HUMAN VACCINES & IMMUNOTHERAPEUTICS
2023, VOL. 19, NO. 1, 2175519
https://doi.org/10.1080/21645515.2023.2175519

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REVIEW

The applications of animal models in phage therapy: An update

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ABSTRACT

The rapid increase in antibiotic resistance presents a dire situation necessitating the need for alternative therapeutic agents. Among the current alternative therapies, phage therapy (PT) is promising. This review extensively summarizes preclinical PT approaches in various *in-vivo* models. PT has been evaluated in several recent clinical trials. However, there are still several unanswered concerns due to a lack of appropriate regulation and pharmacokinetic data regarding the application of phages in human therapeutic procedures. In this review, we also presented the current state of PT and considered how animal models can be used to adapt these therapies for humans. The development of realistic solutions to circumvent these constraints is critical for advancing this technology.

ARTICLE HISTORY
Received 25 August 2022
Revised 24 January 2023
Accepted 25 January 2023

KEYWORDS
Phage therapy; animal infection model; antimicrobial resistance; ESKAPE Pathogens; Endolysin

Introduction

Bacteriophages (phages) are natural predators of bacteria that can recognize, attack, and kill bacterial hosts without harming other bacteria or human cells. Since their independent discovery by Frederick W. Twort (1915) and Félix d'Hérelle (1917), bacteriophages have been essential in various microbiological discoveries, especially in microbial genetics. Bacteriophages, the natural predators of bacteria, have recently been discovered to be effective in modern biotechnology. They've been recommended as antibiotic options for numerous antibiotic-resistant bacterial strains. Phages can potentially be exploited as biocontrol agents in agriculture and the petroleum sector.

Furthermore, phages are employed as vehicles for DNA and protein vaccines, detecting pathogenic bacterial strains, and as a display system for numerous proteins and antibodies.[1] Phages have played an important role in understanding several essential principles in molecular biology since their discovery in the early twentieth century. They were crucial model organisms in searching for the physical nature and function of gene, beginning with Max Delbrück's establishment of the American Phage Working Group and extending to the explication of Francis Crick's central dogma of molecular biology through studies of RNA transcription and protein expression in phage. Phages have been widely used in biotechnology and illuminating fundamental molecular biology concepts. Phage biology provides a wealth of recombinant DNA technologies, clinical diagnostics, and synthetic biology techniques.[2]

Antimicrobial resistance and phage therapy

In 2017, the World Health Organization (WHO) issued a list of 12 antibiotic-resistant priority pathogenic bacteria that threaten human health and need immediate attention. These bacteria, including *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (collectively termed ESKAPE), can cause life-threatening diseases. The absence of effective antibiotics makes public health threat causing exacerbating healthcare problems. Alternative strategies to conventional treatment are needed to curb the global threat of antibiotic resistance.[3–6] One potential alternative strategy is using bacteriophages to prevent and treat antibiotic-resistant pathogens, owing to their high specificity and killing ability.[7] Bacteriophages are the natural enemies of bacteria that can specifically infect and kill the host bacteria. To date, no adverse

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effects of bacteriophages have been reported, making them a safer therapeutic option for clinical use. The safety of phage therapy justifies its use in treating several bacterial infections.[8,9]

The increasing multidrug-resistant bacterial infections have spurred interest in phage therapy among physicians and scientists, and some pharma companies are also involved in phage therapy research. Phage therapy centers and recent clinical trials have produced positive results in treating patients, such as the Eliava institute in the Republic of Georgia, Military Hospitals in Belgium, and Hospitals in Wroclaw, Russia, Novosibirsk, and Poland.

Bacteriophage treatment may influence the immune-inflammatory response to bacterial infection, reduction in C-reactive protein level, and leukocyte counts, with a similar propensity of the erythrocyte-sedimentation rate that can be one of the most promising aspects of phage therapy. Unlike antibiotics, in phage therapy, repeated doses of bacteriophages are not required because bacteriophages remain in the body for longer, and their multiplication depends on the host bacterium. Notably, fewer doses of bacteriophages are needed because the number of bacteriophages increases at the infection site due to their proliferation in the bacterial host. From an immunological point of view, phage therapy is safer because bacteriophages surround our ecosystem; therefore, humans have routine exposure to bacteriophages in day-to-day life. Though bacteriophages are used as therapeutic agents in different parts of the world without any reported adverse effects, the safety of phage therapy applications needs to be evaluated.[10–12] In numerous animal studies, phage therapy is secure.[13–15] Uchiyama et al. found that administering mice with repeated intraperitoneal phage injections seven times per day every four days for two months had no noticeable clinical effects.[13] Mice were intraperitoneally injected with phages, and neither the mice's health nor the main organs of the mice displayed any abnormal histological changes.[16] These results confirm the safety of phages.[17–20]

Another main concern about the safety and efficacy of phage therapy is the innate and adaptive immune response against bacteriophages. Although there are no reports of life-threatening anaphylaxis (immune) reactions during phage therapy treatment, it has been reported that immune responses could neutralize bacteriophages by producing antibodies against them. However, bacteriophage neutralization does not mean there will be a complete treatment failure. With the available sequencing technologies, bacteriophage selection and genetically-modified bacteriophages can tackle these problems. Moreover, tailored delivery systems such as lipid vesicles and nanoparticles can help bacteriophages to be sustainably released in the *in-vivo* environment without eliciting any immune response and neutralizing antibodies.[21–27]

For therapeutic applications, tailed bacteriophages (dsDNA viruses) are preferred and confined to a single order of *Caudovirales*. These tailed bacteriophages are diverse and are the most abundant viruses in nature. As per the recent International Committee on Taxonomy of Viruses (ICTV) classification, the order *Caudovirales* are further divided into 14 families, 73 subfamilies, 927 genera, and 2,814 species (https://talk.ictvonline.org/taxonomy/). All these tailed phages have a head or capsid with a dsDNA molecule; a tail with or without tail fibers. During bacterial contact, the phage makes specific contacts with surface receptors using the tail or tail fibers or both.

Bacteriophage interventions in treating Gram-positive infections

Gram-positive bacteria such as staphylococci (MRSA; Methicillin-Resistant *Staphylococcus aureus*), streptococci (DRSP; Drug-Resistant *Streptococcus pneumoniae*), and enterococci (VRE; Vancomycin-Resistant Enterococci) are prevalent and cause life-threatening infections in humans. The diseases caused by these bacteria range from mild to severe such as food poisoning to skin and soft tissue infections, endocarditis, pneumonia, osteomyelitis, sepsis, and septic shock. Some other diseases include hospital-acquired infections caused by *S. aureus*, ventilator-associated pneumonia caused by *S. aureus*, community-acquired pneumonia caused by *S. pneumoniae*, catheter-related infections, and urinary tract infections caused by enterococci. Given their clinical importance, antibiotic resistance among Gram-positive bacteria is worrisome. Thus, using phages to treat infections of these bacteria is a welcome intervention. Interestingly, phages targeting many Gram-positive bacteria have been isolated. Some of these are discussed below.

Staphylococcus bacteriophages

Studies on *Staphylococcus*-infecting bacteriophages started in the 1970s, but the in-depth molecular characterization began in the early 21st century. The increasing *Staphylococcus* phage studies can be attributed to the emerging staphylococcal infections that are antibiotic-resistant, in which case phage therapy is an immediate alternative. Most of the characterized *Staphylococcus* phages belong to the *Siphoviridae* family, and most of the characterized lytic phages have mutations in their lysogenic gene. As staphylococcal strains or variants are varied, a bacteriophage cocktail with broad infectivity is favorable for applications. Bacteriophage cocktails have no reported adverse effects when administered orally, topically, intranasally, intravenously, or subcutaneously. Interestingly, it has been found that many *Staphylococcus* phages can exhibit broad-host-range activity, infecting at least 20 strains.[28–30] A study by Peng et al. 2019 showed that phages ϕMR001 and ϕSA012 could infect 101/104 and 76/104 healthcare- and community-associated MRSA strains, respectively. A study by Kishor et al.[31] showed that a phage cocktail containing seven bacteriophages at 10^{12} PFU/ml could cure osteomyelitis in rabbits. They further reported that in the chronic group periosteal reactions, arthritis persisted, but the wound was healed, and the infection site was sterile, as shown by radiological features. A study by Leiman et al.[32] showed that AB-SA01, a *Staphylococcus* phage cocktail containing three *Myoviridae* phages, could kill 94.5% of 401 clinical *S. aureus in vitro*. *In vivo* studies in mice showed that AB-SA01 had efficacy in curing acute lung infections, and no phage-resistant mutants were observed. The same AB-SA01 bacteriophage cocktail was used in human trials (first-in-humans, phase 1) by Ooi et al.,[19] in which the safety and tolerability were tested in 9 patients by administering intranasally. The nine patients with recalcitrant chronic rhino sinusitis responded well to the treatment, and AB-SA01 was found to be safe up to doses of 3×10^9 PFU for 14 days, at which time 2/9 patients had eradicated *S. aureus* infection. In another study, *Staphylococcus* phages vB_Sau_Clo6, vB_Sau_CG, and K were found to infect

88%, 96%, and 86% of MRSA (n = 47) strains tested, respectively.[33] *Staphylococcus* bacteriophage preparations are commercially available for treatment, including (I) AB-SA01 by AmpliPhi Biosciences Corp. The US, (II) Staphylococcal bacteriophage by Eliava Institute, Georgia, (III) PhagoBurn and Phosa by Pherecydes pharma, France.[19–36]

Streptococcus bacteriophages

Streptococcus phages are one of the least studied bacteriophages, only representing about 5% of phage genomes in the NCBI nucleotide database. Though the number of studies performed using animal models is few, we will review some of the characterized *Streptococcus* bacteriophages. The two pneumophages Dp-1 and Cp-1 are one of the first lytic phages isolated against *S. pneumoniae* that belongs to *Siphoviridae* and *Podoviridae*, respectively. The *pneumoniae* phage MS1 was isolated from the upper respiratory tract of the infected patient, and it was found to be closely related to Dp-1. The lytic bacteriophage A25 is a well-studied bacteriophage isolated against *S. pyogenes*. The novel bacteriophage, J × 01, was found to infect *S. agalactiae* and belongs to the family *Siphoviridae*. The phage M102AD was found to infect *S mutants* which belong to *Siphoviridae* and are closely related M102 phage. All the known *Streptococcus thermophilus* bacteriophages belong to the *Siphoviridae* family, but their applications are still under exploration. There is a void in *Streptococcus* phage research, and the number of animal studies is also scarce. Considering the infections caused by Streptococcal species in humans, there is a scope for future bacteriophage therapy research.[37–42]

Enterococcus bacteriophages

The enterococcal phages are mostly bound within the two species, *Enterococcus faecalis* and *E. faecium*. The characterized lytic bacteriophages infecting *E. faecalis*, such as ϕEF24C (*Myoviridae*), EFRM31 (*Siphoviridae*), and EFAP1 (*Siphoviridae*), are known to have a short lifecycle and *in-vitro* efficacy. The siphovirus vB_EfaS_AL3 infects *E. faecalis* with a genome of about 40kb. The *Enterococcus* bacteriophage, vB_EfaS_HEf13 infecting *E. faecalis*, was found to reduce the bacterial load in the human dentin *ex vivo* infection model. The efficacy of two Enterococci bacteriophages, vB_EfaS-Zip infecting *E. faecium* and vB_EfaP-Max infecting *E. faecalis*, was tested in *in vitro* collagen wound model (CWM), and it was found that the phage cocktail was effective in removing multi-species biofilms. In another study, a bacteriophage cocktail (EFDG1 AND EFDG1^{r}) produced an additive effect against VRE *E. faecalis* strain. As the root canal treatment is getting tedious due to VRE *E. faecalis* infections, these enterococcal phages such as EFDG1, phiEF24C, IME-EF1, and EFLK1 can be used alone or as cocktails to prevent recurrent *E. faecalis* infections.[43–47]

Bacillus and Listeria bacteriophages

Other bacteriophages infecting Gram-positive bacteria include *Bacillus* bacteriophages and *Listeria* bacteriophages. The characterized *Bacillus* bacteriophage phi29 is one of the smallest known dsDNA podovirus. The three bacteriophages, Negev_SA, Carmel_SA, and Tavor_SA, have been shown to be highly effective against *B. anthracis*. A *Myoviridae* phage vB_BceM-HSE3 was found to reduce *B. cereus* infections in *in-vitro* models. A *Listeria* phage P100 was found to control *Listeria monocytogenes* contamination in food products. There is excellent potential for bacteriophage therapy as a therapeutic candidate against Gram-positive bacterial infections. The growing antibiotic crisis has caused the opening of phage therapy, as the drug-resistant infections caused by MRSA, DRSP, and VRE are life-threatening.[48,49] Table 1

Table 1. Animal studies to evaluate bacteriophages' efficacy in treating Gram-positive bacterial infections.

Target bacteria	Bacteriophage preparation	Model organism	Outcome	References
S. aureus	Five *Myoviridae* bacteriophages at 10^9 PFU/ml	Peri-prosthetic joint infections in rats	• Decreased inflammation within joints when treated with bacteriophage and vancomycin	50
S. aureus	Two *Myoviridae* bacteriophages and encapsulated	Soft-tissue infections in rats	• The transfer of some-entrapped bacteriophage cocktails saved all the infected animals	51
S. aureus	One *Myoviridae* and one *Podoviridae* bacteriophage at 10^9 PFU/ml	Mastitis in mice	• The highest intramammary phage titer was achieved without spreading systematically	52
S. aureus	Two bacteriophages at 10^9 PFU/ml	Abscesses in mice	• Reduced the bacterial load and weight of abscesses	53
S. aureus	Seven bacteriophages at 10^{12} PFU/ml	Acute and chronic osteomyelitis in rabbits	• A bacteriological cure was observed with no side effects in six weeks	31
S. aureus	Three *Myoviridae* phages (AB-SA01) at 10^8 PFU/ml	Mice	• Phages were safe and well-tolerated	35
S. aureus	One *Siphoviridae* bacteriophage at MOI of 0.1	Bacteremia in mice	• Intraperitoneal administration of phages saved mice from bacteremia	54
S. aureus	One *Myoviridae* bacteriophage at 10^{10} PFU/ml	Mice	• Dose-dependent recovery of mice from lethal bacterial infections	55
S. aureus	One phage at 10^{10} PFU/ml	Septicemia in mice	• Intranasal application rescued the infected mice	56
S. pneumoniae	Phage SP-SQ1 at 10^9 PFU/ml	Pneumococcal infections in mice	• Treated mice were recovered after 48h of treatment	57
E. faecalis	Two bacteriophages (poloxamer) at 10^9 PFU/ml	Root canal infections in rat	• A 99% reduction in bacterial load was observed	58
E. faecalis	One bacteriophage at 10^{10} PFU/ml	Human dentin *ex vivo* model	• Effective bacteriophage activity was noted	45
E. faecalis	Two bacteriophages at 10^8 PFU/ml	Peritonitis mice model	• Single phage cocktail treatment was enough to eliminate 100% of mortality	59

describes the recent bacteriophage-based preclinical studies performed to treat Gram-positive bacterial infections.

Bacteriophage interventions in treating Gram-negative infections

Most common pathogenic Gram-negative bacteria include *E. coli*, *K. pneumoniae*, *E. cloacae*, other Enterobacterials, *P. aeruginosa* and *A. baumannii*, which can cause deadly infections and mortality in humans. These bacteria cause severe local and systemic infections such as urinary tract infections, bacteremia, sepsis, bloodstream infections, respiratory tract infections, and other life-threatening nosocomial infections. The use of antibiotics in the treatment of Gram-negative infections is failing because of the intrinsic and acquired resistance toward commonly used antibiotics. The common antibiotic-resistance bacteria are carbapenem-resistant *Klebsiella*, carbapenem-resistant *Acinetobacter*, colistin-resistant *E. coli* and multidrug-resistant *Pseudomonas*.[60] To overcome the bacterial resistance problem, alternative therapies are being prescribed, mainly phage therapy and a combination of bacteriophages and antibiotics. Several animal studies have been performed to establish the potential of bacteriophages in treating infections caused by Gram-negative pathogens.

Pseudomonas bacteriophages

Controlled phage therapy can cure drug-resistant infections in the post-antibiotic era. A study performed by Shiley et al. using an *in vitro* human lung model (*Pseudomonas* lung infection) showed antimicrobial activity. It increased interleukin 6 (IL-6) and tumor necrosis factor (TNF) production, demonstrating the antimicrobial efficacy as well as the immunological response of the phage therapy.[61] *P. aeruginosa* is known to cause infections by forming biofilms. It has been proven that some phages can penetrate pseudomonal biofilms, which is one of the advantages of conventional therapy.[62–64] A single dose of a virulent bacteriophage vB PaeP-SaPL rescues bacteremic mice infected with multidrug-resistant *P. aeruginosa*.[65] In mice, the lung infection model of *P. aeruginosa* study performed by Pabary et al. showed that phages were effective in controlling the spreading of infection and can also decrease the bacterial load post-treatment.[66] The combination of antibiotic-phage therapy was used to cure the relapsing periprosthetic joint infection of the knee and chronic osteomyelitis caused by MDR *P. aeruginosa*. The eradication of *Pseudomonas* infection was observed with no side effects.[67] When phage OMKO1 was used with ceftazidime to treat a chronic *P. aeruginosa* infection of an aortic Dacron graft, a single application appeared to resolve the infection with no signs of recurrence. Other recent studies also demonstrate that bacteriophages are excellent candidates for treating *P. aeruginosa* infections.[67–69]

Acinetobacter bacteriophages

As an ESKAPE pathogen, nosocomial infections caused by MDR *Acinetobacter* are tedious to cure in healthcare settings. Animal studies in wound infection models of *A. baumannii* showed that phages, either alone or in cocktails, could reduce the bacterial load and recovery of mice/rats even with a single dose.[70,71] The mice lung infection model of carbapenem-resistant *A. baumannii* found that after intranasal injection of bacteriophages, the mouse recovered from lethal *A. baumannii* infections.[72] A study by Zhou et al. showed that two *Myoviridae* phages could recover the larvae (*G. mellonella*) from infections caused by carbapenem-resistant *A. baumannii*.[73] *Acinetobacter* phage Abp1 was found to recover infected mice from pan-drug resistant AB, and the cytotoxicity studies showed no detectable toxicity in HeLa or THP-1 cells.[74] Human phage therapy trials successfully treated *A. baumannii* pancreatic pseudocyst infection, indicating that the intravenous administration of phages could recover patients from *A. baumannii* infections.[75]

Other Enterobacteriaceae phages

Enterobacteriaceae includes the deadliest pathogens, and the WHO priority list contains both *E. coli* and *K. pneumoniae* at the highest rank among antibiotic-resistant bacteria. Studies showed that a single dose of bacteriophage could cure *K. pneumoniae* in a murine burn wound infection model.[76] The phage cocktail prepared using three phages infecting *E. coli*, *K. pneumoniae*, and *Enterobacter* was found to cure multiple bacterial infections in a *Galleria* larvae model.[77] In the mice lung infection model of *K. pneumoniae*, Cao et al. demonstrated that the administration of bacteriophages intranasally could decrease the bacterial load in the lungs and increase the survival of mice in a dose-dependent manner.[78] Dufour et al. conducted a study in a mouse infection model of *E. coli* and administered ceftriaxone and phages separately after a two-hour post-bacterial injection. It was found that the phage-treated mice showed a 100% survival rate and demonstrated that bacteriophages have better efficacy than antibiotics in reducing the bacterial load and increasing the survival rate.[79] Bacteriophage cocktails have been gaining more attention recently because of their ability to target multiple strains, species, or genera. A recent study using bacteriophages infecting *E. coli*, *K. pneumoniae*, *Haemophilus influenzae*, *P. aeruginosa*, *Citrobacter freundii*, and *Moraxella catarrhalis* showed that the prepared phage cocktails (polyvalent) have the potential to cure bacteremia in mice models. Treating mice with bacteremia after 45 min to 24 h post-infection could recover the mice from infection.[80] Some other bacteriophages infecting Gram-negative bacteria including *Salmonella* phages, *Enterobacter* phages, *Shigella* phages, *Proteus* phages, *Serratia* phages, etc.[77–84] Though phage therapy against Gram-negative bacteria shows promising results, more studies on these bacteriophages would give insights into their therapeutic effects. Table 2 describes the recent phage-based preclinical studies performed for the treatment of Gram-negative bacterial infections.

In vivo animal trials of phage therapy

Nematode (*Caenorhabditis elegans*), common fruit fly (*Drosophila melanogaster*), wax moth (*Galleria mellonella*)

Table 2. Animal studies to evaluate bacteriophages' efficacy in treating Gram-negative bacterial infections.

Target Bacteria	Bacteriophage preparation	Model Organism	Outcome	References
P. aeruginosa	Bacteriophage PEV20, inhalable powder at 2×10^7 PFU/mg	Mouse lung infection model	• Bacterial load in the lungs was decreased in 24 hours, showing the feasibility of a pulmonary delivery	85
P. aeruginosa	Six bacteriophages at MOI of 0.05, 0.1, 1.0	Respiratory lung infection in mice	• The highest MOI reduced the bacterial load within 6 hour	86
	Six bacteriophages at MOI of 25, 8	*G. mellonella*	• Higher phage concentrations proved to be effective, and prevention was established.	86
P. aeruginosa	A bacteriophage KPP12 at 5×10^8 PFU	Murine infection model of keratitis	• Suppression of neutrophil infiltration and bacterial clearance in the infected cornea	87
P. aeruginosa	Two bacteriophages at 1.2×10^9 PFU	Lung infection in Murine model	• Bacteriophages were effective in decreasing both inflammation and bacterial load	66
A. baumannii	One phage at 1.2×10^{10} PFU/ml	Rat wound infection model	• In bacteriophage-treated animals, bacteriophages decreased the number of bacteria in wound infection	70
A. baumannii	Four bacteriophages at 5×10^9 PFU	Mouse wound infection models	• The Bacteriophage cocktail reduced the bacterial load drastically	71
A. baumannii	One bacteriophage at 10^7, 10^8, 10^9 PFU/mouse	Immunocompromised mouse model	• Intravenous injection effectively rescued mice from *Acinetobacter* lung infection	72
A. baumannii	One bacteriophage at 5×10^8 PFU	Mouse local and systemic infection model	• Phage Abp1 effectively reduced bacterial load in the wound and systemic infections	74
K. pneumoniae	One bacteriophage at 10^{10} PFU/ml	Murine burn wound infection model	• A single phage application recovered infected mice	76
K. pneumoniae	One bacteriophage at 10^4 PFU/ml	*G. mellonella* infection model	• Multiple doses of bacteriophages were required to obtain 100% larval survival	77
K. pneumoniae	One phage at 2×10^9 PFU/mouse	Mice infection model	• In bacteriophage-treated groups, bacterial load decreases, and the lung lesion improves due to bacteriophage treatment	78
E. coli	One phage at 1×10^8 PFU/mouse	Intravenous mouse model	• Infected mice were recovered when bacteriophages were administered within 3 hours post-infection	88
E. coli	Two phages, 536_P1 at 10^8 PFU/mouse	Mice infected with a bioluminescent strain	• Intranasal administration obtained 100% survival	79
	536_P7 10^8 PFU/mouse	Mice infected with a ventilator-associated strain	• The occurrence of new variant strains reduced the recovery rate	79
E. coli	One bacteriophage at 10^4 PFU/ml	*G. mellonella* infection model	• Multiple doses of phages were required to obtain 100% larval survival	77

and zebrafish (*Danio rerio*) are among the most commonly used invertebrate or lower vertebrate models for phage therapy, while chicken (*Gallus gallus*), rabbit (*Oryctolagus cuniculus*), hamster (*Mesocricetus auratus*) and mouse are for higher vertebrates.[89]

Infection in nematodes is simple because their nutritional source is bacteria; thus, pathogens primarily colonize the intestine, and phages can be delivered through the same route. *Caenorhabditis elegans* models have been used to evaluate the efficacy of phage therapy for *Salmonella enteritidis* and *S. aureus* infections. In both cases, bacteriophage administration increased the survival of infected larvae significantly. The health of recovered nematodes was confirmed by their ability to produce healthy progeny 100 hours after phage treatment. These results show that *C. elegans* can be a useful animal model for assessing the efficacy of phage therapy.[90,91]

Insects have a high potential among non-vertebrate infection models due to their complex innate immune system, which is very similar to mammals. *D. melanogaster* was used in two studies to assess the therapeutic effect of phages against *P. aeruginosa* infections.[92] Lindberg et al.[93] conducted the first study in which they investigated the pharmacokinetics and potential toxicity of phages on their own. Healthy flies were given phage solutions mixed with corn meal-dextrose medium. The presence of live bacteriophages in the lysates of the flies at various time points after treatment demonstrated that the phages survived and were not degraded in the gastrointestinal system. This suggests that oral administration can be effective in animal models, highlighting the intriguing possibility of testing phage oral administration in *D. melanogaster*. Furthermore, the lack of lethality after phage administration suggests that phage treatments are safe and nontoxic. Heo et al.,[94] compared the effects of phage administration on *P. aeruginosa* infection in mice and *D. melanogaster*. They thought to use two infection models in order to confirm phage antibacterial activity against *P. aeruginosa*, which activates different virulence factors depending on the host. Given the promising potential of *D. melanogaster* as a simple, rapid and inexpensive animal model for studying bacterial infection and phage therapy, a guided protocol has recently been established to evaluate the antibacterial efficacy of new bacteriophages against *P. aeruginosa* infection in this model.[95]

G.mellonella is another invertebrate used for microbial infection and phage therapy. Different bacteriophages were efficiently administered in *G. mellonella* larvae to treat *Burkholderia cepacia* infection in a study by Seed et al.[96] The authors also investigated whether the protective effect observed in the treated larvae was due to bacteriophage action or the host's immune system reaction triggered by the phage injection. Heat-inactivated phages activated the immune system but did not improve larval survival, indicating that antibacterial action depended on active phage multiplication.

Interestingly, two separate studies in wax moth larvae reported the prophylactic efficacy of phage cocktails when injected or orally administered.[96] In the first study, Nale et al.[97] found that adding a four-phage cocktail capable of disrupting *C. difficile* biofilm to larvae food increased survival while preventing bacterial colonization. Forti et al.[86] found that the six-phage cocktail originally used to prevent *P. aeruginos*a infections in *G. mellonella* effectively counteracted lung infections in mice. This finding demonstrated that the same bacteriophages could function in both invertebrates and vertebrates.

Zebrafish is gaining popularity as a model for studying host-bacterial interactions, particularly in its larval stage.[98] The embryos' developed innate immune system, genetic tractability, and optical transparency make them useful for studying aspects of infectious diseases unavailable in traditional animal models. Recently, some zebrafish models were created for research purposes – bacterial infections such as *E. faecalis* and *P. aeruginosa* for phage therapy.[99,100]

In a study using zebrafish embryos, systemic infection was accomplished by injecting bacteria into the embryos. Following circulation, phage was administered via the same route. The success of treatment was demonstrated by the increased survival of infected zebrafish embryos, their recovery from bacterial infection-induced morphology changes, and a reduction in the bacterial burden on homogenized embryos after plating. This vertebrate model validates phage therapy's efficacy in a short (five days) and low-cost manner, demonstrating the survival and effectiveness of phages in an aquatic model delivered in the blood.[89]

The use of invertebrates and smaller vertebrates, like zebrafish, has many benefits for research, including decreased expense and experiment time. However, the use of higher vertebrate models to apply phage therapy to humans cannot be discarded. For instance, oral phage administration was used in birds as prophylaxis or post-infection treatment to combat infections such as salmonellosis, colibacillosis and campylobacteriosis, which are significant economic health issues for poultry worldwide.[101,102] The use of encapsulated phages of specific virion regions, such as the tail spike domain, to enhance phage therapy in chickens was also investigated in some studies.[103–105] Given the significance of applying phage therapy using birds as animal models, a procedure to assess phage effectiveness using a chicken embryo infected with colibacillosis was recently developed.[106]

Rabbits have also been used to model *S. aureus* wound infection and phage delivery.[107] Rabbits naturally have *S. aureus* infections, as humans do, but unlike mice. This makes rabbits a good animal model to research the spread of these bacteria. Subcutaneous injections provided the bacterial infection, which led to abscesses. Phage delivery to bacteria was done simultaneously or right after but at the same site. Animals were slaughtered four or six days after infection to assess the bacterial load in the abscess area and the efficiency of phage therapy.[89]

Phage treatment was tested in a rabbit model of *S. aureus* infection in another study published by Kishor et al.[31] and discussed by Abedon.[108] Although the authors established the feasibility of phage therapy to cure bacterial illness, the rabbit model differed from the patient's circumstance, in which bacterial infection was chronic, and phage therapy was used after traditional techniques failed. Yen et al.[109] established a prophylactic impact of phages to prevent or minimize bacterial infection in newborn mouse and rabbit models infected with *Vibrio cholerae*.[109]

Nale et al.[110] reported that hamsters infected with *Clostridium difficile* and orally treated with a phage cocktail had a higher survival rate. Temperate phages were used in this investigation due to a shortage of virulent lytic phages infecting *C. difficile*. As a result, it was unsuitable for therapeutic use. However, the authors demonstrated how combining multiple phage types could lessen their negative influence.[110]

Murine models are the most commonly employed animals for phage therapy research. Because they resemble humans, they have been utilized to demonstrate the efficacy of traditional phage therapy and to examine the interactions between phages and the host immune system.[65,66,86,111,112]

Overall, animal models have helped to learn more about bacteriophages' efficacy and mechanisms of action *in vivo*. Invertebrates and vertebrates demonstrate the effectiveness of such medicines in less expensive, faster and more ethical ways than human clinical trials. The development of vertebrate animal models to test phages may provide a more comprehensive understanding of the mechanisms prompting host immunological and inflammatory responses to phages, which is one of the most pressing concerns about the applicability of phage therapy to people. Several ways have been attempted to increase and intensify phage activity, highlighting the promise potency of bacteriophages or their enzymes in human therapeutics. Among these are the potential to improve antibacterial action by boosting phage transport, the use of phages in cocktails, the mixing of phages with antibiotics, and the use of phages for prophylactic therapies. These strategies can be (and inexpensively) carried out in animal models before being translated into humans.[89]

These are all (or mostly) uncontrolled clinical trials, which limits the ability to draw clear conclusions about safety and efficacy compared to placebo-controlled blinded trials.

Furthermore, because one of the most important goals of current phage therapy is to rapidly find phages capable of counteracting bacterial infection in compassionate research, animals can gesture the safety of specific phages before patient treatment. Because the custom-made usage of phages cannot currently be regulated, phage pre-screening in animals for tailored therapeutics should at least mitigate some of the issues.[113] Figure 1 shows the bacteriophage therapy in the animal infection models, and Figure 2 shows various routes of phage administration in the mice infection model.

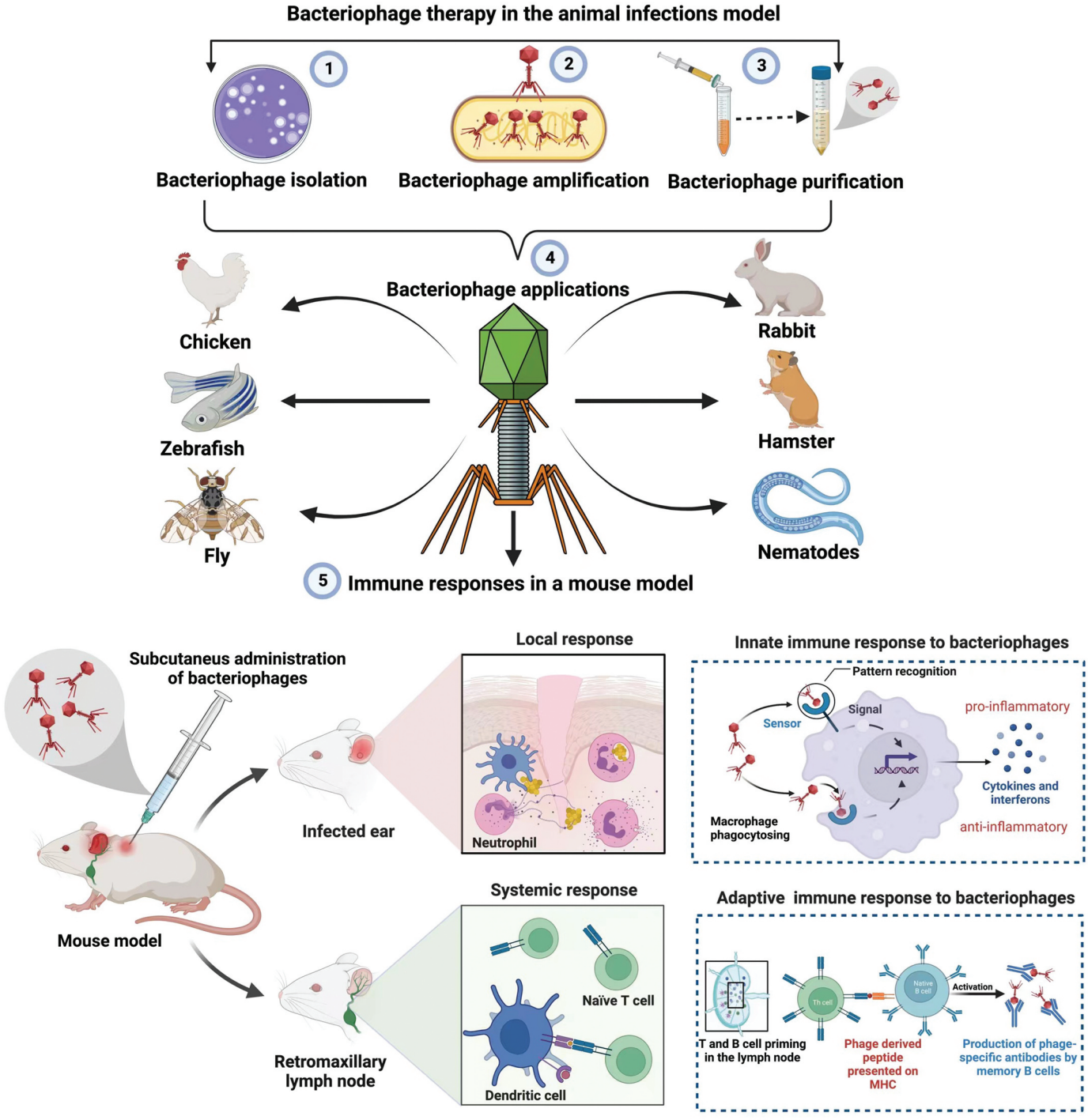

Figure 1. Figure shows the bacteriophage isolation process, animal infection models, and the immune responses to phages in a mouse model. The figure was created with Biorender.com.

For these reasons, animal models are essential in researching possible phage therapeutics and bringing them to human medicine.[89]

Recent phage therapy clinical trials and status

Currently, phage therapy is only being tested in a few clinical trials, with most of them in phase I and some preparations undergoing phase II trials.[114] In a recent clinical trial, bacteriophages were used for treating urinary tract infections in 113 patients undergoing transurethral resection of the prostate. The study showed the safety of bacteriophages, but bacteriophage treatment was not superior to standard-of-care antibiotic treatment, suggesting the need for extensive sample size studies with robust protocol design.[115] The main reasons for the limited use of bacteriophages in therapy include the lack of proper regulatory guidelines and little public awareness.

Bacteriophage cocktails are gaining therapeutic interest because of their efficacy in killing many bacteria. Recently, a cGMP-prepared bacteriophage cocktail saved a patient's life

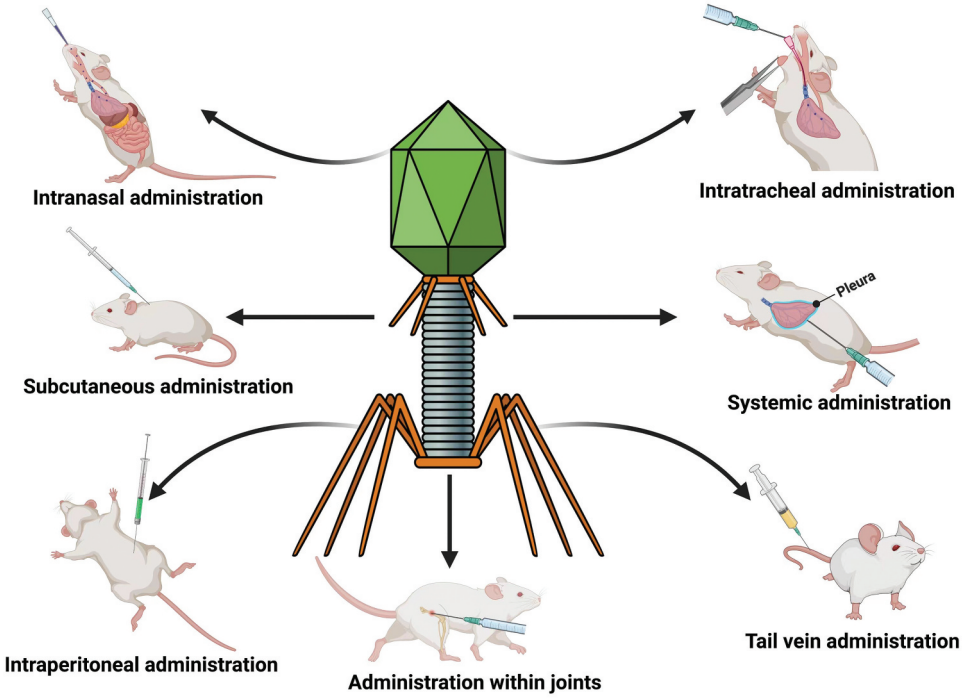

Figure 2. Representation of various routes of phage administration in the mice infection model. The figure was created with Biorender.com.

from an otherwise deadly *A. baumannii* infection.[116] In another study, an 88-year-old patient suffered hospital-acquired pneumonia spurred on by carbapenem-resistant *A. baumannii*. A single phage in combination with tigecycline and polymyxin E was consistently administered to the patient for 16 days. The therapy cleared the pathogen, and the patient's lung function improved clinically.[117]

Several recently completed clinical trials have provided important phage therapeutics lessons. One of the most illuminating clinical trials of the last decade focused on *P. aeruginosa* infection of burn wounds.[114] The topical treatment with a fixed cocktail of 12 antipseudomonal phages was compared to 1% sulfadiazine silver emulsion cream in the open-label, controlled trial. Despite being interpreted as a negative study because it was terminated for futility before reaching full enrollment, this study provided several key lessons for future studies. The first is that the phages in the treatment regimen must be active against the treated organisms. All patients with topical isolation of *P. aeruginosa* from a burn were eligible to participate in the trial, regardless of whether their specific *P. aeruginosa* strain was susceptible to the phages in the cocktail used or not. A post-hoc analysis revealed that a subset of patients with organisms sensitive to phages in the treatment regimen benefited clinically. Second, the study demonstrated the importance of assessing phage-phage interactions before combining them in a phage cocktail and critically assessing phage stability between the production line and the bedside. To the investigators' credit, these issues were detailed in the clinical trial report and now serve as guidance for all subsequent studies.[118]

Another recent clinical trial using the T4 phage to treat acute bacterial diarrhea orally in Bangladesh showed how crucial it is to comprehend pharmacokinetic and pharmacodynamic issues before beginning large-scale clinical trials.[17] The goal of this study was to use T4 coliphages to treat infantile diarrhea that was thought to be brought on by enteropathogenic *Escherichia coli*. After an interim analysis showed no clinical benefit, the study was stopped. In this interim analysis, the researchers found that the phage regimen's multiplicity of infections failed to consistently cause a self-sustaining replicative cycle in the gut lumen of people treated.[118] Another investigation of using a fixed phage cocktail against *Staphylococcus aureus* bacteremia demonstrated the difficulties in studying serious infections with binary outcomes. Thirteen patients were diagnosed with *Staphylococcus aureus* and administered a three-phage cocktail intravenously twice daily for 14 days.[119] The cocktail was safe, and all patients in the study found it safe and well-tolerated. More than half (62%) of the patients showed clinical improvement following phage therapy, while the other half had various issues unrelated to the phage. Although the study added evidence that phages could be administered parenterally to critically ill patients, the study also highlighted the difficulties. They were demonstrating advantages over current standard-of-care therapeutics. Regardless of these "failures," the stage is now set for a new era of clinical trials to determine antimicrobial and clinical properties of phages within a framework that builds on these early findings, experiences and principles of antibiotic development that have contributed to the body of knowledge evidence supporting current antimicrobial therapy. After all is said and done, phages are just "living antibiotics."[120]

A leading scientist of clinical phage therapy, professor Jean-Paul Pirnay, published the successful clinical results of the patient treated with phages. They present the case of a toddler who developed drug-resistant *Pseudomonas aeruginosa* sepsis following liver transplantation. For 86 days, he

received intravenous bacteriophage-antibiotic combination therapy. Without antibody-mediated phage neutralization, this salvage therapy was well tolerated. It was linked to objective clinical and microbiological improvement, allowing for liver retransplantation and the complete resolution of all infections. *In vitro*, phage-antibiotic synergies were observed. Bacterial phage resistance did not result in therapeutic failure, which could be attributed to phage-induced virulence tradeoffs, which were investigated in various experimental models.[121] Their successful clinical results show new hope and confidence in clinical phage therapy.

Another scientist who works on synthetic phages, Professor Yingfei Ma of the Shenzhen Institute of Advance Technology, Chinese Academy of Sciences research group, treated an 88-year-old Chinese man with natural phages who developed carbapenem-resistant *A. baumannii* pneumonia in the hospital. A personalized lytic-specific single-phage preparation, in combination with tigecycline and polymyxin E, was continuously nebulized in the patient for 16 days. The treatment was well tolerated, resulted in pathogen clearance, and improved the patient's lung function.

This case shows phage therapy's clinical therapeutic efficacy and safety.[122]

Professor Graham F. Hatfullf, a phage scientist at the University of Pittsburgh, USA, treated a 15-year-old girl with phages. Following bilateral lung transplantation, the patient with cystic fibrosis and a disseminated *Mycobacterium abscessus* infection was treated with a three-phage cocktail. Genome engineering and forward genetics were used to create effective lytic phage derivatives that kill the infectious *M. abscessus* strain. Intravenous phage treatment was well tolerated and linked to objective clinical improvements such as sternal wound closure, improved liver function, and effective improvement of infected skin nodules. In the clinical treatment, phage administration had no adverse effects.[123]

Professor Graham F. Hatfullf published another clinical study of phage therapy. The study presented a case of refractory cutaneous disseminated *Mycobacterium chelonae* infection in a patient with seronegative arthritis. The patient was successfully treated with antimicrobial, surgical, and single bacteriophage therapy. The patient developed neutralizing antibodies against the bacteriophage but has improved with negative biopsies and no evidence of bacterial resistance to the phage.[124]

A clinical study of phage therapy was recently reported. A male with treatment-refractory *Mycobacterium abscessus* pulmonary infection and severe cystic fibrosis lung disease received two mycobacteriophages intravenously. The phages were engineered to be more effective against *M. abscessus* and were chosen as the most effective against the subject's bacterial isolate. Using molecular and metabolic assays in conjunction with clinical assessments, evidence of phage-induced lysis was discovered in the context of compassionate use. *M. abscessus* isolates showed genetic stability before and after phage treatment, with a general decline in diversity and no increased resistance to phage or antibiotics. Anti-phage neutralizing antibody titers to one phage increased over time, but this did not prevent clinical improvement during treatment. On day 379, the subject received a lung transplant, and systematic culturing of the explanted lung revealed no *M. abscessus*. The study described the successful results of clinical phage therapy of *M. abscessus*.[125]

Kutter et al. detailed previous clinical trials involving phage therapy, which included those conducted in Georgia and Poland.[126] Two phage therapy clinical trials used as examples throughout the literature are worth mentioning: the safety of phages for treating venous leg ulcers[127] and the safety and efficacy in chronic otitis.[128] Rhoads and colleagues reported no adverse effects with the administration of phages in a small phase I trial in patients with venous leg ulcers.[129] Wright et al. demonstrated the efficacy and safety of anti-Pseudomonal phages against MDR-*P. aeruginosa*-dominated late-stage recurrent otitis. These are among the first human-controlled clinical trials in the Western world.[128] Several clinical trials have recently been registered (https://clinicaltrials.gov/and https://globalclinicaltrialdata.com/).

Helen et al. recently published a comprehensive review article.[130] They found 13 modern clinical or safety trials published between 2005 and 2021. The trials took place in Bangladesh and/or Switzerland (n = 6), the US (n = 2), France and/or Belgium (n = 2), Australia (n = 1), Georgia (n = 1), and the United Kingdom (n = 1). Four of the 13 trials were identified as 'phase I,' two as 'phase I/II,' and seven had no formal identification. Clinical and safety studies have consistently shown that using naturally occurring phage for therapy via various delivery methods is safe. Clinical investigations also reveal that phage is effective when the appropriate amount of the proper phages is supplied to the right location to treat infections containing enough susceptible bacterial cells. However, earlier clinical trials found it difficult to match this constellation of elements. It is hoped that future trials will yield the convincing results that the field has anticipated. Meanwhile, the data on the safety and efficacy of phage therapy is deemed sufficient for continued compassionate use when antibiotics cannot meet clinical needs.[131]

Several uncontrolled case studies show positive clinical outcomes. On the other hand, clinical failures are likely underreported, and the few randomized controlled trials conducted have failed to demonstrate benefit. Thus, under any circumstances, no recommendation can be made to support the routine clinical use of phage therapy; much is unknown about the efficacy of phage therapy and potential reasons for failure, such as dosing, frequency of dosing, duration of treatment, routes of administration, interactions with antibiotics, interactions with other phages, the emergence of phage resistance, inadequate phage delivery, and superhost immune response. Even though more clinical research studies are required, there is a lack of (and need for) standardized assays for phage therapy and phage quantification methods, as well as a wide range of potential clinical indications, the safety and tolerability of phages, the prerequisites for safe administration of phage therapy, current regulatory pathways for expanded access, and the requirements for safe administration of phage therapy.[132]

The conclusion of phage therapy clinical trials is that phage treatment is generally safe, with a low incidence of adverse effects via various routes of administration. Although phage therapy appears to be a promising strategy in the fight against

difficult-to-treat infections and antimicrobial resistance, high-quality trials are urgently needed to improve our understanding of the treatment's long-term outcome.[133] Significant effort is required to identify success predictors and design phage clinical trials leading to more widespread use.[134]

The current phage therapy status, hurdles, and limitations

Although phage therapy has shown promising efficiency in addressing infections of bacterial pathogens, it still has several limitations, including a narrow host range, a lack of relevant regulations, and the lack of pharmacokinetic data. Lin et al.[135] recently provided a comprehensive review of phage therapy's current status and limitations and summarized existing solutions for these limitations. Among others, they suggested establishing a national standard scheme for personalized phage therapy and clarifying the standard operating procedure of the clinical application of phage therapy. If these solutions are considered, there would be an improvement in the outcomes of phage therapy and clinical trials.

Bacteriophages have a limited targeting range

The bacteriophage cleavage spectrum is too narrow due to high specificity. Bacteriophages typically act on a limited number of bacteria genera and species and thus cannot target all pathogenic strains of a single bacterial species.[136] Bacteriophages help treat diseases caused by a single bacterium, but clinical cases are frequent infections caused by various pathogenic bacteria. As a result, it is frequently difficult for specific bacteriophages to exert the desired therapeutic effect.[136]

The lysogenic phenomenon occurs when some lysogenic phages cannot lyse the host bacteria and inhibit the lytic effect of other phages on their host bacteria after integration. The viral genome replicates with the host DNA in lysogenicity, either as a free plasmid-like state or after integration into the bacterial chromosome.[137] A more serious issue is that bacteriophages in the lysogenic state can transmit toxins and antibiotic resistance genes to bacteria. Unlike protein drugs, whose activity and purity can be determined using specific antibody titers, the composition of phage therapy preparations are more complex, containing both proteins and nucleic acids. As a result, assessing its quality and curative effects is difficult.[138]

The absence of necessary phage therapy policies

There aren't any policies or rules regarding the use of PT in clinical settings.[139] Appropriate regulatory guidelines can open doors for promoting this promising treatment. The opinions of European stakeholders were thoroughly analyzed by Verbeken et al., who also discussed the necessity of changing the regulatory framework to account for PT.[140] Whether PT development takes place on an industrial or patient-specific scale, a hospital-based scale is a crucial factor to consider. They promoted the creation of a new, exclusive European regulatory framework for PT. Additionally, the potency of isolated phage preparations varies because there is no established standard for phage isolation and purification. Using bacteriophages in clinical settings is not a standard practice.[135]

Bacterial resistance to phages

Several studies on the emergence of bacteriophage-resistant strains suggested that if a single bacteriophage is used repetitively for a long time, bacteria evolve phage-resistant strains through natural selection.[141] This is part of a long-term evolution in bacteria of anti-bacteriophage strategies such as adsorption inhibition, restriction-modification systems, injection blocking, abortion infection, superinfection immunity, and the Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated (CRISPR-Cas) system.[142] Bacteriophage-bacteriophage interactions are reduced as a result of adsorption resistance. Bacteriophages and bacteria both die during abortion infection. CRISPR-Cas is a component of the adaptive immune system that provides bacteria and archaea adaptive immunity against foreign invaders such as plasmids and bacteriophages.[143]

CRISPR and Cas proteins work together to form an overall system that interferes with foreign nucleic acids in bacteria and archaea. The CRISPR-Cas system has at least two stages: adaptation, in which cells acquire new spacer sequences from exogenous DNA, and interference, in which newly acquired spacers are used to target and cleave invasive nucleic acids. By adding or deleting gaps in host cells and mutations or deletions in phage genomes, the CRISPR-Cas system contributes to the continuous evolution of bacteriophages and bacteria.[137,144]

Limited data on the pharmacokinetics of phages

Standardizing PT preparations is difficult because the dosage description is still unknown. Furthermore, the administration and dosage of PT directly impact its effects, making the clinical application of PT difficult. Bacteriophages are almost entirely composed of proteins and DNA or RNA, so they are easily degraded when interacting with human metabolisms, such as in the liver or stomach, when confronted by the mammals' immune system.[145] According to related pharmacokinetic studies, a quarter of bacteriophage infusions lasted 36 hours after treatment, but their effective concentration was diluted by body fluids.[146]

Oral administration has been the best option for both humans and animals. Furthermore, compared to other drug administration methods, it is relatively simple and comfortable, with low immunogenicity.[147] Bacteriophage particles enter the systemic circulation after passing through the stomach, intestine, and intestinal mucosa during oral administration. As a result, the gastrointestinal system is regarded as the primary barrier in preventing bacteriophage infiltration of tissue.[148] Furthermore, the mammalian circulatory system effectively removes bacteriophages from the blood, making maintaining sufficient bacteriophage concentrations to destroy the target bacteria difficult.[149]

During phage therapy, phage resistance can emerge

The potential quick growth of phage-resistant bacterial variations, which could obstruct successful treatment outcomes, is one of the critical concerns with phage therapy. According to experimental data, up to 80% of studies that focused on the intestinal milieu and 50% of studies that used sepsis models found phage-resistant variations. Three of the four clinical trials that documented the emergence of phage resistance described the observation of phage-resistant variants in human investigations. Bacteria can resist phage infection through diverse strategies, including phage receptor blockade, extracellular matrix production, competitive inhibitor production, phage DNA entry prevention, restriction-modification systems and infection prevention systems.[150] Interestingly, studies have shown that bacterial mutations confer phage resistance and may also result in fitness costs in the resistant bacterium, which may be advantageous for the host. Therefore, efforts should be made to create approaches for monitoring and avoiding phage resistance.[141]

The immune system responses to phage therapy

Bacteriophages and their products are non-self-antigens. Thus, it is unsurprising that the immune system can recognize and launch reactions that could conceivably lessen the benefits of administering phages. Experimental studies in both animals and humans have shown immune response to phages, although there are variances depending on the phage strain, the delivery method and the amount of prior exposure. In a study, when T7 phage survival in the blood of healthy and immunocompromised mice was compared, it was discovered that phage titers remained constant for a long time in animals with severe combined immunodeficiency. 99% of phages were removed in healthy mice within 60 min of the injection. As phage titers were steady in the B-cell-deficient mice, the development of particular antibodies appeared to be the primary cause of phage clearance from blood.[116]

Dabrowska et al.,[130] who evaluated the antigenicity of the proteins constituting the *E. coli* T4 phage head surface in humans, found that particular antibodies could be discovered in more than 80% of enrolled persons, even though none had received phage therapy. Although not fully shown, it is likely that the immune response elicited by phages has a minor or no effect on the potential bacterial killing of phage administration. Bacterial lysis happens before the induction of a particular antibody. Furthermore, with a few exceptions, phage delivery is not related to tissue damage, an increase in pro-inflammatory cytokines, or an increase in reactive oxygen species (ROS).[130] Another study found that giving mice phage T4 and its head proteins intraperitoneally did not affect the production of cytokines, interferon, tumor necrosis factor, monocyte chemoattractant protein, gamma-induced monokine, granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor.[151,152] Hwang et al. discovered similar results when they evaluated the safety of an *E. coli* phage cocktail administered orally to rats for four weeks.[153] Carmody et al. investigated the efficacy of intranasal phage therapy in a mouse model of *Burkholderia cenocepacia* lung infection and found that bacterial density, macrophage inflammatory protein-2, and tumor necrosis factor were not increased but were considerably reduced in treated mice lungs compared to untreated controls. On the other hand, data collected in humans appear to support the notion that, even if present, the immunological response to phages is not clinically significant.[154]

Kaźmierczak et al. conducted a comparative immunogenicity study of two therapeutic bacteriophages, A3R and 676Z, active against *Staphylococcus aureus* and routinely used in patients at the Phage Therapy Unit in Poland. In a murine model, a comparison of the overall ability of whole phages to induce specific antibodies revealed typical kinetics of IgM and IgG induction by these two phages. Further research revealed that antibodies specific to ORF096 neutralize the phages' antibacterial activity in studies of protein-specific sera. None of the studied proteins plays a particular role in the induction of specific antibodies in humans; thus, none potentially affects the effectiveness of A3R and 676Z. No evidence was found of increased specific immune responses to the investigated proteins in patients who received phage therapy.[155] Phages can cause the production of neutralizing antibodies as well as phage-specific ones. However, their influence on therapeutic efficacy seemed minimal and did not prevent phage therapy from having a positive outcome. There is a "therapeutic window" because there is a difference between the specific immune response induced by high doses of parenterally administered phages (as in an animal model) and the response seen in patients treated with bacteriophages.[155]

Before, phages were considered spectators who only indirectly affected immunity through their effects on the mammalian microbiome. It is now known, however, that phages directly affect immunity in ways that are typically anti-inflammatory. Through phagocytosis and cytokine production, phages can influence innate immunity, but they can also affect adaptive immunity through effects on antibody production and effector polarization. Phages may thus significantly impact the outcome of bacterial infections by modulating the immune response.[156]

Approaches to overcome the limitations of phage therapy

Host range of bacteriophages

The problem of a limited host range can be addressed in a variety of ways, including the use of phage mixtures,[157] the establishment of a phage library,[158] and extensive screenings.[142] A phage mixture is analogous to several drug therapies. Different bacteriophages in a mixture can infect various bacterial strains that may be present following a specific diagnosis.[149] McVay treated burned mice with a bacteriophage mixture, significantly reducing their mortality.[159] A phage library is an isolated phage collection. These bacteriophages have specific properties and can be used as phage preparations or as unexpanded phage reserves to match newly isolated specific target bacteria.[157] The extensive screening of bacteriophages involves using a variety of hosts to identify bacteriophages that use common surface receptors to cleave a variety of pathogens, such as bacteriophages that target multiple isolates of two

different pathogenic bacteria.[160] It can help expand the host range of a single bacteriophage and solve the host spectrum problem by utilizing many bacteriophages.[149] Expanding the host range of a single phage can also be accomplished by using genetic engineering techniques to modify a portion of the phage responsible for host binding or by cloning a second alternative or different version of these proteins involved in host binding into a single phage.[161] The T7 phage, as a genetically modifiable biological nanoparticle, holds promise for biomedical imaging probes, therapeutics, drug and gene carriers, and detection tools.[162] In general, a phage mixture with more than one phage type to attack bacteria may be preferable in terms of efficacy. However, a phage library may be preferable for phage-host specificity based on direct matching with a specific target pathogen.[163]

The exclusion of temperate bacteriophages is one of the principles for preventing lysogenicity. To eliminate the immune effects of infected lysogenic bacteria on similar bacteriophages, PT must be achieved by lytic phages that have been highly purified. Bacteriophages can encode enzymes that hydrolyze peptidoglycans, causing cell walls to degrade and infect or release offspring viruses in host cells. Endolysin is the enzyme that dissolves peptidoglycan from within. Compared to direct bacteriophage therapy, endolysin's therapeutic effect in treating bacterial diseases is easy to evaluate. This reduces the difficulty of assessing quality. Research has recently focused on synthesizing and transforming lysin-coding genes into antimicrobial peptides, improving the original bacteriophage's antibacterial activity.[164]

Implementation of relevant policies, regulations and guidelines

Several meetings on PT supervision and regulation have been held to promote the development of PT.[140,165] Since their discovery, bacteriophages have been widely used in Eastern Europe and the former Soviet Union; thus, therapeutic bacteriophages have been integrated into healthcare systems.[166] PT's open policy promotes its rapid development. As a result, relevant regulatory standards must be issued on time. Furthermore, bacteriophages are isolated and purified in various ways, but all methods involve the same steps: environmental samples are collected and tested for the presence of bacteriophages. Standardized bacteriophage purification has been considered several times.[167] As a result, a national standard scheme for personalized PT should be established. Furthermore, the standard operating procedure for the clinical application of PT should be clarified, including the recruitment of PT patients, the establishment of phage libraries, the isolation and identification of pathogens, the screening of effective phages for pathogens, the preparation of phage formulations, management strategies, approaches to bacteriophage preparations, the monitoring of PT efficacy, and the detection of the emergence of pathogens.[168]

Combination of dosage guidelines to tackle phage resistance in bacteria

Bacteriophages can be used with other antimicrobials, such as antibiotics, due to the introduction of anti-bacteriophage strains. The greatest approach to combating phage resistance is utilizing bacteriophages with antibiotics. This is also a step toward switching from antibiotic therapy to PT, hastening the growth of the PT research sector. The effectiveness of combining bacteriophages and antibiotics has been demonstrated in numerous investigations.[169]

Similarly, Oechslin et al. found that ciprofloxacin and a bacteriophage worked in synergy to effectively treat experimental endocarditis in rats caused by *Pseudomonas aeruginosa* to prevent the formation of anti-phage mutants.[170] Similar to this, clinical examples showed that multidrug-resistant *Staphylococcus aureus* infections in radiation-exposed patients might be successfully treated with a wound healing solution including ciprofloxacin and bacteriophage polymers.[117] Additionally, we know biofilm pairings can increase bacterial resistance to antibiotics, and numerous studies have demonstrated that bacteriophages and antibiotics together can lower the bacterial density in biofilms.[171] Undoubtedly, several bacteriophages express anti-CRISPR proteins to circumvent the CRISPR-Cas immunity and avoid bacterial resistance. These proteins block the resistance mechanism. Prioritizing amongst bacteriophages can be employed to increase the antibacterial activity of bacteriophage combinations to limit phage resistance emergence.[172] Antimicrobial peptides are innate immune components with broad-spectrum antibacterial action found in practically all organisms. Some studies had demonstrated a potent synergistic effect when bacteriolysin LysH5 and nisin were used to treat *Staphylococcus aureus*.[173,174]

The success of phage therapy, which uses bacteriophages to treat multiple drug-resistant bacterial infections, depends on the carefully chosen antibiotics used in combination therapy. Vashisth, Medhavi, et al. reported and tested the combination of different antibiotics with the bacteriophages. Using time-kill curve assays and counting the number of viable bacterial cells left after the experiment, the synergy assessment of these phages with gentamicin and tetracycline was carried out to validate this antagonistic effect. When phage-antibiotic combination groups were compared to phage-only treatment groups, a rise in bacterial turbidity was seen. This study concludes that bacteriophages' therapeutic potential may be decreased by antibiotics targeting the bacterial protein biosynthetic machinery. Therefore, the such combination must undergo careful screening before being used in combination treatment regimens.[175]

Evaluation and optimization of phage delivery channels

Despite numerous advances in phage preparation for clinical applications, each route of administration presents challenges. These include phage preparation stability, target-site specific delivery, and antibody-mediated phage inactivation and clearance by the recipient's reticuloendothelial system. Particularly, not only are phages unable to penetrate tissues, but the immune system can clear phage particles, and phage proteins are rapidly degraded by enzymes or inactivated by the stomach's low pH. Loh et al. have briefly explained and addressed the issue of Phage delivery and encapsulation strategies before phage therapy can be considered reliable standard therapy.

They further described efficient and targeted phage delivery methods, including phage encapsulation.[6]

Optimizing medication delivery channels must consider whether the bacteriophage can survive in the body and go to all sections of the body. If it is local, infection occurs via systemic circulation, and the bacteriophage persists in a cycle long enough to get to the afflicted location. If the phage gets delivered to the intestine, it must be consumed to survive until it enters the circulatory system.[104,113,115,118,119,176,177] The encapsulation of phages in a natural biopolymer matrix, for example, is one method utilized as a barrier against the stomach environment to prevent the inactivation of bacteriophages after intake; this ensures bacteriophage effectiveness.[178] Related research revealed that liposome-encapsulated phages could be efficiently maintained in the body and stay safe until they are released. Upon arrival in the stomach, the gut wall temporarily protects phages from bile salts and excretion clearance.[179] Furthermore, the dose can be increased or decreased over a brief period to prevent bacteriophage inactivation or loss before antibodies reach the target microorganisms.[10,180]

A broader view of phage-related research

With the advent of antibiotic resistance, the number of untreatable bacterial infections has increased rapidly. The unavoidable adaptation of microbes to antibiotics, regardless of their origin (natural, semi-synthetic, or nature-inspired synthetic origin), has necessitated the need for alternative antibacterial agents, such as therapeutic phages or antimicrobial peptides.[181] *In-vivo* efficacy of bacteriophages to cure bacterial infections has been established using animal infection models. Many preclinical studies included mice as model animals with different infection models, and the efficacy of different routes of phage administration has also been evaluated. However, most animal studies have focused on the antibacterial efficacy of bacteriophages and lack substantial studies on the immunological response of the bacteriophage. Immunological modulation of the host animals *via* bacteriophages may provide conclusive evidence about the therapeutic outcomes and aid the design of future treatment strategies.

Phage delivery strategies have also been employed in recent years to treat gastrointestinal tract, skin, lung, and urinary tract infections and showed better antibacterial response than bacteriophage preparations alone. However, the pharmacokinetics, immunomodulatory properties, large-scale production and sterilization are critical knowledge gaps in the use of these delivery systems in clinical applications. Apart from technological advancements, a global legal and regulatory framework is yet to be established for bacteriophages and their delivery systems.[182]

While studies on preparations containing one or more phage types have received much attention, the interest in phage-derived endolysins is also increasing.[183] The prominent advantage of endolysin therapy is the lack of bacterial resistance. Interestingly, many studies have demonstrated the potential of endolysins as alternative therapeutic options for treating multidrug-resistant bacterial infections.[184–187] Endolysins are bacteriolytic enzymes that can kill bacteria with an activity broader than the bacteriophage. However, the specificity of endolysins is still under investigation.[188] Several strategies, such as molecular engineering and encapsulation of endolysins, are under exploration to improve the activity, biodistribution and half-life of endolysins.[182,189] Many bacteriophage researchers believe phage therapy or phage-encoded proteins can completely replace chemical antibiotics. However, the future will likely see a co-existence of both strategies, with phage therapy as an additional weapon against the bacteria, possibly used as a combination with antibiotics.[190,191] So far, such a combination therapy has tremendously succeeded in treating bacterial pathogens because the bacteria cannot simultaneously develop resistance against antibiotics and phages.

In Table 3, we provided the outcomes of endolysins administration against bacterial pathogens in some animal models. Although bacteriophage-related research has seen a renaissance over the past years, some aspects still require more attention, such as (i) the development of phage-resistant bacteria, (ii) the interaction of phages with the human immune system (during long-term treatments of infections, for example), (iii) the development of genetically modified phages for therapy, and (iv) a global regulatory framework for the production and clinical application of bacteriophages. In addition, there are limited animal studies to prove the efficacy of phage therapy in some bacteria, such as *Streptococcus*, *Enterococcus* and *Mycobacterium*. To establish a preclinical therapeutic efficacy, a simple animal model such as *G. mellonella* and *C. elegans* can be used for these pathogens.[77,219] In Table 4, advantages and limitations of phage therapy are presented.

Future perspective

Phage therapy has remarkable potential as an option for controlling antibiotic-resistant infections. However, there are many obstacles to establishing phage therapy as mainstream medicine. Here we discuss five essential strategies or challenges to be addressed to establish phage therapy in western medicine. [A] **Phage socialization**: Education and awareness play an important role in accepting and distributing any new therapeutic model within a population. Poor medicine literacy can lead to the misuse of medicine, mainly due to the spread of nonfactual information (as seen in antibiotic therapy). The rebirth of phage therapy is seeing increased therapeutic applications and patient and physician awareness. A strong and direct outreach is essential to capitalize on this situation in which stakeholders – researchers, physicians, policymakers, regulatory authorities, government officials, and the public should be included. Public awareness can be improved through social media platforms and regular campaigns. [B] **Accessibility and availability**: A successful medicine or therapy should always reach the suffering population and be available when in need. Currently, the accessibility of phage therapy is extremely low, and limited therapy centers are located in Georgia, Poland, Russia and USA. Therefore, the health care cost is high and is affordable only for the high-class population. Though compassionate-use programs are underway in many countries, including Australia, Canada, China, Japan, the UK and the USA, they are limited to critically-ill cases. Easy accessibility is also related to patient education and

Table 3. Outcomes of endolysins administration in animal models.

Endolysin	Animal model	Target Bacteria	Outcome	References
LysP53	Mice	*A. baumannii*	• After 1 hour of treatment with 100 µg/mL, bacteria were reduced by 5 logs • Higher decolonization efficacy in the mouse model of burn infection	185
PlyC	Tested in human and mouse models	*Streptococcus spp.*	• A powerful antibacterial endolysin was tested in a human and a mouse model • PlyC is immunogenic but does not cause hypersensitivity • PlyC-challenged mice developed PlyC-specific IgE in an animal model but not in humans • No unwanted effects, including hypersensitivity, were observed in the animals	192
LysSS	Mice	*A. baumannii*	• With an intraperitoneal injection of LysSS (125 ug/ml), 40% of mice were rescued from *A. baumannii* systemic infection	193
ClyC	Mice	*S. aureus*	• It reduced the bacterial loads in the infected mice organs by 2 Log10 (CFU/mL)	194
ClyH	Mice	*S. aureus*	• In a mice infection model, one dose of ClyH kept mice safe from death as a result of MRSA infection	195
ClyF	Mice	*Methicillin-resistant S. aureus*	• In mice bacteremia and wound infection models, a single treatment of ClyF showed good MRSA removal activity	196
ClyJ-3	Mice	*S. pneumoniae*	• Superior to the parental enzyme ClyJ • 100% survival compared to the control group	197
ClyJ	Mice	*S. pneumoniae*	• 100% and 20% of mice survive when treated one and three hours after infection, respectively • Up to 1000 µg/mouse, there are no side effects	198
ClyR	Mice	*S. agalactiae*	• ClyR has no harmful effects on the mice • In mice, repeated injections of ClyR elicited an immunological response • The immunized serum, however, was unable to neutralize ClyR lytic activity	199
ClyR	Rat	*S. Sobrinus* and *S. mutants*	• Compared to the control group, the ClyR showed a 56% reduction in caries-related dentinal lesions	200
ClyV	Mice	*S. agalactiae*	• The treatment survival rate is 100% • The ClyV has no side effects up to 800 µg/mouse	201
SAL200	Rodent and Dogs	*S. aureus*	• In rodent, single- and repetitive toxicity studies, intravenous administration of SAL200 showed no evidence of toxic effects • No abnormal observations in the dog repetitive toxicity test	202
Cpl-1and Pal	Mice	*S. pneumoniae*	• According to the safety investigation results, IgG levels in mice exposed to endolysins increased • However, IgE levels remained low, suggesting a low probability of hypersensitivity or allergic reactions • No adverse health consequences in mice • Good safety and toxicity profile	203
CPl-711	Mice	*S. pneumoniae*	• Independent of strain and treatment dosage • It showed an activity of 2-log reduction in the nasopharyngeal carriage • The lysis activity was better than parental endolysin Cpl-1	204
Ply5218	Mice	*S. suis*	• The survival rate was 80–90% with rapid treatment • The survival rate was 70–80% with delayed treatment	205
Ply5218	Piglet	*S. suis*	• After seven days, no bacteria were in the blood in the treatment group (compared to 4 logs in the control group) • The treatment group had lower body temperature, clinical assessment, and pro-inflammatory cytokines	205
PL3 and Cpl-711	Zebrafish	*S. pneumoniae*	• The treatment survival rate of PL3 is 50% • The treatment survival rate for Cpl-711 is 44.4%	206
Cpl-711	Mice	*S. pneumoniae*	• The Cpl-711 survival rate is 58% • The Cpl-711 has a 100% survival rate in synergy combination with cefotaxime	207
CF-301	Rabbit	*S. aureus*	• It resulted in eight logs reduction in MRSA growth	208
CF-301	Rat	*S. aureus*	• It resulted in a 0.48 log reduction in MRSA growth in the bone infection model • Showed synergistic activity with daptomycin, combined with daptomycin, showed a 1.56 log reduction	209
S25-3LYS-his	Mice	*S. aureus*	• It resulted in a 1 to 2-log reduction in *S. aureus* on the skin	210
ElyA1	Mice	*A. baumannii*	• *A. baumannii* in the lungs was reduced by 0.5 logs	211
Ply6A3	Mice	*A. baumannii*	• The survival was 70% after endolysin therapy • After treatment, the white blood cell counts, IL-10, and procalcitonin levels were lowered	212
LysGH15	Mice	*S. aureus*	• The endolysin LysGH15 showed a 2.8 log reduction of *S. aureus* on the skin • It showed synergistic activity with apigenin and showed a 3.3 log reduction • The wound healing was improved after treatment with LysGH15	213
PlyPa91	Mice	*P. aeruginosa*	• The endolysin PlyPa91 showed 70% survival rates after intranasal plus intratracheal treatment • It showed a 20% survival rate after two intranasal treatments	214
Plya03 and PlyPa91	Mice	*P. aeruginosa*	• The endolysin PlyPa03 showed >2 log reduction of *P. aeruginosa* on the skin • The endolysin PlyPa91 showed a 1 log reduction of *P. aeruginosa* compared to the control treatment	214
LysRODI	Mice	*S. aureus* and *S. epidermis*	• The LysRODI enhanced mammary gland health • The LysRODI showed a 2–3 log bacterial reduction	215
TSPphg	Mice	*S. aureus*	• The endolysin TSPphg showed a 3 logs reduction of *S. aureus* on the skin • It showed faster-wound healing and similar outcomes with kanamycin therapy	216
PyS2-GN4	Mice	*P. aeruginosa*	• The endolysin PyS2-GN4 25 mg/kg therapy showed 100% survival	217
LysB	Mice	*M. ulcerans*	• The endolysin LysB reduced bacteria in footpads by 1 log compared to the control.	218

Table 4. Advantages and limitations of phage therapy.

Advantages of phage therapy	Limitations of phage therapy
• Because they are highly specialized, bacteriophages don't affect beneficial bacteria	• When the particular species of infecting bacteria is unclear, or there are multiple infections, phages' high specificity is a disadvantage
• Bacteriophages are good medicine	• Bacteria can also develop phage resistance
• They grow at the infection site until there are no more bacteria	• Because phages multiply as long as bacteria are there, it takes just a few phages in an inaccessible region in the body to trigger healing in some conditions.
• Phage cocktails can kill bacteria resistant to one type of phage.	• Phages are relatively large in comparison to chemical molecules. As a result, the locations in the body they can access must be carefully defined
• Bacteriophages are readily available everywhere.	• Phage therapy appears best suited for infected sites, such as wounds, where phages can be easily applied
• Finding new phages is simple, even when needed for resistant bacteria	• There have been far too few pharmacological investigations that have shed light on these issues
• Bacteriophages are also effective against bacteria that have developed antibiotic resistance	• Bacteria have an immune system that degrades the genetic material of invading phages
• Antibiotic-resistant bacterial pathogens are particularly deadly. At the same time, some resistant bacteria developed during phage treatment are less virulent and can be controlled by the immune system	• Only the ideal phages can defeat the bacterial immune system
• Individual phage components, such as phage-derived endolysin, can also be employed as antimicrobials	• Bacterial pathogens embedded within human cells may be inaccessible to phages
• Despite extensive testing, no bacterial resistances have emerged thus far to endolysins	
• Phages have a limited range of activity	• The human immune system recognizes phages injected into the bloodstream
	• They are quickly excreted, and the body produces antibodies against the phages after a certain period. As a result, a single phage type can only be used once for intravenous treatment
	• Not all phages are quickly eliminated. Furthermore, variants that can persist in the blood for an extended period can be chosen. Antibodies do not appear for one to two weeks.
	• Phages are complex organisms that can transfer toxin genes between bacteria, as compared to chemical molecules.
	• This risk can be reduced by strictly lytic phages, sequencing phage hereditary material, and performing toxicity tests.
• Significantly safe	• The shelf life of a phage deviates and should be evaluated and measured
• Greater tolerance	• Antibiotics are easier to administer than phages
	• To correctly prescribe and use phages, a physician must receive specialized training
• Simple to administer	• Issues with pharmaceutical preparation formulation and stability
• Less costly	• Immune system reaction to bacteriophages results in decreased activity

treatment success. [C] **Regulatory framework**: The establishment of legal frameworks on the production, preliminary testing, clinical trials, use, and surveillance of phage therapy will be strongly supported by public awareness, accessibility, and education. Country-wise regulatory frameworks can be developed based on medical, industrial and constitutional systems. [D] **Manufacturing pipeline**: The production of therapeutic phages is complicated and expensive, hindering availability and applicability. Complete phage manufacturing can be divided into small assignments such as isolation, purification, screening, testing, production, storage, and transport, which create an economic activity for the new start-ups and job opportunities for the experts. [E] **Clinical trials**: The lack of regulated preclinical and clinical trials is a major obstacle. Phage therapy finds more success in clinics than in corresponding clinical trials. Recently, phage therapy has been approved as an investigational new drug (IND) for life-threatening cases. Still, the modern medical research model needs approval for a successful regulatory path (*in vitro* discovery, animal testing, safety trials, and efficacy trials). The need for controlled clinical trials controlled by placebo or compared to standard-of-care treatment.

Conclusion

Antibiotic resistance is one of the major global healthcare threats endangering the efficacy of available antibiotics. Exploring bacteriophage therapy as a standard clinical strategy to treat infections could be a way out of this crisis. The contribution of animal studies in the approval of medicinal drugs or therapeutics is inevitable. This review sheds light on using animal models to evaluate the efficacy of phages as therapeutics and the necessary regulations in transforming phage research from *in vitro* to preclinical-clinical trials and medical products. Considering the importance of bacteriophages in molecular biology, diagnostics and drug delivery, this review summarizes the applications of phages in therapy and vaccinology. Though our understanding of phage genomes has been improvised in the technological era, a lot needs to be studied about the immunological and pharmacological aspects for which animal models will be inevitable. We also observed some obstacles in establishing phage therapy in the 21st century and addressed them with possible solutions to improve the future of phage therapy research.

Acknowledgments

The authors (FMK, VSG, GKO, and NO) sincerely thank Prof. Hongping Wei and Prof. Hang Yang (Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China) for their guidance. The figures were created with Biorender.com.

Authors' contributions

FMK, PM, and VSG conceptualized the idea and data collection and wrote the original draft. NM, GKO, and NO reviewed and edited the manuscript. TA and GH conceptualized the idea and reviewed and edited the final draft. FMK and GH also contributed funds for this work. All the authors read and approved the final version for publication.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work is supported by a Research Fund for International Young Scientists from the National Natural Science Foundation of China (NSFC) to Dr. Fazal Mehmood Khan of Grant number: 32250410292. This work is financially supported by the National Natural Science Foundation of China [81960353 and 82172238], the Science and Technology Support Project of Guizhou Province [2020-1Y332], Sanming Project of Medicine in Shenzhen[SZSM202111020], Science and Technology Cooperation Project of Zunyi Science and Technology Bureau [HZ-2019-50] and Health Commission Science and Technology Fund Project of Guizhou Province [gzwjkj2019-1-151].

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