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Review

Bacteriophage therapy as an alternative treatment for human infections. A comprehensive review

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ABSTRACT

Bacteriophages, or phages, are viruses that infect bacteria. They were discovered around a century ago and have been used ever since for therapeutic purposes, particularly in former Soviet Union countries. Their use in Western countries was abandoned after the discovery and broad use of penicillin. The rising problem of antimicrobial resistance has revived interest in bacteriophage therapy. The aim of this article is to provide a comprehensive review of all aspects of natural phage therapy.

1. Introduction

Antimicrobial resistance is a major and increasing global health problem. The World Health Organization (WHO) highlighted in its first global report on antibiotic resistance the danger of an upcoming post-antibiotic era, where simple infections could once again be lethal [1]. The Centre for Disease Control (CDC) estimates that at least 23 000 deaths per year in the USA are a result of infection by resistant bacteria [2]. In Europe, growing levels of antibiotic resistance are being reported, particularly in countries with existing high levels of multidrug resistance, thereby limiting therapeutic options [3]. On the other hand, pharmaceutical companies are showing a declining interest in developing and producing novel antibacterial agents, mostly because the antibacterial market is less profitable than other markets [4]. The possibility of developing therapeutic products that are alternatives to antibiotics could be a great help in the fight against antibiotic resistance. Bacteriophages and bacteriophage-based products have the potential to become one of the most successful alternatives [5].

2. Bacteriophage Biology

Bacteriophages, or phages, are viruses that infect bacteria. Bacteriophages are in every environment containing their bacterial hosts and play an important role in many biological processes; they are supposed to be the most abundant organisms on the planet [6]. Most of the bacteriophages (specifically 96% of those currently identified) are classified in the order of *Caudovirales*, which are tailed, have double-stranded DNA and are further classified in the families of *Siphoviridae, Myoviridae* and *Podoviridae* [7]. Phages usually infect their bacterial hosts in a species- or even strain-specific manner. They can be divided into virulent and temperate phages based on their life cycle. Virulent phages produce the lytic cycle where the phage attaches itself to its bacterial host, injects its genome, reproduces by seizing the host's molecular machinery and finally lyses the host cell, concurrently releasing its progeny [8]. Most of the lytic phages use two kinds of protein to destroy their host cell, the holins and the lysins. The holins perforate the bacterial cytoplasmic membrane and work as a synergy tool for the endolysins, which are responsible for the destruction of the bacterial cell wall [9]. Temperate phages differ in that they infect their host by initiating a lysogenic cycle, where the phage genome remains dormant as a prophage, replicates along with its host and occasionally bursts into a lytic cycle under a specific trigger [8]. Lysogeny and prophages can be beneficial to bacteria as they can encode genes for antibiotic resistance or other

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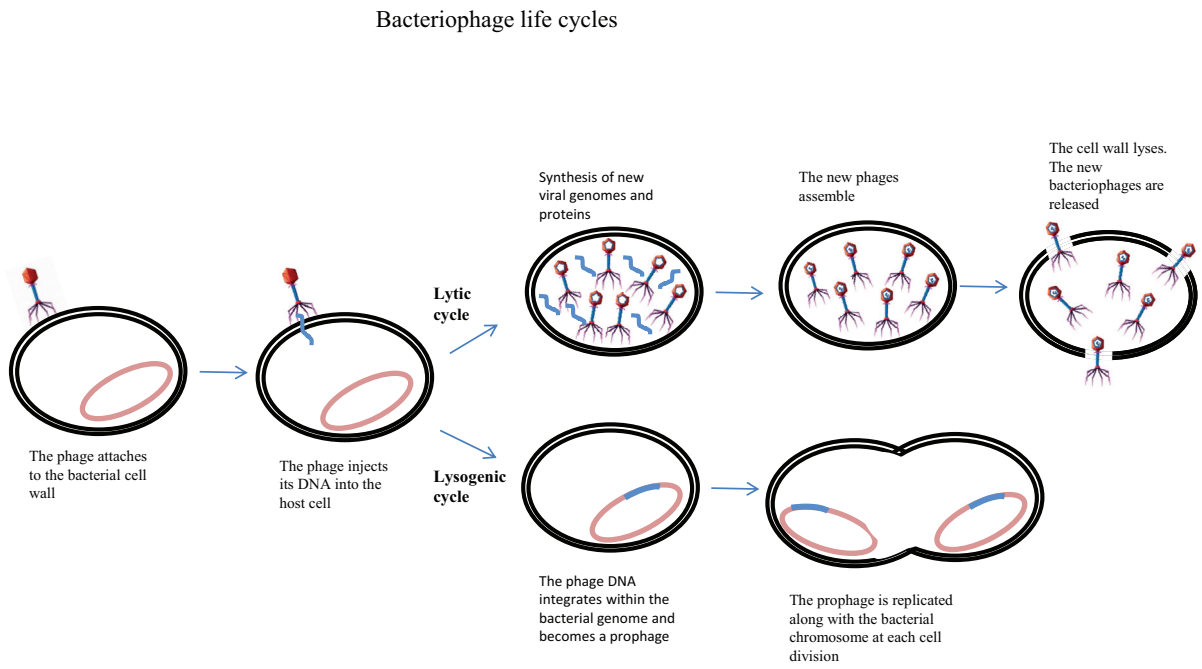

Fig. 1. Overview of the main differences between the lytic and the lysogenic bacteriophage life cycle.

virulence factors [10]. For that reason, only lytic phages should be used for bacteriophage therapy [11]. The main differences between the lytic and the lysogenic life cycle can be seen in Fig. 1.

3. Phage therapy history

In 1915, Frederick Twort, a British physician, was the first to describe the potential existence of an "ultra-microscopic virus" that could harm bacteria. His speculation arose after encountering difficulties with his vaccinia preparations with contaminating bacteria. He noticed "glassy and transparent" spots within the colonies of the contaminating microbes, which appeared to be zones of dead bacteria. Twort gave some possible explanations for the phenomenon, including the possibility of the presence of a microscopic virus, but he was not able to confirm his hypothesis [12]. Two years later, in 1917, Felix d'Herelle, a French-Canadian microbiologist, observed a similar phenomenon in stool cultures of patients recovering from bacillary dysentery. Unlike Twort, d'Herelle was convinced that the cause of bacterial antagonism was the presence of an "invisible microbe", a virus which he called bacteriophage [13]. After the discovery of bacteriophages, the idea of phage therapy was taken with enthusiasm and was implemented in many cases, but its application subsequently subsided after the discovery of antibiotics and their use in the Second World War, flourishing only behind the "iron curtain" [14]. In this setting, most of the historical knowledge about the practice of phage therapy is associated with two places: the Hirszfeld Institute in Wroclaw, Poland and the Eliava Institute in Tbilisi, Georgia. The main focus of the Eliava Institute has been the production and therapeutic use of phage cocktails (e.g. pyophage and intestiphage) that target specific pathogenic bacteria. On the other hand, the Hirszfeld Institute supports the development of a more individualized approach to phage therapy preparations. Both institutes have a history of publishing convincing success rates with phage therapy, yet without the corroboration of properly controlled clinical trials [15]. Further information on the past Soviet experience with phage therapy can be reviewed in articles by Nina Chanishvili [16,17]. Apart from their utilization in human therapeutics, bacteriophages have played an important role in the field of molecular biology research, mostly highlighted by the discovery of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its associated nucleases (CRISPR/Cas) bacterial adaptive immune system and its possible use in various implementations of gene editing [18].

4. Bacteriophage choice and preparation for therapeutic purposes

Since the discovery of bacteriophages, many efforts in phage therapy have produced good results; however, other phage therapy studies, particularly those conducted before the extensive use of antibiotics, have proved unsuccessful. The major factors contributing to the failure of phage therapy have been inappropriate phage selection, preparation and storage [19]. Bacteriophages can be isolated for therapeutic purposes from any environmental source containing the target pathogen, with sewage probably the richest phage source [20]. For clinically-relevant bacteria in the hospital setting, waste water and sewage that are directly connected to the hospital environment seem to be the prime source of phage isolation [21]. On-demand phage isolation against these clinically important pathogens is possible, although it seems to be variably dependent on the bacterial host: it is more difficult to isolate lytic bacteriophages for some hosts (e.g. *Staphylococcus aureus*) than others (e.g. *Pseudomonas aeruginosa*) [22]. The most direct way to isolate a bacteriophage is to sterilize an environmental sample to remove unwanted microorganisms, then plate it to the bacterial host strain culture to evaluate the formation of plaques [19]. More detailed technical instructions for bacteriophage isolation can be found in a recently published book by Azeredo and Sillankorva [23]. After isolation, a bacteriophage to be used for therapeutic purposes should be characterized and genetically sequenced [19]. Bacteriophage selection is crucial for therapeutic phage preparation. The most important criteria for selecting a suitable bacteriophage are phage specificity, efficacy and the avoidance of adverse effects; therefore, the selected bacteriophage should efficiently absorb and be lytic for the target bacterial host [20]. The use of temperate phages is not appropriate for therapeutic phage preparations, as lysogeny can amplify the virulence of target bacteria and induce phage resistance. Moreover, many temperate phages

are associated with genetic transduction, in which genetic material is moved from one bacterial host to another, hence the bacterial hosts can acquire unwanted genes [19]. The development of bacteriophage resistance is another major concern of phage therapy, and different strains of bacteria may differ in their phage-susceptibility. Thus, modifying the host range of a phage therapeutic preparation could greatly impact its success [24]. To ensure the desirable host range of a phage formulation, mixtures containing two or more bacteriophages, called "phage cocktails", have been developed [25]. The in-situ survival of the bacteriophage after administration is also important for the efficacy of phage therapy. Techniques to select long circulating phages or to modify phage immunogenicity to decrease clearance by the cells of the reticulo-endothelial system (RES) have been employed to address this [24]. Any phage therapy product must be manufactured in accordance with Good Manufacturing Practice (GMP), which provides very strict rules for sterilization and purification. Methods of filtration and centrifugation of phage lysates to separate the bacteriophages from other components are warranted. More thorough purification is needed for lysates of Gram-negative bacteria to remove bacterial endotoxin, and a more rigorous purification process is needed in applications for systemic administration [26]. Phage preparations should be stored correctly: phages consist of protein structures and are vulnerable to several factors, including high temperature, harmful pH, organic substances and mechanical stresses. Several techniques, including encapsulation, have been developed to improve phage preparation storage [27]. For phage therapy to be effective, bacteriophages must be delivered to the target site and maintain therapeutic levels for a sufficient time. Phage administration can be enteral, topical, inhaled or injected, with enteral administration by far the most common [28]. An estimated minimum of 10 phage virions for every bacterial cell is needed to significantly reduce a bacterial population. Therefore, an estimated 10^8 bacteriophages/mL is required to achieve a successful rate of bacterial reduction, although this estimate does not consider the active phage replication in the site of infection [29]. The current medicinal regulation pathways are cumbersome for phage therapy products and an adapted framework is needed to make phage therapy realistic [30].

5. Phage therapy safety

Phages are generally considered safe, based on their abundant nature and our constant exposure to them in the environment, and because they have been used extensively in some parts of the world with no reports of harmful events. Despite this optimistic point of view, the safety of phage therapy must be verified by modern scientific experiments [31]. The safety concerns of phage therapy include the possible impact of bacteriophages on body tissues and non-target microbiota, bacteriophages can modify their bacterial targets by expressing virulence genes or by transducing DNA between bacteria, and bacteriophages may induce immunological reactions [14,32]. Phage products can contain harmful products, such as endotoxin, during the formulation process but this can be tackled using various purification methods [19,26]. Bacteriophages have high specificity and usually infect only a few strains of a bacterial species; therefore, they have minimal impact on normal gut microflora [33]. The potential of bacteriophages to encode virulence factors or transduce unwanted genes can be circumvented by avoiding the use of temperate phages. Current methods of phage genome sequencing enable these matters to be properly addressed [32]. A few phase I/II clinical trials have been conducted to date. No significant adverse reactions have been noted in any of these studies, which are presented in a review article by Vandenheuvel et al. [34]. Interestingly, even the systemic administration of bacteriophages via the intravenous route seems to be safe, according to a literature review by Speck and Smithyman [35]. Objections for the intravenous use of phages are based on: the rapid removal of bacteriophage from the bloodstream by the RES; the possible massive release of endotoxins due to the rapid lysis of bacteria; the possibility of immune responses, such as the production of anti-phage antibodies, which would reduce the efficacy of phage therapy; or more serious adverse immune reactions, like anaphylaxis. However, anaphylaxis has not been reported in the setting of phage therapy [35].

6. Immune response to phage therapy

A major issue concerning the safety and efficacy of phage therapy is the immunological response towards bacteriophages, which comprises the adaptive and the innate immune responses [36]. As mentioned above, major life-threatening immune reactions, such as anaphylaxis, have not been reported in phage therapy [32,35]. However, immune system reactions can neutralize phages and thus reduce their antibacterial efficacy. The production of anti-phage antibodies in patients receiving phage therapy has resulted in phage inactivation [37,38], although a high rate of phage inactivation does not necessarily mean treatment failure [38]. The route of administration seems to be important for the impact of anti-phage activity of sera, with the local route of administration showing higher anti-phage activity than the oral [37]. In a murine model studied by Hodyra-Stefaniak et al., an antibody-complement cooperation was shown to eliminate phage activity, mimicking the immune response to eukaryotic viruses [39]. This could imply a link between adaptive and innate immunity against bacteriophages. In the same study, aspects of innate immunity were valued in a model of systemic inflammatory response syndrome (SIR) in mice, showing an increased phage clearance in the spleen and blood of SIR mice compared with controls [39]. The effect of phage therapy on phagocytosis has also been studied. Weber-Dabrowska et al. have shown an increased level of immature neutrophils and a decreased level of neutrophil phagocytosis in patients receiving phage therapy compared with controls in an in-vitro study. These changes, however, did not affect the outcome of therapy [40]. Later studies by Jonczyk-Matysiak et al. demonstrated that experimental phage therapy did not decrease intracellular killing of bacteria in the phagocytes of patients with chronic bacterial infections, so phage therapy did not harm phagocyte bactericidal activity [41]. An in-vivo study of a murine model conducted by Przerwa et al. showed that phagocytosis in the granulocytes of infected mice treated with the homologous phage was increased compared with in controls. Moreover, phage therapy may reduce oxidative stress by reducing reactive oxygen species (ROS) [42].

7. Phage therapy studies in animal models

In 2017, the WHO published a list of bacteria for which new antibiotics are urgently needed [43]. The group of pathogens named by the acronym "ESKAPE" *(Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa* and *Enterobacter spp*.) represents the most common causes of severe hospital-acquired infections, particularly in critically ill patients [44]. Several animal studies have shown promising results of phage therapy in treatment of infections with these pathogens. In an experimental model of imipenem-resistant *Pseudomonas* bacteremia in mice, Wang et al. showed that intraperitoneal injection of phages rescued bacteremic mice, even when administration was delayed for up to 3 h [45]. Similarly, Watanabe et al. pointed out that phage administration induced significant protection against *P. aeruginosa* in a mouse model of gut-derived sepsis [46]. Fukuda et al. demonstrated high efficacy in *P. aeruginosa* elimination after bacteriophage eye-drop administration in a murine model of *Pseudomonas* keratitis [47], while more recently, Pabary et al. showed

Table 1
Overview of clinical trials conducted on bacteriophage therapy.

Clinical trial	Trial phase	Target bacterium	Phage(s) used	Observations	References
Polish case studies	I/II	Several multidrug-resistant bacteria	Hirszfeld Institute phage collection	No adverse events; up to 40% rate of good response to phage therapy	66
Phage therapy on venous leg ulcers	I	*Escherichia coli; Staphylococcus aureus; Pseudomonas aeruginosa*	Intralytix phage cocktail WPP-201	No adverse events observed	68
Phage therapy against chronic ear infections	I/II	*Pseudomonas aeruginosa*	Biocontrol phage cocktail Biophage-PA	No adverse events; significant improvements observed in the phage-treated group	69
Phage therapy against diarrhea	I/II	*Escherichia coli*	T4 coliphage cocktail; Microgen ColiProteus phage cocktail	No adverse events; terminated due to lack of therapeutic effect	70
Phagoburn project: Phage therapy on burn wounds	I/II	*Escherichia coli; Pseudomonas aeruginosa*	Pherecydes Pharma phage cocktails against *E.coli* and *P. aeruginosa*	Not yet published	71

that phage delivery significantly reduces bacterial load and systemic spread of infection in an experimental *P. aeruginosa* lung infection in mice [48]. Studies conducted by Kusradze et al. and Regeimbal et al. demonstrated positive results of bacteriophage application in *A. baumannii* wound infection models in mice [49,50]. Hua et al. demonstrated that intranasal administration of phages protected neutropenic mice from lethal carbapenem-resistant *A. baumannii* lung infection [51]. Topical application of phages has proven to be efficient in treatment of *K. pneumoniae* infection in a murine burn wound model, as reported by Kumari et al. [52]. Hung et al. demonstrated that bacteriophages administered orally or intraperitoneally protect mice from death and reduce liver damage in an intragastric model of *K. pneumoniae* infection [53], while Cao, Wang et al. showed that intranasal phage treatment of *K. pneumoniae* lung infection in a murine model increased survival in a dose-dependent manner and reduced bacterial burden in infected mouse lungs [54]. Promising results were also shown in an extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* bacteremia murine model studied by Wang et al., in which phage treatment rescued mice from lethal bacteremia, with the timing of post-infection phage administration important for survival rates [55]. A recent study in a mouse model of *E. coli* pneumonia, conducted by Dufour et al. demonstrated a 100% survival rate of phage-treated mice and showed a bacteriophage efficacy that was similar to ceftriaxone in terms of survival rate and bacterial load of the lungs. Phages and ceftriaxone were respectively administered 2-h post bacterial inoculation [56]. Similarly encouraging results have been shown in animal studies of Gram-positive bacterial infections. Phage therapy has demonstrated satisfactory efficacy in murine bacteremia models of vancomycin-resistant *E. faecium* [57] and vancomycin-resistant *Enterococcus faecalis* [58]. Likewise, indicative studies of phage therapy against *S. aureus* (including MRSA) infections in animal models have shown positive results in protecting against abscess formation [59], protection against fatal bacteremia [60,61] and chronic osteomyelitis [62].

8. Phage therapy in humans

There are numerous publications on successful phage therapy in humans. Two recent case reports, one concerning a patient with *P. aeruginosa* septicemia [63] and the other concerning a patient with *P. aeruginosa* aortic graft infection [64], demonstrated a favorable outcome after bacteriophage therapy. Unpublished data from Georgian clinical trials organized by the Eliava Institute are reviewed by Kutateladze and Adamia. In these data, high treatment efficacy of an anti-staphylococcal phage preparation is highlighted, with about 70% and 55% rate of favorable outcome in the treatment of staphylococcal infections and staphylococcal sepsis, respectively [65]. Data from the Phage Therapy Unit in the Hirszfed Institute, Wroclaw, Poland are promising, with about 40% rate of good response to phage therapy in case series of patients with various difficult-to-treat infections between the years 2008 and 2010 [66]. In a case series conducted by Fish et al., a total of nine patients with difficult-to-treat diabetic foot ulcers with concomitant *S. aureus* infection, were successfully treated with a commercially available Georgian staphylococcal phage preparation [67]. Although phage therapy has been around for almost a century and regardless of the promising experience from Polish, Georgian and other former Soviet reports and the recently renewed interest, this form of therapy lacks modern, properly controlled, double-blind clinical trials. Unfortunately, only a handful of phage therapy clinical trials have so far been conducted [34]. A phase I safety trial on phage therapy of venous leg ulcers concluded that phages did not harmfully affect the healing of leg ulcers [68]. A small controlled, double-blind clinical trial of phage therapy in chronic otitis patients demonstrated significant clinical improvement in the phage-treated group compared with the placebo group, and no adverse effects were reported [69]. More recently, a randomized trial of per os phage therapy in *E.coli*-associated diarrhea in Bangladeshi children showed no significant differences between therapeutic groups and was discontinued; there were no significant side effects [70]. The most highly anticipated results are those of the Phagoburn project, a multicenter trial in France, Belgium and Switzerland that aims to evaluate the efficacy of phage therapy preparations against *E. coli* and *P. aeruginosa* in burn victims [71]. The most significant clinical trials of bacteriophage therapy performed so far are presented in Table 1. Phage therapy could be particularly useful in patients with chronic multidrug-resistant infections, e.g. cystic fibrosis patients, who usually develop chronic multidrug-resistant *P. aeruginosa* pulmonary infections. A recent review by Rossitto et al. summarizes the benefits and disadvantages of phage therapy in in vitro and in vivo models of multidrug-resistant *P. aeruginosa* infection, and highlights the need for further research in lung models for phage therapy clinical trials to be a realistic future goal for cystic fibrosis patients with such infections [72].

9. Phage-derived proteins and engineered bacteriophages

The potential of bacteriophage applications in the fight against bacterial pathogens can be expanded beyond their utilization as

naturally occurring phages. As mentioned above, bacteriophages use proteins called lysins to hydrolyze and degrade the bacterial cell wall. These endolysins can be used as antibacterials, particularly against Gram-positive bacteria; the Gram-negative outer membrane provides protection to the endolysin-susceptible peptidoglycan layer [73]. To improve the efficacy of lysins against Gram-negative bacteria, several studies have investigated endolysins that have a natural ability to degrade Gram-negative bacteria by disorganizing the outer membrane, the combination of lysins with other agents that can destabilize the outer membrane, or the genetic engineering of the endolysins [74]. Moreover, bacteriophages can be genetically engineered with different methods to provide various forms of antimicrobial activity [75]. Possible antimicrobial implementations of engineered bacteriophages include methods to enhance antibiotic activity [76], to reverse antibiotic resistance [77], or to create sequence-specific antimicrobials, mostly highlighted by the exploitation of the CRISPR/Cas immune system [78,79]. Methods of engineered phage-mediated reversal of antibiotic resistance can also be used as a prevention measure before an actual infection occurs; these methods include sensitizing bacterial populations on hospital surfaces or on the skin flora of medical professionals [80,81].

10. Conclusion

Bacteriophage therapy has a long history, from early enthusiasm, through abandonment because of the broad use of antibiotics, and renewal of interest due to increasing antibiotic resistance. Despite the promising reports of phage therapy in certain parts of the world, more modern randomized double-blind controlled clinical trials are needed to prove the safety and efficacy of phage therapy. Issues like bacteriophage choice, isolation, preparation, purification, storage and pharmacology should be addressed individually and researched in depth. Moreover, regulations for production of bacteriophage preparations are obstacles for implementation of phage therapy, as the current regulatory rules for medicinal products are challenging for phage therapy products [82]. Despite the hurdles, bacteriophages are potentially suitable alternatives for treatment of bacterial infections in the era of rising antimicrobial resistance [5,33]. Apart from natural phages, phage-derived endolysins and engineered bacteriophages can also work as effective antimicrobials [5,73,75].

Declaration of interest

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Competing Interests

No conflicts of interest.

Ethical Approval

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