

Review

Phage Therapy for Management of Multi-drug Resistance: Unleashing Nature's Tiny Warriors to Combat Bacterial Infections

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Abstract

The prevalence of multi-drug resistant (MDR) bacterial strains renders conventional antibiotics ineffective, thus, bacteriophages are gaining popularity as attractive solutions for bacterial infections through phage therapy. This mini-review provides an overview of the diverse approaches in administering phage therapy, the development of phage therapy techniques, and its prospects and challenges for future clinical applications in treating MDR bacterial infections. Selected journal articles derived from several databases were screened using the Cochrane Risk of Bias Tool and the Newcastle-Ottawa Scale to ensure the reliability of the sources of literature. Several studies demonstrated that the success of phage therapy against the various MDR bacteria primarily depends on selecting an appropriate bacteriophage because of its high specificity in its host range. Recent advances in phage therapy include efficient strategies for isolation and characterization, genetic modification, phage formulation, therapeutic approaches, and clinical trials. While there is promise in employing phage-antibiotic synergy in managing MDR infections, gaps in the bacteriophage selection, resistance development, and pharmacokinetics must be addressed to apply phage therapy on a larger scale. Future research is most likely geared to focus on genetically engineering bacteriophages to overcome these limitations.

Keywords: antibiotics, antimicrobials, bacteriophage, genetic engineering, therapy

Резюме

Разпространението на мулти-лекарствено-резистентни (МЛР) бактериални щамове прави конвенционалните антибиотици неефективни, поради което бактериофагите набират популярност като атрактивни решения за бактериални инфекции чрез фагова терапия. Този мини-обзор предоставя общ преглед на различните подходи при прилагането на фагова терапия, разработването на техники за фагова терапия и нейните перспективи и предизвикателства за бъдещи клинични приложения при лечение на МЛР бактериални инфекции. Избрани статии от списания, получени от няколко бази данни, бяха прегледани с помощта на инструмента за риск от пристрастия на Кокрейн и скалата Нюкасъл-Отава, за да се гарантира надеждността на източниците на литература. Няколко проучвания показват, че успехът на фаговата терапия срещу различните МЛР бактерии зависи предимно от избора на подходящи бактериофаги поради високата му специфичност в неговия гостоприемник. Последните постижения във фаговата терапия включват ефикасни стратегии за изолиране и характеризиране, генетична модификация, фагово формулиране, терапевтични подходи и клинични изпитвания. Въпреки че има обещание за използване на синергия между фаги и антибиотици при управлението на MDR инфекции, пропуските в селекцията на бактериофаги, развитието на резистентност и фармакокинетиката трябва да бъдат отстранени, за да се приложи фаговата терапия в по-голям мащаб. Бъдещите изследвания най-вероятно са насочени към генетично инженерни бактериофаги, за да се преодолеят тези ограничения.

Introduction

The success of penicillin's drug development

by Alexander Flemming in 1930 established the clinical relevance of antibiotics and sparked re-

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Acta Microbiol. Bulg. 2025; 41(02). <https://doi.org/10.59393/amb25410203>

search into natural products that inhibit microbial growth (Hutchings *et al.*, 2019). Since then, modern medicine has relied on antimicrobials to prevent and treat various infectious diseases (Dadgostar, 2019; Bakkeren *et al.*, 2020). However, the overuse and misuse of antibiotics in human and veterinary medicine, coupled with inadequate infection control, poor surveillance, population growth, migration, and poor sanitation, have led to the rise of multi-drug resistant (MDR) bacteria (Sekyer and Asante, 2018; Bakkeren *et al.*, 2020). These pathogenic strains generate resistance either by spontaneous mutations in internal chromosomal genes or by acquiring exogenous plasmid via horizontal gene transfer that can render antibiotics ineffective by secreting bacterial enzymes that chemically alter the antibiotic molecule, modifying its target site, and reducing cell permeability and efflux (Munita and Arias, 2016; Murray *et al.*, 2022).

As the world is in its post-antibiotic era, antimicrobial resistance poses a global public health concern, claiming 700,000 lives in 2014 (Sekyer and Asante, 2018). Without intervention, its predicted mortality will reach 10 million by 2050, which compels the scientific community to formulate and develop a solution against MDR bacterial infections (Bassetti *et al.*, 2017).

Recently, phage therapy has received renewed attention as a therapeutic alternative to conventional antibiotics (Cui *et al.*, 2019). Its development during the 1950s was halted due to the insufficient understanding of phage biology (Abedon, 2018). These self-replicating bacterial viruses are highly specific, which makes them selective against pathogenic strains and are amenable to genetic engineering - making them attractive solutions against MDR strains and biofilms (Nobrega *et al.*, 2015; Ghosh *et al.*, 2019). Further, their ubiquitous nature makes them a cost-effective strategy. Since the current period offers low-cost whole-genome sequencing, automated equipment for detecting the growth of microbes, and efficient high-throughput methods for screening hundreds or thousands of samples simultaneously, the characterization of test phage has advanced immensely (Kortright *et al.*, 2019). Phage therapy has been applied to treat infections in the urinary, respiratory, and gastrointestinal tract in several animal models for pre-clinical trials, wherein it was evaluated to be clinically safe (Międzybrodzki *et al.*, 2012; Melo *et al.*, 2020).

The primary goal of this paper is to assess the progress and future potential of phage therapy as an alternative to traditional antibiotics in treating

MDR infections. Specifically, the paper aims to: (1) explore the mechanisms of phage therapy, (2) examine recent developments in phage therapy and their relevance to case studies and clinical trials, and (3) evaluate its benefits and challenges for the future management of MDR infections in comparison to conventional antibiotics.

Overview of Bacteriophages

Bacteriophages, otherwise known as phages, are viruses that infect bacteria as their hosts. Similar to typical viral characteristics, they do not possess the ability to independently replicate which calls for the need of a bacterial host to facilitate reproduction. They are tiny with sizes ranging from 50-200 nm and hold genetic material (DNA or RNA) encapsulated within a protein capsid. Phages usually display host specificity and a single phage may infect various species within a genus, as well as a majority of strains within a species and occasionally one or several isolates of a species. According to Hatfull *et al.* (2022), the bacterial inclination of any specific phage is known as the host range, which can be classified as narrow (low number of bacterial isolates assist in replication) or broad (large number of diverse species or genre support replication).

Phages commonly bind to particular receptors located at the surface of a bacterial cell, inject their genetic information into the host cell, and proceed either with the integration of this information into the bacterial genome (temperate phages) and subsequently performing vertical gene transfer from mother to daughter cell, or by seizing the cellular machinery of the host cell to generate new phage progeny which leads to cell lysis (lytic phages). A phage may undergo two replication strategies namely: lytic or lysogenic (Lin *et al.*, 2017), as shown in Fig. 1.

These microorganisms are extremely ubiquitous and abundant in the environment. In fact, ocean water possesses approximately 10^7 phage particles per milliliter, and an estimated roughly 10^{31} overall phage particles are found in the biosphere – surpassing the bacterial population by around a factor of ten (Hendrix *et al.* 1999; Mushegian, 2020). The phage population is both ancient and dynamic, and the persistent occurrence of infections periodically results in a complete change in the phage population (Hendrix, 2002).

Despite their small size, a multitude of phages varying in structures and sizes can be easily seen under an electron microscope. A large bulk of the phages perceived in the environment belong to the order *Caudovirales*. In terms of their morphology,

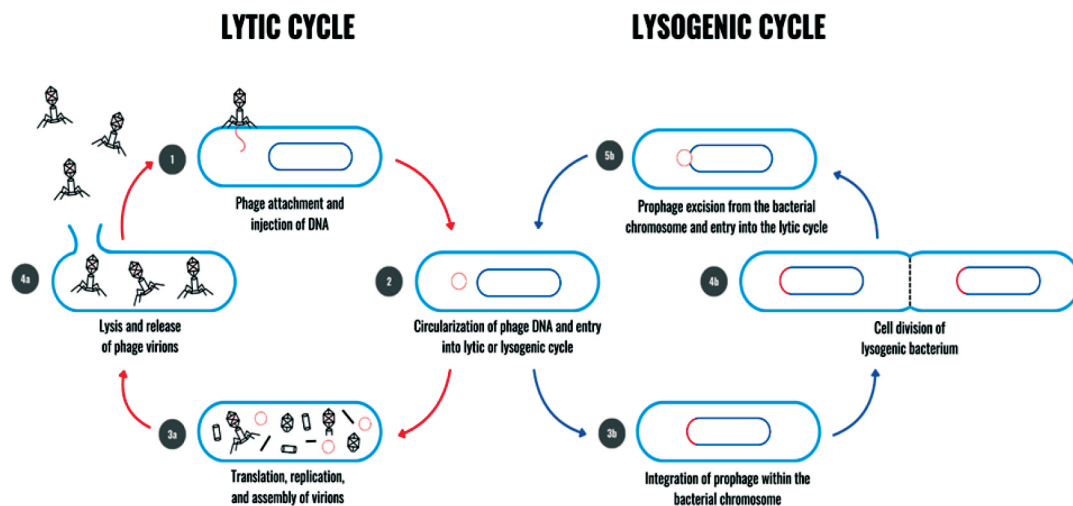


Fig. 1. Bacteriophage life cycles (adapted from Batinovic *et al.* 2019; Alsubhi 2021; Hatfull *et al.* 2022; Louten 2023).

phages like these generally possess a dsDNA-containing head and a tail wherein the tip functions to recognize receptors. The order consists of three families (depending on the type of tail): *Myoviridae* (contractile tails), *Podoviridae* (short, stocky tails), and *Siphoviridae* (long, non-contractile tails) (Hatfull *et al.*, 2022).

History of phage therapy

The concept of employing phages in the realm of therapeutics to combat bacterial infections has been around for over 100 years. However, the prevalence of infections caused by MDR pathogens resulted in the rise of the popularity of phage therapy. Dating back to the preliminary observations made on phage-mediated bacterial cell lysis, its nature and therapeutic value have always been frowned upon. Numerous authors suggest that phages were discovered during the late 19th century, thus information also presents that the independent discovery of phage was in the year 1915-1917 by Twort (Abedon, 2014). The earliest clinical trials of using phages as antibacterial agents began soon after the discovery and the first paper was published as early as the year 1921 (Abedon, 2014). Frederick Twort was the first to delineate the area of lysis linked to phage infection in 1915, but the individual behind the identification of the origin of such a phenomenon, associated the plaques to bacterial viruses, as well as in the invention of the term “bacteriophage,” (“bacteria eater”) was Felix d’Herelle. The same individual was also the one who created the concept of therapeutic use of phages and is known to be responsible for the initial recorded clinical utilization of phage at the Paris-based hospital – Hôpital des Enfants-Malades – in 1919 wherein phages were employed as a treatment for bacterial dysentery in four pediatric patients (Chanishvili, 2012). Although there was a series of

successful trials, d’Herelle’s primary experiments were infamous for lacking rigorous control, and his research was subjected to heated debate in the scientific community (Wittebole *et al.*, 2014). Nonetheless, he persisted in pioneering the use of phage therapy in treating several diseases such as the bubonic plague, cholera, and dysentery with a number of commercial phage production plants and phage therapy centers scattered all over India and Europe in the early 20th century (Sulakvelidze *et al.*, 2001). A 1931 phage therapy trial for cholera treatment conducted in the Punjab area of India, constituted a cohort of 73 experimental subjects and 118 control subjects. It was then noted by d’Herelle that a 90% mortality rate decreased, specifically 5 in the experimental group and 74 in the control group (Chanishvili, 2012). d’Herelle as well as other entrepreneurs were involved in the attempted commercialization of phage manufacture in the United States and Brazil, composed of phage preparations for *E. coli*, *Staphylococcus*, *Streptococcus*, among others. These were transported all over the world to amenable clinicians, yet the treatment outcomes differed in terms of success. The deficiency in soundness made antibiotics in Western medicine seem like a better option (Sulakvelidze *et al.*, 2001).

The erroneous results during the initial and many of the trials involved in phage therapy can be correlated to a substandard comprehension of the biological characteristics of phages. Unsophisticated storage and purification protocols yielded low concentrations of active phage and bacterial antigen contaminants, and those that did not hold the ability to infect the bacteria of interest were employed in the treatment process. Moreover, the conveyance of phages to the infection zone was discredited as a consequence of the medical constraints at the time. An example of this is that the

role assumed by the innate immune response of a patient in the elimination of active phage and the reduction of phage therapy effectiveness was not noted until lately; it may be a contradicting physiological mechanism (Hodyra-Stefaniak *et al.*, 2015). Hence, phage therapy was extensively disregarded by the vast majority of Western medicine following the establishment of pharmaceutical antibiotics in the 1940s. The only anomaly to such a case is in Eastern Europe and post-Soviet states as the substantial use of phage therapy has been observed as a treatment for antibiotic-resistant infections engendered by a variety of infectious bacteria namely: *E. coli*, *Klebsiella*, *Pseudomonas*, and *Staphylococcus* (Morozova *et al.*, 2018).

Mechanism of action and phage therapy approaches

Phage therapy works by administering virulent phages to eliminate pathogenic bacteria quickly. This type of therapy incorporates bioengineered phages, purified lytic phages, and lytic phages to infect and cause bacterial lysis at the infection site. These phages, including their lytic proteins, can mainly provide benefits as a treatment for MDR bacteria, which can be delivered by itself or augmented with antibiotics (Hibstu *et al.*, 2022). There exist various approaches to phage therapy administration, and they are as follows: (1) monotherapy or single phage, (2) combination therapy or phage cocktails, and (3) phage-antibiotics synergy (PAS). *Monophage vs phage cocktails*

Most phage treatments have been conducted in the laboratory using animal models as proof of concept (Górski *et al.*, 2020). The rapid establishment of bacterial phage resistance, even in small groups of bacteria, could render monophage treatment ineffective. In a natural environment, phages and the bacteria they infect will evolve and adapt to remain infectious or develop resistance. Even though the ability of phages to surmount bacterial resistance through evolution is an additional advantage over antibiotic therapy, the process may need to be improved before monophage therapy can be used clinically (Gordillo Altamirano and Barr, 2019). Another issue with monophage therapy is that the etiological agent and the phage must be a perfect match. This is typically performed in a laboratory, but the lytic characteristics of phage *in vitro* and *in vivo* are not always identical (Gordillo Altamirano and Barr, 2019).

The capacity of a monophage to eliminate a specific bacterial infection depends in part on the number of bacterial species it can attack. This

is comparable to how the spectrum of activity of a chemical antibacterial drug affects a variety of bacterial species. In general, monophages cannot be used as the initial defense against bacterial diseases. (Abedon *et al.*, 2021). Increasing the range of action beyond what a monophage is capable of can be achieved by designing a phage cocktail that consists of at least more than one bacteriophage to target various types of bacteria. (Chan and Abedon, 2012).

Phage cocktails could be designed to prevent bacteria from developing phage resistance. They can target a single bacterial strain, multiple bacterial species, and multiple strains of a single species, wherein their activity can be quantified depending on their breadth and depth (Abedon *et al.*, 2021). In designing phage cocktails, Abedon and colleagues (2021) emphasized how it is necessary to consider the breadth of the impact rather than its depth. Since bacteriophages in combination can interfere with one another upon coinfection, it is crucial to remember that phage cocktails should not be random mixtures of different bacteriophages when developing cocktails against a single species or genus, as interference may have both synergistic and antagonistic effects (Merabishvili *et al.*, 2018). On the other hand, the microbial composition of target infection sites must be precisely defined through epidemiological surveys, and the frequency of various bacterial species during different infections at the reception site must be considered for designing cocktails that target multiple species or genera as highlighted by Merabishvili and colleagues (2018). The downside of employing phage cocktails is that they could significantly impact non-target microbes and be more expensive (Nobrega *et al.* 2015).

Phage cocktail therapy furnishes a broader target range of these treatment modalities and poses a lower risk of developing phage resistance in bacteria over monophage therapy. It is worth noting, however, that combination therapy substantially increases the likelihood of gene exchange, the development of phage resistance for all active phages in the cocktail, and the difficulty of determining inflammatory effects (Chan *et al.*, 2013). Studies have demonstrated that commercially available phage preparations, such as monophage Sb-1 and polyphage PYO, effectively prevent and eliminate MRSA biofilm formation *in vitro* (Tkhilaishvili *et al.*, 2020). Kifelew *et al.* (2020) evaluated the lytic potential of a phage cocktail combining AB-SA01 and AB-PA01 against *S. aureus* and *P. aeruginosa* strains in both planktonic and biofilm forms. The

results showed that the phage cocktail effectively lysed both strains, even in the presence of biofilms from non-susceptible species. Li *et al.* (2020) investigated the effectiveness of phage combination therapy (cocktails) in treating infections in immunodeficient hosts, where immune signaling is absent and immune response is at a basal level. The model assumes wild-type and host-range mutant phages target phage-sensitive and phage-resistant bacteria, respectively. The research aims to optimize phage delivery schedules using optimal control theory (2D-OC) and practical approximations for clinical application. In their study, it was found that the minimal phage dosage required for bacterial elimination decreases with higher basal-level immune density and optimal phage delivery schedules were identified through 2D-OC and practical treatment approximations. In immunodeficient models, where immune response is absent, phage-resistant bacteria tend to grow exponentially without proper control. However, using a phage cocktail and adjusting the dosing schedule based on immune levels enhances treatment efficacy, even in low immune response cases, which shows the importance of tailored phage therapy strategies. Limitations of the model, including its focus on acute infections and deterministic assumptions, are acknowledged, and future work may explore stochastic control, chronic infections, and multiple phage strains. This framework provides valuable insights for advancing phage therapy and its application in other therapeutic areas involving replicating agents. One study evaluated the effectiveness of phage therapy against MDR *Klebsiella pneumoniae* isolated from diabetic foot ulcers (DFU). Four bacteriophages (KP1, KP2, KP3, KP4) were isolated from animal feces and sewage. While bacterial regrowth occurred with single phages, a phage cocktail showed significantly better antibacterial activity ($p < 0.05$) and prevented regrowth. The findings suggest that phage cocktails are more effective than individual phages in treating MDR *K. pneumoniae* infections in DFU, offering a potential alternative to antibiotic treatment (Jokar *et al.*, 2023).

Phage-antibiotics synergy

Phages can be used solely or mixed with some probiotics, antibiotics, or synbiotics (Łusiak-Szelachowska *et al.*, 2022). Morrisette *et al.* (2020), enumerated three general advantageous mechanisms and interactions of phage-antibiotic combinations: (1) Phage-antibiotic synergy (PAS); (2) Biofilm eradication; and (3) Phage-antibiotic seesaw effect. The proposal of combining phages

with antibiotics is due to a suggestion that their efficacy and efficiency would increase. Numerous studies highlighted that certain antibiotics increase the biosynthetic capabilities of bacteria while phages are finding ways to accelerate their own division mechanisms.

Phage-antibiotic synergy was first observed in animal models and *in vitro* (Segall *et al.*, 2019). There are numerous mechanisms behind the PAS based on its interactions with specific bacterial strains. The mechanisms may involve increasing the antibiotic susceptibility of bacteria through the help of antibiotics by (1) decreasing the Minimum Inhibitory Concentration (MIC), (2) accelerated phage amplification, (3) decreasing the occurrence of mutants, (4) depolymerization of cell walls for increased cell penetration of antibiotics, and (5) increased susceptibility of the bacteria due to the presence of both the antibiotics and the phage (Nobrega *et al.*, 2015).

The phage-antibiotic synergy (PAS) may increase the amplification of bacteriophages inside the host cell (Nobrega *et al.*, 2015). The PAS also escalates the sensitivity of the bacteria to antibiotics and may decrease the number of mutants that are resistant to antibiotics and phages (Nobrega *et al.*, 2015). For example, the *P. aeruginosa* phage cocktail PAM2H along with meropenem, ciprofloxacin, or gentamicin decreased the number of resistant *P. aeruginosa* cells and increased the number of susceptible cells by 81%, 56%, 63%, and 31% (Nobrega *et al.*, 2015). A recently published study was able to explore the molecular mechanisms behind PAS using a hypervirulent *K. pneumoniae* strain and phage H5 combined with ceftazidime (CAZ). The results show that the combination suppresses bacterial metabolic activity more effectively than phage alone, altering bacterial population evolution. A point mutation (wcaJG949A) was selected by phage H5, promoting carbohydrate metabolism and phage resistance, but the phage-CAZ combination counter-selected this mutation, imposing a higher fitness cost. This led to changes in metabolic pathways, such as the phosphotransferase system and ascorbate metabolism, contributing to resistance suppression (Qin *et al.*, 2024). These findings provide novel insights into how PAS alters bacterial adaptation, offering a novel approach to optimize phage-antibiotic therapy. Another study optimized PAS therapy to combat MDR *K. pneumoniae* strains. The researchers selected broad-host-range phages, optimized their formulations, and combined them with last-line antibiotics, including

colistin and tigecycline, to enhance therapeutic efficacy. PAS treatment significantly reduced bacterial growth in 64%-75% of 88 clinical MDR *K. pneumoniae* strains, with a notable decrease in resistance emergence from 55.66% to 3.75%. In murine models, optimized PAS therapy also led to a significant reduction in bacterial burden compared to antibiotic-only treatments. The findings demonstrate PAS therapy's potential not only for improving treatment outcomes but also for reducing the emergence of antibiotic resistance. Key strategies for optimizing PAS therapy include: (i) selecting broad host-range phages targeting various MDR strains, (ii) optimizing phage formulations for better lytic capacity and host specificity, and (iii) combining phages with effective last-line antibiotics. This optimized PAS approach could delay resistance evolution and deepen our understanding of bacterial pathogenesis and genetic mechanisms related to antibiotic-phage interactions (Zhao *et al.*, 2024).

The structural polysaccharides in bacteria including the LPS or lipopolysaccharide and the PG or peptidoglycan and the EPS or exopolysaccharides found in biofilms exhibit crucial roles in maintaining the integrity of bacteria against foreign invaders and as well as maintain their bacterial virulence. The depolymerization of bacterial polysaccharides happens when the phages are added and the glycan polymers are broken down as the depolymerases of phages assist. Depolymerases of phages may either become a component within a virion or a soluble protein after the lysis of the host cell as the phage matures. The depolymerase includes different types such as endorhamnosidases, endosialidases, hyaluronate lyases, alginate lyases, and peptidoglycan hydrolases (Nobrega *et al.*, 2015). Through this mechanism, the cell penetration of antibiotics via diffusion increases because of the membrane breakdown.

However, despite all the advantages some antagonistic relationships between the phages and the antibiotics can also be observed but this phenomenon is rare (Nobrega *et al.*, 2015). There is also an existence of “non-active” synergisms of bacteriophages and antibiotics ((Łusiak-Szelachowska *et al.*, 2022). For instance, a recent study investigated phage-antibiotic combinations against MDR bacteria, including *S. aureus*, *Enterococcus faecium*, and *P. aeruginosa*, using protein synthesis inhibitors and cell envelope-active agents. The results showed that combinations of phages with linezolid (LZD) and minocycline (MIN) were antagonistic for *S. aureus* and *E. faecium*, while phages combined with

daptomycin (DAP) and ceftaroline (CPT) exhibited synergy. For *P. aeruginosa*, phage-azithromycin (AZM) was antagonistic, phage-gentamicin (GEN) was additive, and phage-cefepime (FEP) was synergistic. Phage growth kinetics were also impacted by the antibiotics, particularly LZD and MIN, suggesting that some antibiotics may interfere with phage efficacy through changes in phage growth dynamics. This shows the complexity of phage-antibiotic interactions, emphasizing the need for further studies to optimize combination therapies. This concurs with the review of Fungo *et al.* (2023) which posited that even though PAS shows potential against MDR pathogens, optimal combinations remain unclear, specifically, PAS efficacy against ESKAPE pathogens highlights key factors: treatment order, dose, mechanisms of action, and clinical applicability. The lack of consistency in current PAS regimens suggests the need for individualized approaches. Indeed, more focused experimental studies to address these gaps and refine PAS protocols are needed to advance its clinical potential.

Advancements in phage therapy

The promise of bacteriophage therapy has immensely progressed over the years due to heightened mediatization and research attention given to the tool as a novel treatment approach to address MDR bacterial infections (Pirnay, 2020). Recent notable advancements in the therapy have been developed such as efficient strategies for isolation and characterization, genetic modification, phage formulation, and therapeutic approach, as well as clinical trials.

Bacteriophage isolation and characterization techniques

The early phase of bacteriophage therapy applies significant procedures such as the isolation of bacteriophages with beneficial traits, and their subsequent characterization. The success of the therapy greatly relies on the application of these procedures to ensure proper identification and development of phages. Early techniques include the enrichment protocol for isolation developed by Felix d’Herelle, culture-based detection methods (*e.g.* plaque testing, routine test dilution, culture lysis, and spot testing), and characterization procedures for bacteriophage morphology through electron microscopy and efficiency of plating by plaque assay. These long-established procedures are limited, strenuous, and time-consuming which highlights the importance of newly developed isolation and characterization methods in phage therapy (Hyman, 2019).

Current phage isolation protocols have sig-

nificantly progressed and accelerated due to the use of modern systems for metagenomics (*e.g.* viral metagenomics and single-virus genomics), and high-throughput sequencing. Viral metagenomics is a modern approach to analyzing the whole viral genetic material isolated from a wide range of environments (Díaz-Muñoz *et al.*, 2017). It involves the optimization of phage isolation processes (viral-like particle recovery, purification, and concentration) from various environmental samples by “phage spiking” with the use of a set titer of known phages and diversion of main purification routes such as polyethylene glycol precipitation and tangential flow filtration into many advanced extraction routes. Optimization improved the number of phage particles isolated and phage DNA obtained in contrast with traditional methods (Hayes *et al.*, 2017). Another, single-virus genomics is an approach that allows isolation and complete sequencing of single viral particles in nature. It is associated with flow cytometric techniques for sorting individual viruses, genome amplification by multiple displacement amplification, and whole genome sequencing. The use of this technique paved the way for the genome of a single phage to be assessed in detail in terms of essential characteristics (Hayes *et al.*, 2017). The

development of these game-changing metagenomic techniques is vital in phage therapy as they enable direct sequencing and analysis of phage genomes and maximized evaluation of diverse populations of phages (Pires *et al.*, 2016).

The establishment of high-throughput next-generation sequencing has greatly transformed the technology for gene sequencing wherein it paved the way for consistent and regular generation of metagenomic data from different environments. In particular, whole-community metagenomic sequencing permits host genome sequencing to confer various information on host-phage dynamics and the prevalence of prophages. Accumulated information from metagenomic studies resulted in the development of several machine-learning tools and software used to identify new phage species which are vital in phage therapy. In the last ten years, different mainly utilized phage identification tools that operate locally are described in Table 1 (Ho *et al.*, 2023).

Identified bacteriophages are subjected to characterization procedures to determine their properties and effectiveness against bacterial species of interest. Over time, advanced strategies for characterization based on high-throughput sequenc-

Table 1. Selected metagenomic phage identification tools to identify and predict phage sequences

Tool	Brief Description and Applications
DeepVirFinder	Predicts sequences via k-mer-based deep learning method and enables heightened viral identification at all contig lengths
Kraken2	K-mer-based metagenomic classifier tool that assigns taxonomic labels, and detects viruses through the application of a database
MetaPhinder	Determines phage sequences in organized contigs and mean nucleotide identity by employing BLAST hits to multiple phage genomes
viralVerify	Module of the MetaviralSPAdes tool; Naïve Bayes classifier elicited from Hidden Markov models protein hits is used to classify contigs
PPR-Meta	Ascertains the identity of phages and plasmids by using deep learning convolutional neural networks approach
Seeker	Applies a neural network that integrates a long short-term memory model regardless of its phage sequence motifs or pre-selected features
VIBRANT	Retrieves prophages and emphasizes auxiliary metabolic genes and pathways by deep-learning neural network based on protein signatures
VirFinder	Primal machine learning tool that applies K-mer signatures to identify viral contigs
VirSorter	Predicts and identifies phage by reference-based and reference-free methods; relies on probabilistic models and protein homology searches
VirSorter2	Extension of VirSorter; assesses “viralness” of phage genomic features by applying machine learning to enhance the detection of viral diversity

ing approaches have been developed to ensure an efficient selection of phages to be used in the therapy. In particular, whole-genome sequencing enables extensive assessment of phage genomes, recognition of essential phage traits (e.g. antibiotic resistance and virulence), and confirmation of the absence of toxin genes (Khan Mirzaei and Nilsson, 2015). In addition, comparative genomics techniques which involve the comparison of whole genome sequences of various species are currently applied to allow phage classification into distinct categories and phage identification based on relatedness, host range, and functional traits (Khan Mirzaei and Nilsson, 2015; Hyman, 2019). Furthermore, a tool called pulse-field gel electrophoresis is now employed to allow direct measurement of phage genome size. It is applied for restriction fragment length polymorphism analysis which involves the isolation of several phages and mainly differentiates the enzyme digested DNA to reveal the difference of every phage (Hyman, 2019).

Bacteriophage engineering techniques

Technologies designed to genetically manipulate phages have progressed promptly, and their application is vital for the development of more functional phage therapies. As phage modification enables experts to improve phage therapeutic capacity, infectivity, and bactericidal potential, novel engineering techniques provide ways to synthetically create phages that are more stable and safe for use in therapies. Different phage and phage component editing strategies that help conquer previous limitations in phage use are aided by data on phage genome sequencing and studies on host-phage interactions (Pires *et al.*, 2016).

Several notable phage engineering techniques have been developed within the decade. Major techniques include Bacteriophage Recombineering of Electroporated DNA (BRED) and the clustered regularly interspaced short palindromic repeats associated-Cas (CRISPR-Cas) systems. BRED is a technique that involves co-electroporation of donor dsDNA and bacteriophage DNA into electrocompetent bacterial cells that express proteins (such as RecE/RecT-like protein) that encourage homologous recombination. It allows gene deletion, insertion, and/or replacement to induce point mutations in the genome of the phage. The use of BRED has developed phages collected at greater frequencies of around 10-15% and can be screened using only a few numbers of polymerase chain reactions (Pires *et al.*, 2016; Chen *et al.*, 2019). Aside from BRED, some studies genetically modify bacteriophages us-

ing a 5-Site Directed Mutagenesis Kit (New England Biolabs). In one study, genetically modified *fd* bacteriophages were engineered to display variants of the Cu(II)-binding peptide HGFANVA, forming filamentous structures with observed thicknesses of 2–5 nm via atomic force microscopy (AFM) (Korkmaz *et al.*, 2024). Peptide-modified phages could be engineered to selectively target cancer cells, MDR bacteria, or inflammatory sites, and may serve as delivery vehicles for drugs, vaccines, or therapeutic enzymes, enabling targeted therapy with reduced side effects.

CRISPR-Cas systems employ methods of (a) CRISPR adaptation, (b) RNA biogenesis, and (3) CRISPR-Cas interference. The approach involves homologous recombination between the DNA of bacteriophage and plasmid, followed by gene targeting by CRISPR-Cas technology used for counterselection of wild phages, and to ensure the retention of recombinant phages. CRISPR-Cas-based engineering approaches used in phages have allowed intricate editing such as resistance gene removal and therapeutic payload integration to promote greater efficacy. A recent review argues that the CRISPR-Cas system offers a more precise and efficient approach to generating genetically modified bacteriophages, surpassing conventional methods that often involve extensive screening and low success rates. CRISPR-Cas9 has successfully modified phage tail fibers to enhance infectivity and broaden host range, while CRISPR-Cas10 facilitates the removal of virulent genes and expansion of host specificity in staphylococcal phages. These advancements show CRISPR's potential for rapidly developing targeted therapeutic phages, with significant implications for military and clinical applications (Kovacs *et al.*, 2024). Further, the scarless Cas9-assisted recombineering (no-SCAR) system has been demonstrated as an effective tool for genome editing of bacteriophages, particularly in expanding their host range. In a recent study, phages from the *Podoviridae* (PHB20), *Myoviridae* (PHB13), and *Siphoviridae* (SP01) families were edited using the no-SCAR system. The system showed varying antiviral activity across different phages, with PHB13 exhibiting a selective antiviral response when specific sgRNAs were applied. The most significant result was obtained with PHB20, where the tail fiber gene (ORF40) was swapped to broaden its host spectrum. This modification enabled PHB20 to target MDR *E. coli*. By replacing the phage's tail fiber gene with sequences derived from environmental water samples or metagenom-

ic databases, the engineered phage became capable of infecting both the original host and new MDR *E. coli* strains. The engineered polyvalent phages retained the lytic activity of the original phages and effectively killed MDR *E. coli* (Song *et al.*, 2023).

Other vital advanced methods for phage engineering include *in vitro* phage genome refactor-ing, cell-free transcription-translation systems, and yeast-based phage genome assembly (Hymes, 2018; Chen *et al.*, 2019). Furthermore, the application of strategies in synthetic biology is beneficial for phage therapy as it allows the creation of synthetic phage genomes that have desirable customized characteristics. Some of the strategies include the bacteriophage genome de novo synthesis, non-natural nucleotide integration, and genetic circuit addition (Lajoie *et al.*, 2013).

Bacteriophage formulation, delivery, and clinical trials

Numerous studies prioritize the optimization of phage formulation and delivery with an aim to maintain stable phages with longer shelf life and better survival capacity in the course of formulation production. Advanced phage formulation mainly centers on encapsulation methods such as liposomal encapsulation (liquid), emulsification (semi-solid), hydrogels (semi-solid), spray-drying (powder), electrospinning (nanofibers), and freeze-drying (powder) which involve phage coating as protective measures. These phage formulation methods serve to control and direct targeted phage delivery to infected locations which is essential in phage therapy as it optimizes therapeutic activity. For phage delivery, novel methods like biofilms and phage-loaded nanoparticles are continually investigated to enhance phage efficacy (Drulis-Kawa *et al.*, 2012). Pharmaceutical nanoparticles, including nanospheres, nanocapsules, and materials like carbon nanotubes and dendrimers, are solid carriers with diameters under 100 nm, often designed to be either biodegradable or non-biodegradable. These nanoparticles can be produced via various methods such as emulsion-solvent evaporation, double emulsion, salting out, diffusion, and solvent displacement. For instance, Gondil *et al.* (2020) investigated Cpl-1-loaded chitosan nanoparticles for combating antibiotic-resistant *S. pneumoniae*, demonstrating biocompatibility and hemocompatibility with no cytotoxic effects. Another study used thiolated phages to induce gold nanoparticle aggregation, which enabled rapid detection of Gram-negative bacterial infections and determination of antibiotic susceptibility profiles. The assay relied on

the phage-bacteria interaction between chimera viruses of the *Inoviridae* family and Gram-negative bacteria (Peng *et al.*, 2020). Abdelsattar *et al.* (2022) combined silver nanoparticles with phage ZCSE6 to inhibit *Salmonella* growth. The study reported a significant decrease in the MIC and MBC values when combined with the phage (from 6.25 to 12.5 µg/ml to 1.5 µg/ml), showing that nanoparticle-phage combinations may be an effective infection control method. Another recently published paper reported that the combined use of T7 phages and phage-presented Ag Nanoparticles showed significant synergistic effects in eradicating *E. coli* biofilm (Szymczak *et al.*, 2024). Alipour-Khezri and colleagues (2024) also explored the combined efficacy of green-synthesized zinc oxide nanoparticles and bacteriophages PB10 and PA19 in combating *P. aeruginosa* biofilms. The combination significantly reduced biofilm formation and bacterial viability, repressed quorum-sensing gene expression, and mitigated the cytotoxic effects of zinc oxide nanoparticles on human cells, demonstrating the potential for enhanced anti-biofilm therapies. The encapsulation of bacteriophages protects them from stomach acid in oral delivery systems. However, challenges remain in maintaining phage activity during storage and after encapsulation due to temperature, pH, and solvent sensitivity. Tailored encapsulation methods are required for different phage strains.

Different phage formulation and delivery techniques are applied for lytic and non-lytic phages. In particular, lytic bacteriophages utilize liquid phage filtrate, tablets, and formulas wherein delivery is by parenteral route for systemic infections, oral route for gastrointestinal infections, and local route for topical infections. On the other hand, non-lytic phages apply formulation by injections, formulas, and aerosols wherein delivery is by parenteral route for systemic infections, oral route restricted to proteolysis, and a local route for topical infections (Rosner and Clark, 2021).

With the progress in phage isolation, manipulation, and formulation techniques, many clinical trials have been done for the assessment of the safety and efficacy of phage therapy approaches against particular infections. This step is essential to determine the clinical usefulness and acceptance of phage therapy for clinical practice (Pirnay *et al.*, 2018). Remarkable examples and trials include the (a) effectiveness and safety assessment of topical phage therapy in people with chronic diabetic foot infections done in Wroclaw, Poland which revealed

good results in full or pronounced infected wound curing, (b) effectiveness and safety assessment of phage therapy in people with urinary tract infections linked to antibiotic-resistant pathogenic bacteria that reached a phase I to II in Belgium which reported - high success percentage to remove infections and improve symptoms, (c) numerous tolerability, effectiveness and safety assessment of phage therapy in people with serious lung infection associated to cystic fibrosis - a promising result of a case study showed the lowering of bacterial concentration in infected sputum and in the necessity of antibiotic intake, and (d) therapy investigation as bacterial sepsis remedy (Hraiech *et al.*, 2015; Duplessis and Biswas, 2020). Other applications in humans include a cure for cholera, and skin bacterial infections linked to *P. aeruginosa* and *S. aureus* (Principi *et al.*, 2019). These successful clinical trials have paved the way for further progression of phage therapy, yet the tool is still experimental which still promotes extensive research to guarantee ideal use, address existing limitations, and ensure maximized applicability of phage therapy.

Phage therapy clinical trials in humans

Table 2 shows some of the recent trials of using phage therapy in humans. These clinical trials show the potential of bacteriophage therapy in managing chronic bacterial infections, particularly in cystic fibrosis (CF) and antibiotic-resistant conditions. The WRAIR-PAM-CF1 trial (NCT05453578) is evaluating a 4-component anti-*P. aeruginosa* bacteriophage mixture (4×10^7 to 4×10^9 PFU) in 72 CF patients across 16 U.S. centers. The trial aims to assess safety and bacterial reduction. In addition, the completed SWARM-P.a. study (NCT04596319) tested AP-PA02, a multi-phage cocktail, demonstrating a sustained two-log bacterial load reduction. Effective lung delivery via nebulization was confirmed, with minimal systemic absorption, supporting its safety and efficacy. These findings suggest a pathway for larger trials targeting both CF and non-CF bronchiectasis (NCFB).

The Yale Phage Therapy trial (NCT04684641) validated personalized phage therapy for chronic *P. aeruginosa* infections in CF patients. Meanwhile, trials like Personalized Bacteriophage Therapy (NCT05177107) and TP-102 (NCT04803708) have shown potential for treating diabetic foot ulcers and osteomyelitis. These studies highlight phage therapy's capacity to reduce bacterial burden and aid in tissue healing. Phage cocktails such as LBP-EC01 (NCT04191148) for *E. coli* urinary tract infections and AP-SA02 (NCT05184764) for

S. aureus bacteremia demonstrated enhanced efficacy when combined with antibiotics. Emerging therapies, including VRElysin™ (NCT05715619) for vancomycin-resistant *Enterococcus* and Mycobacteriophage POSTSTAMP (NCT06262282) for mycobacterial infections, indicate expanding clinical applications. Optimizing delivery mechanisms and addressing bacterial resistance are critical next steps. Ongoing trials will determine the long-term efficacy and durability of these therapies in chronic and MDR infections.

Phage therapy and antibiotic therapy comparison

The continuous development of phage therapy is motivated by the urgent public health concern associated with the rise of MDR bacteria. Since conventional antibiotics have adverse effects and several species have grown resistant to different classes of antibiotics, the establishment of new alternatives like phage therapy is highly important. Potential differences in efficacy and safety of the two approaches - phage therapy and antibiotic therapy - lie in their distinction in terms of mechanism of action, specificity, biofilm penetration, and availability (Górski *et al.*, 2021).

Traditional antibiotic therapy acts to hinder growth or eliminate bacterial species by targeting essential properties like replication of DNA replication and synthesis of cell walls, while phage therapy makes use of bacteriophages in nature to infect and destroy bacteria (Davies and Davies, 2010). In terms of specificity, antibiotics offer a wide range of spectrum (such as pathogenic and beneficial ones) and are not specific to one strain, while phage therapy utilizes highly specific targeting by phages which enables targeted treatment and lowered damage to the host's microbial. Another comparative category is called biofilm penetration wherein antibiotic therapy struggles, while phages are able to penetrate and infect bacterial species in a gene therapy. Lastly is clinical availability and applications. Antibiotic therapy is widely accessible and available worldwide brought by substantial research and creation, while phage therapy is evidently less widespread and remains experimental. Overall, each approach offers varying advantages, evolutionary capabilities, self-amplification, as well as limitations. With more studies, phage therapy is said to have great potential as a valuable tool in the fight against antibiotic resistance and the treatment of bacterial infections (Schooley *et al.*, 2017; Pirnay *et al.*, 2018).

Table 2. Overview of clinical trials investigating phage therapy (Source: clinicaltrials.gov)

Intervention	Target Infection	Clinical Trial ID	Status	Latest Update
Walter Reed Army Institute of Research - PAM-Cystic Fibrosis1 (WRAIR-PAM-CF1)	Cystic Fibrosis chronically colonized with <i>P. aeruginosa</i>	NCT05453578	Recruiting	2024
AP-SA02 Adjunct to Best Available Antibiotic Therapy	Bacteremia due to <i>S. aureus</i>	NCT05184764	Recruiting	2024
Mycobacteriophage (POSTSTAMP)	Cystic Fibrosis and non-tuberculous mycobacteria pulmonary disease	NCT06262282	Enrolling by invitation	2024
AP-PA02 Multi-Phage Therapeutic Candidate for Inhalation	Cystic Fibrosis and chronic pulmonary <i>P. aeruginosa</i> infection	NCT04596319	Completed	2024
Inhaled AP-PA02	Non-Cystic Fibrosis Bronchiectasis and Chronic Pulmonary <i>P. aeruginosa</i> Infection (Tailwind)	NCT05616221	Completed	2024
VRElysin™	Vancomycin-resistant <i>Enterococcus</i>	NCT05715619	Recruiting	2023
Personalized Bacteriophage Therapy	Diabetic foot osteomyelitis	NCT05177107	Recruiting	2023
Yale Phage Therapy	Cystic Fibrosis with chronic <i>P. aeruginosa</i> airway infections	NCT04684641	Completed	2023
Anti-Staphylococcus aureus Bacteriophages	Hip or knee prosthetic joint infection due to <i>S. aureus</i> treated with DAIR and antibiotic therapy	NCT05369104	Recruiting	2022
TP-102	Diabetic foot ulcers with <i>P. aeruginosa</i> , <i>S. aureus</i> , and/or <i>Acinetobacter baumannii</i>	NCT04803708	Completed	2022
LBP-EC01 Phage Cocktail	Lower urinary tract colonization caused by <i>E. coli</i>	NCT04191148	Completed	2022

Advantages of phage therapy

Bacteriophage therapy offers various advantages as an alternative treatment for bacterial infections. This ability can be attributed to the characteristics and abilities of the phages themselves. Its most essential set of traits includes availability in nature, targeted specificity, versatility, independent amplification, and lower-level resistance establishment.

Phage therapy offers significant advantages due to the natural abundance and diversity of bacteriophages in terrestrial, aquatic, and human environments, making it cost-effective. Their easy engineering allows for targeted solutions to antibiotic limitations (Principi *et al.*, 2019). Additionally, phages' high specificity enables them to target bacterial species or strains, including resistant ones. This precision minimizes adverse effects and

reduces collateral damage to beneficial bacteria and human health (Luong *et al.*, 2020). Moreover, phage therapy is acknowledged for its safety and tolerability brought about by its adaptability and low chance of developing resistance (Principi *et al.*, 2019). Phages are considered “bactericidal agents”, wherein infecting a bacterial host, bacteria are less likely to gain their viabilities (Loc-Carillo and Abedon, 2011). This prevents the host from developing into a resistant strain. On the other hand, some types of antibiotics are bacteriostatic, wherein they may induce the development of resistance in some strains (Loc-Carillo and Abedon, 2011).

Antibiotic resistance can develop through bacterial mechanisms that respond to specific doses of antibiotics, influenced by metrics like Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC). In contrast, phages

used as biocontrol agents exhibit auto-dosing, amplifying as long as their bacterial hosts are present (Łusiak-Szelachowska *et al.*, 2022). This allows them to target and replicate at infection sites, even at low doses. Additionally, combining antibiotics with phage therapy, known as Phage–Antibiotic Synergy (PAS), offers promising advancements in combating bacterial resistance.

Challenges and future directions of phage therapy

MDR are generally resistant to most antibiotics used by conventional treatment options. The ability of bacteriophages to specifically target and kill bacteria can be utilized as an alternative therapeutic method against the increasing prevalence of infections caused by MDR bacteria. While the clinical trials of bacteriophage therapy yielded positive results, there are still several challenges that must be resolved to achieve its full potential. The major challenges are discussed below, along with the possible solutions to overcome such limitations as well as the future directions of phage therapy.

Bacteriophage selection

The success of the therapy against the MDR bacterial strain is primarily dependent on the selection of an appropriate bacteriophage. As already mentioned, bacteriophages are highly specific in their host range, with most of them only infecting or colonizing a particular species of bacteria (Chan and Abedon, 2012; Drulis-Kawa *et al.*, 2012). The relatively narrow range of host compatibility of bacteriophages offers both advantages and challenges to such an approach. Although the advantage of having relatively few compatible hosts includes the preservation of beneficial bacterial strains, the utilization of specific bacteriophages is limited only to a small number of bacterial strains (Chan and Abedon, 2012). Therefore, the establishment of sufficient and comprehensible phage databases, as well as the development of efficient screening methods, is necessary, particularly in the formulation of bacteriophage with a diverse host range. However, even if scientists secured a collection of “ready-to-use” bacteriophage vectors, identifying the bacterial pathogen is still crucial. Furthermore, this could be hindered by several factors such as the severity of infection, cost, and limited availability of clinical laboratories (Drulis-Kawa *et al.*, 2012). Thus, the selection of bacteriophages to effectively target the intended MDR pathogens in infections remains a hurdle for

most cases.

Development of resistance against bacteriophages

The interaction between bacteriophages and bacteria could likely drive co-evolutionary processes that may influence their tolerance to each other. Similar to antibiotic resistance, MDR bacteria are very capable of developing resistance against bacteriophage. Resistance of bacteria occurs through various mechanisms such as phenotypic shifts, point mutations in surface bacteriophage receptors, acquisition of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) spacers, and formation of biofilms (Chanishvili, 2016; Torres-Barceló, 2018). The development of bacterial resistance towards the administered bacteriophages compromises the efficacy of the phage therapy and may lead to the persistence or reoccurrence of the bacterial infection. Therefore, in designing effective phage therapeutic strategies, it is important to consider the factors involved in the evolution of bacterial resistance. Moreover, the selection of long-term effective phages that could minimize the possible impact of resistance development is vital. Lastly, future research must be able to give much-needed information on the evolutionary and molecular consequences of phage therapy in MDR bacterial infections (Torres-Barceló, 2018).

Bacteriophage pharmacokinetics and delivery

In the context of phage therapy, pharmacokinetics refers to the dynamic movements or behavior of bacteriophages as they pass through the body, particularly during the processes of absorption, distribution, metabolism, and excretion (Dąbrowska and Abedon, 2019). Understanding these mechanisms is a crucial step in the optimization of their therapeutic efficacy.

One of the pharmacokinetics obstacles associated with phage therapy is the accessibility of the target bacteria within the body. Bacteriophages can be administered via oral delivery, injection, or direct application to the infection sites. Among these routes, oral delivery is known as the most convenient and most likely to be accepted by the patients. Although this method is effective in treating gastrointestinal infections, the bacteriophage is susceptible to inactivation due to the acidic and proteolytic environment of the gut (Malik *et al.*, 2017). On the other hand, the intravenous route of administration is more efficient and faster in terms of the introduction of bacteriophage in the systemic circulation. However, the administered bacteriophage could be subjected to various clearing and immune mecha-

nisms provided by the innate immune system. They can be cleared in the bloodstream by the reticulo-endothelial system or be neutralized by pre-existing antibodies (Malik *et al.*, 2017; Dąbrowska and Abedon, 2019; Dhungana, 2021). In terms of phage distribution, the penetration of bacteriophage varies from organ to organ. Although bacteriophages, in general, can easily gain access to a wide variety of tissues, it is still unknown if they can remain at a sufficient level or concentration at the sites of bacterial infections. Therefore, the potential for therapeutic success in bacteriophage therapy could be reinforced by molecular engineering of the phage virions (Dąbrowska and Abedon, 2019).

Future direction of phage therapy

The emergence of MDR bacterial infections is recognized as a major public health concern. Thus, the future direction of phage therapy, as a novel and alternative therapeutic approach against MDR bacteria, should be directed toward the improvement of its safety, efficacy, and specificity. This can be done by exploring the fields of combination therapies, phage engineering, and synthetic biology.

Combination therapy involves the utilization of bacteriophages along with conventional treatments such as antibiotics and other immune-stimulating agents. PAS is a phenomenon in which phages are especially effective against MDR bacteria when combined with antibiotics (Diallo and Dublanchet, 2022). The use of PAS is a great approach for the management of bacterial resistance against antibiotics and phages. PAS can overcome the limited host range of bacteriophages allowing for broader coverage of pathogenic bacterial strains. Several studies have reported that PAS can effectively reduce bacterial densities as well as limit the production of bacterial virulence factors (Torres-Barceló, 2018; Li *et al.*, 2021). Although several studies have explored the *in vitro* effects of PAS and gained positive results, further studies, and optimization must be done to fully utilize this combination as a routine therapeutic option against MDR bacterial infections.

Advances in technology, particularly in synthetic biology, could open a lot of opportunities that may enable the modification of natural phage properties to overcome the current limitations of phage therapy. Recombination-based approaches are the most feasible way to engineer phage genomes to acquire the desired mutations. Through genetic engineering, researchers can potentially expand the host range of bacteriophages, overcoming the

limitations of specificity. Moreover, the technology could also be used to overcome the bacterial resistance mechanisms of MDR bacteria against bacteriophage by designing counter-resistance systems. Finally, the creation of synthetic phage via genetic engineering can potentially solve the problems associated with the human innate immune response, phage-clearing mechanism, and regulatory and production challenges (Barbu *et al.*, 2016; Lenneman *et al.*, 2021).

Conclusion

Phage therapy offers hope to overcome the challenge of antibiotic resistance emergencies. While they may not be fully able to replace antibiotics, the advantageous properties of phages establish their salience as a vital addition to the antimicrobial arsenal. With the ongoing advancements in synthetic biology, phage therapy is predicted to expand its practice and relevance in the field of medicine. Further studies that commit to the development of a clear selection criterion for bacteriophage and genetic engineering of phage components for minimized resistance against MDR infections and improved, phage-derived drug administration are highly recommended.

Acknowledgments

The authors gratefully acknowledge the support provided by the Division of Biological Sciences, College of Arts and Sciences, University of the Philippines Visayas, during the preparation and writing of this manuscript.

References

- Abdelsattar, A. S., T. A. Hakim, N. Rezk, W. M. Farouk, Y. Y. Hassan, S. M. Gouda, A. El-Shibiny. (2022). Green synthesis of silver nanoparticles using *Ocimum basilicum* L. and *Hibiscus sabdariffa* L. extracts and their antibacterial activity in combination with phage ZCSE6 and sensing properties. *J. Inorg. Organomet. Polyme. Mat.* **32**: 1951–1965. DOI: 10.1007/s10904-022-02234-y.
- Abedon, S. T. (2014). Phage therapy: eco-physiological pharmacology. *Scientifica* **2014**: 1–29. DOI: 10.1155/2014/581639.
- Abedon, S. T. (2018). Phage therapy: various perspectives on how to improve the art. *Methods Mol. Biol.* **1734**: 113–127. DOI: 10.1007/978-1-4939-7604-1_11.
- Abedon, S. T., K. M. Danis-Wlodarczyk, D. J. Wozniak. (2021). Phage cocktail development for bacteriophage therapy: toward improving spectrum of activity breadth and depth. *Pharmaceuticals* **14**: 1019. DOI: 10.3390/ph14101019.
- Alipour-Khezri, E., A. Moqadami, A. Barzegar, M. Mahdavi, M. Skurnik, G. Zarrini. (2024). Bacteriophages and green synthesized zinc oxide nanoparticles in combination are efficient against biofilm formation of *Pseudomonas aeruginosa*. *Viruses* **16**: 897. DOI: 10.3390/v16060897.

- Bakkeren, E., M. Diard, W.-D. Hardt (2020). Evolutionary causes and consequences of bacterial antibiotic persistence. *Nat. Rev. Microbiol.* **18**: 479–490. DOI: 10.1038/s41579-020-0378-z.
- Barbu, E. M., K. C. Cady, B. Hubby (2016). Phage therapy in the era of synthetic biology. *Cold Spring Harb. Perspect. Biol.* **8**: a023879. DOI: 10.1101/cshperspect.a023879.
- Bassetti, M., G. Poulakou, E. Ruppe, E. Bouza, S. J. Van Hal, A. Brink. (2017). Antimicrobial resistance in the next 30 years, humankind, bugs and drugs: a visionary approach. *Intensive Care Med.* **43**: 1464–1475. DOI: 10.1007/s00134-017-4878-x.
- Chan, B. K., S. T. Abedon (2012). Phage therapy pharmacology phage cocktails. *Adv. Appl. Microbiol.* **78**: 1–23. DOI: 10.1016/B978-0-12-394805-2.00001-4.
- Chan, B. K., S. T. Abedon, C. Loc-Carrillo (2013). Phage cocktails and the future of phage therapy. *Future Microbiol.* **8**: 769–783. DOI: 10.2217/fmb.13.47.
- Chanishvili, N. (2012). Phage therapy - history from Twort and d'Herelle through Soviet experience to current approaches. *Adv. Virus Res.* **83**: 3–40. DOI: 10.1016/b978-0-12-394438-2.00001-3.
- Chanishvili, N. (2016). Bacteriophages as therapeutic and prophylactic means: summary of the Soviet and post-Soviet experiences. *Curr. Drug. Deliv.* **13**: 309–323. DOI: 10.2174/156720181303160520193946.
- Chen, Y., H. Batra, J. Dong, C. Chen, V. B. Rao, P. Tao (2019). Genetic engineering of bacteriophages against infectious diseases. *Front. Microbiol.* **10**: 954. DOI: 10.3389/fmicb.2019.00954.
- Cui, Z., X. Guo, T. Feng, L. Li (2019). Exploring the whole standard operating procedure for phage therapy in clinical practice. *J. Transl. Med.* **17**: 373. DOI: 10.1186/s12967-019-2120-z.
- Dadgostar, P. (2019). Antimicrobial resistance: implications and costs. *Infect. Drug. Resist.* **12**: 3903–3910. DOI: 10.2147/idr.s234610.
- Davies, J., D. Davies (2010). Origins and evolution of antibiotic resistance. *Microbiol. Molec. Biol. Rev.* **74**: 417–433. DOI: 10.1128/mmb.00016-10.
- Dhungana, G., R. Nepal, M. Regmi, R. Malla (2021). Pharmacokinetics and pharmacodynamics of a novel virulent *Klebsiella* phage Kp_Pokalde_002 in a mouse model. *Front. Cell. Infect. Microbiol.* **11**: 684704. DOI: 10.3389/fcimb.2021.684704.
- Diallo, K., A. Dublanchet (2022). Benefits of combined phage–antibiotic therapy for the control of antibiotic-resistant bacteria: a literature review. *Antibiotics* **11**: 839. DOI: 10.3390/antibiotics11070839.
- Díaz-Muñoz, S. L., R. Sanjuán, S. West (2017). Sociovirology: conflict, cooperation, and communication among viruses. *Cell Host Microbe* **22**: 437–441. DOI: 10.1016/j.chom.2017.09.012.
- Dąbrowska, K., S. T. Abedon (2019). Pharmacologically aware phage therapy: pharmacodynamic and pharmacokinetic obstacles to phage antibacterial action in animal and human bodies. *Microbiol. Mol. Biol. Rev.* **83**: 10–1128. DOI: 10.1128/MMBR.00012-19.
- Drulis-Kawa, Z., G. Majkowska-Skrobek, B. Maciejewska, A.-S. Delattre, R. Lavigne (2012). Learning from bacteriophages - advantages and limitations of phage and phage-encoded protein applications. *Curr. Protein Pept. Sci.* **13**: 699–722. DOI: 10.2174/138920312804871193.
- Duplessis, C. A., B. Biswas (2020). A review of topical phage therapy for chronically infected wounds and preparations for a randomized adaptive clinical trial evaluating topical phage therapy in chronically infected diabetic foot ulcers. *Antibiotics* **9**: 377. DOI: 10.3390/antibiotics9070377.
- Fungo, G. B. N., J. C. W. Uy, K.L.J. Porciuncula, C.M.A. Candelario, D.P.S. Chua, T. A. D. Gutierrez, D. M. D. Papa (2023). “Two is better than one”: The multifactorial nature of phage-antibiotic combinatorial treatments against ES-KAPE-induced infections. *Phage* **4**: 55–67. DOI: 10.1089/phage.2023.0007.
- Ghosh, C., P. Sarkar, R. Issa, J. Haldar (2019). Alternatives to conventional antibiotics in the era of antimicrobial resistance. *Trends Microbiol.* **27**: 323–338. DOI: 10.1016/j.tim.2018.12.010.
- Gondil, V. S., T. Dube, J. J. Panda, R. M. Yennamalli, K. Harjai, S. Chhibber (2020). Comprehensive evaluation of chitosan nanoparticle based phage lysin delivery system; a novel approach to counter *S. pneumoniae* infections. *Int. J. Pharm.* **573**: 118850. DOI: 10.1016/j.ijpharm.2019.118850.
- Gordillo Altamirano, F. L., J. J. Barr (2019). Phage therapy in the postantibiotic era. *Clin. Microbiol. Rev.* **32**: 2. DOI: 10.1128/cmr.00066-18.
- Górski, A., J. Borysowski, R. Międzybrodzki, R. (2020). Phage therapy: towards a successful clinical trial. *Antibiotics* **9**: 827. DOI: 10.3390/antibiotics9110827.
- Górski, A., J. Borysowski, R. Międzybrodzki (2021). Bacteriophage interactions with epithelial cells: therapeutic implications. *Front. Microbiol.* **11**: 631161. DOI: 10.3389/fmicb.2020.631161.
- Hatfull, G. F., R. M. Dedrick, R. T. Schooley (2022). Phage therapy for antibiotic-resistant bacterial infections. *Annu. Rev. Med.* **73**: 197–211. DOI: 10.1146/annurev-med-080219-122208.
- Hayes, S., J. Mahony, A. Nauta, D. van Sinderen (2017). Metagenomic approaches to assess bacteriophages in various environmental niches. *Viruses* **9**: 127. DOI: 10.3390/v9060127.
- Hendrix, R. W., M. C. M. Smith, R. N. Burns, M. E. Ford, G. F. Hatfull (1999). Evolutionary relationships among diverse bacteriophages and prophages: all the world's a phage. *Proc. Natl. Acad. Sci.* **96**: 2192–2197. DOI: 10.1073/pnas.96.5.2192.
- Hendrix, R. W. (2002). Bacteriophages: evolution of the majority. *Theor. Popul. Biol.* **61**: 471–480. DOI: 10.1006/tpbi.2002.1590.
- Hibstu, Z., H. Belew, Y. Akelew, H. M. Mengist (2022). Phage therapy: a different approach to fight bacterial infections. *Biologics Targets Ther.* **16**: 173–186. DOI: 10.2147/btt.s381237.
- Ho, S. F. S., N. Wheeler, A. Millard, W. van Schaik (2023). Gauge your phage: benchmarking of bacteriophage identification tools in metagenomic sequencing data. *Microbiome* **11**: 84. DOI: 10.1186/s40168-023-01533-x.
- Hodyra-Stefaniak, K., P. Miernikiewicz, J. Drapała, M. Drab, E. Jończyk-Matysiak, D. Lecion, Z. Kaźmierczak, W. Beta, J. Majewska, M. Harhala, et al. (2015). Mammalian host-versus-phage immune response determines phage fate *in vivo*. *Scient. Rep.* **5**: 14802. DOI: 10.1038/srep14802.

- Hraiech, S., F. Bregeon, J.-M. Rolain (2015). Bacteriophage-based therapy in cystic fibrosis-associated *Pseudomonas aeruginosa* infections: rationale and current status. *Drug Des. Dev. Ther.* **9**: 3653. DOI: 10.2147/dddt.s53123.
- Hutchings, M. I., A. W. Truman, B. Wilkinson (2019). Antibiotics: past, present and future. *Curr. Opin. Microbiol.* **51**: 72–80. DOI: 10.1016/j.mib.2019.10.008.
- Hyman, P. (2019). Phages for phage therapy: isolation, characterization, and host range breadth. *Pharmaceuticals* **12**: 35. DOI: 10.3390/ph12010035.
- Hynes, A. P., G. M. Rousseau, D. Agudelo, A. Goulet, B. Amigues, J. Loehr, D. A. Romero, C. Fremaux, P. Horvath, Y. Doyon, C. Cambillau, S. Moineau (2018). Widespread anti-CRISPR proteins in virulent bacteriophages inhibit a range of Cas9 proteins. *Nat. Commun.* **9**: 2919. DOI: 10.1038/s41467-018-05092-w.
- Jokar, J., R. O. Saleh, N. Rahimian, A. Ghasemian, G. Ghaznavi, A. Radfar, E. Zarenezhad, S. Najafipour (2023). Antibacterial effects of single phage and phage cocktail against multidrug-resistant *Klebsiella pneumoniae* isolated from diabetic foot ulcer. *Virus Genes* **59**: 635–642. DOI: 10.1007/s11262-023-02004-z.
- Khan Mirzaei, M., A. S. Nilsson (2015). Isolation of phages for phage therapy: a comparison of spot tests and efficiency of plating analyses for determination of host range and efficacy. *PLoS one* **10**: e0118557. DOI: 10.1371/journal.pone.0118557.
- Kifelew, L.G., M. S. Warner, S. Morales, L. Vaughan, R. Woodman, R. Fitridge, J. G. Mitchell, P. Speck (2020). Efficacy of phage cocktail AB-SA01 therapy in diabetic mouse wound infections caused by multidrug-resistant *Staphylococcus aureus*. *BMC Microbiol.* **20**: 204. DOI: 10.1186/s12866-020-01891-8.
- Kortright, K. E., B. K. Chan, J. L. Koff, P. E. Turner (2019). Phage therapy: a renewed approach to combat antibiotic-resistant bacteria. *Cell Host Microbe* **25**: 219–232. DOI: 10.1016/j.chom.2019.01.014.
- Korkmaz, N., S. Himawan, M. Usman, S. Baik, M. Kim (2024). Bacteriophage engineering for improved copper ion binding. *Macromol. Biosci.* **24**: 2300354. DOI: 10.1002/mabi.202300354.
- Kovacs, C. J., A. Antonacci, A. Graham, F. Jessup, W. Rankin, B. Brasko, *et al.* (2024). Comparing methods to genetically engineer bacteriophage and increase host range. *Mil. Med.* **189**: e1488–e1496. DOI: 10.1093/milmed/usae226.
- Lajoie, M. J., A. J. Rovner, D. B. Goodman, H. R. Aerni, A. D. Haimovich, G. Kuznetsov, J. A. M. Mercer, H. H. Wang, P. W. Carr, J. A. Mosberg, N. Rohland, P. G. Schultz, J. L. Jacobson, J. Rinehart, G. M. Church, F. J. Isaacs (2013). Genomically recoded organisms expand biological functions. *Science* **342**: 357–360. DOI: 10.1126/science.1241459.
- Lenneman, B. R., J. Fernbach, M. J. Loessner, T. K. Lu, S. Kilcher (2021). Enhancing phage therapy through synthetic biology and genome engineering. *Curr. Opin. Biotechnol.* **68**: 151–159. DOI: 10.016/j.copbio.2020.11.003.
- Li, G., C. Y. Leung, Y. Wardi, L. Debarbieux, J. S. Weitz (2020). Optimizing the timing and composition of therapeutic phage cocktails: a control-theoretic approach. *Bull. Math. Biol.* **82**: 75. DOI: 10.1007/s11538-020-00751-w.
- Li, X., Y. He, Z. Wang, J. Wei, T. Hu, J. Si, G. Tao, L. Zhang, L. Xie, A. E. Abdall, G. Wang, Y. Li, T. Teng (2021). A combination therapy of phages and antibiotics: two is better than one. *Intern. J. Biol. Sci.* **17**: 3573–3582. DOI: 10.7150/ijbs.60551.
- Lin, D., B. Koskella, H. Lin (2017). Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World J. Gastrointest. Pharmacol. Ther.* **8**: 162. DOI: 10.4292/wjgpt.v8.i3.162.
- Loc-Carrillo, C., S. T. Abedon (2011). Pros and cons of phage therapy. *Bacteriophage* **1**: 111–114. DOI: 10.4161/bact.1.2.14590.
- Luong, T., A. C. Salabarria, D. R. Roach (2020). Phage therapy in the resistance era: where do we stand and where are we going? *Clin. Ther.* **42**: 1659–1680. DOI: 10.1016/j.clinthera.2020.07.014.
- Lusiak-Szelachowska, M., R. Międzybrodzki, Z. Drulis-Kawa, K. Cater, P. Knežević, C. Winogradow, K. Amaro, E. Jończyk-Matysiak, B. Weber-Dąbrowska, J. Rękas, *et al.* (2022). Bacteriophages and antibiotic interactions in clinical practice: what we have learned so far. *J. Biomed. Sci.* **29**: 23. DOI: 10.1186/s12929-022-00806-1.
- Malik, D. J., I. Sokolov, G. K. Vinner, F. Mancuso, S. Cinquerui, G. T. Vladislavjevic, M. R. J. Clokie, N. J. Garton, A. G. F. Stapley, A. Kirpichnikova (2017). Formulation, stabilisation and encapsulation of bacteriophage for phage therapy. *Adv. Colloid Interface Sci.* **249**: 100–133. DOI: 10.1016/j.cis.2017.05.014.
- Melo, L. D., H. Oliveira, D. P. Pires, K. Dabrowska, J. Azere-do (2020). Phage therapy efficacy: a review of the last 10 years of preclinical studies. *Crit. Rev. Microbiol.* **46**: 78–99. DOI: 10.1080/1040841X.2020.1729695.
- Merabishvili, M., J. P. Pirnay, V. De Vos (2018). Guidelines to compose an ideal bacteriophage cocktail. *Bacteriophage Therapy. Methods Mol. Biol.* **1693**: 99–110. DOI: 10.1007/978-1-4939-7395-8_9.
- Międzybrodzki, R., J. Borysowski, B. Weber-Dąbrowska, W. Fortuna, S. Letkiewicz, K. Szufnarowski, Z. Pawełczyk, P. Rogóż, M. Kłak, E. Wojtasik, *et al.* (2012). Clinical aspects of phage therapy. *Adv. Virus Res.* **83**: 73–121. DOI: 10.1016/b978-0-12-394438-2.00003-7.
- Morozova, V. V., V. V. Vlassov, N. V. Tikunova (2018). Applications of bacteriophages in the treatment of localized infections in humans. *Front. Microbiol.* **9**: 1696. DOI: 10.3389/fmicb.2018.01696.
- Morrisette, T., R. Kebriaei, K. L. Lev, S. Morales, M. J. Rybak (2020). Bacteriophage therapeutics: a primer for clinicians on phage–antibiotic combinations. *Pharmacotherapy: J. Hum. Pharmacol. Drug Ther.* **40**: 153–168. DOI: 10.1002/phar.2358.
- Munita, J. M., C. A. Arias (2016). Mechanisms of antibiotic resistance. *Microbiol. Spectr.* **4**: 481–511. DOI: 10.1128/microbiolspec.vmbf-0016-2015.
- Murray, C. J., K. S. Ikuta, F. Sharara, L. Swetschinski, G. R. Aguilar, A. Gray, *et al.* (2022). Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet* **399**: 629–655. DOI: 10.1016/S0140-6736(21)02724-0.
- Mushegian, A. R. (2020). Are there 1031 virus particles on Earth, or more, or fewer? *J. Bacteriol.* **202**: e00052–20. DOI: 10.1128/jb.00052-20.
- National Library of Medicine. (n.d.). ClinicalTrials.gov. U.S. National Institutes of Health. Retrieved November 28, 2024, from <https://clinicaltrials.gov/>.

- Nobrega, F. L., A. R. Costa, L. D. Kluskens, J. Azeredo (2015). Revisiting phage therapy: new applications for old resources. *Trends Microbiol.* **23**: 185–191. DOI: 10.1016/j.tim.2015.01.006.
- Peng, H., R. E. Borg, A. B. Nguyen, I. A. Chen (2020). Chimeric phage nanoparticles for rapid characterization of bacterial pathogens: detection in complex biological samples and determination of antibiotic sensitivity. *ACS Sensors* **5**: 1491–1499. DOI: 10.1021/acssensors.0c00654.
- Pires, D. P. P., H. G. Oliveira, L. D. R. Melo, M. A. Cerqueira, J. Azeredo (2016). Bacteriophage-encoded depolymerases: their diversity and biotechnological applications. *Appl. Microbiol. Biotechnol.* **100**: 2141–2151. DOI: 10.1007/s00253-015-7247-0.
- Pirnay, J. P. (2020). Phage therapy in the year 2035. *Front. Microbiol.* **11**: 1171. DOI: 10.3389/fmicb.2020.01171.
- Pirnay, J., G. Verbeke, P. Ceyssens, I. Huys, D. De Vos, C. Ameloot, A. Fauconnier (2018). The magistral phage. *Viruses* **10**: 64. DOI: 10.3390/v10020064.
- Principi, N., E. Silvestri, S. Esposito (2019). Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Front. Pharmacol.* **10**: 513. DOI: 10.3389/fphar.2019.00513.
- Qin, K., X. Shi, K. Yang, Q. Xu, F. Wang, S. Chen, *et al.* (2024). Phage-antibiotic synergy suppresses resistance emergence of *Klebsiella pneumoniae* by altering the evolutionary fitness. *Mbio* **15**: e01393-24. DOI: 10.1128/mbio.01393-24.
- Rosner, D., J. Clark (2021). Formulations for bacteriophage therapy and the potential uses of immobilization. *Pharmaceuticals* **14**: 359. DOI: 10.3390/ph14040359.
- Schooley, R. T., B. Biswas, J. J. Gill, A. Hernandez-Morales, J. Lancaster, L. Lessor, J. J. Barr, S. L. Reed, F. Rohwer, S. Benler, A. M. Segall, *et al.* (2017). Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob. Agents Chemother.* **61**: 10–1128. DOI: 10.1128/aac.00954-17.
- Segall, A. M., D. R. Roach, S. A. Strathdee (2019). Stronger together? Perspectives on phage-antibiotic synergy in clinical applications of phage therapy. *Curr. Opin. Microbiol.* **51**: 46–50. DOI: 10.1016/j.mib.2019.03.005.
- Sekyere, J. O., J. Asante (2018). Emerging mechanisms of antimicrobial resistance in bacteria and fungi: advances in the era of genomics. *Future Microbiol.* **13**: 241–262. DOI: 10.2217/fmb-2017-0172.
- Song, J., Z. Liu, Q. Zhang, Y. Liu, Y. Chen (2023). Phage Engineering for targeted multidrug-resistant *Escherichia coli*. *Int. J. Mol. Sci.* **24**: 2459. DOI: 10.3390/ijms24032459.
- Sulakvelidze, A., Z. Alavidze, J. Morris (2001). Bacteriophage therapy. *Antimicrob. Agents Chemother.* **45**: 649–659. DOI: 10.1128/aac.45.3.649-659.2001.
- Szymczak, M., J. A. Pankowski, A. Kwiatek, B. Grygorciewicz, J. Karczewska-Golec, K. Sadowska, P. Golec (2024). An effective antibiofilm strategy based on bacteriophages armed with silver nanoparticles. *Scient. Rep.* **14**: 9088. DOI: 10.1038/s41598-024-59866-y.
- Tkhilaishvili, T., L. Wang, C. Perka, A. Trampuz, M. Gonzalez Moreno (2020). Using bacteriophages as a trojan horse to the killing of dual-species biofilm formed by *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus*. *Front. Microbiol.* **11**: 695. DOI: 10.3389/fmicb.2020.00695.
- Torres-Barceló, C. (2018). Phage therapy faces evolutionary challenges. *Viruses* **10**: 323. DOI: 10.3390/v10060323.
- Wittebole, X., S. De Roock, S. M. Opal (2014). A historical overview of bacteriophage therapy as an alternative to antibiotics for the treatment of bacterial pathogens. *Virulence* **5**: 226–235. DOI: 10.4161/viru.25991.
- Zhao, M., H. Li, D. Gan, M. Wang, H. Deng, Q. E. Yang (2024). Antibacterial effect of phage cocktails and phage-antibiotic synergy against pathogenic *Klebsiella pneumoniae*. *Msystems* **9**: e00607-24. DOI: 10.1128/msystems.00607-24.