

Bacteriophage-based therapy in cystic fibrosis-associated *Pseudomonas aeruginosa* infections: rationale and current status

Sami Hraiech^{1,2}
Fabienne Brégeon^{1,3}
Jean-Marc Rolain¹

¹Institut Hospitalo-Universitaire Méditerranée Infection, URMITE CNRS IRD INSERM UMR 7278, ²Réanimation Médicale – Détresses Respiratoires et Infections Sévères, APHM, CHU Nord, ³Service d'Explorations Fonctionnelles Respiratoires, APHM, CHU Nord, Marseille, France

Abstract: Pulmonary infections involving *Pseudomonas aeruginosa* are among the leading causes of the deterioration of the respiratory status of cystic fibrosis (CF) patients. The emergence of multidrug-resistant strains in such populations, favored by iterative antibiotic cures, has led to the urgent need for new therapies. Among them, bacteriophage-based therapies deserve a focus. One century of empiric use in the ex-USSR countries suggests that bacteriophages may have beneficial effects against a large range of bacterial infections. Interest in bacteriophages has recently renewed in Western countries, and the in vitro data available suggest that bacteriophage-based therapy may be of significant interest for the treatment of pulmonary infections in CF patients. Although the clinical data concerning this specific population are relatively scarce, the beginning of the first large randomized study evaluating bacteriophage-based therapy in burn infections suggests that the time has come to assess the effectiveness of this new therapy in CF *P. aeruginosa* pneumonia. Consequently, the aim of this review is, after a brief history, to summarize the evidence concerning bacteriophage efficacy against *P. aeruginosa* and, more specifically, the in vitro studies, animal models, and clinical trials targeting CF.

Keywords: pneumonia, pulmonary infection, bacterial infection, multidrug resistance

Introduction

Pseudomonas aeruginosa is one of the first pathogens to infect the airways of cystic fibrosis (CF) patients. In the CF lung, a genetic lack of functioning CF transmembrane regulators in the apical membrane of respiratory epithelial cells results in a change in the airway environment that is favorable to *P. aeruginosa* infection. During the evolution of CF-induced respiratory tract disease, *P. aeruginosa* progressively takes the first place among bacteria recovered from pulmonary samples, and up to 80% of CF adults suffer from chronic *P. aeruginosa* infection.¹ Chronic pneumonia resulting from *P. aeruginosa* colonization threatens patients with a deterioration in lung function and shortens their life expectancy,² especially when mucoid phenotypes are involved.³ Although infection with *P. aeruginosa* may be cured if treatment is started early,⁴ no antibiotic is able to eradicate an established chronic lung infection. Finally, CF patients are exposed to systemic and inhaled broad-spectrum antibiotic treatments early in their life,⁵ which in turn has led to a dramatic emergence of multidrug-resistant (MDR) bacteria and extremely resistant strains of *P. aeruginosa*.⁶ Thus, the “pipeline” of new antibiotics is running dry,⁷ and physicians are facing the threat of untreatable infections.

New antimicrobial molecules or therapeutic strategies against *P. aeruginosa* infections, especially MDR strains, are under investigation.^{8,9} Bacteriophage-based therapy

Correspondence: Jean-Marc Rolain
IHU Méditerranée Infection, URMITE
CNRS IRD INSERM UMR 7278, 27 Bd
J Moulin, 13385 Marseille Cedex 05,
France
Tel +33 4 86 13 68 28
Email jean-marc.rolain@univ-amu.fr

appears as a promising “innovative” treatment and deserves a focus.¹⁰ Numerous bacteriophages active against *P. aeruginosa* have been identified,¹¹ and a variety of these phages are efficient against *P. aeruginosa* strains from CF patients.¹² While the first multicenter randomized controlled study (NCT02116010) evaluating the efficacy and safety of phages against *P. aeruginosa* wound infections in burn patients will soon begin, several steps remain before bacteriophage-based therapies can be applied to CF patients.

After a brief reminder of the history of bacteriophage use, this review summarizes the current data available and the perspectives regarding bacteriophage-based therapy in the treatment of *P. aeruginosa* pneumonia in CF patients. In the absence of consistent clinical data in this field, we will enlarge the topic to in vitro and animal studies focusing on CF bacterial flora.

Historical context of bacteriophage therapy: a pendulum effect?

Despite not being demonstrated at the time, Briton Ernest Hanbury Hankin's observation in India in 1896 of antibacterial activity against *Vibrio cholerae* from the water of the Jumna and Ganges rivers was probably the first experiment due to bacteriophage activity. Later, at the beginning of the 20th century, the concept of an independent microorganism able to infect and lyse bacteria – namely, the bacteriophage – was first co-described independently by two microbiologists, Felix d'Herelle of France and Frederick William Twort of Great Britain. They both questioned whether the agent responsible for bacterial lysis was a bacterial virus destroying bacteria. However, at the time, bacteriophages remained invisible. Soon after, following successful assays on animals as well as himself, d'Herelle pioneered the use of phage preparations to treat patients suffering from *Shigella* dysentery in France and cholera epidemics in India.¹³ D'Herelle moved then to Georgia, and his collaboration with the scientist George Eliava probably explains why Georgia became the main center of bacteriophage development with the Tbilisi Institute of Bacteriophages, founded in 1923 by Eliava.

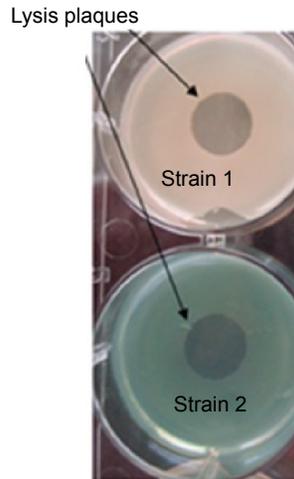
Despite controversy about “the therapeutic value of lytic filtrates”, bacteriophages were largely produced in the 1940s. In Paris, d'Herelle founded the Laboratoire du bactériophage and commercialized various cocktails of bacteriophages. In the USA, bacteriophages were industrially produced mainly for the treatment of traumatic or postoperative wound infections. However, at the end of the

40s, difficulties in maintaining industrial amounts of phage production, treatment failures, and the emergence of antibiotics marked the end of bacteriophage use in Western countries. In the Soviet Union, however, the extensive use of bacteriophage-based therapy persisted, and most of the literature concerning bacteriophages comes from case reports or studies from former USSR countries and Eastern Europe. These studies reported successful treatment of various infections, including *Staphylococcus aureus* and *P. aeruginosa*, but publications were confined to a small area and, because they lacked control groups, these studies were too weak to convince the rest of the world about phage efficacy and safety.

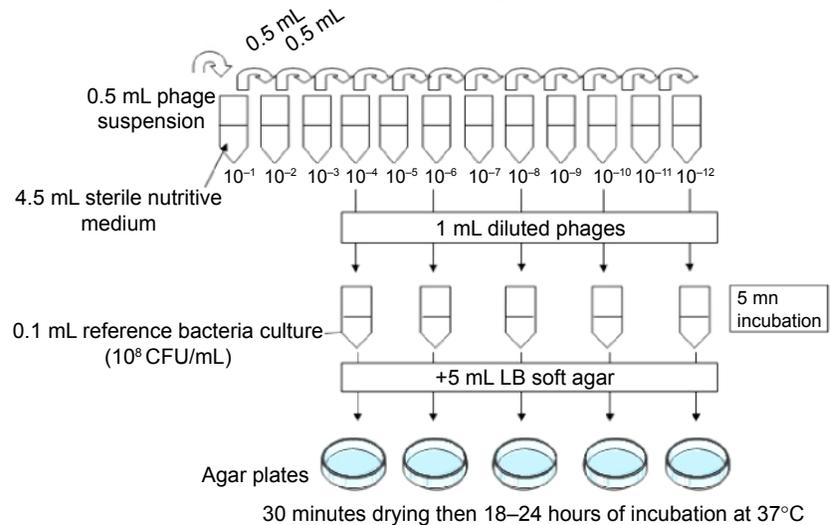
In the last two decades, bacteriophages regained the interest of scientists in Western countries who face the emergence of MDR bacteria and a decrease in new antibiotics. Characterization of bacteriophages then occurred.

Bacteriophages are ubiquitous organisms present in high numbers in the water, soil, and animal-associated ecosystems. Most of the bacteriophages described to date belong to Caudovirales, which is itself composed of Myoviridae, Siphoviridae, and Podoviridae. These bacteriophages share an icosahedral capsid containing a double-stranded linear DNA and a caudal part with structures for adhesion to bacteria. Lytic phages lead to bacterial death and are weakly implicated in horizontal gene transfer (responsible for antibiotic-resistance gene transfer). This is contrary to the lysogenic bacteriophages¹¹ that can integrate into the bacterial genome as prophages and be responsible for virulence factor transfer. Easy to extract, phage isolation is rapid and cost-effective so that any bacterial infection could, in theory, be controlled by bacteriophage-based therapy (Figures 1 and 2). However, the efficacy of the isolated bacteriophages needs verification prior to use, and results obtained in vitro cannot determine effectiveness in vivo, especially when the lung is concerned.

It is only recently that the first well-designed clinical trials were launched and showed encouraging results, mainly for wound infection in burn patients, ulcers,^{14,15} and chronic otitis.¹⁶ The interest in bacteriophages is so significant that bacteriophages have been elected one of the seven weapons to fight against antibiotic resistance by the US National Institute of Allergy and Infectious Diseases.¹⁷ In 2014, the first multicenter randomized controlled trial (France, Belgium, and the Netherlands) in humans obtained funding from the European Commission and the trial will be starting soon in patients with wound infections due to *Escherichia coli* and

A Test for phage activity against two target bacterial strains**B**

Titration of a phage-containing master stock according to double-agar overlay plaque assay



→ Read the limit dilution with lysis result



Figure 1 In vitro steps before phage use for in vitro or in vivo models.

Notes: (A) Specific bacterial activity: the *Pseudomonas aeruginosa* strains of interest (eg, Strain 1 and Strain 2) are co-cultured on agar plates with a phage suspension to verify the lytic activity of the phages against known bacterial strains. (B) Phage suspension is titrated with the double agar overlay plaque assay according to Gratia's method.⁶⁸ Briefly, serial dilutions of a phage suspension are added to bacteria broth in dilute agar or agarose matrix ("top agar" or "overlay"). After uniform mix, solidification is obtained on standard agar plates ("bottom agar" or "underlay"). After incubation, lysis is visualized as clear areas called plaques and corresponding to decreased density of bacterial lawn due to the lytic activity of the phages. Extrapolation of the initial phage suspension contents is obtained by extrapolation from the last dilution giving a plaque. Phage quantity is therefore expressed as plaque-forming units.

Abbreviations: LB, Luria-Bertani; CFU, colony-forming units.

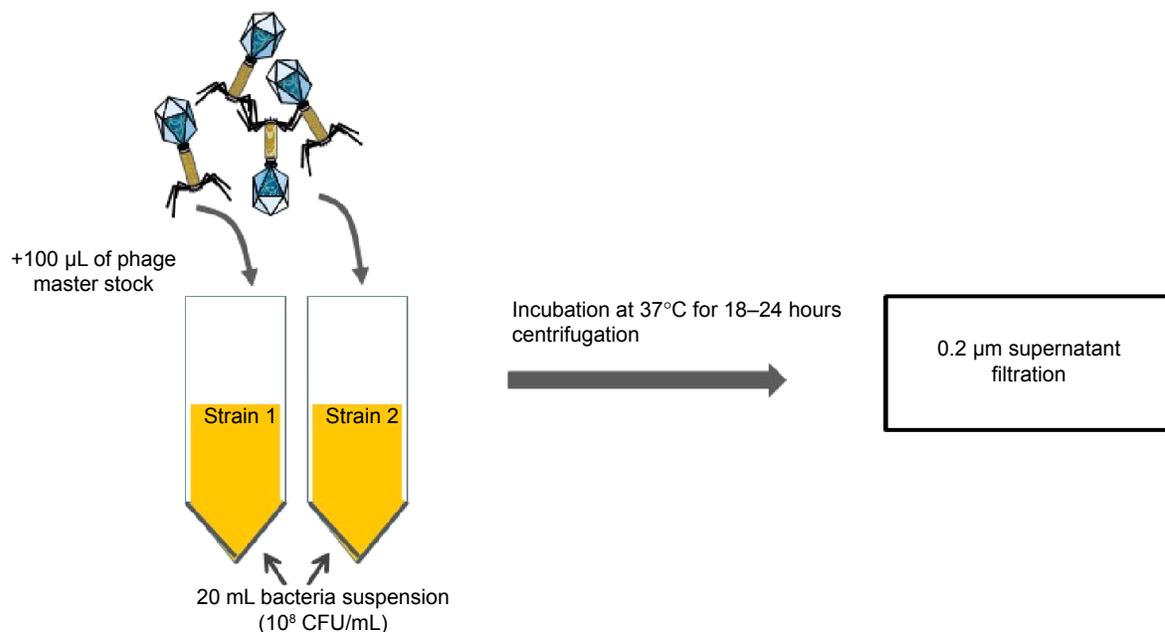


Figure 2 Example of *Pseudomonas aeruginosa*-specific high-titer phage-production protocol. Target strains of *P. aeruginosa* are incubated separately for 24 hours in the early exponential phase ($OD_{600\text{ nm}} = 0.1$) with a known phage cocktail at a 0.035 multiplicity of infection (= volume of phage suspension/volume of bacterial suspension). After 24 hours, the obtained lysates are centrifuged at $5,000 \times g$ for 30 minutes at 4°C . Supernatant is filtered through a $0.2\ \mu\text{m}$ membrane and stored at 4°C .

Abbreviations: CFU, colony-forming units; OD, optical density.

P. aeruginosa to compare phage therapy with silver sulfadiazine (NCT02116010).

Bacteriophage therapy to treat *P. aeruginosa* infections in CF patients: a new paradigm?

P. aeruginosa is a ubiquitous environmental agent. It is also a commensal in humans and is recovered largely in the gut and sometimes the skin. This bacteria acts as an opportunistic agent in CF patients.¹⁸ One of the major characteristics of *P. aeruginosa* is its ability to develop a biofilm, favoring the development of a niche and conferring a relative protection against host defenses and antibiotics. Moreover, the development of a mucoid phenotype of *P. aeruginosa* is favored.¹⁹ The biofilm formation associated with *P. aeruginosa* colony development contributes to antibiotic treatment failure,²⁰ a feature that was first ascribed to the ability of the biofilm matrix to restrict antimicrobial penetration.²¹ Other mechanisms explaining antibiotic treatment failure include the establishment of slow-growing antibiotic-resistant subpopulations within the biofilm (persister cells)²² and biofilm-specific gene expression.²³ As an alternative to antibiotics, bacteriophages have the advantage of targeting a precise bacterial species. It is known that a specific bacteriophage exists for each specific bacterium. Bacteriophages also produce enzymes active against bacteria. As an example, the endolysin Cpl-1 was delivered by inhalation and was effective to rescue mice with fatal pneumococcal pneumonia.²⁴

Given the large amounts of bacteria in CF airways, the CF patient respiratory microbiota is a source of novel phage discovery. It is also plausible that the disease by itself could be a source of new virulent bacteriophages that could be interesting tools to control the CF infectious biomass. Bacteriophages have the ability to penetrate easily into the *P. aeruginosa*-associated biofilm and are also able to produce natural enzymes that hydrolyze the biofilm,²⁵ arguing for their use in chronic *P. aeruginosa* infections in CF patients. As several strains of the same bacterial species often coexist in the same patient, treatment with one bacteriophage at a time may fail, and therefore, cocktails of phages have been developed. For example, the Georgian Pyophage cocktail, synthesized at the Eliava Institute (Tbilisi, Georgia), is a mixture of phages directed against five bacterial species that predominate in purulent infections, including *P. aeruginosa*, and is used for phage therapy.²⁶

Prior to their use in humans, experiments using bacteriophage-based therapy were conducted in vitro and in animal experiments, as described following.

In vitro proofs of concept of the efficacy of bacteriophages against *P. aeruginosa* strains from CF patients

The main in vitro antibacterial effects of bacteriophages against clinical and nonclinical strains of *P. aeruginosa* proven in vivo are summarized in Table 1.

Susceptibility of CF-associated *P. aeruginosa* to bacteriophages

Observation of bacteriophages from the sputum of CF patients²⁷ has opened the way to extensive research. Following the isolation of a clinical strain of *P. aeruginosa* in a patient sample, the existence of a virulent (lytic) phage targeting this isolate can be assessed from a library of already known phages or can be found de novo. A recent in vitro study assessed the antibacterial efficacy of bacteriophages against CF strains of *P. aeruginosa*.²⁸ Six different phages were purified from the Georgian Pyophage cocktail, including four genera of bacteriophages with some active against *P. aeruginosa*.²⁹ Phages were tested against 47 strains isolated from CF sputum collected in several French hospitals. Of these 47 strains, 33 were lysed with at least one bacteriophage of the cocktail. The strains that were resistant to the first cocktail prompted an investigation to find new bacteriophages. Two novel viruses were isolated in sewage water from France and the Ivory Coast. The combination of the preexisting different cocktails of phages with the newly identified viruses allowed a larger spectrum against strains of *P. aeruginosa*. This work suggests that it would be possible, from clinical samples from CF patients, to constitute a new cocktail composition with enlarged antibacterial targets. This is particularly promising considering that clinical strains of *P. aeruginosa* might be more susceptible to bacteriophages than environmental strains.³⁰

Very recently, a French team¹² worked on *P. aeruginosa* strains from CF patients and showed that a specific cocktail of ten phages was able to reduce bacterial growth. The originality of this work was the direct application of the virions on the recovered sputum, suggesting that the phages were active against bacteria in their original environment.

Phage resistance in CF strains of *P. aeruginosa*

One of the hypotheses to explain phage susceptibility and the virulence of clinical strains issued from CF patients are the clinical characteristics of such strains. Indeed, strains infecting CF in a chronic manner might be more susceptible

Table 1 Summary of the main in vitro antibacterial effects of bacteriophages against clinical and nonclinical strains of *Pseudomonas aeruginosa*

Strain(s) targeted	Bacterial conditioning	Including MDR strains	Bacteriophage(s) tested	Anti-pseudomonal effect observed	Study
47 CF patients' strains	Bacterial culture on growth medium	Yes	Cocktail from "pyophage" with six bacteriophages	Lytic activity against 70% of strains	Essoh et al ²⁸
48 samples from CF patients' sputum	Patients' sputum	Yes	Cocktail of ten bacteriophages	Decrease in the bacterial growth	Saussereau et al ¹²
Mucoid strains from CF patients	Bacterial culture on growth medium	NA	Phage PT6	Alginase secretion with alginate degrading activity	Glonti et al ⁴³
PAOI	Bacterial culture on growth medium	No	Phage LUZ7	Synergy with streptomycin for inhibition of growth	Torres-Barceló et al ⁴⁷
PAOI	Bacterial culture on growth medium	No	Phage PB-1 + tobramycin	Reduction in tobramycin resistance in combined treatment	Coulter et al ⁴⁸
PAOI	Bacterial culture on growth medium	No	Engineered T7 phage with <i>ailA</i> gene	Quorum quenching: inhibition of biofilm formation	Pei and Lamas-Samanamud ⁴⁹
Nine clinical strains (mucoid and non-mucoid)	Bacterial culture on growth medium	NA	Cocktail of two newly isolated phages (ϕ MR299-2 and ϕ NH-4)	Growth reduction and destruction of biofilm	Alemayehu et al ⁵⁵

Abbreviations: CF, cystic fibrosis; MDR, multidrug resistant; NA, data not available.

to bacteriophages than strains responsible for intermittent acute infections.³¹ This could be particularly true with strains colonizing the lungs of CF patients for more than 2 years, probably because their adaptation to the lung environment is associated with a less virulent phenotype with decreased growth and increased susceptibility to bacteriophages. This "fitness cost" has been described with the acquisition of multiple antibiotic resistance.³² However, the association between the susceptibility of CF isolates of *P. aeruginosa* toward phages and their clinical characteristics (and, in particular, the duration of lung colonization) was not found in another study.¹² This particularity remains to be explored.

The mechanisms of bacterial resistance to bacteriophages could involve loss or significant alteration of receptors or overproduction of exopolysaccharides such as alginates.³³ Bacteriophages have been reported to be largely present in CF lungs. Their identification from lung samples was assessed, but little is known about their densities or biological activity in situ. Chronic CF lungs could be dominated by lysogenic prophages that reside mostly within bacterial genomes but can occasionally convert to the lytic phenotype.³⁴ In their lysogenic form, phages might have a relatively small role in regulating bacterial densities despite their prevalence. Moreover, temperate bacteriophages have been implicated in horizontal gene transfer. They have been shown to confer selective beneficial traits to a range of *P. aeruginosa* hosts.²⁹ For example, phage ϕ CTX infection of the PAS10 strain results in conversion of the bacteria to a toxigenic strain.³⁵ The genome sequencing of the *P. aeruginosa* hypervirulent strain LESB58 revealed that it shares 95% similarity with the laboratory strain PAO1. However, the LESB58 core genome is punctuated by five prophage clusters probably implicated in its highly pathogenic potential.³⁶ LESB58 mutants, with disrupted prophage genes, exhibited ten- to 1,000-fold decreased competitiveness in a rat model of chronic lung infection compared to wild-type LESB58. The choice of lytic phages seems therefore crucial for their use in CF pulmonary infections.

Biofilm inhibition

One of the main advantages of bacteriophage-based therapy against *P. aeruginosa* is its supposed efficacy against the biofilm.^{37,38} Bacterial biofilms are populations of cells adherent to an abiotic or biotic surface that can grow to be several millimeters thick.³⁹ Biofilms formed by *P. aeruginosa* have been found not only on medical implants such as catheters⁴⁰ but also on native airways from CF patients.⁴¹ Biofilms have been shown to be highly tolerant to antimicrobials, a feature

that was first ascribed to the ability of the biofilm matrix to restrict antimicrobial penetration.²¹ Other mechanisms that have been described include the establishment of slow-growing, antibiotic-resistant subpopulations within biofilms (persister cells)²² and biofilm-specific gene expression.²³ In CF patients, the development of a biofilm confers to bacteria a resistance to antibiotics as well as an inhibition of polynuclear chemotaxis.

There are several advantages of bacteriophages over antibiotics in treating biofilm-associated infections. First, in contrast to antibiotics passing over the surface of a biofilm, bacteriophages infect bacteria at the surface and will then replicate, generating a high concentration of antibacterial agents at the site of the infection. Bacteriophages can also infect persister cells and can become active and destroy them when reactivation occurs. Moreover, bacteriophages can produce enzymes that dissolve the biofilm matrix or induce enzyme production by the bacterial host.⁴² This has been shown in vitro using clinical mucoid strains of *P. aeruginosa* from CF sputum. Hanlon²⁵ and Glonti et al⁴³ showed that a bacteriophage-derived enzyme was able to depolymerize the alginic acid capsule of *P. aeruginosa*. The alginase produced by these bacteriophages may have the potential to increase the well-being of CF patients by facilitating the expectoration of sputum, accelerating phagocytic uptake of bacteria, and perturbing bacterial growth in biofilms. The ability of bacteriophages to weaken and even destroy the biofilm matrix can also aid the penetration of other agents, raising the possibility of a synergistic combination with antibiotics. Because *P. aeruginosa* is important for producing the biofilm matrix, such synergy could also extend to other species present within a polymicrobial biofilm. While those species might not be targeted by the bacteriophage, they could become more susceptible to antibiotics.

Synergy with antibiotics

One major concern with *P. aeruginosa*-bacteriophage activity against biofilms is that the bacteriophages have essentially been shown to decrease the biomass of a biofilm but not to eradicate it.⁴⁴ As a consequence, several works have investigated the role of bacteriophages in combination with antibiotics to restore antibiotic activity.^{45,46} Recently, Torres-Barceló et al demonstrated the synergy of bacteriophages and streptomycin in the inhibition of the in vitro growth of *P. aeruginosa*.⁴⁷ They showed that the efficacy of bacteriophages associated with streptomycin was superior to the addition of each treatment separately. Moreover, the combination was able to decrease the bacterial growth

rebound observed at the end of a single treatment with either bacteriophages or streptomycin. The best synergy was obtained when streptomycin was added 12 hours after the bacteriophage treatment. The resistance to streptomycin and to bacteriophages was also lower in the combined therapy. These results concerning the decrease in antibiotic resistance in combined phage-antibiotic therapies were also recently confirmed using tobramycin and bacteriophages, with a 60% reduction in tobramycin resistance.⁴⁸

Quorum-sensing inhibition

Quorum-sensing inhibition is one of the therapeutic options against *P. aeruginosa* infections. Hydrolyzing acyl-homoserine-lactones, lactonases have been shown to decrease in vitro biofilm production and in vivo virulence of *P. aeruginosa*.⁹ A recent study showed that bacteriophages can be genetically modified to produce a lactonase implicated in *P. aeruginosa* biofilm production inhibition. This newly engineered bacteriophage induced a significant decrease in bacterial count as well as an important inhibition of quorum-sensing activity.⁴⁹

Proof of concept in animal models of pneumonia

The first use of animal experiments to assess the efficacy of bacteriophage-based therapy was related by d'Herelle against *Pasteurella multocida*. In 1919, while epidemic fowl cholera was responsible for poultry mortality in France, he gave water that contained phages active against the bacterium responsible for the disease to the non-diseased animals and noticed that phage-pretreated animals did not contract the infection. In the same way, he obtained efficacy against hemorrhagic septicemia in cattle. D'Herelle pursued animal studies to assess the interaction of phages with the mammalian immune system. He observed that guinea pig leukocytes incubated in the presence of *Shigella* bacteria and bacteriophages had enhanced phagocytic power compared to incubation with the bacteria alone. Later, and until recently,⁵⁰ studies in guinea pigs and mice produced discordant data, suggesting that the effect of phages on mammalian phagocytic activity against bacteria may depend on numerous factors, including the concentration of the phages, the bacterial species, the duration of the experiment, and the type of phagocytic cell studied.

Animal studies are of special interest to assess bacteriophage toxicity and diffusion in mammalian organisms. Routes and delay of bacteriophage penetration in vertebrates have been extensively evaluated in various animal species and are reviewed in detail by Dabrowska et al.⁵¹ Briefly, whatever

the route of inoculation, bacteriophages exhibit a rapid bloodstream spread and multiorgan diffusion ability, including into the lung and the brain. After any oral, nasal, aerosol, intra-uterine, skin, or other route of administration, the delay before detection of phages in the blood is short (15 minutes to 1 hour), except after intranasal administration, which has been reported to take approximately 24 hours to diffuse into systemic circulation. No toxicity, organ dysfunction, or lesion was observed with these different routes of administration. After a single dose, bacteriophage clearance takes several days. As one could expect, the persistence of bacteriophages in an animal's body is enhanced when concomitant infection with sensitive bacteria exists.

The subject of respiratory tract infections started to be questioned in the 2000s. Huff and colleagues conducted a series of experiments to treat *E. coli* respiratory infections in broiler chickens.⁵² They demonstrated that an aerosol spray given just after bacterial inoculation with 10^4 colony-forming units (CFU) was able to reduce the mortality from 50% to 20%. Approximately 10 years later, a few animal studies were specifically designed to assess clinically relevant endpoints in pneumonia with the benefits of recent experimental techniques including in vivo light imaging and fluorescent confocal microscopy. However, experimental data from in vivo animal studies are still scarce in the field of pneumonia, and those that do exist are from studies mainly conducted in mice with *P. aeruginosa*,^{53–56} *Klebsiella pneumoniae*,⁵⁷ or *Burkholderia cenocepacia*.⁵⁸ As far as we are aware, only two studies focused on CF and *P. aeruginosa*^{54,55} (Table 2).

Whatever the animal species or the bacteriophage, all reports converge to state that the efficacy of bacteriophage-based therapy in lung infection is time and dose dependent. Indeed, nearly all studies assessing this endpoint show that efficacy relies on the time frame of the treatment (within the first 2 hours postinfection), and preventive administration should be considered (24 hours before bacterial challenge).⁵³ In a lethal mouse model of pneumonia using an MDR *P. aeruginosa* strain from CF patients, Morello et al⁵⁴ reported that a single intranasal administration of the bacteriophages PAK-P or P3-CHA resulted in survival rates of 90%–100% and reduced pathological damage when given within 2 hours postinfection. In addition, a preventive effect was obtained with the bacteriophage P3-CHA when given 4 days prior to infection, showing 100% protection at a dose of 3×10^8 plaque-forming units.

The bacteriophage-to-bacterium ratio appears to influence treatment efficacy. For example, in the preliminary part of a study published by Debarbieux et al⁵³ all mice

Table 2 Bacteriophage-based therapy in animal models of *Pseudomonas aeruginosa* pneumonia

Animal model	Route	Dose	Duration of treatment	Strain	Bacteriophages tested	Time of evaluation of the effect	Endpoint(s)	Study
Mouse pneumonia	Intranasal	2×10^9 to 5×10^9 PFU/mL (50 μ L/animal)	Single dose 2 hours after infection	Clinical strain from CF patient	Cocktail of two newly isolated phages (ϕ MR299-2 and ϕ NH-4)	4–8 hours after treatment	Increased lung bacterial clearance	Alemayehu et al ⁵⁵
Mouse lethal pneumonia	Intranasal	3×10^7 or 3×10^8 PFU	Single dose 2 hours after infection	MDR <i>P. aeruginosa</i> strain from CF patient	PAK-P or P3-CHA bacteriophage	18 hours after treatment	Reduced mortality Reduced lung damage	Morello et al ⁵⁴
Mouse lethal pneumonia	Intranasal	3×10^8 PFU	Single dose 4 days before infection	MDR <i>P. aeruginosa</i> strain from CF patient	PAK-P or P3-CHA bacteriophage	4 days after preventive treatment	Preventive effect: 100% survival	Morello et al ⁵⁴
Mouse lethal pneumonia	Intranasal	10:1 phage to bacteria ratio (10^8 PFU)	Single dose 2 hours after infection	PAK bioluminescent <i>P. aeruginosa</i> strain	PAK-P1 bacteriophage	6–24 hours after infection	Reduced mortality Reduced lung inflammation	Debarbieux et al ⁵³
Mouse lethal pneumonia	Intranasal	10:1 phage to bacteria ratio (10^8 PFU)	Single dose 24 hours before infection	PAK bioluminescent <i>P. aeruginosa</i> strain	PAK-P1 bacteriophage	24 hours after preventive treatment	Preventive effect: 100% survival	Debarbieux et al ⁵³

Abbreviations: CF, cystic fibrosis; MDR, multidrug resistant; PFU, plaque-forming unit.

treated intranasally with a phage-to-bacterium ratio of 1:10 died within 5 days following infection with 10^7 CFU *P. aeruginosa*, whereas mice treated with ratios of 1:1 and 10:1 survived until 12 days. Similarly, other studies using *K. pneumoniae* or *B. cenocepacia* showed the efficacy of bacteriophage-based therapy in reducing lung bacterial burden when given early at high amounts.^{58,59}

Very recently, efficacy with delayed treatment was reported,⁶⁰ which is of major interest for its clinical relevance. In an immunocompromised (cyclophosphamide-induced leukopenia) mouse model of antibiotic-resistant *Burkholderia cepacia* from CF lung infection, Semler et al showed efficacy of a bacteriophage therapy (five bacteriophage strains including Myoviridae and Podoviridae) delivered via aerosol 24 hours postinfection.⁶⁰ Two days after treatment (3 days postinfection), bacterial lung culture decreased by 2.5 to 4.0 log, the aerosol route showing better results than the intraperitoneal route. Liposomes can also be used as a delivery vehicle for phages with the advantage of delayed efficiency, as recently reported in a mouse model of *K. pneumoniae* lobar pneumonia with both therapeutic and prophylactic administration.⁵⁷

Whether bacteriophages are still efficient against mucoid strains is a major concern. Alemayehu et al⁵⁵ assessed the effect on the biofilm of two bacteriophages active against *P. aeruginosa*, the newly isolated bacteriophage NH-4 and the bacteriophage MR299-2, both by in vitro and in vivo experiments. For their in vivo study, after isolation of several *P. aeruginosa* strains from CF patients' sputum, they lux tagged a mucoid strain and a non-mucoid strain. BALB/c mice were infected intranasally (approximately 10^7 CFU/mouse), and after 2 hours, animals were inoculated with a phage-mix suspension at approximately 10^8 plaque-forming units per animal. Bioluminescent imaging performed from 2 to 6 hours after infection (maximal level at 6 hours) showed a significant reduction in fluorescence in the phage-treated mice, regardless of whether they were infected with a mucoid or non-mucoid strain. These data offer interesting perspectives for future treatment of CF patients who often chronically harbor mucoid strains.

Summary

Numerous animal studies argue for a preventive or curative treatment of *P. aeruginosa* lung infection with bacteriophages, even when mucoid strains and biofilm formation are concerned. However, the preferred route of administration, the dose, the duration of treatment, co-treatment with antibiotics, and the choice of a single agent or of a host-adapted

or preexisting cocktail are still unclear. In the absence of standardization, comparison between studies is sometimes difficult. In addition, because studies in small animals have a short time of observation compared to chronic lung infections in humans, the question of whether bacteriophage-based therapy may favor transduction of virulence factors would need to be assessed in vivo. CF patients often have polymicrobial lung colonization and even anaerobic flora perhaps hidden by *P. aeruginosa*.⁶¹ To our knowledge, no animal study has yet assessed the value of bacteriophage-based therapy in targeting multiple bacterial species.

Importantly, the clearance and diffusion of therapeutic agents deep in the respiratory airways of CF patients need to be considered. The effectiveness of bacteriophages to treat infected CF-like mouse lungs would be interesting to assess in, for example, the recent mouse model of pulmonary deficiency developed by Zhou et al.⁶² Additionally, before delivery to patients, animal models would be of great interest to determine how bacteriophages interact with eukaryotic cells by, for example, using intravital imaging technology.

Clinical studies and therapeutic perspectives

Safety and efficacy of bacteriophages

Studies from Eastern countries have offered sufficient evidence to support the use of bacteriophages in controlled studies in Western countries. The prerequisite to these studies, and, more largely, to open the door of the therapeutic arsenal to bacteriophage-based therapy, is the affirmation of their safety. Indeed, the major concern is that bacteriophages are viruses, and it is difficult to override this image of human pathogens. However, phage safety and efficacy have been recently investigated.⁶³ In that study, the authors designed a cocktail of lytic phages active against *P. aeruginosa* and *S. aureus* clinical isolates from burn patients with infected wounds. Prior to its clinical use, the cocktail was tested for multiple parameters including stability, pyrogenicity, sterility, cytotoxicity, antibacterial efficacy with specific activity against the targeted bacteria, lytic nature, the absence of temperate bacteriophages, and the absence of toxin-coding genes. This first step was recently supported in the Evaluation of Phage Therapy for the Treatment of Escherichia Coli and Pseudomonas Aeruginosa Wound Infections in Burned Patients – “Phagoburn” – study by the European Ethics Committees, and its funding was provided by the European Commission. Other controlled studies using bacteriophages in the treatment of infected wounds¹⁴ and chronic otitis¹⁶

demonstrated encouraging results and showed the safety of the bacteriophages.

Clinical data in CF patients

The experimental treatment of pneumonia or airway bacterial colonization in CF patients is until today limited to case reports.⁶⁴ For example, in a 5-year-old child with failure of multiple antibiotic therapies and chronic airway colonization by *P. aeruginosa*, aerosolized bacteriophages from the Pyophage cocktail (from the Georgian Eliava institute) targeting *P. aeruginosa* were used three times a day, 6 to 10 days per month during 3 consecutive months. The authors describe an improvement of the clinical status with facilitation of expectoration and weight gain. After the third round of aerosols, *P. aeruginosa* was no longer identified in the patient's sputum. More recently, between 2007 and 2010, a study was performed on eight CF patients in Tbilisi. Patients received bacteriophages as aerosols for 6 to 10 days along with conventional antibiotic treatment, mucus thinners, and vitamins. The authors reported a decrease in the concentration of bacteria in the sputum of patients and a decrease in the need for antibiotic treatments.⁶⁵ These studies are encouraging, and it seems that bacteriophages are able to reduce *P. aeruginosa* infections. However, due to their lack of standardized design and the few number of patients included, it is difficult to draw conclusions on the effectiveness of bacteriophages for this indication. Larger clinical trials, especially with randomized designs, are necessary to determine the efficacy of bacteriophage-based therapy in *P. aeruginosa* pneumonia in CF patients.

One of the main elements underlined by these studies is the therapeutic potential of bacteriophage-based therapy in CF patients using a local application by the nebulized route. Indeed, this route of administration of antibiotics is commonly used in CF patients because of the high local concentration of the agents at the infection site and the supposed low systemic diffusion. In this context, dry-powder systems for inhalation have been developed and tested⁶⁶ with a phage cocktail targeting the *B. cepacia* complex and *P. aeruginosa*. This technique offers the possibility of obtaining a lyophilized solution containing phages with controlled titers and lytic activity. Using a mouth-throat replica, the nebulization technique allowed a significant inhaled fraction of bacteriophages to be distributed throughout the experimental system. Nebulization of a lyophilized solution of bacteriophages could be a potentially attractive alternative to the traditional inhaled chemotherapeutic treatments of patients with CF colonized by *P. aeruginosa*.

Limits

Although the clinical data and technical perspectives regarding bacteriophage treatment in CF pneumonia seem particularly interesting, the scarcity of the literature concerning specifically CF strains and patients limits for now the conclusions that can be drawn for phage therapy for this indication. Indeed, few clinical data are available. In the past, one of the main limits to the development of bacteriophage use in Western countries was the report of therapeutic failures. Thereby, the antibacterial efficacy of bacteriophages in CF patients can only be supposed from clinical case reports and from animal and in vitro studies. The only well-designed clinical studies in existence focus on wound infections, so extrapolation to the respiratory tract seems hazardous. On the other hand, bacteriophages, especially in their lysogenic lifestyle, contribute significantly to bacterial genome alterations and could be vehicles of the resistome in CF.⁶⁷ In addition, the question of whether bacteria develop resistance against phages requires more profound investigations.

Conclusion and perspectives

At the time of a scarcity of new antibiotics against MDR infections, bacteriophages are potential new anti-infectious treatments that may be of particular interest against bacterial flora from CF patients given the frequency of MDR bacteria in this population. Today, evidence is growing to support bacteriophage-based therapy in such patients, especially in *P. aeruginosa* infections. Phage cocktails targeting *P. aeruginosa* are already available and new phages can easily be identified in the environment. Phage specificity against clinical strains from CF sputum has been proven, as well as phages' possible synergy with antibiotics. Phage activity against biofilms, which are particularly involved in CF persistent infections, is also of particular interest. The results of animal studies seem to agree with in vitro findings. The inhaled route of administration has been demonstrated to be suitable for bacteriophage aerosol administration. With the development of molecular biology, bacterial genome sequencing may allow the identification of putative target genes associated with bacteriophage activity. With the beginning of the first randomized controlled study using bacteriophage treatments in infected wounds and the demonstration of their safety in clinical use, the road seems open for the first clinical trials evaluating the efficacy of bacteriophages in the treatment of *P. aeruginosa* infections in CF patients. However, many features remain to be clarified, such as the route of administration, the timing of treatment in the evolution of chronic

infections, the standardization of delivery (dose, frequency), administration with antibiotics, and the risk for the emergence of lysogenic forms with horizontal gene-transfer abilities. Even if the main limitation is that no well-designed clinical study has been conducted, physicians and researchers cannot ignore bacteriophages in the search for innovative treatments for *P. aeruginosa* in CF patients.

Disclosure

The authors declare no conflicts of interest in this work.

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