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Phage therapy: An alternative to antibiotics in the age of multi-drug resistance

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Abstract

The practice of phage therapy, which uses bacterial viruses (phages) to treat bacterial infections, has been around for almost a century. The universal decline in the effectiveness of antibiotics has generated renewed interest in revisiting this practice. Conventionally, phage therapy relies on the use of naturally-occurring phages to infect and lyse bacteria at the site of infection. Biotechnological advances have further expanded the repertoire of potential phage therapeutics to include novel strategies using bioengineered phages and purified phage lytic proteins. Current research on the use of phages and their lytic proteins, specifically against multidrug-resistant bacterial infections, suggests phage therapy has the potential to be used as either an alternative or a supplement to antibiotic treatments. Antibacterial therapies, whether phage- or antibiotic-based, each have relative advantages and disadvantages; accordingly, many considerations must be taken into account when designing novel therapeutic approaches for preventing and treating bacterial infections. Although much is still unknown about the interactions between phage, bacteria, and human host, the time to take phage therapy seriously seems to be rapidly approaching.

Keywords: Bacteriophage, Bacteriophage therapy, Phage, Phage therapy, Endolysin, Lysin, Multidrug resistance, Antibiotic resistance, Phage safety, Methicillin-resistant *Staphylococcus aureus*

Core tip: Phage therapy is widely being reconsidered as an alternative to antibiotics. The use of naturally-occurring phages to treat bacterial infection has a contentious history in western medicine. However, the emergent landscape of phage-based antimicrobials has advanced well beyond traditional methods. In this rapidly evolving field, novel technologies such as bioengineered chimeras of phage-derived lytic proteins show potential as a new class of antibacterial pharmaceuticals. This review aims to provide a topical perspective on the historical context of phage therapy, in order to highlight modern advances in phage research and innovations in the field.

INTRODUCTION

Almost a decade before the discovery of penicillin, the controversial practice of phage therapy was being developed as a treatment for bacterial infections. Phages, short for bacteriophages, are bacteria-specific viruses that have been used as a treatment against pathogens such as *Shigella dysenteriae* as early as 1919[1]. With an estimated 10^{31}-10^{32} phages in the world at any given time[2], they make up the most abundant biological entity on Earth and play a crucial role in regulating bacterial populations; phages are responsible for the death of approximately 20%-40% of all marine surface bacteria every 24 h[3]. Much of the controversy surrounding phage therapy was due to poor documentation of use and variable success. The complications in implementing phage therapy stemmed from how little was known about phages at the time of their discovery. In fact, the nature of their existence was a topic of contention until they were visualized in the 1940's after the invention of electron microscopy[4]. A number of logistical and technical obstacles in developing phage therapy led to its widespread abandonment after the discovery of antibiotics.

The advent of pharmaceutical antibiotics in the mid-20^{th} century, along with a better understanding of disease and sanitation, revolutionized healthcare and drastically improved both quality of life and life expectancy in the industrialized world. In 1900, life expectancy for men and women in the United States was 46 and 48, respectively, and the major causes of death were infectious diseases, many of which were bacterial (*e.g.*, cholera, diphtheria, typhoid fever, plague, tuberculosis, typhus, scarlet fever, pertussis, and syphilis)[5]. Antibiotics helped usher in a new era in medicine, rapidly becoming an indispensable medical tool with 262.5 million treatment courses prescribed in the United States in 2011 alone (842 prescriptions per 1000 persons) and an estimated 100000-200000 tons of antibiotics used globally between medicine, agriculture, and horticulture each year[6,7]. Antibiotic

resistance genes encoding for bacterial resistance to common antibiotics, including β-lactams, aminoglycosides, chloramphenicols, and tetracycline, are posing a major threat to current medical treatment of common diseases, and these genes now appear to be abundant in the environment[8]. The spread of antibiotic resistance genes carries a unique danger in that many antibiotics have diminishing efficacy against common infections, particularly the difficult-to-treat nosocomial infections caused by the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.).

Admonitions of a return to "the pre-antibiotic era" have become increasingly common and regulatory organizations such as the Centers for Disease Control (CDC) and WHO have declared antibiotic resistance a threat to global health[9,10]. The CDC estimates antibiotic-resistant infections result in 2 million illnesses and at least 23000 deaths a year, with many more dying from conditions complicated by antibioticresistant infections, costing the United States $55 billion annually[7]. According to the United Kingdom government's 2016 Review on Antimicrobial Resistance, an estimated 700000 people die each year globally from resistant infections with a projected cost of $100 trillion and a death toll of 10 million by 2050[7]. In the United States, methicillin-resistant *S. aureus* (MRSA) infections alone account for more deaths than HIV/AIDS and tuberculosis combined[11]. Since the discovery of antibiotics, there has been a steady stream of novel antibacterial pharmaceuticals in what has been dubbed the "antibiotic pipeline". However, due to the rate at which bacteria evolve resistance to antibiotics, there has been less commercial interest in the research and development of novel compounds. In the years of 1983-1987, there were 16 new pharmaceutical antibiotics approved by the Food and Drug Administration (FDA) for use in the United States, this number has steadily trended downwards and between 2010-2016 only 6 new antibiotics were approved[12]. At the end of the antibiotic pipeline is the carbapenem class of antibiotics, often reserved as the "last resort" due to their adverse effects on health. Beginning in 2000, the incidence of carbapenem-resistant, hospital-acquired *K. pneumoniae* infections began to increase in the United States; due to the lack of treatment options, these infections are associated with a 40%-50% mortality rate[13]. Reaching the end of the antibiotic pipeline could signal a shift in the global culture of infectious disease treatment and some claim is the imminent return to a pre-antibiotic era of medicine.

On September 21, 2016, the United Nations General Assembly convened to discuss the problem of antibiotic resistance and deemed it "the greatest and most urgent global risk"[14]. In the hunt for alternative strategies for prophylaxis and control of bacterial infection, one of the more popular suggestions involves revisiting the practice of phage therapy. Proponents of phage therapy tout several major advantages that phages have over antibiotics such as host-specificity, self-

amplification, biofilm degradation, and low toxicity to humans[15,16]. Owing to the development of analytical tools capable of studying these small biological entities (approximately 25-200 nm in length), such as next-generation sequencing and electron microscopy, the field of phage biology is only now reaching maturity. These technological advancements have ushered in a renaissance of phage therapy research as indicated by a wave of recent human clinical trials and animal research. To fully evaluate the viability of phage therapy, one must also consider the role of the indigenous gut phageome in human health and disease. However, this complex story is only beginning to unfold and will not be included in this review (for current literature review see Wahida, Ritter and Horz[17] 2016). This review aims at discussing historical use of phage therapy and current research on the feasibility of phage-based infection control with a focus on multidrug-resistant infections.

PHAGE BIOLOGY BASICS

Phages are simple, yet incredibly diverse, non-living biological entities consisting of DNA or RNA enclosed within a protein capsid. As naturally-occurring bacterial parasites, phages are incapable of reproducing independently (*i.e.*, non-living) and are ultimately dependent on a bacterial host for survival. Phages typically bind to specific receptors on the bacterial cell surface, inject their genetic material into the host cell, and then either integrate this material into the bacterial genome (so-called “temperate” phages) and reproduce vertically from mother to daughter cell, or hijack the bacterial replication machinery to produce the next generation of phage progeny and lyse the cell (so-called “lytic” phages). Upon reaching a critical mass of phage progeny, which can be anywhere from a few to over 1000 viral particles, depending on environmental factors, the lytic proteins become active and hydrolyze the peptidoglycan cell wall, releasing novel phage to reinitiate the lytic cycle[18,19].

Most phages are infectious only to the bacteria that carry their complementary receptor, which effectively determines lytic phage host range[20]. Host specificity varies among phages, some of which are strain-specific, whereas others have demonstrated the capability of infection across a range of bacterial strains and even genera[21,22]. Bacteria have evolved numerous mechanisms to resist infection by lytic phages, and phages have an equally impressive diversity of mechanisms for breaking this resistance. For bacteria, this can include alteration or loss of receptors and integration of phage DNA into the clustered regularly interspaced palindromic repeats/CRISPR associated system (CRISPR/Cas) system[23], while for phage this can include recognition of new or altered receptors and anti-CRISPR genes[24]. The most common lytic phages associated with human pathogens and the gut microbiota are in the orders *Caudovirales*, commonly known as “tailed phages” which contain double-stranded DNA genomes, and *Microviridae*, which are tailless, single-

stranded DNA viruses[25,26].

In contrast to lytic phages, lysogenic phages integrate their genetic material into the bacterial chromosome in the form of an endogenous prophage (less commonly phage DNA can remain separate as a plasmid but still be stably transmitted across bacterial generations). The bacterial lysogen then propagates the prophage with each cell division. Environmental stressors on the bacterial host are capable of inducing the lysogenic phage from the latent prophage form, triggering a transition to the lytic cycle and the release of phage progeny into the environment. When incorporating their genetic material into the bacterial genome, prophage-encoded genes become available for transcription by the host. Up to 18 prophages have been found in one bacterial genome, as in the food pathogen *Escherichia coli* (*E. coli*) O157:H7 strain Sakai[27], with prophage-encoded genes comprising up to 20% of bacterial chromosomal content[28]. Prophage genes can be beneficial to the bacterial host and can encode for virulence factors (*e.g*., diphtheria toxin, shiga toxin, and botulinum toxin), metabolic genes, and antibiotic resistance genes (*e.g*., β-lactamases)[29-32]. Phage biologists now recognize that phage lifecycles can fall on a spectrum between lytic and lysogenic with pseudolysogenic, chronic, and cryptic lifecycles as examples of recent classifications[19,33]. Conventional phage therapy relies on strictly lytic phages, which obligately kill their bacterial host. For treatment, lytic phages are compiled into preparations called “phage cocktails” which consist of multiple phages proven to have *in vitro* efficacy against the target pathogen.

HISTORY OF PHAGE THERAPY

Although the idea of using bacterial viruses therapeutically against bacterial infections has recently gained traction in response to the emergence of multidrug-resistant pathogens, the practice has been around for nearly a century. Since the initial observations of phage-induced bacterial lysis, the biological nature of phage, as well as their therapeutic value, has been controversial. Frederick Twort first described the characteristic zone of lysis associated with phage infection in 1915, but it was Felix d’Herelle who identified the source of this phenomenon, attributed the plaques to bacterial viruses, and coined the term “bacteriophage” (literally “bacteria-eater”). It was also d’Herelle who conceived of the idea to use phages therapeutically and is responsible for the first documented clinical use of phage in 1919 at the Hôpital des Enfants-Malades in Paris where phages were successfully used to treat 4 pediatric cases of bacterial dysentery[1]. Despite several successful trials, d’Herelle’s early experiments were notorious for being poorly controlled and his research was vigorously disputed by the scientific community[3]. Nevertheless, d’Herelle continued to pioneer phage therapy with the treatment of dysentery, cholera, and the bubonic plague in the early 20th century with a series of phage therapy centers and commercial phage production plants throughout

Europe and India[34]. One 1931 trial of phage therapy as a treatment for cholera in the Punjab region of India involved a cohort of 118 control subjects and 73 experimental subjects who received phage treatment; d'Herelle observed a 90% reduction in mortality with 74 lethal outcomes in the control group and only 5 in the experimental group[1].

Along with d'Herelle, several other entrepreneurs attempted to commercialize phage production in Brazil and the United States with phage preparations for *Staphylococcus*, *Streptococcus*, *E. coli*, and other bacterial pathogens[34]. These preparations were shipped throughout the world to willing clinicians but treatment was met with mixed success; this lack of reliability, in large part, added to the preference for antibiotics in western medicine[1].

Many mistakes were made during these early trials of phage therapy and most can be attributed to a poor understanding of the biological nature of phages. Rudimentary purification and storage protocols resulted in low titers of active phage and contamination from bacterial antigens, and phages that lacked infectivity for the target bacteria were used for treatment. Furthermore, delivery of phage to the site of infection was confounded by the medical limitations of the day. For example, the role of the patient's innate immune response in removing active phage and diminishing the efficacy of phage therapy was only observed recently as a potentially confounding physiological mechanism[35]. As a result, phage therapy was widely dismissed by most of western medicine after the introduction of pharmaceutical antibiotics in the 1940's. The exception to this is in the former Soviet Union and Eastern Europe where clinical phage therapy has been used extensively to treat antibiotic-resistant infections caused by a range of infectious bacteria such as *Staphylococcus*, *Pseudomonas, Klebsiella*, and *E. coli*[36,37].

PHAGE AGAINST CLINICALLY SIGNIFICANT PATHOGENS

Recent investigations using animal models have explored phage treatment against a range of clinically significant pathogens. When challenged with gut-derived sepsis due to *P. aeruginosa*, oral administration of phage saved 66.7% of mice from mortality compared to 0% in the control group[38]. In a hamster model of *Clostridium difficile* (*C. difficile*)-induced ileocecitis, a single dose of phage concurrent with *C. difficile* administration was sufficient prophylaxis against infection; phage treatments post-infection saved 11 of 12 mice whereas control animals receiving *C. difficile* and clindamycin died within 96 h[39]. Phage combinations also significantly reduced *C. difficile* growth *in vitro* and limited proliferation *in vivo* using a hamster model[40]. Intraperitoneal administration of a single phage strain was sufficient to rescue 100% of mice in bacteremia models using vancomycin-resistant *E. faecium*[41], extended spectrum β-lactamase producing *E. coli*[42], and imipenem-

resistant *P. aeruginosa*[43]. Phage cocktails have also been used to treat antibiotic-resistant *P. aeruginosa* infections of the skin, lungs, and gastrointestinal tract in animal models[38,44]. Additional animal studies show similarly promising results for multidrug-resistant *E. coli* O25:H4-ST131[45], *Vibrio parahaemolyticus*[46], *S. aureus*[44,47], and *A. baumanii*[38]. There is even an indication that phage are capable of restoring antibiotic sensitivity in antibiotic-resistant bacteria, as in the case of multidrug-resistant *P. aeruginosa*[48].

Human trials for phage therapy have taken place for almost a century at several institutes in Eastern Europe, the most famous of which are the Eliava Institute of Bacteriophage and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland. The Eliava Institute has extensively used phage in preclinical and clinical treatment of common bacterial pathogens such as *S. aureus*, *E. coli*, *Streptococcus* spp., *P. aeruginosa*, *Proteus* spp., *S. dysenteriae*, *Salmonella* spp., and *Enterococcus* spp.[49]. Effective applications range from surgical to gastroenterological, both therapeutic and prophylactic. In a six patient case series of antibiotic-unresponsive diabetic foot ulcers, topical application of *S. aureus*-specific phage was sufficient for recovery in all individuals[50]. In a 1938 clinical trial, 219 patients with bacterial dysentery (138 children and 81 adults) were treated solely with a phage cocktail consisting of a variety of phage targeting *Shigella flexneri*, *Shigella shiga*, *E. coli*, *Proteus* spp., *P. aeruginosa*, *Salmonella typhi*, *Salmonella paratyphi* A and B, *Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp.; cocktails were administered both orally and rectally. Within 24 h, 28% of patients with blood in their stools were relieved of this symptom, with a further 27% showing improvement within 2-3 d. Overall, 74% of the 219 patients showed improvement or were completely relieved of symptoms[51]. Additionally, during a 1974 typhoid epidemic, a cohort of 18577 children was enrolled in a prophylactic intervention trial using typhoid phages. Phage administration resulted in a 5-fold decrease in typhoid incidence compared to placebo[49]. The potential for phage therapy has yet to be fully realized since phages tend to be more effective against the target pathogen when used in combination with antibiotics[52], a treatment option that has not yet been investigated in humans.

Currently there are no phage therapy products approved for human use in the EU or United States. However, in the food industry, there are several commercial phage preparations used for biocontrol of bacterial pathogens that are approved by the FDA under the classification of “generally considered as safe.” These preparations are used against *Salmonella* spp., *Listeria monocytogenes*, MRSA, *E. coli* O157:H7, *Mycobacterium tuberculosis*, *Campylobacter* spp., and *Pseudomonas syringae*, among others[53-56]. Phages also have potential value for pathogen detection, an example of which is using bioluminescent reporter phage to detect *Bacillus anthracis*[56]. In 2011 there was an estimated 48 million cases of food poisoning in the United States alone[55]. Evidence suggests that phage

biocontrol can be an effective method for improving food safety at numerous stages in meat production and processing, and also has potential to reduce bacterial contamination in fruits, vegetables, and dairy products[55]. These investigations into phage biocontrol in food production, as well as recent placebo-controlled human trials that demonstrated the safety of oral phage administration[57-60], are gradually beginning to fill the knowledge gap in phage therapy safety. The evidence on phage safety will continue to strengthen with further randomized, double-blind, and placebo-controlled phase I/II clinical trials of phage therapy, such as the one that established both safety and efficacy in treating chronic otitis caused by antibiotic-resistant *P. aeruginosa*[61].

Innovations in the gene editing tool CRISPR/Cas have created novel opportunities for phage therapy. One example of which is the use of bioengineered phage to deliver a CRISPR/Cas programmed to disrupt antibiotic resistance genes and destroy antibiotic resistance plasmids[62]. These phages may be applied to hospital surfaces to reduce frequency and spread of antibiotic resistance genes. The field of bioengineered phages is still in its infancy but will undoubtedly yield many invaluable technologies such as this (Table 1).

Table 1.

Published findings on phage therapy in humans and in animal models

Causative agent	Model	Condition	Oral	Result summary[1]	Ref.
Shigella dysenteriae	Human	Dysentery	Oral	All four treated individuals recovered after 24 h	[1]
Vibrio cholerae	Human	Cholera	Oral	68 of 73 survived in treatment group and only 44 of 118 in control group	[1]
Pseudomonas aeruginosa	Murine	Sepsis	Oral	66.7% reduced mortality	[38]
Clostridium difficile	Hamster	Ileocecitis	Oral	Co-administration with *C. difficile* prevented infection	[39]
	Hamster	Ileocecitis	Oral dose every 8 h for 72 h	92% reduced mortality	[39]
Vancomycin-resistant Enterococcus faecium	Murine	Bacteremia	i.p.	100% reduced mortality	[41]
β-*lactamase producing Escherichia coli*	Murine	Bacteremia	i.p.	100% reduced mortality	[42]
Imipenem- resistant P. aeruginosa	Murine	Bacteremia	i.p.	100% reduced mortality	[43]
Acinetobacter baumannii, P. aeruginosa and *Staphylococcus aureus*	Murine	Sepsis	i.p.	Animals protected against fatal dose of *A. baumannii* and *P. aeruginosa* but not *S. aureus*	[44]

Causative agent	Model	Condition	Oral	Result summary[1]	Ref.
Escherichia coli	Murine	Meningitis and Sepsis	i.p. or s.c.	100% and 50% reduced mortality for meningitis and sepsis, respectively	[45]
MDR Vibrio parahaemolyticus	Murine	Sepsis	i.p. and oral	92% and 84% reduced mortality for *i.p*. and oral routes, respectively	[46]
S. aureus	Rabbit	Wound infection	s.c.	Co-administration with *S. aureus* prevented infection	[47]
MDR S. aureus	Human	Diabetic foot ulcer	Topical	All 6 treated patients recovered	[50]
Unclassified bacterial dysentery	Human	Dysentery	Oral	Phage cocktail improved symptoms of 74% of 219 patients	[51]
Salmonella typhi	Human	Typhoid	Oral	In cohort of 18577 children, phage treatment associated with 5-fold decrease in typhoid incidence compared to placebo	[49]
Antibiotic-resistant *P. aeruginosa*	Human	Chronic Otitis	Oral	Phage treatment safe and symptoms improved in double-blind, placebo-controlled Phase I/II trial	[61]

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[1]Reduced mortality is for phage-treated groups and are relative to 100% mortality in control animals, unless otherwise specified. MDR: Multi-drug-resistant; i.p.: Intraperitoneal

injection; s.c.: Subcutaneous injection.

DEVELOPMENT AND APPLICATION OF PHAGE-DERIVED LYTIC PROTEINS

Among the most promising of advances in phage therapy is the isolation of phage-encoded lytic enzymes, which are functionally similar to the antimicrobial eukaryotic enzyme lysozyme. Genes for phage lytic enzymes are expressed by the bacterial host during the lytic cycle and assist the phage by hydrolyzing the cell wall to release viral progeny. The discovery and analysis of these proteins opens the possibility for the development of novel phage-based pharmaceuticals.

Two major protein classes are employed by the majority of phage species during the lysis of the bacterial host. One of which is the transmembrane protein holin and the other is a peptidoglycan cell wall hydrolase called endolysin (lysin). These two proteins work together in triggering the lysis of the bacterial cell. The holin protein acts as a molecular “clock” in the lytic cycle. During the process of viral assembly within the cytoplasm, holin molecules accrue in the cell membrane. At the end of the lytic cycle the holin proteins trigger an opening on the cytoplasmic side of the cell membrane, allowing the lysin proteins to access and hydrolyze the cell wall[63]. Although both of these enzymes are present across the majority of phage species, there is huge structural and biochemical variability and therefore little conservation between phage species. Each phage can encode for several unique lysin and holin enzymes, some of which are highly specific but others can exhibit broad-spectrum activity between strains and even between species, as in the case of recently discovered lysin ABgp46. ABgp46 has the ability to lyse several gram-negative and multidrug-resistant pathogens, including *A. baumannii*, *P. aeruginosa*, and *Salmonella typhimurium*[64].

Phage lysins alone are capable of bacterial cell lysis, whereas holins are not; therefore lysins have received a lot of attention as potential antimicrobial agents. These proteins are fast acting, potent, and inactive against eukaryotic cells. Lysins have successfully saved mice from bacteremia caused by multidrug-resistant *A. baumannii*[65], *Streptococcus pneumoniae*[66], and MRSA[67], among others[63]. Combining phage lysins and antibiotics may be more effective at eliminating infections than by using antibiotics alone, as demonstrated *in vitro* and *ex vivo* in a colon model using *C. difficile*. [68]. Not all lysins show equal therapeutic potential, however, as demonstrated by Gilmer et al[69] who identified a uniquely potent lysin, PlySs2, which was highly effective against a range of pathogenic *Streptococcus* and *Staphylococcus* species, including MRSA, and was fully functional after 10 freeze-thaws. A single dose administered intraperitoneally to mice in a mixed *S. pyogenes* and MRSA bacteremia model provided a significantly higher survival rate than treatment with 3

previously characterized lysins[69]. A recent study exploring the isolation and application of phage proteins has revealed that lysins are even capable of crossing epithelial cell membranes to eliminate difficult to treat intracellular infections of *S. pyogenes*[70]. Phage lysins can also disrupt vegetative cells as demonstrated with the *B. anthracis* lysin PlyG which is capable of attacking endospores of bacillus, a distinct advantage over antibiotics[71]. Lysins can also be mass produced through common recombinant techniques. The gene for bacteriophage-derived cysteine, histidine-dependent amido hydrolase/peptidase (CHAPK) has been cloned and inserted into *E. coli* to be overexpressed for purification. Not only is the CHAPK lysin highly effective against MRSA, but it can disperse *S. aureus* biofilms[72].

Efforts to optimize lysins through bioengineering have yielded some promising results. Yang et al[73] produced a novel chimeric lysin, by combining the active site of a lysin with a cell wall binding domain, that was capable of saving mice challenged with MRSA bacteremia. Research on chimeric lysin enzymes is still in the early stages, but some of these modified lysins have also been shown to prevent death from *S. pneumoniae* bacteremia[74] and prevent development of methicillin-sensitive *S. aureus* endophthalmitis in a mouse model[75]. Since lysins act by enzymatically cleaving the bacterial cell wall, they are inherently less effective against gram-negative bacteria which have an impermeable lipopolysaccharide outer membrane. In an attempt to broaden lysin activity to target gram-negative pathogens, several researchers have begun to bioengineer artificial lysin molecules, termed Artilysins, that are capable of penetrating the outer membrane. Some of these lysins are created by combining the active site of the lysin enzyme with lipopolysaccharide-destabilizing peptides which allows the molecule to penetrate the outer membrane. So far Artilysins have been shown to decolonize *P. aeruginosa* in a nematode gut model and protect human keratinocytes when challenged with *A. baumannii*[76].

Adding to the appeal of lysins as antibacterial agents, it is widely considered to be unlikely that bacteria will evolve resistance to lysins due to the fact that they target sites on the peptidoglycan cell wall critical for bacterial viability[63]. Engineered recombinant phage lytic proteins would be far easier to mass produce and administer than preparations of actual phage, which can be limited by a short shelf life, removal by the reticuloendothelial system of the host, and the potential for generating neutralizing antibodies[35]. Future potential for phage lysin application includes combination therapy of lysins in conjunction with antibiotics, which has been shown to be more effective than antibiotics or lysins alone against pathogens such as MRSA and *C. difficile* in mice[77-79] (Table 2).

Table 2.

Recently published findings on phage lytic enzymes

	Lytic enzyme	Model	Target pathogens	Result summary	Ref.
Phage-derived lysins	ABgp46	*In vitro*	MDR *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium*	Cross-inoculation significantly reduced bacterial density	[64]
	PlyF307	Murine	MDR *A. baumannii*	*i.p*. treatment rescued mice from lethal bacteremia	[65]
	Cpl-1	Murine	*Streptococcus pneumoniae*	*i.p*. treatment rescued mice from lethal pneumonia	[66]
	Cocktail of 6 distinct lysins	*In vitro* and murine *in vivo*	MRSA	Effective against biofilms *in vitro* and protected mice from lethal sepsis	[67]
	PlyCD	*In vitro* and *ex vivo*	*Clostridium difficile*	Reduced *C. difficile* colonization	[68]
	PlySs2	Murine	*Streptococcus pyogenes* and MRSA	i.p. treatment reduced mortality from lethal bacteremia	[69]
	PlyG	*In vitro*	*Bacillus anthracis*	Eliminated *B. anthracis* spores and vegetative cells	[71]

	Lytic enzyme	Model	Target pathogens	Result summary	Ref.
Bioengineered chimeric lysins	CHAPK	*In vitro*	MRSA	Eliminated MRSA and dispersed biofilms	[72]
	ClyH	Murine	MRSA	Treatment rescued mice from bacteremia	[73]
	Cpl-711	Murine	*S. pneumoniae*	Treatment rescued mice from bacteremia	[74]
	Ply187	Murine	*Staphylococcus aureus*	Prevented bacterial endophthalmitis	[75]
	Artilysins	Nematode gut	*P. aeruginosa*	Decolonized *P. aeruginosa* from gut	[76]
		Human keratinocytes	*A. baumannii*	Protected cells from bacterial challenge	[76]
Lysin and antibiotic combination therapy	CF-301	Murine	MRSA	Lysin treatment was most effective when combined with vancomycin or daptomycin	[77]
	MR-10	Murine	Burn wound infection	Lysin treatment was most effective when combined with minocycline	[78]

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MDR: Multi-drug-resistant; *i.p.*: Intraperitoneal injection; MRSA: Methicillin-resistant *S. aureus*.

PHAGE THERAPY *VS* ANTIBIOTIC THERAPY

Both antibiotics and phages function as antibacterials that disrupt bacterial colonies through lysis or inhibition, yet several key differences make each antibacterial more or less appropriate depending on the situation.

Safety

Adverse reactions to antibiotics are well documented and include instances of anaphylaxis, nephrotoxicity, cardiotoxicity, hepatotoxicity, and neurotoxicity, as well as a number of gastrointestinal and hematological complications[80]. The majority of adverse reactions; in these rare instances the anaphylaxis is associated with specific classes of antibiotics or is the product of high tissue concentrations[81-83]. In contrast to the comprehensive literature on antibiotic safety, phage therapy has only recently gained attention by western medicine and, as a result, much of the available information on phage safety is new. Although oral phage administration is generally considered to be safe[57-60], a major consideration for phage therapy is the translocation of phage across the intestinal epithelium where they subsequently circulate within the blood[84]. Some data show that phage translocation may benefit the host by downregulating the immune response to indigenous gut microbe antigens through the inhibition of interleukin-2, tumor necrosis factor, and interferon gamma production[84]. Other studies discovered a host innate immune response aimed at removing phage after administration in mice[35,85]. While the pros of phage therapy likely outweigh the cons in non-immunocompromised patients, the immunological response to phage may be indicative of the potential for an adverse reaction in immunocompromised patients, which could hypothetically worsen a patient's condition. There is currently no consensus on this possibility as other researchers argue it is unlikely phage therapy will elicit such an adverse reaction in immunocompromised patients[86].

Additional complications include the possibility that phage cocktails induce a state of intestinal barrier dysfunction, otherwise known as “leaky gut”. Tetz and Tetz used a mouse model to demonstrate that oral administration of a commercial Russian phage cocktail was capable of increasing intestinal permeability and elevating serum levels of inflammatory circulating immune complexes in the blood, which are associated with a number of pathological conditions[87]. However, another study observed no significant increase in cytokine levels in response to phage treatment[88]. The potential for phage therapy to disrupt normal intestinal barrier function would have serious implications for several disorders recently linked to intestinal barrier dysfunction such as Crohn’s disease, inflammatory bowel disease, and type 1 diabetes[87]. Pincus et al[89] found that the

inflammatory response to phage was dependent on site of infection. Clearly, many of the safety concerns with phage therapy still need to be addressed. It is likely that the physiological response to phages also differs between individuals and is dependent on the specific phage strains used. To determine the safety of phage treatments in regards to human health, future investigations will need to focus on human clinical trials as much of the current research on the immunological response to phage is limited to animal models.

Specificity

In stark contrast to antibiotics, phages tend to be specific towards both species and strain. In certain situations this can be a major advantage, considering the well-documented, collateral effects of broad-spectrum antibiotics on commensal gut microbes, which are notorious for secondary outcomes such as antibiotic-associated diarrhea and *C. difficile* infection[90]. Other consequences of antibiotic perturbations in the gut microbial community include risk of asthma, obesity, and diabetes[91-93]. The current understanding of collateral damage due to phage therapy is limited, but, compared to antibiotics, phage therapy has been reported to result in less perturbation of the gut microbiome while still effectively reducing gut carriage of pathogens such as *Shigella sonnei* and uropathogenic *E. coli*[94,95].

While strain and species specificity of antibacterial compounds offers many advantages, it comes with a number of inherent constraints. By targeting a single pathogen, phage therapy could be less effective against infections such as infected burn wounds, which are often colonized by more than one strain of bacteria[96]. This can be accounted for by creating phage cocktails infective against a range of known pathogens, but the success of this approach depends on knowledge of which pathogens are being treated. Logistically, host specificity significantly impacts treatment development and testing, and also limits the possibility of large-scale production and distribution, a distinct advantage of broad-spectrum antibiotics. Bourdin et al[15] cross-inoculated phages from 2 distinct geographic regions (Mexico and Bangladesh) against diarrhea-associated *E. coli* from the same regions and found that phage showed high strain specificity to the *E. coli* of their indigenous region. In a randomized clinical trial, Sarker et al[60] administered a commonly used Russian *E. coli* phage cocktail to a cohort of 120 Bangladeshi children with microbiologically-proven enterotoxogenic *E. coli* diarrhea. No improvement of clinical outcome was observed in patients receiving the phage cocktail compared to placebo[60]. These findings are in line with the *in vitro* work that suggests phage cocktails are better adapted to local bacterial populations[15], and bacterial host range can be restricted both spatially and temporally[97]. In an *in vitro* cross-inoculation of a phage cocktail against shiga toxin-producing *E. coli* O157:H7, lysis occurred in

isolates of both human and bovine origin, which suggests a potential for regional phage cocktails for both clinical and agricultural settings[98]. Latz et al[99] found that phages targeting antibiotic-resistant bacteria are more likely to be found within the environment of the infected patient, which, in this case, was the hospital effluent where the antibiotic-resistant bacteria were isolated. Regional specificity may therefore be advantageous when looking for phages that target specific bacterial strains.

Regional specificity may be helpful in finding phages with the greatest infectivity towards the target pathogen, this would especially benefit regions with limited access to antibiotics. Together, the mounting evidence for the local adaptivity of phage suggests that regulatory pipelines must also be rapidly adaptable (*i.e*., allowing for the replacement or addition of phages into cocktails without requiring further clinical trials) for phage therapy to work on a global scale.

Biofilm penetration

Antibiotic therapy is highly effective with planktonic bacteria, such as *V. cholerae* and *Yersinia pestis*, yet is limited in treating biofilm-based bacterial infection[100]. Phages, however, are equipped with enzymes (*e.g*., EPS depolymerase) on the exterior of the capsid that degrade the extracellular polymeric substances (EPS) and disperse bacterial biofilms, allowing the phage to access bacteria embedded within the EPS matrix[83]. The phage progeny released upon completion of the lytic cycle propagate the dispersal of the biofilm through the removal of biofilm-embedded bacteria in subsequent layers[83,101]. In order to penetrate dense biofilms, high doses of antibiotics are typically required to observe any inhibition of bacterial growth, yet complete eradication is rare and regrowth of colonies begins after the end of antibiotic treatments[102,103]. Although low concentrations of many antibiotics are generally considered non-toxic, high concentrations can result in tissue toxicity[83]. Gabisoniya et al[104] at the Eliava Institute of Bacteriophages in Tbilisi, Georgia found that the application of phages on *in vitro* colonies of the pathogen *P. aeruginosa* not only prevented additional biofilm formation by the pathogen but also degraded existing biofilm. Phage treatments have eliminated biofilms formed by *L. monocytogenes, P. aeruginosa*, and *Staphylococcus epidermidis* on the surface of medical devices[22]. These findings are highly relevant to the problem of persistent infections caused by implanted medical devices such as catheters, lenses, and prostheses where biofilm formation is common.

Phage cocktails

Due to the massive diversity of environmental phages, designing a phage cocktail is substantially

more complicated than designing a regimen for combination antibiotic therapy. Composition of the phage cocktail is critical for the success of phage therapy. Factors in the construction of a phage cocktail are beyond the scope of this review and have been thoroughly discussed elsewhere[105], but one of the major logistical challenges is whether to approach phage therapy with a standardized or a customized cocktail. Customizing phage cocktails to each infection is time consuming and costly but, on the other end of the spectrum, a "one-size-fits-all" approach may not provide the strain specificity required for favorable clinical outcomes[105]. Other considerations are the collateral effects of phages on the indigenous microbiota, a topic that has not yet been fully explored[88,94,95]. In cocktail design, one must also take into account phage lifecycle. Lysogenic phages appear to be very common in the indigenous gut microbiota, with prophages comprising the majority of the gut virome[25]. Some therapeutically promising lysogenic phages effectively silence virulence genes in pathogenic bacteria or provide genes for short chain fatty acid metabolism, whereas other lysogenic phages supplement genes for virulence and antibiotic resistance[29,30,106].

Antibiotic resistance genes have been collected from the phage fraction of DNA in wastewater and have been reported to persist longer in phages than in bacteria[107]. Antibiotic resistance genes are also present in the phage fraction of human fecal samples and antibiotic treatment in mice enriches the abundance of phage-encoded antibiotic resistance genes, indicating a possible role for phages as a reservoir for antibiotic resistance genes[30-32]. The hypothetical potential for lysogenic phages to complicate existing infections through the horizontal transfer of antibiotic resistance genes to infectious bacteria largely excludes them from consideration for most phage cocktails. Yet, Regeimbal et al[106] demonstrated the possibility for an innovative application of lysogenic phages by designing an "intelligent" 5 phage cocktail that eliminated *A. baumannii* skin wound infection in a mouse model. This intelligent phage cocktail was composed of 4 phages that were incapable of lysing the *A. baumannii* host and 1 phage that only inhibited growth *in vitro*. The growth-inhibiting phage targeted capsulated *A. baumannii*, selecting for the loss of the capsule. The removal of the capsule, a known virulence factor, decreased the virulence of the bacterium and made it susceptible to lysis from the 4 additional phages[106]. This type of cocktail design represents the beginning of novel treatment options for eliminating bacterial infections that are resistant to conventional treatment. Lysogenic phages have many intriguing properties that may be useful for this type of *in situ* manipulation of individual bacterium, and potentially the human gut microbiome metagenome[108], but first much more needs to be known about the role of lysogenic phages in the human gut phageome for this to be done safely and effectively.

CONCLUSION

The available literature on the use of phages and phage-derived proteins for combating bacterial infections, specifically those of multidrug-resistant bacteria, increasingly shows promise for the prospect of phage therapy as either an alternative or a supplement to antibiotics. However, discrepancies in recent findings on the immunomodulatory effects, the host range, and the potential for horizontal gene transfer make it abundantly clear that we need a better understanding of the interaction between phage, microbiome, and human host before implementing phage therapy on a large scale. Phage lysins may thus be a much more practical therapeutic tool for their decreased immunological potential, among other reasons such as ease of production, purification, and storage. Despite the promising preliminary findings on phage and phage-derived lytic proteins, it is more than likely that no panacea for antibiotic-resistant infections will arise. The increased efficacy of antibacterial agents when used in conjunction implies that therapy using some combination of phage, phage-derived lytic proteins, bioengineered phage, and/or antibiotics will be necessary for addressing the growing problem of antibiotic-resistant infections.

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