Phage Therapy: a Step Forward in the Treatment of *Pseudomonas aeruginosa* Infections

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Antimicrobial resistance constitutes one of the major worldwide public health concerns. Bacteria are becoming resistant to the vast majority of antibiotics, and nowadays, a common infection can be fatal. To address this situation, the use of phages for the treatment of bacterial infections has been extensively studied as an alternative therapeutic strategy. Since *Pseudomonas aeruginosa* is one of the most common causes of health care-associated infections, many studies have reported the *in vitro* and *in vivo* antibacterial efficacy of phage therapy against this bacterium. This review collects data of all the *P. aeruginosa* phages sequenced to date, providing a better understanding about their biodiversity. This review further addresses the *in vitro* and *in vivo* results obtained by using phages to treat or prevent *P. aeruginosa* infections as well as the major hurdles associated with this therapy.

Bacteriophages, or phages, are bacterial viruses that were discovered almost a century ago (1, 2). These viruses are considered to be among the most ubiquitous and diversified organisms on Earth and are believed to exist wherever their hosts thrive (3–6). According to their life cycles, phages can be distributed into two classes: virulent phages (strictly lytic) and temperate phages (7). Lytic phages adsorb to the host cell surface, inject and replicate their DNA, and then induce host cell lysis, resulting in the release of progeny phages that can start another round of infection (7). Temperate phages generally integrate their genome into the host chromosome or sometimes maintain it as a plasmid which is transmitted, by cell division, to the daughter cells (7, 8). For therapeutic purposes, only lytic phages are of interest.

The use of phages as antimicrobial agents was proposed in 1917 by Félix d'Hérelle, and the early results reported were promising (2, 9, 10). However, after the arrival of the golden era of antibiotics, together with the scientific controversy regarding the use of phages due to poorly controlled trials, the interest in phage therapy decreased sharply (11). Antibiotics were cheap and extremely effective against bacterial diseases and thus were considered the perfect solution to fight bacterial diseases. Consequently, following World War II, phage therapy was abandoned in the Western world but kept being used in Poland and the Soviet Union (11–13). The inadequate use of antibiotics has significantly increased the emergence of multidrug-resistant (MDR) bacteria, and new alternative strategies for antibiotherapy are highly desired by the worldwide scientific community (14–16). Consequently, a renewed interest was given to phages and they have been again come to be considered a good option for the treatment of bacterial infections (17–20).

Bacteriophages specific for the *Pseudomonas* genus were first described in the middle of the 20th century (21, 22), and there are currently (27 January 2015) 137 completely sequenced *Pseudomonas* phage genomes found in public databases. Since *Pseudomonas aeruginosa* is one of the most problematic opportunistic pathogens involved in hospital-acquired infections (23–26), large fractions of the phage application studies and genome sequencing projects have been focused on this bacterium (3, 5, 27–33).

This review gathers information about the huge diversity of *P. aeruginosa* phages, and it further describes *in vitro* and *in vivo* phage therapy studies designed to defeat this problematic bacterium that have been reported throughout the years.

OVERVIEW OF *P. AERUGINOSA* CLINICAL IMPACT: VIRULENCE FACTORS AND ANTIMICROBIAL RESISTANCE

P. aeruginosa is a Gram-negative opportunistic bacterial pathogen frequently present in diverse environments such as water, soil, and plants (34, 35) that is able to infect different organisms, including plants, animals, and humans (34, 36, 37). This opportunistic bacterium is one of the most common causes of health care-associated diseases, including pneumonia, bloodstream infections, urinary tract infections, and surgical site infections, especially in patients with compromised host defenses (34, 35, 37, 38). Most of the infections caused by *P. aeruginosa* are strikingly difficult to treat using conventional antibiotic therapies, since this microorganism displays high intrinsic resistance to a wide range of antibiotics, including aminoglycosides, fluoroquinolones, and β-lactams (37), which results in significant morbidity and mortality rates (35). According to the U.S. Centers for Disease Control and Prevention, it is estimated that 51,000 health care-associated *P. aeruginosa* infections occur each year in the United States alone (38). About 13% of these infections are multidrug resistant, with roughly 400 deaths per year attributed to such infections (38). In addition to possessing intrinsic resistance mechanisms (37, 39, 40), *P. aeruginosa* also has other mechanisms that confer resistance to antibiotics (Fig. 1). For instance, this bacterium quickly develops acquired resistance as a consequence of mutations or through acquisition of antibiotic resistance genes (37, 39). Fur-
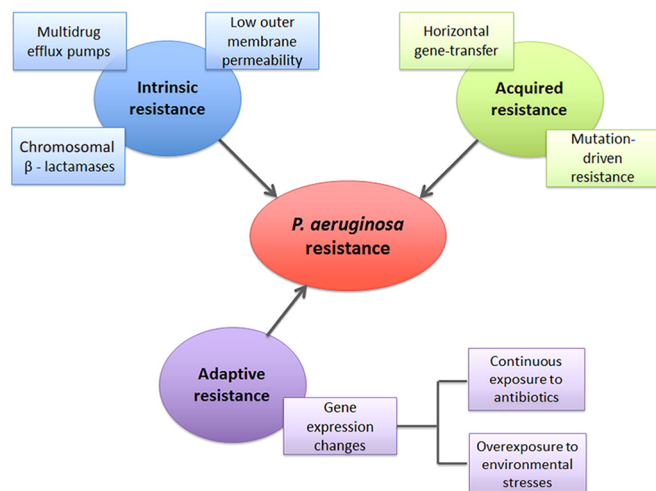
FIG 1 *P. aeruginosa* resistance mechanisms.

thermore, the inadequate use of antibiotics or exposure to different environmental conditions or stresses has induced the development of adaptive resistance (37, 41).

P. aeruginosa possesses an arsenal of virulence factors enabling it to invade host cells and circumvent host defenses (35, 42). These mechanisms of virulence include secreted factors such as proteases, elastase, pyocyanin, exotoxin A, phospholipase C, and exoenzyme S and also cell-associated factors such as lipopolysaccharide (LPS), flagella, and pili (35, 43). Many of these virulence factors are controlled by a cell-cell communication mechanism known as quorum sensing (QS), which allows bacteria to produce these factors in a cell density-dependent manner (35, 43, 44).

P. aeruginosa also has an ability to adhere to surfaces, such as those of medical devices or epithelial cells, and to form biofilms consisting of microbial communities embedded in a matrix of extracellular polymeric substances (EPS) (45–49). Biofilms are frequently associated with chronic infections, since their structure confers protection of bacterial cells from antimicrobial agents (50).

All of these virulence factors and resistance mechanisms contribute to increased *P. aeruginosa* pathogenicity, resulting in infections that are consequently very hard to treat (23, 37, 39).

P. AERUGINOSA BACTERIOPHAGES

To date, 94.2% of the 137 known phages targeting the *Pseudomonas* genus have been found to belong to the *Caudovirales* order (see Table S1 in the supplemental material), which comprises three families of double-stranded DNA (dsDNA) phages differing in the characteristics of the phage tail: *Podoviridae*, with a short and noncontractile tail; *Myoviridae*, with a long and contractile tail, and *Siphoviridae*, with a long and noncontractile tail (3, 5, 51). So far, only 8 nontailed *Pseudomonas* phage species have been sequenced: 2 belonging to the *Inoviridae* family (single-stranded DNA [ssDNA] phages) (52, 53), 2 belonging to the *Leviviridae* family (ssRNA phages) (54, 55), and 4 belonging to the *Cystoviridae* family (dsRNA phages) (56–58).

According to the data collected, 85% of the sequenced *Pseudomonas* genus phages belonging to the *Caudovirales* order are specific for the *P. aeruginosa* species (Fig. 2A) and the clear majority (approximately 60%) of these are lytic phages whereas 21.8%

are temperate and 18.2% are unclassified (see Table S1 in the supplemental material). Among the *P. aeruginosa* lytic phages, 41% belong to the *Myoviridae* family and 38% belong to the *Podoviridae* family, and the least representative family is the *Siphoviridae* (20%). Only 1% of the lytic *P. aeruginosa* phages are nonclassified (Fig. 2B). The genome size distribution of *P. aeruginosa* lytic phages by family is fairly divergent (Fig. 2C), with *Myoviridae* phages comprising genome sizes ranging from 64.1 kb to 309.2 kb whereas the *Podoviridae* and *Siphoviridae* phage genomes are much smaller and are in the ranges of 41.6 to 74.9 kb and 34.5 to 61.1 kb, respectively. *P. aeruginosa* lytic phages have been isolated from different sources all over the world (Fig. 2D and E); nevertheless, it seems that sewage, including hospital and wastewater treatment plant sewage, is the best choice for their isolation (56%).

IN VITRO PHAGE TRIALS

Many *in vitro* studies have been conducted in recent years to evaluate the potential of phages against *P. aeruginosa* clinical isolates, including multidrug-resistant (MDR) strains, in planktonic cultures or even biofilms.

Fu et al. studied the effect of lytic phages in the prevention of *P. aeruginosa* biofilm formation in hydrogel-coated catheters. For this, they pretreated catheters with *Myoviridae* phage M4 for 2 h prior to bacterial inoculation and observed a 2.8-log reduction in the number of viable cells compared with control (untreated) catheters after 24 h of biofilm formation. However, a regrowth of biofilms in phage-treated catheters was observed at between 24 and 48 h of biofilm formation. The phage-resistant biofilm isolates were recovered from the pretreated catheters, and, in accordance with their susceptibility profiles with respect to other phages, a cocktail of five phages was developed. The pretreatment of catheters with this cocktail resulted in a 3-log reduction of biofilm cell populations after 48 h, compared with untreated catheters (32).

Pires et al. used a broad-host-range phage for *P. aeruginosa* biofilm control (59). Although significant reductions in the number of viable cells were observed after 6 h of biofilm treatment, a resurgence in the number of biofilm cells was observed after 24 h of biofilm infection, indicating the emergence of phage-resistant phenotypes (59).

A different approach for the treatment of bacterial infections is the combination of phages with other antimicrobials. Recently, Torres-Barceló et al. reported treatment using a combination of *Podoviridae* phage LUZ7 and streptomycin against *P. aeruginosa* PAO1. An exponential-growth culture of *P. aeruginosa* was challenged with either single or combined treatments, and the bacterial population density was tracked over 70 h. The combined therapy revealed a positive synergism resulting in a bacterial density lower than that seen after the addition of each single treatment (60).

Knezevic et al. also studied the antimicrobial efficacy of therapy consisting of *P. aeruginosa*-specific phages belonging to the *Podoviridae* and *Siphoviridae* families combined with subinhibitory concentrations of gentamicin (an aminoglycoside), ceftriaxone (a cephalosporin), ciprofloxacin (a quinolone), and polymyxin B (a polypeptide) (61). In their work, the effectiveness of the combined treatment was determined by the time-kill curve method and only the combination of the ceftriaxone with one of the phages used in the study revealed a synergistic effect (61). Another work reported
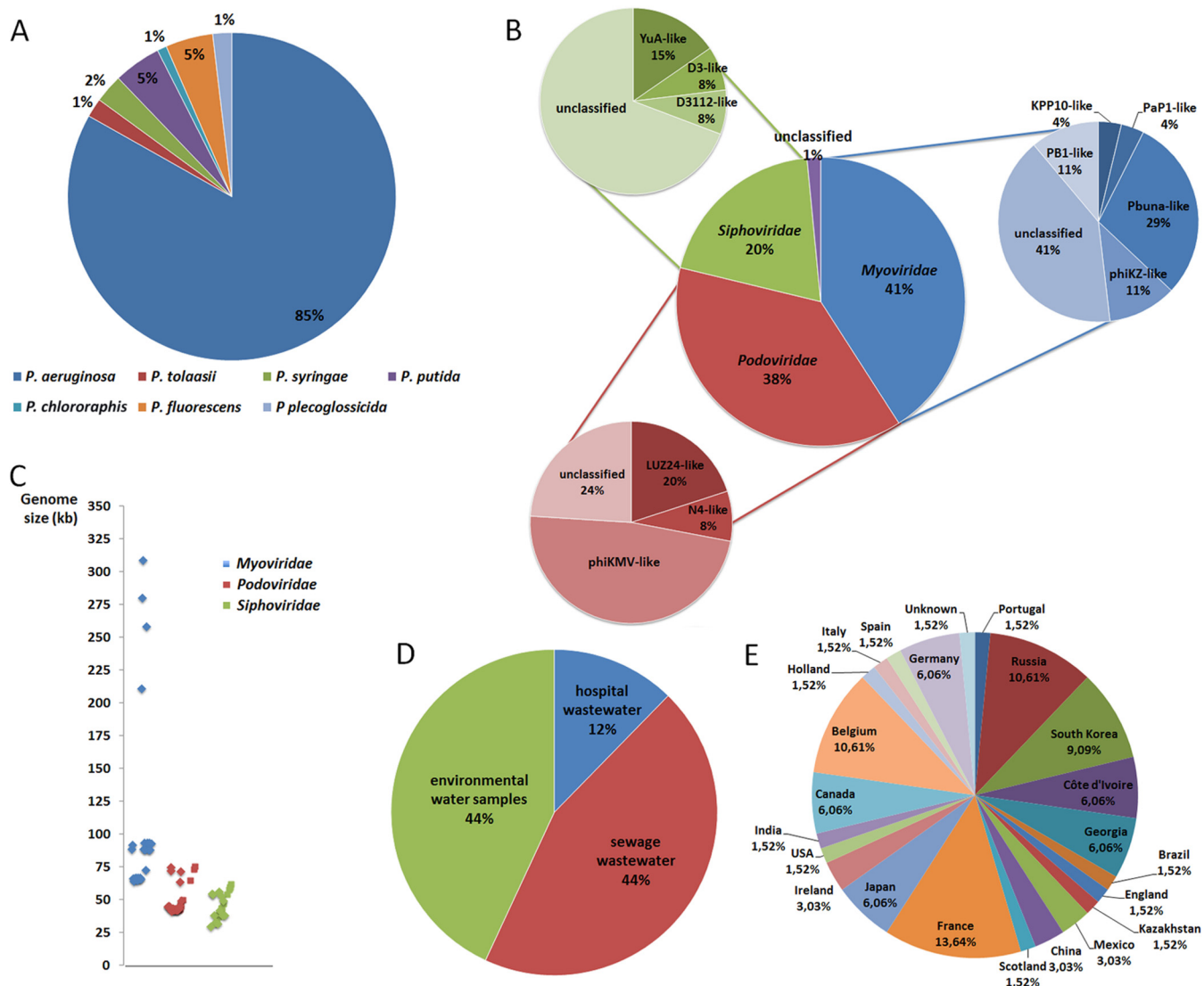
FIG 2 (A) Distribution of *Caudovirales* phages targeting the *Pseudomonas* genus by *Pseudomonas* species. (B) Distribution of *P. aeruginosa* lytic phages by family and genus of phages. (C) Genome sizes of *P. aeruginosa* lytic phages. (D) Isolation sources of *P. aeruginosa* lytic phages. (E) Distribution of *P. aeruginosa* lytic phage isolates by country.

a treatment consisting of lytic RNA phages combined with chlorine against *P. aeruginosa* biofilms that resulted in enhanced efficacy in the reduction of biofilm formation and biofilm eradication using the combined therapy compared with the use of each therapy alone (62).

The combination of two or more phages with different host ranges in a single suspension—a phage cocktail—has been reported to be more effective than the use of a single phage alone (63–65). Accordingly, Hall et al. studied the effect of using one, two, or four phages, either sequentially or simultaneously, against *P. aeruginosa* PAO1 planktonic cultures (66). Multiphage therapy was revealed to be more efficient in reducing the bacterial density, and simultaneous application of phages was consistently equal or superior to sequential application with respect to efficacy (66).

CLINICAL TRIALS OF PHAGES IN ANIMAL MODELS

The efficacy and safety of phage therapy have been analyzed using *in vivo* animal models. Most of the *P. aeruginosa* phage therapy

clinical animal trials that have been reported in the last decade were conducted in mouse models and have shown extremely encouraging results (Table 1).

Overall, phage therapy has significantly contributed to control and even prevention of *P. aeruginosa* infections *in vivo* (Table 1). Regarding the clinical trials in mouse models, most of the reported cases showed survival rates that ranged between 80% and 100% after phage treatment, and intraperitoneal or intranasal administration seemed to be the most efficient route of administration.

The great potential of phage therapy was also observed in catfish and dogs, since phages were able to successfully treat ulcerative lesions and chronic otitis, respectively. Phages were also able to increase the life span of both *P. aeruginosa*-infected wax moth larvae and *P. aeruginosa*-infected *Drosophila melanogaster*. Overall, the *in vivo* studies (Table 1) have demonstrated that broad-host-range phage cocktails potentiate the efficacy of phage therapy to control *P. aeruginosa* infections.

TABLE 1 Summary of the most relevant studies with targeted *P. aeruginosa* phage therapy in animal models[a]

Phage(s) (taxonomy)	Procedure	Result(s)	Reference
Mouse			
KPP12 (*Myoviridae*)	Phage at an MOI of 100 was applied in the corneal surface of mice 30 min after infection, and the eyes were examined 1, 3, and 5 days after phage application	The treated mice showed only slight corneal opacities on day 1 which gradually faded by day 5 after treatment; the infected control mice had most of the corneas perforated on day 5	67
ØNH-4 (*Myoviridae*) and ØMR299-2 (*Podoviridae*)	A phage mix suspension with an MOI of 10 was administered intranasally 2 h after infection	The use of the two phages together was able to reduce *P. aeruginosa* levels by at least 3 to 4 log units from murine lungs in 6 h	68
P3_CHA (*Myoviridae*)	Two doses of phage (MOI of 1 and MOI of 10) were applied intranasally 2 h after the onset of infection in the case of curative treatment or 4 days before infection in the case of preventive treatment	The curative phage treatment allowed over 95% survival of mice, and the preventive treatment resulted in 100% survival in the 16 days of the expt	31
PA1Ø (*Siphoviridae*)	Phages were administered via intraperitoneal route in normal mice (MOI of 1, 10, and 100) and neutropenic mice (MOI of 10)	Immunocompetent mice treated with phage achieved 80% to 100% survival rates, and no viable bacteria were found in organ samples after 48 h of the phage treatment; phage treatment was inefficient in neutropenic mice infected with *P. aeruginosa* PAO1 even though it was able to extend their lives for 12 h	69
PAK-P1 (*Myoviridae*)	Phages were applied intranasally 2 h after bacterial infection onset (MOI of 0.1, 1, and 10), with expts performed for up to 12 days, or phages were used as a preventive treatment 24 h before infection (MOI of 10), with expts carried out during 16 days	100% of the mice treated with an MOI of 10 survived until the end of the expt; in the preventive treatment, 100% of pretreated mice survived until the end of the expt, whereas 100% of untreated mice died within 2 days	30
MPK1 (*Myoviridae*) and MPK6 (*Podoviridae*)	Two different doses of phages (MOI of 1 and 10) were tested by administration through i.m. and i.p. routes after 6 to 12 h postinfection	The survival rate of mice achieved after 48 h of treatment with MK1 phage was up to 80% for i.m. administration or 100% for i.p. administration; the results with phage MK6 were significantly lower (about 20% for i.m. and 70% for i.p.)	70
Cocktail of 3 phages: ATCC 12175-B1, ATCC 14203-B1, and ATCC 14205-B1	A phage cocktail containing 1×10^8 PFU of each phage was administered i.m., i.p., or s.c.	A single dose of phage cocktail significantly decreased the mortality of thermally injured *P. aeruginosa*-infected mice from 6% survival without treatment to 22% to 87% survival with treatment, depending on the route of administration	29
KPP10 (*Myoviridae*)	To evaluate the efficacy of phages against gut-derived sepsis, 1×10^{10} PFU was orally administered to each mice 1 day before, 1 day after, or 6 days after *P. aeruginosa* inoculation	Phage significantly protected mice from mortality, achieving a survival rate of 66.7% for the phage-treated group (1 day after infection) vs 0% for the control group; mice treated with phage also had lower numbers of viable *P. aeruginosa* cells in their blood, liver, and spleen	33
Catfish			
MBL (*Podoviridae*)	A phage suspension (10^{10} PFU/ml) was applied on the infected skin lesion with a cotton swab	Phage therapy effectively cured the ulcerative lesions in infected fish after 8–10 days of treatment, with a 7-fold reduction of the lesion compared with untreated fish	28
Wax moth larvae			
14/1 (*Myoviridae*); φKZ (*Myoviridae*); PNM (*Podoviridae*); PT7 (*Myoviridae*)	Phages were applied by injection in the abdomen and three treatments were tested: phage φKZ applied alone, all phages applied sequentially, and all phages applied simultaneously in a cocktail	Phage therapy increased the life span of infected wax moth larvae: the avg time to death increased from 12.67 h to 26.67 h, 27.33 h, and 33.33 h, respectively, for larvae treated with φKZ, the multiphage sequence, and the phage cocktail; high phage/bacterium ratios completely cleared otherwise-lethal infections	66
Dog			
NA	Dogs with chronic otitis were treated with a single dose of a phage cocktail (6 phages), containing 1×10^5 PFU of each phage, by direct application into the external auditory canal	48 h after treatment, the clinical score and *P. aeruginosa* counts of all isolates from ears significantly decreased (mean score decrease, 30.1%; mean count decrease, 67%)	71

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TABLE 1 (Continued)

Phage(s) (taxonomy)	Procedure	Result(s)	Reference
NA	A dog with *P. aeruginosa* chronic bilateral otitis externa was treated with 400 PFU of phage instilled into the right auditory canal	Before phage treatment, the ears were similarly erythematous, with copious discharge; 27 h after treatment, the right ear was dry and did not appear inflamed, while the left ear (not treated) remained unchanged; the left ear was then also treated with phage, which dramatically improved its condition	72
Drosophila melanogaster MPK1 (*Myoviridae*) and MPK6 (*Podoviridae*)	The antibacterial efficacy of phages administered by feeding at a concn of 5×10^7 PFU was evaluated in *Drosophila melanogaster* systemic infection	Both phages significantly delayed the PAO1-induced killing, although MPK1 persisted longer than MPK6 in uninfected *D. melanogaster* tissue samples	70

a MOI, multiplicity of infection; NA, information not available; i.m., intramuscular; i.p., intraperitoneal; s.c., subcutaneous.

CLINICAL TRIALS OF PHAGES IN HUMAN PATIENTS

The therapeutic use of phages started in Paris in 1919 when d'Herelle used oral phage preparations to treat bacterial dysentery (73). Patients treated with a single dose of phage preparation started to recover within 24 h of treatment (11, 20). Later, d'Herelle also introduced treatment with intravenous phage for invasive infections (74). However, the first report about the use of phages to treat bacterial infections in humans was published in 1921 by Bruynoghe and Maisin (9). In that study, phages were administered by injection to treat staphylococcal skin disease and clinical improvements were clearly visible within 24 to 48 h (9). Following those early reports, many other studies have shown that phages can be used successfully for therapeutic purposes (75, 76).

Even after the arrival of antibiotics, phage therapy continued to be extensively used to treat bacterial infections in eastern Europe and the former Soviet Union, and the Eliava Institute in Tbilisi (Georgia) was one of the crucial centers (11, 77). The increasing problem of bacterial resistance to antibiotics revitalized interest in phage therapy, and many clinical trials in humans have been conducted (72, 78–82).

The use of phages against *P. aeruginosa* has also been examined in clinical trials. For instance, the efficacy and safety of a therapeutic phage preparation (Biophage-PA) were reported by Wright et al. (82). According to those authors, 12 patients with antibiotic-resistant *P. aeruginosa* chronic otitis who were treated with a single dose of phage preparation and followed at 7, 21, and 42 days after treatment (82) showed significant clinical improvements compared to 12 placebo group individuals. Furthermore, no related side effects or local systemic toxicities were observed, which highlights the safety of phage therapy (82).

Previously, Sivera Marza et al. reported the successful topical use of phage to treat a burn patient who had been colonized by *P. aeruginosa* months after skin grafts had been applied (72). In this case, the purified phage preparation, adsorbed to filter paper discs, was placed in the patient's colonized burned areas, and 3 days later, *P. aeruginosa* was no further isolated, resulting in a subsequent successful grafting (72).

Merabishvili et al. described a quality-controlled small-scale production of a phage cocktail (BFC-1) for use in human clinical practice (83). The cocktail was designed to treat *P. aeruginosa* and *Staphylococcus aureus* burn wound infections, and the quality control parameters of the phage cocktail, such as stability, pyrogenicity, sterility, and cytotoxicity, were evaluated (83). The BFC-1 phage cocktail was then topically applied in the wounds of nine acute burn patients with MDR *P. aeruginosa* and/or *S. aureus* burn wound colonization (84). Although that study did not enable any conclusion regarding the efficacy of the phage cocktail, no adverse effects or clinical abnormalities were observed after phage application (84).

CURRENT CHALLENGES FACED BY PHAGE THERAPY

Despite the remarkable positive results obtained by phage therapy, there are still some major concerns regarding the use of phages in clinical practice. The fast development of bacterial resistance to phage, the immune response that induces the production of antibodies neutralizing phage action, and the safety concerns regarding phage preparations that might contain bacterial endo- and exotoxins, as well as the limited spectrum of activity, are some of the major drawbacks of phage therapy that limit its broad application. Some of these bottlenecks are already being addressed by the introduction of molecular biology techniques that enable the genetic engineering of phage genomes. For example, phages have been genetically engineered to enhance their performance against biofilms (85), to improve antibiotic activity (86, 87), and to expand their host ranges (88–91). The use of well-defined and purified phage preparations is also crucial for therapeutic applications and for regulatory approval (83, 92). Furthermore, a deep understanding of the phage-host interaction, phage diversity, phage dynamics, and genome function is crucial for a better understanding of the limitations of phage therapy and for designing new strategies to overcome it.

CONCLUDING REMARKS

The continuous development of antibiotic-resistant bacteria, together with the current low rate of antibiotic discovery, urgently demands novel and effective strategies to combat bacterial infections. The huge diversity of phages, the easy discovery of new ones, their low-cost production, and their effectiveness against the target bacteria make them a very attractive alternative option to antibiotics.

The *in vitro* and animal model studies reported to date are good indicators that phages effectively constitute a powerful tool to fight *P. aeruginosa* infections. Early work in human patients also showed promising results. Furthermore, phages can be used in combination with antibiotics or other antimicrobials for improved performance.

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