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REVIEW ARTICLE

Bacteriophage as a Novel Therapeutic Weapon for Killing Colistin-Resistant Multi-Drug-Resistant and Extensively Drug-Resistant Gram-Negative Bacteria

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Abstract

Colistin-resistant multidrug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug-resistant (PDR) bacteria are highly lethal and many researchers have tried hard to combat these microorganisms around the world. Infections caused by these bacteria are resistant to the last resort of antibiotic therapy and have posed a major challenge in clinical and public health. Since the production of new antibiotics is very expensive and also very slow compared to the increasing rate of antibiotic resistance, researchers are suggesting the use of natural substances with high antibacterial potential. Bacteriophages are one of the most effective therapeutic measures that are known to exist for use for incurable and highly resistant infections. Phages are highly taken into consideration due to the lack of side effects, potential spread to various body organs, distinct modes of action from antibiotics, and proliferation at the site of infection. Although the effects of phages on MDR and XDR bacteria have been demonstrated in various studies, only a few have investigated the effect of phage therapy on colistin-resistant isolates. Therefore, in this review, we discuss the problems caused by colistin-resistant MDR and XDR bacteria in the clinics, explain the different mechanisms associated with colistin resistance, introduce bacteriophage therapy as a powerful remedy, and finally present new studies that have used bacteriophages against colistin-resistant isolates.

Introduction

In the fight between microorganisms and antibiotics, microorganisms have won; thus, challenging doctors and researchers in treating antibiotic-resistant microorganisms. Various studies have reported that antibiotic-resistant bacteria use various mechanisms to resist different antibiotics, even those considered as last-line antibiotics [1–4]. The emergence of multidrug-resistant (MDR) bacteria, followed by extensively drug resistant (XDR) bacteria, has posed a threat to the lives of patients with infectious diseases. Over the past few years, reports of pan-drug-resistant bacteria (PDR), which are resistant to all classes of antibiotics, have brought up extensive concerns [5]. About 25,000 patients in Europe die each year from infectious diseases caused by MDR bacteria, and more than 50% of cases die due to infections caused by XDR bacteria. Antibiotic-resistant infectious diseases impose a heavy annual cost of about 20 billion US dollars on the worldwide [5]. Today, the world is facing a growing and challenging threat with the emergence of bacteria which are almost resistant to the remaining effective antibiotics [6, 7]. Unfortunately, due to the limited activity

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of pharmaceutical industries in producing new antibiotics, physicians have been urged to use older highly toxic antibiotics such as colistin. For decades, colistin has been used in clinics, mainly against infections caused by MDR and XDR Gram-negative pathogens [8, 9]. Resistance to antibiotics have gradually developed in bacteria following their overuse and in the last decade, these microorganisms have even become resistant to last-line antibiotics such as carbapenem and colistin [10–12]. Resistance to colistin as a last-line antibiotic has been observed in Gram-negative bacteria, and in addition to chromosomal mutations, various other mechanisms have been shown to be involved in colistin resistance that can be transferred from one bacterium to another by horizontal transmission [13]. Therefore, the main problem is that final options for treating resistant pathogens, especially Gram-negative bacteria that cause nosocomial and community-acquired infections, are running out. Hence, it is predicted that antibiotic resistance could become an uncontrollable global catastrophe and the use of alternatives with significant antimicrobial activity, especially specific bacteriophages, has become a priority for researchers [5, 14]. The use of phages is so important that the US Food and Drug Administration (FDA) has approved bacteriophage cocktails as food additives to prevent foodborne bacterial contamination [15]. Phages have been reported to be used for prophylaxis and treatment of antibiotic-resistant bacteria, and a wide range of lytic phages have been tested for their therapeutic potentials against bacterial infections in animals and humans [7]. The results have been promising, raising the attention of researchers to this therapeutic method for eradicating MDR, XDR and PDR Gram-negative bacteria [16]. This review focuses on increased rate of MDR and XDR colistin-resistant Gram-negative bacteria as well as the subsequent challenges associated with these microorganisms in the clinic. Then we review the mechanisms that confer colistin resistance and discuss phage therapy as a promising option to eradicate MDR and XDR colistin-resistant Gram-negative bacteria.

MDR and XDR Challenge

It has been almost a decade since the World Health Organization (WHO) declared "combat drug resistance: no action today, no cure tomorrow". Over time, the development of antibiotic resistance in bacteria has become inevitable. Infections caused by MDR and XDR Gram-negative bacteria contribute to the exacerbation of the infection due to resistance to last-line antibiotics has been reported [12, 17]. MDR isolates are bacteria that are resistant to at least one antibiotic within three or more classes of antibiotics. XDR isolates are bacteria that are resistant to all but one or two classes of antibiotics [18]. The important issue about infections caused by MDR and XDR bacteria is that in addition to employing known mechanisms of antibiotic resistance, repeated or long-term administration of antibiotics could enhance the development of resistance to new compounds [19]. In addition, many of the different mechanisms of antibiotic resistance in these MDR and XDR Gram-negative bacteria are due to the presence of resistance genes that are located on the mobile genetic elements and can be transmitted to other bacteria, leading to the spread of resistance [12]. According to various studies, a high mortality rate is observed due to infections by MDR and XDR isolates. Different mechanisms of antibiotic therapy failure include the external barriers (preventing the entry of drugs into bacteria), genetic transmission of resistance (through plasmids, integrons, transposons, and other mobile genetic elements), natural mutations in antibiotic targets, enzyme-dependent drug alterations, and efflux pumps [5]. According to recent reports, antibiotic-resistant bacterial infections in hospitals account for approximately 400,000 deaths annually [20]. Mortality rate varies depending on the bacterial *spp*., type of infection and the geographical region. The mortality rate of MDR *Pseudomonas aeruginosa* isolates, as one of the main causes of nosocomial infections is estimated to be 18–61% [21, 22]. On the other hand, the mortality rate of infections caused by MDR and XDR *Acinetobacter baumannii* isolates is 21.2% due to bacteremia, 5% in different wards of the hospital (general ward), and 54% in intensive care units (ICU) [23, 24]. Therefore, MDR and XDR bacteria are important factors in increasing mortality and morbidity of patients as well as the high cost of medical care. Antimicrobial drug resistance is reported to add 30–100 billion US dollars to health care costs per year [25]. Moreover, infections caused by MDR and XDR bacteria pose unavoidable problems such as the delayed treatment process, prolonged hospitalization time, and the need to use more toxic antibiotics [12]. Therefore, there is a clear and vital need to develop a new approach for treating infections caused by MDR and XDR pathogens. Although the use of a combination of several antibiotics and the production of new antibiotics have become a priority in combating infections caused by these bacteria, there have not been much success [5]. Therefore, researchers have conducted serious research on non-antibiotic drugs for the treatment of MDR and XDR bacterial infections, and the use of specific phages has been introduced as a main alternative to antibiotics.

Colistin-Resistant MDR and XDR Bacteria

Colistin was discovered about 80 years ago (1940s), however, its use was discontinued by doctors for decades due to having many side effects and the discovery of other less toxic antibiotics. In recent years, the emergence of MDR and XDR bacteria as well as the lack of efficient antibiotics have led to the reuse of colistin against infections [12]. There are currently

two commercially available types of colistin, including colistimethate sodium and colistin sulfate [26]. One monotherapy option against MDR and XDR Gram-negative bacteria isolated from nosocomial infections is colistin. Although colistin has still a good effect on antibiotic-resistant isolates in many places, it has many side effects [27]. After re-use of colistin in the clinic, the main noticed side effect was nephrotoxicity. Studies have confirmed that nephrotoxicity varies from 6 to 58% after intravenous colistin administration. In patients with normal renal function, nephrotoxicity was observed in 10% of cases, but in abnormal kidneys, nephrotoxicity was observed in 27–58% of subjects [28, 29]. Widespread use of colistin in livestock around the world has gradually led to the emergence of colistin-resistant bacteria in livestock, which can be transmitted from animals to humans through food. Increased resistance to this antibiotic has led to a ban on its use in livestock, especially in developed countries, to prevent further development of resistance to colistin [12]. Accordingly, severe side effects of colistin and emergence of colistin-resistant MDR and XDR Gram-negative bacteria have made their employment even more challenging as resistance to last-line antibiotics poses a major challenge for physicians. There have been various reports of colistin-resistant MDR and XDR isolates in some parts of the world, but there no exact pattern of prevalence and rate of resistance to this antibiotic has been demonstrated in all countries. Today, colistin resistance in *K. pneumoniae* isolates is increasing, and mortality rate due to severe infections caused by these isolates range from 25 to 71% [30]. Colistin-resistant MDR, XDR, and pandrug-resistant (PDR] *Acinetobacter spp*. have also been associated with high mortality rate and nephrotoxicity [27]. The emergence of a highly resistant clone of *K. pneumoniae* ST14 with high colistin resistance rate of 37.1% has been reported, posing a serious concern [31]. The emergence of the *mcr-3* gene (a plasmid-mediated colistin resistance gene) was detected in both *mcr-1* and *mcr-2* negative *Escherichia coli* isolates. This plasmid-borne *mcr-3* was transferred to an *E. coli* recipient by conjugation. This resistance plasmid was also identified in *K. pneumoniae* isolate from Thailand, *E. coli* isolate from Malaysia, and *Salmonella enterica* serovar Typhimurium isolate from the USA. According to the ubiquitousness of *Aeromonas* in the environment and the transmission of *mcr-3* between *Aeromonas* and *Enterobacteriaceae* isolates, the broad dissemination of *mcr-3* might be underestimated. As colistin has been widely used in veterinary medicine and is increasingly prescribed for humans, monitoring plasmid-borne colistin resistance in colistin-resistant Gram-negative bacteria is imperative for prevention and control of the spread of *mcr* genes [32]. Colistin resistance has been reported in 21.3% of MDR and XDR *P. aeruginosa* isolates. Among colistin-resistant isolates, a protein profile belonging to OprH as an efflux pump has been reported which differs from the LPS profile [33]. In various studies, *mcr* genes (*mcr*-1 to *mcr*-10), which are involved in colistin resistance in MDR and XDR isolates, have been reported in different bacteria [34–36]. In another study, a novel *mcr* gene, *mcr-10*, was identified in a clinical *Enterobacter roggenkampii* isolate. When plasmid-borne *mcr-10* was cloned into a colistin-susceptible *E. roggenkampii* strain, MIC of colistin showed fourfold increase (from 1 to 4 mg/L) [37]. Colistin resistance in 646 V. *parahaemolyticus* isolates was screened in China, among which 25 (2.5%) showed colistin resistance. The *mcr-1* gene was found in one colistin-resistant isolate. Class A β-lactamase gene *blaCARB-17* and the plasmid-mediated quinolone resistance (PMQR) gene *qnrVC5* were accompanied by *mcr-1* gene in a colistin-resistant *V. parahaemolyticus* isolate [38]. One study identified an MDR *Stenotrophomonas spp.* with high levels of resistance to colistin and meropenem, which was of great concern as no resistance plasmid was found after genome sequencing; however, *mcr-5.3*, *mcr-8.2* and four β-lactamase genes were widely observed. In addition, 12 genes associated with seven types of efflux pumps have been identified which are thought to play a major role in the acquisition and transmission of colistin resistance [39]. Two colistin-resistant and nine heteroresistant *P. aeruginosa* isolates were identified, and among these, the two colistin-resistant isolates showed mutations in PmrB. Also, heteroresistant *P. aeruginosa* showed alterations in the PmrAB regulatory system. The conversion of heteroresistance to resistance must be noted in future clinical surveillance [40]. Two colistin-resistant *E. coli* sequence type (ST) 131 isolates were obtained from peritoneal fluid and abscess of the surgical wound. Genome sequencing revealed the presence of the plasmid-mediated AmpC β-lactamase gene bla_{CMY-2} and the *mcr-1* gene [41]. Importantly, colistin resistance in MDR and XDR isolates can be associated with the presence of predominant resistance genes, such as carbapenemases, beta-lactamases, and metallobetalactamases. The co-transfer of multiple important antimicrobial resistance genes pose a certain challenge for healthcare settings as resistance to colistin and other antibiotics could enhance the menace of infections resistant to all classes of antibiotics [38, 39, 41, 42]. Above studies show that resistance to colistin has been observed in a wide range of Gram-negative bacteria, especially MDR and XDR isolates, and transmission of resistance through transferable mobile genes can increase the resistance rate in *Enterobacteriaceae* and other Gram-negative bacteria. Therefore, it is very important to come up with new solutions to deal with these troublesome resistant bacteria.

The Mechanism of Colistin Resistance

Authoritative literature has introduced five mechanisms of action for colistin. The direct antibacterial activity of colistin is such that the cationic diaminobutyric acid residues of colistin bind to anionic phosphate of lipid A moiety of LPS

in the outer membrane via electrostatic bonds, thereby affecting the outer and inner membranes of bacteria and leading to cell lysis. Colistin also has anti-endotoxin activity, such that it binds to LPS molecules and inhibits the activity of lipid A endotoxin [43, 44]. This leads to the release of tumor necrosis factor-alpha (TNF-α) and interleukin-8 (IL-8), thereby inhibiting shock induction. Vesicle-vesicle contact pathway is another mechanism by which colistin acts on Gram-negative bacteria. Colistin binds to anionic phospholipid vesicles after transiting to the outer membrane, causing the fusion of the inner leaflet of the outer membrane with the outer leaflet of the cytoplasmic membrane, subsequently leading to the loss of phospholipids and bacteria death. In the mechanism of the hydroxyl radical death pathway, colistin leads to the release of reactive oxygen species. This pathway is known as the Fenton reaction, which damages DNA, lipids and proteins, eventually leading to bacterial death. Inhibition of respiratory enzymes by colistin is another mechanism of action in which colistin exerts antibacterial activity by inhibiting vital respiratory enzymes [44]. Unfortunately, MDR, XDR, and PDR isolates of Gram-negative bacteria such as *P. aeruginosa*, *E. coli, A. baumannii*, *K. pneumoniae,* and other *Enterobacteriaceae* are globally found to harbor multiple resistance mechanisms. Also, *Morganella morganii, Providencia spp., Serratia marcescens, Proteus spp., Vibrio cholera, Brucella, Edwardsiella spp., Legionella, Chromobacterium, Neisseria spp.,* anaerobic Gram-negative cocci, some *Aeromonas spp., Burkholderia cepacia, Campylobacter,* eukaryotic microbes, and mammalian cells possess intrinsic colistin resistance [44–46]. Mechanisms of colistin resistance may be due to chromosomal mutations and transmissible plasmid genes called *mcr* (*mcr-1* to *mcr-10*) which are able to spread through horizontal transfer between bacteria [26]. At the present time, PCR-based techniques are the most extensively adopted to identified *mcr* genes, however, owing to the presence of various genotypes of *mcr* genes and their capability to undergo horizontal gene transfer; PCR techniques are limited in clinical practice, containing the basic quarantine stations and the primary-care hospitals [47]. The *mcr* genes in Gram-negative bacteria have been identified on various plasmids, including IncY, IncF, IncI2, IncP, IncHI2, ColE10-like ones, and IncX4 [48]. The plasmid-borne *mcr* gene has an interesting mechanism. The product of this gene transfers phosphoethanolamine residues to the main target of colistin which is the lipid moiety of LPS. This leads to alterations in the LPS of Gram-negative bacteria, thereby reducing the bacterial affinity to react with colistin and thus the effectiveness of the drug. It has been reported that the *mcr* gene can also alter the susceptibility of Gram-negative bacteria to antimicrobial peptides and other available antibiotics [49, 50]. In addition, high expression of the *mcr* gene contribute to changes in fitness, growth rate and structural integrity of the outer membrane, which are attributed to the integration of *mcr* into the bacterial membrane and changes in lipid A through its enzymatic activity [50]. In addition to the plasmid gene, another mechanism of resistance to colistin is chromosomal mutations that occur in lipid A synthesis genes including *lpxO2, lpxD*, *lpxC*, and *lpxA*, causing incomplete LPS production. It has also been shown that in case the ISAba11 sequence is inserted into the LPS synthesis genes (*lpx*C and *lpxA*), the bacterium loses the ability of LPS production and therefore, a high level of resistance occurs [51, 52]. In case Gram-negative bacteria are deficient in LPS, less negative charges are present on bacterial surface, leading to the reduced affinity to colistin [53]. Positive charges are suggested as major factors for lipid A changes which are induced by the addition of galactosamine (*naxD*), phosphoethanolamine (pEtN, mediated by chromosomally encoded *eptA* or plasmid-borne *mcr*), and 4-amino-4-deoxy-L-arabinose (L-Ara4N, mediated by *arnBCADTEF-ugd* operon) to lipid A, which decrease the ability of colistin to bind and disrupt the outer membrane [12]. Two-component systems (PhoPQ and PmrAB) are known to be encoded by chromosomes and are responsible for the intrinsic resistance to colistin [54, 55]. The two-component PmrAB system consists of several constituents, including histidine kinase and a response regulator that responds to environmental stimuli. This system enables Gram-negative bacteria to sense and respond appropriately to a variety of environmental conditions, including different pH levels as well as the presence of Mg^{2+}, Fe^{3+}, and Al^{3+} ions. The two-component PmrAB system can confer colistin resistance in Gram-negative bacteria by affecting the expression of genes involved in alterations of lipid A [56–59]. Point mutations in the *pmrA* and *pmrB* genes have been shown to increase the expression level of *pmrAB*, which subsequently leads to altering the bacterial outer membrane, followed by decreased membrane permeability and resistance to colistin [58, 60]. The two-component PhoPQ system is stimulated by environmental factors, such as Mg^{2+}, Ca^{2+}, and cationic antimicrobial peptides (polymyxin, indolicidin, and LL-37), and plays a major role in the virulence and alteration of LPS, as well as increased colistin resistance [12]. One of the most common mechanisms of colistin resistance is mutation of the *mgrB* gene, which regulates negative feedback of the PhoPQ two-component system, activates PhoPQ, and directly increases the expression of the *arnBCADTEF* operon [61]. One of the causes of missense or nonsense mutations in *mgrB* gene is insertion sequence (IS) truncation, among which IS102, IS5 family, IS3-like, IS5-like, and ISKpn14 were related with *mgrB* mutations in clinical samples [12]. Capsule formation is another mechanism of colistin resistance as polymyxin reacts with the capsule polysaccharides by anionic interactions, leading to polymyxin attenuation [62]. Also, regulators of capsule production, such as conjugative pilus

expression (Cpx), and regulator of capsule synthesis (Rcs), are capable of activating efflux pumps that confer resistance to colistin (KpnEF for Cpx and PhoPQ for Rcs regulon) [54, 62]. Colistin resistance by efflux pumps belonging to the RND (resistance-nodulation-cell division) family has been reported. These efflux pumps are composed of membrane fusion protein (encoded by *adeA* gene), which acquires substrate and transports it from cytoplasm or within phospholipid bilayer to the extracellular medium (encoded by *adeB*), and an outer membrane protein channel (encoded by *adeC*) regulated by the *adeR* gene [63]. Other efflux pumps that have been implicated in colistin resistance include *MexXY–OprM, CarO, sapABCDF, acrAB–tolC, kpnEF,* and *emrAB*; however, the mechanisms of colistin resistance by these efflux pumps are not yet clear [61, 64, 65]. Colistin resistance could be obtained via colistin-heteroresistant bacteria. Colistin heteroresistance is an intermediate condition which exhibits a phenotype characterized by the presence of resistant subpopulations among a sensitive population. Colistin heteroresistance phenotypes may account for the unexplained treatment failures and are frequently detected among MDR *A. baumannii, P. aeruginosa,* and *K. pneumoniae* isolates. There are different mechanisms attributed to colistin heteroresistance which include mutations in lipid A biosynthesis genes, *soxRS*-regulated overexpression of the *acrAB-tolC* efflux pump, putrescine/YceI communication, activation of two-component regulatory systems PmrAB, PhoPQ, ParRS, CprRS, and ColRS, as well as biofilm formation [40, 44]. The *lptD* is a vital locus for insertion of the newly produced LPS into the outer membrane, and removal of this locus leads to the complete loss of LPS and increased polymyxin resistance [66]. Deletion mutation in the locus responsible for biotin synthesis has been implicated in polymyxin resistance as biotin is a main co-factor of lipid metabolism. Therefore, higher biotin levels responsible for the increased production of lipid A can increase colistin susceptibility [67, 68]. Another factor contributing to colistin resistance is the overexpression of the outer membrane protein OprH, which binds to negatively charged phosphate groups, ultimately inhibiting the binding of polymyxin. Noteworthy, downregulation of OprD (a porin) is also attributable to polymyxin resistance in *P. aeruginosa* [44]. Intriguingly, a single mutation in *vacJ* contributes to the emergence of colistin-resistant phenotype [69]. The *sodB* and *sodC* genes also contribute to colistin resistance, most probably via the detoxification of reactive oxygen species [70]. Acylation of lipid A portion, which contributes to colistin resistance, could be regulated by *lpxM*. Inactivation of *lpxM* inhibits L-Ara4N modifications and leads to the decreased polymyxin resistance [71]. Bmul_2133 and Bmul_2134 were found to be putative hopanoid biosynthesis genes in *Burkholderia multivorans* which are essential for the stabilization of outer membrane permeability. Therefore these two genes are responsible for polymyxin resistance via a mechanism which is independent of LPS-binding activity [44]. DedA family protein (a membrane transporter) in *Burkholderia thailandensis* is required for colistin resistance. DedA leads to alterations in lipopolysaccharide (LPS) lipid A and the subsequent colistin resistance [72]. For a better comprehension, a summary of the mechanisms of colistin resistance in Gram-negative bacteria is shown in Table 1.

Bacteriophage

Bacteriophages or phages are a group of viruses that act specifically on bacteria and can be used as a therapeutic agent to treat bacterial infections. The basis of phage treatment in infections is the binding of specific phage to the bacterium, which results in rapid lysis of the bacterial cell [5]. In 1896, the British researcher Hankin discovered a biological source in the Indian River Ganges and Jumna that changed the culture of the bacterium that causes cholera. This discovery was possibly the first report of bacteriophage activity [5]. Further reports described bacteriophage as an "anti-Shiga microbe" as it was found in the feces of patients with shigellosis. The curiosity of other researchers led them to believe that the bacterial virus was responsible for the destruction of bacteria [5]. Nevertheless, the use of phages as antimicrobial agents to fight bacterial infections was first introduced almost 90 years ago [74]. It was also suggested that environmental phage counts which have been common heavy greater than 10^6/ml effecting a single host bacterial strain to arrive the rates of bacterial loss [75]. In the last few decades, the production of new antibiotics has no longer been cost-effective as resistance to them has occurred after production. In recent years, with the advent of MDR, XDR, and PDR bacteria as well as the low production of new antibiotics, the reuse of bacteriophages has been noticed [16, 76]. Unique properties of lytic phages in general include absence of inherent toxicity, lack of cross-resistance with antibiotic classes, high selectivity, bactericidal activity, and the ability of proliferation in the presence of pathogenic resistant bacteria [5]. As phages are highly specific to bacteria, unlike broad-spectrum antibiotics, they do not kill the commensal microbiota, which are vital for patients with malnutrition and immunodeficiency. Phages can be stored in a dry powder formulation without the need for a cold chain [77]. Since phages are widely found in a variety of environments, such as sewage effluent, soil, water, hospital effluent, fecal materials, as well as the gastrointestinal tract of humans and animals, they can be rapidly isolated. Consequently, isolation of phages is more cost-effective than the production of antibiotics because phage found in a variety of environments even in special unusual environments such as hot springs [78]. In addition, one of the other factors that put phages under

Table 1 A summary of the different mechanisms that Gram-negative bacteria use for colistin resistance

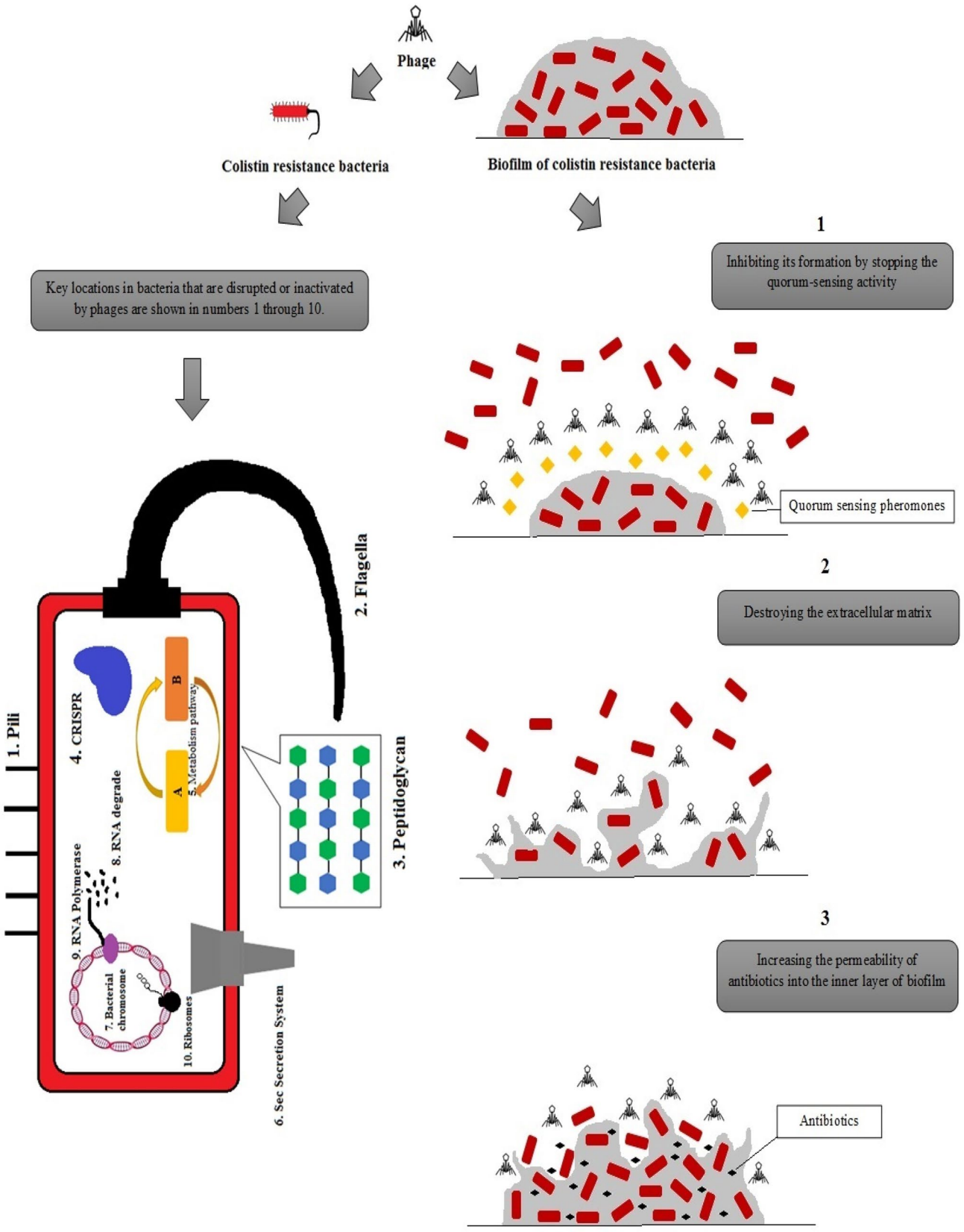
Mode of action of colistin	Mechanism of colistin resistance	Function
Direct antibacterial activity [43, 44] Anti-endotoxin activity [43, 44] Vesicle-vesicle contact pathway [44] Hydroxyl radical death pathway [44] Inhibition of respiratory enzymes [44]	The *mcr* plasmid	Leads to changes in the LPS of Gram-negative bacteria that reduce the affinity to react with colistin and thus leading to the effectiveness of the drug [13, 37, 73]
	Mutations that occur in lipid A synthesis genes	Cause incomplete LPS production and induce less negative charges on the surface [51, 52]
	Insertion of ISAba11 into the LPS synthesis genes	Induces the loss of LPS production and high level of resistance [51, 52]
	Positive charges	Majorly alter lipid A, leading to the decreased ability of colistin binding and prevention of the disruption of outer membrane[12]
	Two-component systems (PhoPQ and PmrAB]	PmrAB leads to colistin resistance by affecting the expression of genes involved in lipid A alterations [56–59] Point mutations in the *pmrA* and *pmrB* genes decrease the membrane permeability and resistance to colistin [58, 60] PhoPQ plays a major role in the virulence and alteration of LPS, and its mutations increase colistin resistance [12]
	Mutation in the *mgrB* gene	Directly increases the expression of the *arnBCADTEF* operon and leads to lipid A changes [61]
	Capsule formation	Leads to polymyxin attenuation [62]
	Efflux pumps	Transport colistin from cytoplasm or within the phospholipid bilayer to the extracellular medium [63]
	Miscellaneous chromosomally encoded colistin resistance genes	These include the *lptD* locus, biotin synthesis locus, OprH protein, OprD porin, *vacJ* locus, *sodB* and *sodC* genes, *lpxM* gene, Bmul_2133 and Bmul_2134, as well as DedA family protein [44, 67–70]
	Colistin heteroresistance phenotype	Mutations in lipid A biosynthesis genes, *soxRS*-regulated overexpression of the *acrAB-tolC* efflux pump, putrescine/YceI communication, activation of PmrAB and PhoPQ two-component regulatory systems, ParRS, CprRS, and ColRS two-component regulatory systems, and biofilm formation [40, 44]

focus is that they can be administered through respiratory, parenteral, gastrointestinal, and topical routes [77, 79]. The absence of side effects, decreased inflammatory responses, and potentially distribution of phages all over the body are the most promising aspects of phage therapy [5]. Different mechanisms of action of phages on both Gram-negative and Gram-positive antibiotic resistant bacteria include the inhibition of peptidoglycan synthesis, engaging the host cell secretion machinery, interference with cellular motility, metabolism, transcription and translation, DNA silencing, RNA degradation, and clustered regularly interspaced short palindromic repeats (CRISPR)-mediated immunity [80, 81]. Phages can eradicate biofilms resistant to antibiotic and environmental stresses by destroying the extracellular matrix, preventing the quorum-sensing mechanism, and increasing the permeation of antibiotics into the inner layers of biofilm structures (Fig. 1) [82]. Phages can also be used as a cocktail, with the benefit of having greater effects on target bacteria and reducing the formation of phage-resistant bacteria. This is because different types of phages can target same species and strains of bacteria [83]. In addition, phages have the ability to destroy biofilms (accumulation of bacterial cells that attach to a surface or to each other and are placed in a self-generated matrix with a high resistance to antibiotics). All of these unique properties of phages mentioned in this section have urged researchers and physicians to consider phage therapy as a potential effective treatment approach. Also, by using the potential of bacteriophages, researchers have achieved significant success in food safety, veterinary medicine, agriculture, industry, and clinical diagnosis (detection and typing of bacteria in human infections) [5]. To date, the use of phages in in vitro, animals, and even

humans with various infections such as antibiotic-resistant chronic rhinosinusitis, necrotizing pancreatitis, and septicemia have been used with promising results. The use of phages against human antibiotic-resistant infections in the clinic proves that phages are suitable weapons against resistant bacteria and can be considered as a promising solution for the treatment of infections caused by bacteria resistant to last-resort antibiotics [5].

Bacteriophages Against Colistin Resistance

Colistin has been mentioned as the latest treatment option for bacteria with high levels of antibiotic resistance, as sometimes is the only effective antibiotic. The global development of colistin resistance in Gram-negative bacteria is alarmingly increasing. Colistin-resistant bacteria are usually resistant to other antibiotics, and they therefore pose a challenge in clinical treatment, public health, and medical interventions [84, 85]. The use of various strategies by researchers to overcome these challenging bacteria has been considered, the most important of which are combination antibiotic therapy, antimicrobial peptides, monoclonal antibodies, nanoparticles, natural compounds, herbal extracts, and phages [12]. In the meantime, of all these treatment options, phage therapy has been used in the clinic, especially in cases infected with MDR, XDR, and PDR superbugs. Accordingly, researchers have identified bacteriophages as a viable alternative to antibiotics in the treatment of infections with superbugs [5]. In a study by Ebrahimi, IsfAB78 lytic phage was examined against colistin-resistant MDR *A. baumannii* clinical isolates. Their results showed that the IsfAB78 phage was able to significantly lyse MDR *A. baumannii* cultures after 40 min and reduced the number of these resistant bacteria in biofilm structures for about 19–87% [86]. In another study, Hao et al. induced colistin resistance in carbapenem-resistant *K. pneumoniae* (colistin-resistant CRKP) isolates by horizontally transferring *mcr-1* gene and inactivating the *mgrB* chromosomal gene. They investigated the effect of lytic phage ϕNJS1 on these isolates. The results showed that the reduction of negative charges on bacterial surface due to colistin resistance led to the increased ϕNJS1 phage adhesion and subsequent infection. Colistin-resistant bacteria were also shown to be more sensitive to the ϕNJS1 phage compared to the wild-type strains when grown in biofilms or moth larvae and during mammalian colon colonization [87]. Shokri et al. aimed at detecting specific phages for the selected MDR, XDR, and PDR clinical isolates of *P. aeruginosa* from waste waters and hospital sewages. They found that phage cocktails (Psu1, Psu2, and Psu3) had antibacterial activity against all MDR, XDR and PDR isolates of colistin-resistant *P. aeruginosa* and completely destroyed the bacterial cells [88]. In another study, Abdelkader et al. used the PMK34 bacteriophage (which encoded an endolysin with potent muralytic activity and was isolated from raw sewage water) against colistin-resistant *A. baumannii*. Two PDR colistin-resistant *A. baumannii* strains exhibited a similar susceptibility to PMK34 phage. Their results indicated that the combination of LysMK34 and colistin decreased the MIC of colistin up to 32-fold, and that colistin-resistant strains were resensitized in Mueller–Hinton broth. Therefore, LysMK34 can be used to maintain the applicability of colistin as a last-resort antibiotic [89]. Manohar et al. investigated the therapeutic features and efficacy of specific phages (from sewage water samples) against *E. coli, K. pneumoniae*, and *Enterobacter* species. The three bacteriophages included myPSH2311 (infecting *E. coli*), myPSH1235 (infecting *K. pneumoniae*), and myPSH1140 (infecting four different *Enterobacter spp.*). Interestingly, phage cocktail decreased the bacterial load from 10^6 to 10^3 CFU/mL within 2 h. All the three characterized phages were detected to have a broad host range activity and the phage cocktail was effective against meropenem- and colistin- (two last-resort antibiotics) resistant bacteria [90]. Some studies have reported the excellent effectiveness of the combination of phage and phage-derived protein with colistin against Gram-negative bacteria with high levels of antibiotic resistance [91, 92]. Blasco et al. investigated the efficacy of ElyA1 and ElyA2 endolysins (from Ab1051Φ and Ab1052Φ phages, respectively) alone and combination with colistin against clinical isolates of MDR Gram-negative bacteria. According to their results, ElyA1 showed antibacterial activity against 25 MDR *P. aeruginosa*, 25 MDR *A. baumannii*, and 17 carbapenemase-producing *K. pneumoniae* isolates (three colistin-resistant strains: *A. baumannii* SOF004b, *P. aeruginosa* AUS034 and *K. pneumoniae* KP2). No antibacterial activity was found in ElyA2. The combination of colistin and ElyA1 decreased the MIC of colistin in all isolates (thus reducing the associated toxicity), except in *K. pneumoniae* [93]. Bernasconi et al. investigated the antibacterial activity of three phages (PYO, INTESTI, and Septaphage) against MDR *E. coli* and *Proteus spp.* isolates from humans, food, and animals. Only four isolates were colistin-resistant, one of which harbored the *mcr-1* plasmid gene. Although Septaphage had no antibacterial activity, 5 of 8 carbapenemase producers and 3 of 4 colistin-resistant isolates were susceptible to PYO and INTESTI, respectively [94]. In another study, Aslam et al. used bacteriophage therapy in two and one lung transplant recipients with life-threatening MDR infections caused by *P. aeruginosa* and *Burkholderia dolosa*, respectively. Two ventilator-dependent lung transplant recipients with large airway complications and refractory MDR *P. aeruginosa* pneumonia (intermediate colistin resistance) received phage therapy. Both cases responded clinically and were discharged from the hospital without the need for ventilator support. Although the third


Phage
Colistin resistance bacteria
Biofilm of colistin resistance bacteria
Key locations in bacteria that are disrupted or inactivated by phages are shown in numbers 1 through 10.
1
Inhibiting its formation by stopping the quorum-sensing activity
Quorum sensing pheromones
2
Destroying the extracellular matrix
3
Increasing the permeability of antibiotics into the inner layer of biofilm
Antibiotics
1. Pili
2. Flagella
3. Peptidoglycan
4. CRISPR
5. Metabolism pathway
8. RNA degrade
9. RNA Polymerase
7. Bacterial chromosome
10. Ribosomes
6. Sec Secretion System
A
B

◂**Fig. 1** Shows the effect of phages on antibiotic-resistant gram-negative bacteria in planktonic and biofilm modes of growth. The effect of phage on antibiotic-resistant biofilms is through three mechanisms including 1) destroying the extracellular matrix, 2) preventing the quorum-sensing mechanism, and 3) increasing the permeation of antibiotics into the inner layers of biofilm structures as illustrated in the right. The release of the planktonic form of antibiotic-resistant bacteria leads to bacterial death by phages through the disruption of 1) pili, 2) flagella, 3) peptidoglycan, 4) CRISPR, 5) metabolic pathway, 6) sec secretion system, 7) bacterial chromosome, 8) RNA degradation, 9) RNA polymerase, 10) ribosomes of bacteria, as marked with numbers on the left side of the figure

patient showed recurrent *B. dolosa* (susceptible to colistin) infection following transplant, the improved consolidative opacities and ventilator weaning were demonstrated via phage therapy. No phage therapy-related side effects were detected in the studied patients. Phage therapy was well related to clinical recovery of lung transplant recipients with MDR Gram-negative bacterial infections that were not responsive to antibiotics [95]. In 2020, researcher screened two phages including vB_AbaM_ISTD and vB_AbaM_NOVI (isolated from wastewaters) for their potential in eradicating 103 clinical *A. baumannii* isolates (three isolates were colistin-resistant). Both phages had fast adsorption rates, high depolymerizing activity, proper growth rates, broad host range, and antibacterial effectiveness against planktonic and biofilm-associated bacteria. Among carbapenem-resistant *A. baumannii* isolates, two colistin-resistant isolates were also sensitive to both NOVI and ISTD phages [96]. Schirmeier et al. measured the inhibitory and bactericidal effects of Artilysin Art-175 (endolysin encoded by bacteriophage) against colistin-resistant *mcr-1*-positive *E. coli* isolates. They observed that Art-175 had a high antimicrobial activity against all *mcr-1* colistin-resistant *E. coli* isolates. Overall, the number of *mcr-1*-positive colistin-resistant bacteria reduced. Also, they demonstrated no cross-resistance between colistin and Art-175 [97]. In another study, Art-175 was used to treat MDR *A. baumannii* isolates. According to the results, Art-175 had high bactericidal properties against all isolates, even those resistant to colistin [98]. Table 2 shows a summary of the above studies confirming the promising effects of phages on colistin-resistant bacteria. Although studies on the miraculous effect of phages and phage-derived protein against colistin-resistant Gram-negative isolates are limited, the presented studies in this review could pave the way for attracting more attention on discovering different phages to counteract colistin-resistant bacteria. Due to the loss of colistin efficiency on these superbugs, doctors have limited treatment options; therefore, phages as new treatment options could be a new hope in the fight against these infections in the future. Not only colistin-resistant isolates are usually resistant to all antibiotics, the ability of biofilm formation in these isolates can exacerbate the challenges associated with their treatment. However, these studies show that phages can show a great potential in destroying biofilms.

Conclusion

Colistin resistance is a critical issue to deal with nowadays. Researchers have introduced novel alternatives to colistin with better efficiency and stability. Bacteriophages, either alone or in a cocktail, have lysing properties against various MDR, XDR, and PDR colistin-resistant Gram-negative bacteria and reduce the bacterial load in both planktonic and biofilm modes of growth. In addition, phage-derived enzymatic proteins have antibacterial activity against Gram-negative bacteria that are resistant to last-line antibiotics. Therefore, phages, with different mechanisms from antibiotics, can be considered as good alternatives to colistin and can even be used along with colistin to fight superbugs in the future due to having synergistic effects. This review represents an important step in the introduction of novel phages that may be considered in the successful treatment of diseases caused by colistin-resistant pathogens. Introducing phages as new agents for last-line antibiotic resistant pathogens requires a strong collaboration among scientists around the world and not only this but additionally the implementation of the required infrastructures.

Table 2 A summary of the therapeutic properties of bacteriophage against infections caused by colistin-resistant Gram-negative bacteria

Author name	Year	Phage	Organism	Result	Ref.
Ebrahimi et al	2020	IsfAB78	Colistin-resistant MDR *A. baumannii*	IsfAB78 phage was able to significantly lyse culture and biofilm of MDR *A. baumannii*	[86]
Hao et al	2019	ϕNJS1	Colistin-resistant CRKP	Colistin-resistant CRKP was also shown to be more susceptible to ϕNJS1 phage compared to wild-type bacteria when grown in biofilms or moth larvae and during mammalian colon colonization	[87]
Shokri et al	2017	Psu1, Psu2, and Psu3	MDR, XDR and PDR isolates of colistin-resistant *P. aeruginosa*	Phage cocktails showed antibacterial activity and completely destroyed the bacterial cells	[88]
Abdelkader et al	2020	PMK34	PDR colistin-resistant *A. baumannii*	PDR colistin-resistant *A. baumannii* strains were susceptible to the PMK34 phage	[89]
Manohar et al	2019	myPSH2311, myPSH1235, and myPSH1140	Meropenem and colistin-resistant *E. coli, K. pneumoniae*, and *Enterobacter spp*.	There was a reduction in bacterial load and efficacy was observed against all bacteria	[90]
Blasco et al	2019	ElyA1 and ElyA2 endolysins [from phage Ab1051Φ and Ab1052Φ]	Colistin-resistant MDR *P. aeruginosa*, MDR *A. baumannii*, and carbapenemase-producing *K. pneumoniae* isolates	ElyA1 illustrated antibacterial activity and combination of colistin and ElyA1 decreased the MIC of colistin in all isolates	[93]
Bernasconi et al	2017	PYO, INTESTI, and Septaphage	MDR carbapenemase-producers, colistin-resistant *E. coli* and *Proteus spp*. isolates	Although Septaphage had no antibacterial activity, carbapenemase producers and colistin-resistant isolates were susceptible to PYO and INTESTI	[94]
Aslam et al	2019	AB-PA01, AB-PA01 m1, Navy phage cocktail1, and Navy phage cocktail2 for patient one, AB-PA01 for patient two, and single lytic phage for patient three	Colistin-resistant MDR *P. aeruginosa* and *B. dolosa*	Phage therapy was well related to clinical recovery in lung transplant recipients with MDR Gram-negative bacterial infections	[95]
Vukotic et al	2020	vB_AbaM_ISTD and vB_AbaM_NOVI	Colistin-resistant *A. baumannii*	Two colistin-resistant isolates were also sensitive to both NOVI and ISTD phages	[96]
Schirmeier et al	2018	Artilysin Art-175	Colistin-resistant *mcr-1*-positive *E. coli* isolates	Art-175 showed a high antimicrobial activity against all *mcr-1* colistin-resistant *E. coli* isolates and the number of bacteria was reduced	[97]
Defraine et al	2016	Artilysin Art-175	MDR colistin susceptible and resistant *A. baumannii*	Art-175 had high bactericidal properties against all isolates, even colistin-resistant isolates	[98]

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