

and environment. Wastes discharged from textile industries, comprising of silver nanoparticle residues (used as antimicrobials) as well as wastewater, which drains after washing of antimicrobial fabrics, are acting as a potential threat to the aquatic ecosystem. Therefore, specific intervention measures for the use of antimicrobials in textiles and coating materials should be established and implemented to mitigate negative impact on the environment (Singer et al. 2016). Environmental regulatory laws should be implemented significantly for the global AMR action plans (Morais et al. 2016). In May 2015, the 68th world health assembly adopted the global action plan on AMR. The goal of the global action plan is to ensure, for as long as possible, continuity of successful treatment and prevention of infectious diseases with effective and safe medicines that are quality-assured. Any antimicrobial used in textiles and coating of surfaces must be nontoxic to human health and environment. Due to the emergence of AMR, antimicrobials, which are synthesized from plants, have been extensively used in field of textiles to reduce the negative impact on environmental problems. The main significance of herbal antimicrobials is that they do not exhibit toxic effects on healthy microbes present on the wearer's skin. The ideal antimicrobial is which produces efficient effect at low dose and negative impact on environment. Besides the "green technologies", the usages of green modified antimicrobial techniques such as chitosan, sericin, and alginate are regenerable and have very low environmental impacts (Uddin 2014). An increasing consumption of antimicrobial finishing fibrous products and regulation for the control of such products provided reasons to appreciate the environment concern associated with finished textiles (Sayed and Korgaonkar 2020). The long-term benefits and potential use of antimicrobials significantly balance the both implementation in textiles and impact on environment. In future, more efforts require increasing the use of green techniques in implementation of antimicrobials to textiles and coating materials.

24.13 Conclusions

Antimicrobial fabrics and coating materials like silver oxide, zinc oxide, quaternary ammonium compound, and graphene look quite promising in providing protection against microbial growth on clothes and thereby directly minimize the risks of infections from them. Along with this, materials like silver-platinum alloy and titanium dioxide confer a good antimicrobial property when used as a coating material especially for medical equipment. All these things seem to be the need of present and future world in combating the problems associated with microbes as well as with the use of antimicrobial medicines. However, leaching of antimicrobial coating materials in the environment sometimes confers AMR to the microbes as well as the cost and durability of antimicrobial textiles are also major concerns. But if we look at the future commercial value and requirements of antimicrobial textiles and coating materials, then it seems like an extensive research needs to be done in overcoming the problems associated with them, and more prominent and eco-friendly compounds should be explored or made along with cost effectiveness of the end products.

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Chapter 25
Nanostrategies Against Rising Antimicrobial Resistance (AMR)-Metallic Nanoparticles as Nanoweapon

Purnima Sharma, Dinesh Goyal, and Bhupendra Chudasama

25.1 Introduction

The term infectivity attributes to the ability of microorganisms (bacteria, parasites, virus, and fungi) to enter and multiply in the body of host causing infections. Infectious diseases have caused millions of deaths and strained the global healthcare system with an unprecedented load. Microbial infections cause nearly nine million deaths annually, which account for 41% of the global disease burden, measured in terms of Disability-Adjusted Life Years (DALYS) (GDB 2015; Hessling et al. 2017). Microbial diseases with their estimated mortality from year 1918 to 2020 are presented in Table 25.1. Infections with most pathogens trigger the immune response inside the host, which inactivates or kills the disease-causing pathogen as first line of defense. However, if the host is unable to do so then external help in the form of antibiotics is required to treat the infectious disease (Pankey and Sabath 2004).

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Table 25.1 Global mortality due to infectious diseases (1918–2020) (Whitworth 2018; Hackett 2018; Bloom and Cadarette 2019; WHO 2019; International Association for Medical Assistance 2019; Coronavirus Outbreak 2021)

Year	Pathogen	Geographic region	Cases/Mortality
1918–1920	Influenza (Spanish flu)	Worldwide	500 million cases and 30 to 100 million deaths
1957–1958	Influenza (Asian flu)	Worldwide	One to two million deaths
1968–1969	Influenza (Hong Kong flu)	Worldwide	500,000 to two million deaths
1960-present	HIV/AIDS	Worldwide, primarily Africa	70 million cases and 35 million deaths
1961-present	Cholera	Worldwide	1.4 to four million annual cases and 21,000 to 143,000 annual deaths
1974	Smallpox	India	130,000 cases and 26,000 deaths
1994	Plague	India	693 suspected cases and 56 deaths
2002–2003	SARS	Originated in China, spread to 37 countries	8098 cases and 774 deaths
2009	Influenza (swine flu)	Worldwide	284,000 deaths
2014–2016	Ebola	West Africa, primarily Guinea, Liberia, and Sierra Leone	28,600 cases and 11,325 deaths
2015-present	Zika	The Americas, Primarily Brazil	Unknown number of cases
2016	Dengue	Worldwide	100 million cases and 38,000 deaths
2017	Plague	Madagascar	2417 cases and 209 deaths
2017–2018	Cholera	Worldwide	1.2 million cases and 2627 deaths
2018	Listeria	South Africa	1060 cases and 216 deaths
2017–2018	Hepatitis A	USA	10,582 cases
2019–2020	Corona virus	Worldwide	155, 192, 083 cases and 3, 244, 581 deaths till 05/05/2021

The word “antibiotic” hails from the word “antibiosis” (meaning against life). Antibiotics are chemical compounds that kill or inhibit the growth of microorganisms. Antibiotics are classified as antibacterial, antifungal, and antiviral agents depending on their target group. However, all antibacterial compounds are commonly referred as antibiotics (Etebu and Arikekpar 2016). For decades, antibiotics have been used to treat various diseases. They are also used in various medical procedures like organ transplant and chemotherapy (Etebu and Arikekpar 2016). Broad-spectrum antibiotics target both the gram-negative and gram-positive

bacteria while narrow-spectrum antibiotics only target one of them (Etebu and Arikekpar 2016). Antibiotics are either bactericidal (lethal to bacteria) or bacteriostatic (causing growth inhibition of bacteria) (Kohanski et al. 2010). They cause cell deaths or the cessation of growth by targeting vital physiology and biochemistry of bacterial cells. Major targets of antibiotics are: bacterial cell walls, cell membranes, DNA and RNA synthesis, protein synthesis, and folic acid (vitamin B9) metabolism (Kohanski et al. 2010). For example, the β-lactam class of antibiotics such as penicillin, cephalosporins, and carbapenem blocks the synthesis of the bacterial cell wall, while antibiotics like tetracycline, aminoglycoside, and macrolide target bacterial ribosomes. Antibacterial action of antibiotics may result in irreversible disruption of the cell integrity by interacting with target molecules through irreversible binding via covalent interaction with enzyme or cellular structure of cells (Kapoor et al. 2017).

Antibiotics have been used extensively for the controlled treatment of infectious diseases and in various surgical procedures like organ transplant and chemotherapy (Wright 2010). Excessive and uncontrolled use of antibiotics has resulted into development of Antimicrobial Resistance (AMR) in both intrahospital and nonhospital strains (Allahverdiyev et al. 2011). Development of antibiotic-resistant strains possesses a serious global threat to the disease management.

Center for Disease Control and Prevention (CDC), World Economic Forum, Infectious Diseases Society of America, and World Health Organization (WHO) have declared antimicrobial resistance (AMR) as one of the "biggest threats to public health globally" (Allahverdiyev et al. 2011; Spellberg et al. 2016). In European Union alone, an estimated 25,000 deaths per annum was caused by AMR (Padiyara et al. 2018). Antibiotic-resistant pathogen-associated hospital-acquired infections (HAIs) cause 99,000 deaths per annum in US (Aslam et al. 2018).

Many intrahospital pathogens have developed resistance against multiple antibiotics resulting in decrease in the efficiency of treatment. Decline in the effectiveness of existing antibiotics has caused higher morbidity and mortality in patients who are infected with multidrug-resistant (MDR) strains (Davies and Davies 2010; Spellberg and Gilbert 2014; González-Candelas et al. 2017). Deaths due to MDR alone are nearly 7,00,000 per annum (Betts et al. 2018). In past two decades, hospitals have witnessed increase in MDR-acquired infections due to the production of β-lactamase which is responsible for development of drug resistance against third generation antibiotics (Blair et al. 2014). Following mechanisms are responsible for antibiotic resistance in microorganisms:

1. Increased efflux of drugs and their reduced uptake.
2. Development of modifying enzymes/drug degrading inside microorganisms.
3. Enzymatic breakdown of antibiotics.
4. Alterations in antibiotic targets wherein new biosynthetic machinery is engaged to alter cell-wall structure.
5. Formation of protective biofilm around the bacteria that prevent its exposure to antibiotics.

Injection of antibiotics as a growth promoter in livestock has increased in recent years resulting in greater MDR strains in animals. Antibiotic consumption in livestock is expected to rise up to 67% by 2030 (Van Boeckel et al. 2015). This could also affect human health, because mobile genetic elements (MGEs) from resistant bacteria make their ways from animals to humans by various means (Molbak 2004; Humphrey et al. 2005).

Development of new and unconventional antibiotics is the need of the hour to overcome challenges put forward by multiple drug resistance (MDR). Metal nanoparticles owing to their high surface-to-volume ratio exhibit enhanced chemical and biological activities (Khurana et al. 2014). Nanoparticles impart toxicity on microorganisms (Table 25.2) by disturbing permeability and respiration of cell walls, inhibit enzymes, deactivate proteins, electrolyte imbalance, interfering with metabolic pathways of cells, damage to the cell membrane, reacting with sulfur-containing proteins and phosphorus-containing compounds such as DNA, induce oxidative stress by generating reactive oxygen species (ROS), generation of free radicals, modify gene expression levels, damage biomolecules like proteins, lipids, and DNA, and prevent biofilm formation (Hemeg 2017; Shaikha et al. 2019; Martinez et al. 2019). Due to the different target sites of metal nanoparticles, the probability of a microorganism developing resistance against metal nanoparticles is very low (Chopra 2007; Harikumar and

Table 25.2 Antibacterial effects of metal nanoparticles on different bacteria (Hemeg 2017)

Nanoparticles	Target bacteria	Antibacterial mechanism
Silver (Ag)	*Acinetobacter baumannii*, *S. typhi*, *Vibrio cholerae*, *B. subtilis*, *S. aureus* (multiple drug resistance), *Escherichia coli*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, coagulase-negative *Staphylococcus epidermis*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Proteus mirabilis*, *Micrococcus luteus*	ROS generation, lipid peroxidation, inhibition of cytochromes of ETC, bacterial membrane disintegration, inhibition of cell wall synthesis, increase in membrane permeability, dissipation of proton gradient resulting in lysis, adhesion to cell surface causing lipid and protein damage, ribosome destabilization, intercalation between DNA bases, disruption of biofilms
Copper (Cu)	*Escherichia coli, Bacillus subtilis, Proteus vulgaris*	ROS generation, disorganization of membrane, inhibition of DNA replication, dissipation of cell membrane potential, lipid peroxidation, protein oxidation, DNA degradation
Zinc oxide (ZnO)	*S. aureus*, *E. coli*, *P. aeruginosa*, *B. subtilis*, *Stenotrophomonas acidaminiphila,* methicillin-resistant *Streptococcus agalactiae*, *E. coli*, *Klebsiella oxytoca*, *S. aureus*, *S. pyogenes,* methicillin-resistant *Staphylococcus aureus*	ROS production, disruption of membrane, adsorption to cell surface, lipids and protein damage, inhibition of microbial biofilm formation

Aravind 2016). Among metals, gold, silver, and copper are known for their antiseptic properties since ancient times (Vimbela et al. 2017). The ligand-based metal (copper complexes) and metal-based formulations of inorganic nanoparticles (barium sulfate) have also showed higher antibacterial activities (Lobana et al. 2020; Sooch et al. 2020).

It has been found that metal nanoparticles exhibit significant toxicity even at low dose levels, where their bulk counter parts were otherwise biocompatible (Morones et al. 2005; Giannousi et al. 2014; Azam and El-Said 2014; Suker and Albadran 2015; Chakraborty and Basu 2017). High active surface area and smaller size of nanoparticles provide them greater biochemical reactivity and membrane permeability (Wang et al. 2017).

Nanoparticle–antibiotic conjugates are promising nanoplatforms to combat bacterial resistance. They help in lowering the dosage by synergistic effect and reduce noxiousness (Sekhon 2010; Pissuwana et al. 2011). Further, these conjugates can increase the concentration of antibiotics at the antibiotic–microorganism contact site (Li et al. 2005). Decrease in the overall drug concentration in the conjugated system makes them less toxic and improves their dosage limits (Chen et al. 2009). Possible mechanisms leading to the synergistic effects in antibacterial activities of metal nanoparticles–antibiotic conjugates are summarized in Table 25.3.

Table 25.3 Mechanisms of antibacterial activities of nanoparticles–antibiotic conjugates on different bacteria (Li et al. 2005; Birla et al. 2009; Banoee et al. 2010; Selvarani 2010; Mandava et al. 2017)

Nanoparticle	Antibiotics	Bacteria	Mechanism
Silver	Kanamycin, tetracycline, streptomycin, ampicillin, amoxicillin, gentamicin, vancomycin, erythromycin	*S. aureus, E. coli, Micrococcus luteus, Salmonella typhi, P. aeruginosa*	Increased antibacterial action of nanoparticles and antibiotics. Ampicillin destroys cell wall and increases penetration of silver nanoparticles Silver nanoparticles act as antibiotic carrier and facilitate approach of hydrophobic antibiotic drugs into the bacterial cell membrane
Zinc oxide	15 antibiotics from different groups	*S. aureus, E. coli*	Increased activity of ciprofloxacin in the presence of ZnO nanoparticles by influencing activity of membrane
Copper	Tetracycline, streptomycin, gentamycin, amoxicillin, ampicillin, ciprofloxacin	*E. coli, M. luteus, Salmonella typhi, Streptococcus pyrogenes, S. mutans*	Increased activity of nanoparticles in the presence of antibiotics either by penetrating membrane or by ROS formation

25.2 Silver Nanoparticles

Silver has historically been widely known due to its antibacterial properties (Chang et al. 2006). Moreover, different salts of silver (Ag) and their by-products are also used as bactericidal agents (Melaiye and Youngs 2005). However, nanosilver greatly enhances the functionality of the material which further increased its applications in various healthcare products including wound dressings, bandages, lotions, ointments, water purifications, and medical devices (Thomas et al. 2007), and also used as protective agents for HIV patients (Shameli et al. 2012). Due to smaller size and greater surface to volume ratio, the silver nanoparticles (AgNPs) hold an extensive contact area with the microbes which makes them a robust bactericidal material. AgNPs showed a broad spectrum of antibacterial properties against a wide range of gram-positive and gram-negative bacteria. The AgNPs have capability to interrupt with cellular functions, including the cell permeation and potentially disrupts the cellular components by reacting with the sulfur-mediated proteins and phosphorus-mediated complexes like deoxyribonucleic acid (DNA) (Pal et al. 2007; Raffi et al. 2008; Ahamed et al. 2010). AgNPs can also interact with the respiratory enzyme system, thereby generating reactive oxygen species (ROS) (hydrogen peroxide (H_2O_2), hydroxyl (OH^-), and superoxide (O_2^-) radicals) that induce oxidative stress which further damages the proteins and nucleic acids (Gurunathan et al. 2018). Therefore, AgNPs have the potential to be widely used as antibacterial agent against drug resistance bacteria such as MRSA. It is estimated that, AgNPs as an antimicrobial agent have an annual demand of 3125 tons/year for medicine and 2800 tons/year in the field of food, hygiene, and water purification (Swathy et al. 2014).

AgNPs have shown enhanced antimicrobial potential in number of studies. Work done by Sondi and Sondi (2004) and Morones et al. (2005) showed that the AgNPs accumulate on the cell wall of *E. coli* that leads to formation of pits. These pits caused a loss of outer membrane integrity, resulting in the release of LPS molecules and membrane proteins and eventually caused cell death. AgNPs can also release silver ions which lead to cell damage. Pal et al. (2007) demonstrated that the surface area to volume ratio of AgNPs and the crystallographic surface structures are important factors that determine the antibacterial activity of AgNPs against gram-negative bacterium *E. coli*. Energy-filtering transmission electron microscopy images revealed that AgNPs caused changes in cell membrane of *E.coli*, resulting in cell death. Studies on AgNPs antibacterial activity against gram-positive *S. aureus* and gram-negative *E. coli* have shown that the growth in case of *E. coli* was inhibited at low AgNPs concentrations than in case of *S. aureus*, and indicated the higher antibacterial activity of AgNPs against gram-negative than gram-positive (Kim et al. 2007). Transmission electron microscopy has revealed that after a few minutes of contact with AgNPs, the cell membrane of *E. coli* cells gets completely disrupted at concentrations as low as 60 μg/mL and resulted into inhibition of bacterial cell growth and multiplication (Raffi et al. 2008). Ayala-Nunez et al. (2009) reported a dose-dependent antimicrobial activity of AgNPs against methicillin-resistant *Staphylococcus aureus* MRSA and non-MRSA by minimum inhibitory

concentration (MIC), minimum bactericidal concentration (MBC). They found that AgNPs are effective bactericidal agents against both MRSA and non-MRSA regardless of the resistance mechanisms that confer importance to these bacteria as an emergent pathogen.

The antibacterial activity and antibacterial mechanism of AgNPs that were studied by Li et al. (2010) on E. coli (ATCC 8739) were investigated by evaluating growth pattern, cell membrane permeability, and morphology following treatment with nanoparticles. The results indicated that 10 μg/mL of AgNPs could completely inhibit the growth of *E. coli* (10^7 cfu/mL cells in liquid medium). AgNPs resulted in the leakage of reducing sugars and proteins by destroying the permeability of the bacterial membranes and induced the respiratory chain dehydrogenases into inactive state resulted in cell decomposition and death eventually. Whereas AgNPs at 50 μg/mL lead to pits and gaps formation as observed by transmission electron microscopy and scanning electron microscopy. They concluded that AgNPs caused structural damage of bacterial cell membrane and suppress the activity of some membranous enzymes which caused bacterial death (Li et al. 2010).

Uniform linoleic acid-capped AgNPs of 12 nm showed antibacterial activities against *S. basillus, S. aureus, and P. aureginosa*. They demonstrated that linoleic acid-capped AgNPs can be used as effective antibacterial agent (Das et al. 2011). Colloidal AgNPs synthesized by green route with controlled size and high stability showed high antimicrobial and bactericidal activity against *E. coli* and *S. aureus* (Dehnavi et al. 2012). In other study, the biologically synthesized AgNPs were shown higher antibacterial potential against gram-negative bacterium *S. typhi* in combination with two standard antibiotics (ampicillin and gentamycin). They showed that higher enhancing effect was observed for ampicillin in comparison to gentamicin due to increased potency of ampicillin-mediated cell wall lysis with combination of AgNPs (Rajawat and Qureshi 2012). This suggested that AgNPs must be increasing the local concentration of antibiotics at the site of action and thus helped in improving the potency of antibiotics. AgNPs showed higher antibacterial potential against gram-negative and gram-positive with decreasing particle size. Lu et al. (2013) prepared AgNPs of different sizes (~5, 15, and 55 nm) by reduction method and found that AgNPs (5 nm) exhibited the highest antibacterial activity against five anaerobic oral pathogenic bacteria *S. mutans, S. sanguis, S. mitis, A. actinomycetemcomitans, F. nuceatum,* and one aerobic bacteria *E. coli*, indicated potential used of AgNPs in oral antibacterial materials in the form of dental restorative material, dental implants, caries inhibitory solution and mouthwash. Similarly AgNPs synthesized with various sizes, i.e., 5, 7, 10, 15, 20, 30, 50, 63, 85, and 100 nm showed bacteriostatic/bactericidal effect in size and dose-dependent manner as determined by the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *E. coli and S. aureus*. AgNPs with less than 10 nm size showed higher antibacterial efficacy whereas AgNPs of 5 nm mediated the fastest bactericidal activity against all the tested strains as revealed through delayed bacterial growth kinetics, corresponding MIC/MBC values, and disk diffusion tests.

The surface charge of AgNPs showed different effects on its antibacterial efficacy. Positively charged AgNPs exhibit the highest bactericidal activity against gram-positive (*S. aureus, Streptococcus mutants,* and *Streptococcus pyogenes*) and gram-negative bacteria (*E. coli* and *P. vulgaris*). The negatively charged AgNPs showed lesser activity while neutral AgNPs show intermediate antibacterial activity against tested strains (Abbaszadegan et al. 2015). The interaction of AgNPs with DNA may cause shearing or denaturation of the DNA and interruption in cell division also (Hsueh et al. 2015; Kumar et al. 2016). AgNPs (10 nm) are attached to the cell wall of cholera and interrupting the cell permeability that leads to bacterial cell death (Gahlawat et al. 2016). AgNPs of different shapes and sizes prepared by solution-based chemical reduction routes showed in vitro antibacterial properties against two types of gram-negative bacteria *P. aeruginosa* and *E. coli* as indicated by Kirby–Bauer disk diffusion susceptibility method. It was noticed that the smallest-sized spherical AgNPs showed better antibacterial activity against both bacterial strains as compared to the triangular and larger spherical-shaped AgNPs (Raza et al. 2016). Similar study was done by Acharya et al. (2018) in which they found bactericidal effects of both spherical and rod-shaped AgNPs against gram-positive (*S. aureus, B. subtilis*) and gram-negative (*E. coli, K. pneumoniae* AWD5, P. *aeruginosa*) bacterial strains.

Liao et al. (2019) reported the antimicrobial effect of AgNPs on clinical isolates of resistant *P. aeruginosa* through determining its minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values, alterations of morphology, and structure by transmission electron microscopy (TEM) and also the analyzed differentially expressed proteins (superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD)). Results revealed that AgNPs had highly bactericidal effect on the drug-resistant *P. aeruginosa* with the MIC range of 1.406–5.625 μg/mL and the MBC range of 2.813–5.625 μg/mL. TEM images showed that AgNPs could enter in bacterial cell and impaired its morphology and structure. The proteomics analyses revealed AgNPs-treated bacteria have high levels of SOD, CAT, and POD. The apoptosis rate of AgNP-treated bacteria was remarkably higher than that of the untreated bacteria. Shu et al. (2020) reported the antibacterial activity of shape-controlled and well-dispersed AgNPs (13.8 nm) which was synthesized by using green method in which biomolecules of reductive amino acids, alpha-linolenic acid, and carbohydrates in yeast extract played a significant role. The AgNPs in combination with antibiotic (ampicillin) reversed the resistance in ampicillin-resistant *E. coli* cells. They found that surface coatings of AgNPs enhanced their affinity toward the bacterial membrane and increased the permeability of the cell wall which further changed the peptidoglycan configuration and finally led to the apoptosis of bacteria. Mahmod et al. (2021) showed antibacterial activity of green synthesized AgNPs against gram-positive (*S. aureus, S. mutans,* and *S. epidermidis*) and gram-negative (*K. pneumoniae* and *P. aeruginosa*) bacteria. They found that gram-negative bacteria were more sensitive than gram-positive bacteria. Results indicated that synthesized AgNPs might be used to develop new antibacterial drug for combating against various diseases.

25.3 Copper Nanoparticles

Copper nanoparticles (CuNPs) have gained increasing attention due to their unique physical and chemical antimicrobial properties (Cioffi et al. 2005). CuNPs have many industrial applications: in gas sensors, high temperature superconductors, solar cells, and wood preservatives (Salvadori et al. 2014). Copper metal is used as an antimicrobial agent since ancient times. Compounds such as $CuSO_4$ and $Cu(OH)_2$ containing copper are used as the inorganic antibacterial agents (Raffi et al. 2010). Copper is a trace element as well as essential micronutrient, which is necessary for growth and maintenance of bone, connective tissue, brain, and heart (Azizi et al. 2017). Deficiency of copper can lead to anemia and improper fetal development during pregnancy. It plays a vital role in oxygen transport and iron homeostasis (Habibovic and Barralet 2011; Vimbela et al. 2017). In 2008, Environmental Protection Agency of US approved copper in medicinal products for human usage (Azizi et al. 2017). CuNPs also help in collagen cross-linking and in bone matrix formation (Klaine et al. 2008). CuNPs and their complexes are now used as antibacterial, antiviral, antifungal, and antifouling agents (Ventola 2015). The antibacterial effect of CuNPs on the bacterial cell involves multiple pathways, including adhesion to gram-negative bacterial cell wall due to electrostatic interaction, effecting protein structure of cell membrane, release of cytoplasmic contents, denaturation of the intracellular proteins, and interaction with phosphorus- and sulfur-containing compounds like DNA, loss of cellular functions, and reactive oxygen species (Raffi et al. 2010).

Antibacterial activities of copper nanoparticles (CuNPs)-based polymer metal nanocomposites were reported by Cioffi et al. (2005). Synthesized nanocomposite was capable of releasing metal species in a controlled manner that inhibits the growth of the organism. The biostatic activities of composites were correlated to the nanoparticle loading. Yoon et al. (2007) studied the antimicrobial activity of AgNPs and CuNPs on *E. coli* and *B. subtilis*. They observed that CuNPs exhibited higher antibacterial activities against gram-positive, *B. subtilis* while AgNPs were more active against gram-negative, *E. coli*. Ruparelia et al. (2008) studied the antibacterial activity of AgNPs and CuNPs on *E . coli, S. aureus and B. subtilis. They reported that AgNPs exhibited higher antibacterial activity against E. coli and S. aureus, while CuNPs were more bactericidal to B. subtilis. CuNPs-loaded* fibers by borohydride induced were tested their antibacterial activities against *E. coli*. These copper nanoparticles loaded fibers can be used in burn/wound dressing (Mary et al. 2009).

A study conducted by Raffi et al. (2010) evaluated the antibacterial activity of CuNPs in liquid and solid medium against gram-negative *E. coli*. They observed formation of cavities/pits in the bacterial cell wall after their interaction with CuNPs. Rispoli et al. (2010) studied the antimicrobial activity of CuNPs against *E. coli* and evaluated effects of pH, temperature, aeration rate, concentration of nanoparticles, and concentration of bacteria. It was concluded that the antibacterial activities of CuNPs not only depend on primary effect of test parameters, but also on the interactive effect of these parameters.

Mullite-based CuNPs were synthesized and their antibacterial activities against pathogenic strains: *E. coli, S. aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Shigella flexneri* were reported. They reported that 70 μg/mL of CuNPs were able to kill all tested microorganisms (Bagchi et al. 2012). Chatterjee et al. (2012) evaluated the antibacterial activity of CuNPs against *E. coli*. They observed increase in the number of filamentous bacteria with increase in the concentration of CuNPs indicating that the antibacterial activity of CuNPs was because of the growth of filamentous bacteria. The synergistic effects of chitosan-CuNPs on gram-positive bacteria (methicillin-resistant *S. aureus* (MRSA) and *Bacillus subtilis*), gram-negative bacteria (*Salmonella choleraesuis* and *Pseudomonas aeruginosa*), and yeast (*Candida albicans*) with nystatin (for yeast), ampicillin (for gram-negative bacteria), and streptomycin (for gram-positive bacteria) were evaluated. They have concluded that CuNPs in combination with antibiotics exhibit higher antibacterial activities against tested bacteria (Usman et al. 2012).

The antibacterial activities of CuNPs and copper oxide nanoparticles on *E. coli, Streptococcus pyogenes, Pseudomonas aeruginosa,* and *S. aureus* showed that CuNPs have higher antibacterial activities as compared to their oxides, indicated CuNPs as potential antibacterial agents than oxide (Karthik and Geetha 2013). Usman et al. (2013) observed that chitosan-coated copper nanoparticles have higher antibacterial activities against gram-negative bacteria than the gram-positive bacteria. Similarly, Shiv and Jong-Whan (2014) evaluated antibacterial activity of CuNPs against gram-positive (*Listeria monocytogenes*) and gram-negative bacteria (*E. coli*). They observed that gram-positive bacteria exhibit higher susceptibility toward CuNPs than the gram-negative bacteria. Figueroa et al. (2004) investigated antibacterial effects of copper, nickel, and bimetallic Cu-Ni nanoparticles on dental pathogens (*S. aureus, E. coli,* and *Streptococcus mutans*). They observed that CuNPs exhibited bactericidal effects while Ni and bimetallic Cu-Ni nanoparticles only show bacteriostatic effects on tested microorganisms.

Kruk et al. (2015) synthesized CuNPs by the reduction of copper ions with hydrazine in SDS aqueous solution and evaluated their antimicrobial activities against standard and clinical strains of methicillin-resistant *S. aureus (MRSA)* and *Candida* species (*C. albicans* and *C. parapsilosis*). Synthesized CuNPs exhibited strong antibacterial and antifungal activities. The antibacterial activities of CuNPs both against standard and clinical strains were stronger than their antifungal activities. CuNPs loaded with regenerated bacterial cellulose (RC) showed antibacterial activity against *S. aureus* (ATCC 6538), *B. subtilis* (ATCC 9372), *C. albicans* (CMCC(F)98,001), *E. coli* (ATCC 25922), and *P. aeruginosa* (ATCC 27853) (Shao et al. 2016). The synergistic effects of metal nanoparticles (silver and copper) with antibiotics (tetracycline and kanamycin) were studied on environmental friendly bacteria (*B. subtilis* and *P. fluorescens*). The antimicrobial activity of tetracycline was improved by 286–346% and 0–28% with silver and CuNPs, respectively whereas the improvement was 154–289% for silver and 3–20% for CuNPs with kanamycin (Khurana et al. 2016).

Ebrahimi et al. (2017) reported the antibacterial activities of CuNPs synthesized by green route against gram-positive (*S. aureus* and *B. cereus*) and gram-negative

(*E. coli* and *K. pneumonia*) bacteria. They observed that gram-positive strains were more susceptible to copper nanoparticles as compared to the gram-negative bacteria. In other study, synergistic effects of CuNPs with antibiotics ampicillin, amoxicillin, gentamicin, and ciprofloxacin were studied against bacterial strains *E. coli, S. typii, M. luteus,* and *S. mutans* (Mandava et al. 2017). Amongst the tested antibiotics, CuNPs-Ampicillin conjugates have highest synergistic antibacterial activities. Zia et al. (2018) synthesized AgNPs and CuNPs by chemical reduction method and studied their antibacterial activity against common human pathogenic bacteria (*S. aureus* and *E. coli*). They observed dose-dependent antibacterial activity of nanoparticles. Both silver and copper nanoparticles exhibited similar antibacterial activities against *S. aureus*, while *E. coli* was more susceptible to AgNPs. Selvarani (2010) investigated the synergistic effects of CuNPs with commercial antibiotics (tetracycline, rifampicin, chloramphenicol, vancomycin, gentamycin, streptomycin, kanamycin, tobramycin, penicillin, and ampicillin) against *B. cereus, E. coli, P. aeruginosa,* and *S. aureus*. Strong synergy of CuNPs was observed with all tested antibiotics.

Mesoporous silica (MSN)-maleamic and MSN-maleamic-Cu nanoparticles were synthesized and their antimicrobial activities on gram-positive and gram-negative bacteria were tested. MICs of *E. coli* and *S. aureus* were 125 μg/mL and 250 μg/mL, respectively. ROS levels in MSN-maleamic-Cu-treated *E. coli* were 40% higher, while in *S. aureus*, it was 30% higher as compared to controls, indicating that ROS plays a vital role in their antibacterial activities (Diana et al. 2019). Jayarambabu et al. (2019) synthesized CuNPs by using green synthesis route and evaluated their antibacterial activity on gram-negative (*E. coli*) and gram-positive (*B. subtilis*) bacteria. They have also observed that gram-positive strains were more susceptible to CuNPs as compared to the gram-negative strains. Kaur et al. (2019) reported synergistic activity of CuNPs with erythromycin, azithromycin, and norfloxacin against gram-positive and gram-negative bacterial strains. In case of antibiotics, the test bacteria (*Klebsiella* sp. and *Pseudomonas* sp.) showed resistance at all tested concentrations. In conjugation with CuNPs, the antibacterial activity of all antibiotics increased suggesting that combination of antibiotics with nanoparticles has significant synergistic antibacterial effects on *Klebsiella* sp. and *Pseudomonas* sp.

Green synthesized CuNPs showed antibacterial activity against *E. coli, Klebsiella pneumoniae, S. aureus*, and *E. faecalis*. They observed antibacterial activities of CuNPs against these pathogens. They observed higher antibacterial activities mainly due to CuNPs than CPE (Das et al. 2020). Yaqub et al. (2020) prepared CuNPs by chemical and biological synthesis and evaluated their synergistic effects with doxycycline against *E.coli* and *P. aeruginosa.* They reported that chemically synthesized CuNPs showed higher antimicrobial activity with doxycycline as compared to green synthesized copper nanoparticles. Jessop et al. (2021) studied antibacterial activities of hybrid composite (metallic copper nanoparticles (CuNPs) and novel cationic π-conjugated polyelectrolyte (CPE)) against gram-negative (*E. coli* and *Salmonella enteritidis*) and gram-positive bacteria (*S. aureus* and *Enterococcus faecalis*).

25.4 Gold Nanoparticles

For many decades, gold has been used in many medicines. Robert Koch was first to explore the biocidal potential of gold (Glišić and Djuran 2014). However, nanoparticles generally show good biocompatibility, strong adsorption ability, and applications in medical imaging, drug delivery owing to their surface, quantum size, and small-size effects (Mukherjee et al. 2016). Gold nanoparticles (AuNPs) have many applications starting from engineering to medicine field in past years (Patra et al. 2015). AuNPs have gained increasing attention due to their optical and electrical properties which further depend on their shape and size of nanoparticles (Verissimo et al. 2016). The biocompatibility of AuNPs made them a potential candidate to be used in the treatment of arthritis and cancer (Jain et al. 2006). Functionalized AuNPs with different biomolecules such as drugs, genes, peptides, and other targeting ligands have been exploited for a wide range of biomedical applications, not only in research and development sector but also in various commercially viable point of care systems viz. cytosensors, immunosensors, drug delivery, cancer imaging, aptasensing, and most advanced theranostics devices (Burygin et al. 2009; Mahato et al. 2019). AuNPs can also sense endocytosis, tumor metabolites, and receptors in cells, under dark-field light-scattering microscopy (Dykman and Khlebtsov 2011). Some AuNPs-based diagnostic kits are under clinical trials. FDA also approvals for AuNP-based in vitro diagnostic systems and clinical trials of AuNPs as cancer and cardiovascular treatments (Mieszawska et al. 2013). AuNPs have been used in the development of quantification of blood glucose, biosensors, toxic metals, disease markers, and insecticides (Liu and Lu 2003) and they also have the potential to degrade and detoxify some toxic pollutants (Lopez et al. 2004; Hernández et al. 2006).

AuNPs with a strongly bound capping (poly-allylamine hydrochloride) and a weakly bound capping agent (citrate) could directly contact with the bacterial (*E. coli*) cell membrane due to its positively charged nature mediated by strong electrostatic attraction to the negatively charged bilayer. The cationic particles are moderately toxic, whereas anionic particles are quite nontoxic in nature (Goodman et al. 2004). Polysiloxane polymers containing embedded methylene blue and AuNPs were shown significant antimicrobial activity against methicillin-resistant *S. aureus* and *E. coli*. The bacterial cell death was due to the light-induced production of singlet oxygen and reactive oxygen species induced by AuNPs (Perni et al. 2009). Antibiotic (gentamicin)–AuNP (15-nm) conjugates showed high antibacterial activity against tested *E. coli* K12 by the agar-well-diffusion method. AuNPs themselves do not have any antimicrobial activity, they may act as drug carriers by increasing surface area and carrying a lot of drug on its surface which further increased antibiotic concentration at the site of bacterium–particle contact and enhanced antibacterial property of antibiotics (Burygin et al. 2009). Amino-substituted pyrimidines-capped AuNPs showed antibacterial activities against multidrug-resistant clinical isolates of *E.coli* and *P. aeruginosa*. The antibacterial mechanism involved targeting the bacteria cell via multiple targets, destabilizing

cell membranes, binding to nucleic acids, inhibiting protein synthesis, and inducing the leakage of cytoplasmic contents, suggested these NPs could probably bypass the resistance mechanisms employed by bacteria to combat conventional antibiotics (Zhao et al. 2010).

Ampicillin-functionalized AuNP (Brown et al. 2012) was functioned as effective broad-spectrum antibacterial agent against gram-negative and gram-positive bacteria. Functionalized AuNP mainly utilizes ampicillin's ability to permeate the outer membrane of bacteria and then nanoparticles enter the bacteria to achieve their antibacterial effects. This combined antibacterial effect can inhibit the growth of multiple-antibiotic-resistant isolates of *P. aeruginosa, E. aerogenes,* and Methicillin-Resistant *S. aureus*. Similarly, dextrose-coated gold nanoparticles (dGNPs) showed bacteriostatic and bactericidal effects against gram-negative (*E. coli*) and gram-positive (*S. epidermidis*) (Badwaik et al. 2012). The antibacterial action of dGNPs caused via disruption of the cell membrane leading to possible leakage of the cytoplasmic contents including nucleic acids. Tiwari et al. (2011) investigated the antibacterial and antifungal activities of 5-fluorouracil-functionalized AuNPs against *Micrococcus luteus, S. aureus, P. aeruginosa, E. coli, Aspergillus fumigates* (*A. fumigates*), and *Aspergillus niger* (*A. niger*). Results showed that AuNPs showed more activity on gram-negative bacteria than gram-positive due to membrane structure difference. AuNPs also showed antifungal activity against tested fungus also. Moreover, according to Wani et al. (2013), AuNPs may act both at the plasma membrane and in the cytoplasm. AuNPs inhibited H + -ATPase-mediated proton pumping and that might alter the normal cell conformation that leads to loss of activity in *Candida* sp.

Lima et al. (2013) reported the antimicrobial effect of AuNPs (5 nm) against *E. coli* and *Salmonella typhi*. They found that AuNPs reduced 90–95% growth of *E. coli* and *S. typhi*. The main factors that influenced the biocidal properties were the roughness and the dispersion of the AuNPs in the medium. The antibacterial efficacy of drugs (acridine derivatives: 9-aminoacridine hydrochloride hydrate (9AA-HCl), acridine yellow (AY), acridine orange (AO), and proflavine (Pro))-coated citrate-stabilized AuNPs studied against gram-positive (*B. subtilis*) and gram-negative bacteria (*E.coli*). AuNPs conjugated 9AA-HCl and AO resulted severe alterations in the cell wall structure that leads to leakage of cellular contents and finally cell death (Mitraa et al. 2014).

Patra and Baek (2015) reported that AuNPs with the standard antibiotics (kanamycin and rifampicin) showed higher antibacterial activity against five food-borne pathogens and demonstrated high synergistic activity. Results showed that AuNPs have antioxidant and antiprotease inhibitory activity also. The antibacterial activities of AuNP–kanamycin conjugates and kanamycin against gram-positive (*S. epidermidis*) and the gram-negative (*Enterobacter aerogenes*) concluded that the MIC values of the conjugate were significantly lower than that of free kanamycin. Similar result was concluded by Rattanata et al. (2016), against the food-borne pathogenic bacterial species *Plesiomonas shigelloides* and *Shigella flexneri* by gallic acid and AuNP–gallic acid. AuNP–gallic acid altered lipids, proteins, and nucleic acids of the bacterial cell membrane of treated bacteria. AuNP–levofloxacin conjugates (27.2 nm) were more efficient than levofloxacin alone and improved the

antibacterial efficacy against *S. aureus*, *E. coli,* and *P. aeruginosa* by 1.94, 2.89, and 1.46 times, respectively (Bagga et al. 2017).

The antibacterial activity of biosynthesized *Nigella arvensis* (NA)-conjugated gold nanoparticles (NA-AuNPs) was determined by well diffusion method against *S. epidermidis*, *B. subtilis*, *S. aureus*, *E. coli*, *Serratia marcescens,* and *P. aeruginosa*. They concluded that NA-AuNPs can be considered as promising antibacterial agents in biomedical applications (Chahardoli et al. 2018). The peptide/triclosan-comodified gold nanoparticles showed better antibacterial effect than pure gold nanoparticles (Wang et al. 2019). Sun et al. (2019) observed that AuNPs cofunctionalized with both bovine serum albumin (BSA) and 4,6-diamino-2-pyrimidinethiol (DAPT) can generate conjugates (Au-DAPT-BSA) with progressive antimicrobial activities against gram-negative bacteria and gram-positive bacteria. Au-DAPT-BSA induced no drug resistance and strengthening the nanomaterial-based bactericides like AuNPs against multiple drug-resistant bacteria (MDR).

The green synthesized AuNPs with average size of 19.45 nm showed antimicrobial potential against a number of pathogenic microorganisms. The green synthesized AuNPs exhibited higher antimicrobial activity toward gram-positive bacteria and gram-negative bacteria (Dongaa et al. 2020). Biogenic AuNPs were synthesized from Brazilian Red *Propolis* hydroethanolic extract and its fractions which showed great potential to produce AuNPs in size range of 8–15 nm. These biogenic AuNPs showed antimicrobial, antifungal activity, and high cytotoxicity at low concentrations suggested that these biogenic AuNPs act as promising candidate in nanomedicine (Botteon et al. 2021).

25.5 Conclusions

Due to the development of multidrug-resistance (MDR) in microorganisms, there is an urgent need for development of new antimicrobial agents that will overcome the threats put up by MDR. Metal nanoparticles owing to their high surface-to-volume ratio exhibit enhanced chemical and biological activities that make them a potential candidate for development of antimicrobial drugs. There are various metal nanoparticles like gold, silver, and copper which possess enhanced antibacterial and cytotoxic activities. Because of the unique capability to target multiple structural sites of organisms randomly, organisms are unlikely to develop resistance against them unlike conventional antibiotics.

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Index

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