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REVIEW

Inhaled phage therapy: a promising and challenging approach to treat bacterial respiratory infections

Elsa Bodier-Montagutelli[a,b,c], Eric Morello[a,b], Guillaume L'Hostis[d], Antoine Guillon [a,b,e], Emilie Dalloneau[a,b], Renaud Respaud[a,b,c], Nikita Pallaoro[a,b], Hélène Blois[c], Laurent Vecellio[a,b,f], Jérôme Gabard[c] and Nathalie Heuzé-Vourc'h[a,b]

[a]Université François Rabelais, UMR 1100, Tours, France; [b]INSERM, Centre d'Etude des Pathologies Respiratoires, UMR 1100, Tours, France; [c]CHRU de Tours, Service de Pharmacie, Tours, France; [d]Pherecydes Pharma, Romainville, France; [e]CHRU de Tours, Service de Réanimation Polyvalente, Tours, France; [f]DTF-Aerodrug, St Etienne, France

ABSTRACT

Introduction: Bacterial respiratory tract infections (RTIs) are increasingly difficult to treat due to evolving antibiotic resistance. In this context, bacteriophages (or phages) are part of the foreseen alternatives or combination therapies. Delivering phages through the airways seems more relevant to accumulate these natural antibacterial viruses in proximity to their bacterial host, within the infectious site.

Areas covered: This review addresses the potential of phage therapy to treat RTIs and discusses preclinical and clinical results of phages administration in this context. Recent phage formulation and aerosolization attempts are also reviewed, raising technical challenges to achieve efficient pulmonary deposition via inhalation.

Expert opinion: Overall, the inhalation of phages as antibacterial treatment seems both clinically relevant and technically feasible. Several crucial points still need to be investigated, such as phage product pharmacokinetics and immunogenicity. Furthermore, given phage-specific features, appropriate regulatory and manufacturing guidelines will need to be defined. Finally, randomized controlled clinical trials should be carried out to establish phage therapy's clinical positioning in the antimicrobial arsenal against RTIs.

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Respiratory tract infection; phage therapy; formulation; biotherapeutics; bacteriophage; antibiotic resistance; aerosol delivery

1. Introduction

Respiratory infections are frequent and life threatening. As a matter of fact, acute respiratory infections are responsible for 4.25 million deaths each year, according to World Lung Foundation's Acute Respiratory Infections Atlas, and are the third cause of deaths in the world (after heart disease and stroke). Pneumonia, a form of acute respiratory infection frequently caused by viruses and bacteria, is the single largest cause of juvenile death in the world, accounting for 15% of deaths among children under the age of 5 years, according to the World Health Organization (WHO). Chronic pulmonary diseases such as chronic obstructive pulmonary disease (COPD) or cystic fibrosis (CF) are also often complicated by acute respiratory infections due to bacteria such as *Pseudomonas aeruginosa*, which accelerate the deterioration of lung function and shorten patients' lifespan. Finally, tuberculosis, a chronic respiratory infection due to a mycobacterium (*Mycobacterium tuberculosis*) affected at least, 9.6 million new persons in 2014 (WHO's global tuberculosis report 2015), leading to 1.5 million deaths.

Although antibiotics revolutionized the management and treatment of patients with respiratory infections, lowering drastically mortality of pneumonia due to *Streptococcus pneumoniae* from 20% to 5%, for example [1,2], it faces a worldwide decline of effectiveness partly due to the growth of resistant infections. Today, antimicrobial resistance is considered as one of the most serious health threats [3]. As previously reported, infections caused by multidrug-resistant bacteria account for approximately 25,000 and 23,000 deaths per year in the European Union and the United States, respectively. Still, these records may be underestimated: indeed, Carlet and Le Coz reported, in 2015 and for France only, more than 12,500 deaths from severe antibiotics-resistant infections [4]. In addition, managing antibiotic-resistant infections is costly, adding considerable pressure on overburdened healthcare systems.

Presently, approximately 40 antibiotics are in development including six against *C. difficile* and 18 against gram-negative bacteria [5]. Although this number sounds impressive, a majority of them will not reach market approval, considering the standard attrition rate of antibiotic molecule development. Assuming that some of them do, many of these new molecules may not prove successful in clinical practice or address unmet medical needs. Moreover, no new class of antibiotics – with new mechanism of action – is emerging. This shows the urging necessity to promote alternatives to antibiotics to fight antibiotic-resistant infections [6]. Among them, bacteriophages (phages) are both natural and non-conventional antimicrobial agents. Used for a long time to treat infections before the advent of antibiotics, then disregarded, they recently gained renewed interest due to their numerous

CONTACT Nathalie Heuzé-Vourc'h nathalie.vourch@meduniv-tours.fr Université François Rabelais, UMR 1100, F-37032 Tours, France; INSERM, Centre d'Etude des Pathologies Respiratoires, UMR 1100, F-37032 Tours, France

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Article highlights

- Inhaled phage therapy has shown successful for the treatment of RTIs in several preclinical models.
- Appropriate manufacturing and regulatory guidelines are currently lacking for phage products.
- Stresses generated during formulation and aerosolization processes can lead to a loss of phages antibacterial activity.
- Phages sensitivity to stresses varies among and within morphological families, which must be considered when formulating phage cocktails.
- The paucity of literature does not allow to define a 'best-suited' aerosolization device for phages.

This box summarizes key points contained in the article.

advantages over antibiotics: bactericidal effect, low inherent toxicity, high selectivity, lack of cross-resistance with antibiotic classes and self-multiplication in the presence of the bacterial host.

After setting the rationale to treat respiratory tract infections (RTI) by bacteriophages and describing bacteriophage biology, this review will highlight the strengths and limitations of phage therapy and finally focus on their delivery by inhalation. Considerations on formulation and administration devices will be discussed, enlightening the promises and challenges for successful inhalation of phages.

2. Respiratory tract infections: new therapeutics are needed

2.1. Pathophysiology of lung infections

More than 10,000 L of air per day are ventilated over the 100 m^2 surface of human lungs. As a consequence, airborne particles are continuously inhaled and in contact with the airway epithelial cells. Airborne particles are not only inorganic materials but they can also contain intact microorganisms. Thus, lungs are a portal for potential pathogens and infectious attacks.

The respiratory system can be divided into two parts: the conducting airways (comprising the upper respiratory tract, trachea, and bronchi) and the respiratory part (mainly comprising the alveoli). An infection of the conducting airways is called trachea-bronchitis and leads to purulent secretions, clinical signs of an infection, which is not considered as pneumonia (Figure 1(a)). In contrast, pneumonia refers to an infection of the respiratory part of the lung. It leads to the consolidation of the alveolar structures filled with inflammatory exudates and degraded cell products. The infected alveoli are poorly aerated and thus cannot participate in the gas exchange between blood and air (Figure 1(a)). We will thereafter focus on pneumonia, as the prescription of antibiotic agents in trachea-bronchitis is a subject of debate.

Pneumonia can be split into two groups: (i) pneumonia acquired outside healthcare units, so-called community-acquired pneumonia, and (ii) healthcare-associated pneumonia. These groups differ in causal pathogens and patient types. Basically, community-acquired pneumonia involves non-immunocompromised patients infected by virulent pathogens, which are usually sensitive to first line anti-infectious treatments. On the contrary, healthcare-associated pneumonia implies hospitalized patients, more likely infected by multidrug-resistant organism(s) (Figure 1(b)).

2.2. Causal agents

Community-acquired pneumonia is a public health issue associated with significant morbidity, mortality, and cost. It accounts for 3–5 cases for 1000 person-years and is the

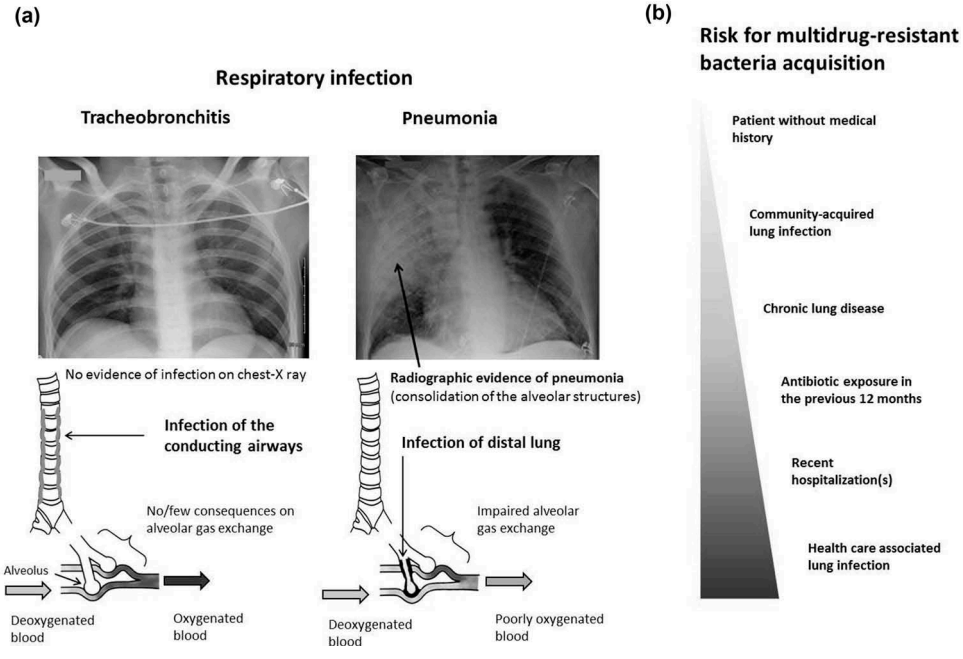

Figure 1. Lung infections: anatomical characteristics (a) and risk factors for multidrug-resistant bacteria acquisition (b).

leading infectious reason for admission in emergency care units [7]. The most common pathogens identified in adults with community-acquired pneumonia are human rhinovirus, influenza virus, and *S. pneumoniae* [8]. Interestingly, the detection of more than one pathogen is frequent. In patients with positive microbiologic diagnosis, 62% have one or more viruses, 29% have bacteria, 7% have both bacteria and virus, and 2% have a fungal or mycobacterial pathogen [8]. It is worth noting that among patients with evidence of community-acquired pneumonia, pathogens could not be detected in more than 60% of cases. Tuberculosis, a subclass of community-acquired pneumonia caused by *M. tuberculosis*, is among the most common infectious diseases and a frequent cause of death worldwide, although not constituting a major threat in industrialized countries.

Among healthcare-associated pneumonia, ventilator-associated pneumonia (VAP) has been perfectly described. VAP affects 10–30% of patients under mechanical ventilation in intensive care units [9,10], resulting in 13% mortality [11]. The major pathogens of healthcare-associated pneumonia and VAP include *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter* species, and *Staphylococcus aureus* [12,13]. The increasing antimicrobial resistance of these pathogens has pointed out the failure of current antimicrobial treatments. Despite the implementation of prevention strategies, the evolution of resistant strains remains an uncontrollable phenomenon.

2.3. Antibiotic resistance issues

In 1943, more than 10 years after its discovery by Alexander Fleming (1928), penicillin started to dramatically change the management of patients with pneumonia, offering for the first time a cure. Later on, antibiotics stopped pneumonia for being a mass killer. Today, there is little information on the outcome of patients with untreated pneumonia. Still, the example of patients with untreated pneumonia due to ethical considerations highlights the obvious efficacy of anti-microbial treatments: 90% of patients with dementia withheld from antibiotic for ethical reasons died within 1 month, versus 27% for similar patients maintained on antibiotic treatment [14]. Based on these facts, envisioning a world without antibiotics is unrealistic. However, the emergence and increasing incidence of infections caused by antibiotic-resistant bacteria are real, as revealed by the WHO's 2014 report on global surveillance of antimicrobial resistance, urging counter-acting responses.

The emergence of antibiotic-resistant bacteria was initially restricted to hospitals. According to the Centers for Disease Control and Prevention (CDC) [3], numerous antibiotic-resistant bacteria are encountered in healthcare-associated pneumonia and represent serious threats. The following figures, concerning the United States, are striking: 63% of the Acinetobacter strains responsible for healthcare-associated pneumonia in critically ill patients under mechanical ventilation have become strongly resistant to antibiotics. The same way, about 13% of healthcare-associated *P. aeruginosa* infections are multidrug resistant, leading to 400 deaths each year in the United States [15]. Other multidrug-resistant pathogens causing healthcare-associated pneumonia include methicillin-resistant *S. aureus* (MRSA).

Nowadays, antibiotic resistance is also observed in community-acquired pneumonia. Indeed, multidrug-resistant gram-negative pathogens become increasingly prevalent in the community, particularly with extended-spectrum beta-lactamase-producing and carbapenem-resistant *Enterobacteriaceae* (mainly *E. coli* and *Klebsiella* species). Some carbapenem-resistant *Enterobacteriaceae* have become resistant to most available antibiotics. Finally, although tuberculosis is treatable and curable in most cases, the causal agent can become 'extensively drug-resistant' and thus challenging to treat.

3. Bacteriophages: a natural solution against bacterial infections

Bacteriophages are ubiquitously present throughout the biosphere, particularly in feces, soil, oceans, and sewage. These viruses selectively infect bacterial prokaryotic cells to propagate. Once their genome is injected into the bacterial host, phages can either enter a lytic cycle associated with virus replication, remain in an unstable carrier state (pseudolysogeny), enter a lysogenic cycle (integration as a prophage in the bacterial genome), or evolve as a defective cryptic prophage. Lytic bacteriophages are preferred candidates as antibacterial therapeutic agents, due to their ability to destroy bacterial cells during their replication cycle. For this reason, this review will focus mainly on this phage category.

3.1. Bacteriophages' biology

Bacteriophages are usually highly specific viruses, infecting only a few to numerous strains of a single bacterial species. The great majority of described lytic phages (96%) belong to the order of Caudovirales, others being grouped into unclassified families so far. Caudovirales consist of three families, Myoviridae (Figure 2(a)), Podoviridae (Figure 2(b)), and Siphoviridae (Figure 2(c)). To date, most lytic phages bearing a therapeutic potential belong to them.

The classification of bacteriophages relates mostly to the morphological and physicochemical properties of the virion, the nature of its nucleic acid, and is increasingly supplemented by genomic data [16]. The first three classification criteria of viruses are in the following order:

- The nature of the nucleic acid (single- or double-stranded DNA or RNA);
- The shape of the capsid (tubular or icosahedral);
- The presence or absence of envelope (peplos).

Phages belonging to the Caudovirales order have a linear double-stranded DNA and are non-enveloped; most of them display contractile, non-contractile or short tails (see Figure 2).

3.2. Phages' antibacterial activity and bacterial resistance to phages

The lytic life cycle ultimately results in the assembly of phage components within the host and bacterial lysis releasing the

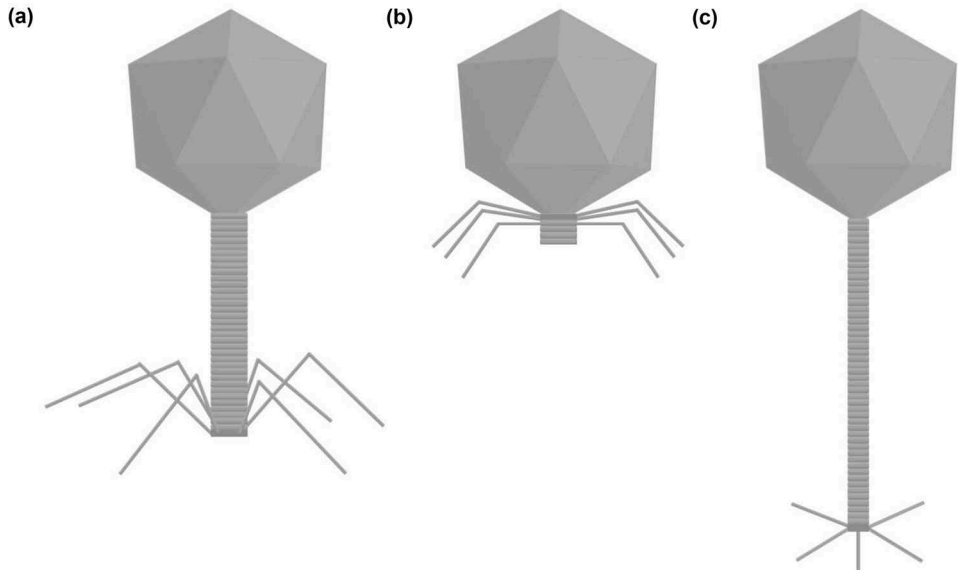

Figure 2. Caudovirales phages belong to three families: (a) *Myoviridae*, with a contractile tail. (b) *Podoviridae*, short-tailed viruses. (c) *Siphoviridae*, bearing a long non-contractile tail.

progeny [17]. Lytic phages are relevant anti-bacterial therapeutic candidates because they are bactericidal, replicate where bacteria are located, regulate their dose, carry low inherent toxicity, have a low impact on the natural microbiome due to high specificity, and can disrupt biofilms for some of them [18,19]. Besides, no cross-resistance between phages and antibiotics has been described to date to our knowledge. This might be due to different pharmacodynamical properties, requiring the simultaneous occurrence of multiple mutations for bacteria to become resistant to both antibacterial agents in case of dual therapy. Finally, compared to antibiotics, phages present a low toxicity toward environment because of their non-chemical nature and ubiquitous presence in natural ecosystems.

Bacteriophages and their bacterial hosts live side-by-side in the same environments, evolving in a co-evolutionary equilibrium [20,21]. Because bacteriophages will eventually kill their host cell, there is a strong pressure for bacteria to develop defense mechanisms against phage attacks. On the other hand, because phages are obligate intracellular parasites, replicating only inside living bacteria, they face a strong pressure to adapt and fit bacterial resistance mechanisms.

Similar to antibiotics, bacterial evolution toward phage resistance is expected. However, the use of multi-phage therapy, that is, simultaneous administration of more than one phage type [18,22,23], might limit such a risk.

4. Phage therapy: revival of a therapeutic approach

4.1. Overview of current phage therapy

4.1.1. Past and present of phage therapy in human medicine

Phage therapy has been used for a long time, encountering numerous therapeutic successes in the Eastern Europe. It has been prescribed in a wide range of indications, such as dermatology, ophthalmology, pulmonology, urogenital tract, or burn infections. Single phages or phage cocktails were delivered parenterally, orally, or locally, that is, directly onto the infected site [19].

Rapidly disregarded after World War II due to the advent of antibiotics and the lack of knowledge on phage biology, phage therapy has been recently revisited with more robust and better designed clinical trials, to face antibiotic resistance. In recent years, several case reports and observational studies have supported the interest of phage therapy against various bacteria [19,23–29], but only a few of them were related to RTIs. A few phase I/II clinical trials are currently being conducted, yet none of them relates to lung pathologies [29–31]. The literature on RTI treatment with phages, although poor, has been recently nicely reviewed elsewhere [27] and highlights the relevance of phage therapy to treat such infections.

4.1.2. Challenges for phage therapy

Obviously, phage therapy has regained interest in the recent years and the increasing number of companies in this research field is attesting it. Still, the future of phage therapy depends on switching from 'research-based case study treatment' to 'patient-wide commercial drug', with randomized controlled clinical trials. Preclinical studies are also required to provide solid supportive safety and effectiveness data as a prerequisite for acceptance and approval by regulatory agencies. Furthermore, it is mandatory to improve the knowledge about phage pharmacology and manufacturing [18,23–25].

Phages are often available as aqueous suspensions. Little is known about the impact of formulations on phage efficacy and stability. For instance, to our knowledge, there is no accelerated aging test method available: in other words, shelf-life studies must be conducted in real time and assess different formulations. Phages delivery has been achieved through different routes, some of them being better than others depending on the targeted infectious site. Each administration route has its own technical challenges and may lift different immune responses. Phage products for human use also face manufacturing issues according to the current

European and others Pharmacopeias; new quality control assays have to be developed to adapt to the replicative nature of this medicinal product. For instance, phages amplification on gram-negative bacteria during the upstream process generates endotoxins. Thus, the downstream process must allow a reduction in endotoxin levels, in agreement with the current guidelines and the purposed routes of administration. In addition, the paucity of information on phage pharmacokinetics limits the extrapolation of animal studies to human usage. The success of phage therapy depends on defining the best doses, the best timing, and administration route: indeed, unlike most other medicinal products, phages own the feature of replicating as long as their targeted bacteria are present. Pharmacokinetics must be characterized for each phage or phage cocktail, and may depend on numerous parameters including the host–bacteriophage ratio and the delivery route [32,33]. Although rarely discussed, immunogenicity may be one of the major hurdles for phage therapy because neutralizing antibodies will render phages inactive upon repeated dosing. An excellent review on phage-dependent modulation of the immune system can be found in Górski et al. [34]. Herein, we would like to focus on two recent studies. The first one showed that immunization in humans may depend on various factors, such as the route of administration, the phage dose, and the phage itself [35]. The second one was carried out in a model of systemic inflammatory response syndrome consecutive to exposure to bacterial endotoxin, mimicking the innate immunity boost occurring during bacterial infections in innate and adaptive immunity on phage pharmacokinetics (PK). The results showed that innate immunity and neutralizing anti-phage antibodies are boosted by pathogen-associated molecular patterns (PAMP) produced by bacteria [33]. Overall, this highlights the complexity of the host's immune response to phage, particularly in the presence of the targeted bacteria. Though inhibition of phages may occur after long-term treatment, it would be valuable to document immunogenicity in clinical trials to adapt the appropriate regimen and medical applications.

4.2. Phage therapy in RTIs

4.2.1. Proof of concept of phage therapy to treat RTIs

Among antibiotics alternatives and/or complementary strategies, phage therapy has recently become one of the most investigated for the treatment of RTIs. Indeed, several studies assessed the ability of bacteriophages to treat lung infections in animal models as well as in humans [27,36–39]. The different animal studies with experimental phage therapy for RTIs are summarized in Table 1. They were carried out on either mouse or mink models. In general, these studies relied on the isolation from the environment of bacteriophages lysing the targeted host, which is usually a clinical strain used to induce lung infection in the model. The isolated phages are then assessed to characterize their lytic properties (host range, burst size, etc.) using classical microbiological assays. Phages' morphotype and family are usually determined with the help of electronic microscopy and proteomic approaches. Finally, toxicity, stability, and the ability of identified lytic bacteriophages to treat animals after bacterial lung challenge is assessed by measuring various parameters (i.e. inflammation, bacterial and phage clearance in organs, behavior, and survival). All of these studies clearly report beneficial use of bacteriophages to treat RTIs with no adverse effect of administration even in the absence of bacteria (Table 1). Successful treatments highly depend on the phage, dose, and administration timing. Optimal protection is generally obtained using the highest doses (multiplicity of infection – MOI – typically comprised between 1 and 100) and the earliest applications after bacterial challenge, irrespective of phage morphology, size, or host range. Compared to human RTIs, preclinical models usually mimic only acute infections. Further evaluation in chronic lung infection models may be of interest to address both efficacy and immunogenicity. Overall, preclinical results might be considered with care because the bacterial load is hypothetical, the therapy is often delivered rapidly after infection and some routes are unsuited (intraperitoneal) for humans or not appropriate (intranasal) to achieve a high delivery into the lungs. However, taken together, they pinpoint very useful critical technical parameters for the implementation of phage therapy in human care. Indeed, types of phage preparation, delivery route as well as regulation aspects have been and are still largely discussed (see [19,22,40–42] and references therein).

4.2.2. Topical delivery of phages by inhalation

When considering the optimal delivery route for RTI treatment with phages, local delivery of phages through the airways, directly into the lungs, by inhalation seems the most relevant: it may lead to the highest quantity of active bacteriophages in close vicinity of the targeted bacteria. As shown in Table 1, most of the studies tested topical (intranasal) delivery of phages in RTI models and demonstrated efficacy. Interestingly, two studies reported efficient treatments of *P. aeruginosa* in mink and *B. cepacia* in mouse using nebulization [36,45], a relevant method for local delivery in humans. In contrast, little data comparing topical versus systemic routes for the delivery of phages in RTI models is available; results are contradictory (Table 1). To determine the most relevant phage delivery route for treating RTIs in humans, we compared pulmonary delivery to intravenous administration in an acute lung infection animal model using *P. aeruginosa* [44], both routes being feasible in the clinical setting. As shown in Figure 3, we found a substantial benefit of delivering phages directly into the lungs rather than systemically. Our findings support the rationale to deliver phages locally into the lungs to treat RTIs.

PK parameters depend on the route of administration. Several preclinical studies assessed phage clearance in the lungs after local delivery, in the presence or absence of the targeted bacteria [39,44]. However, these studies are not sufficiently documented to elaborate a mathematical model to determine PK parameters. Further studies will be required both in uninfected and infected animals to characterize phage PK precisely, help transposing results to humans, to finally determine the best schedule and regimen for phage therapy.

Table 1. Experimental phage therapy on lung disease induced in mammal models.

Targeted bacteria and strain name	Bacteriophages			Optimal protection (vs. control)	Bacteriophages delivery		Comments	Reference
	Name	Morphotype	Efficient dose[a]		Route	Minimal time post-infection		
Pseudomonas aeruginosa								
PAK	PAK_P1	*Myoviridae*	MOI 1 (1.10^7 pfu)	100% survival	Intranasal	2 h		[37]
	PAK_P1, PAK_P2, PAK_P3, PAK_P4, PAK_P5, CHA_P1, LBL3, PhiKZ LUZ19	*Myoviridae* *Podoviridae*	MOI 1–10 (1.10^{7-8} pfu)	100% survival	Intranasal	2 h	Compared correlation between *in vitro* and *in vivo* efficiency	[43]
CHA	CHA_P3	*Myoviridae*	MOI 10 (3.10^7 pfu)	>90% survival	Intranasal	2 h	Prophylactic administration tested	[44]
D9	YH-6	*Podoviridae*	MOI 1 2.10^7 pfu	100% survival	Intranasal	2 h		[38]
PA5-1-1	PPA-ABTNL	*Podoviridae*	MOI 100 (1.10^9 pfu)	100% survival	Ultrasonic nebulization	2 h	Mink infection model Innocuity tested	[45]
NH57388A (mucoid) MR299 (nonmucoid)	φNH-4 φMR299-2	*Myoviridae* *Podoviridae*	MOI 10 (1–2.10^8 pfu)	Decreased luminescence	Intranasal	2 h		[46]
Escherichia coli								
536 PDP302	536_P1, 536_P7 and adapted 536_P7	*Myoviridae*	MOI 3 (1–2.10^8 pfu)	75–100% survival	Intranasal	2 h	Antibiotic comparison and combination	[47]
Klebsiella pneumoniae								
MDRKP 1513	1513	*Siphoviridae*	MOI 10 2.10^9 pfu	80% survival	Intranasal	2 h		[48]
B5055	SS	*Podoviridae*	MOI 100 ($2,5.10^{9-11}$ pfu)	Bacterial cleared in 5 days vs. 10 days	Intraperitoneal	Concomitant	Toxicity tested Antibiotic comparison and combination	[49]
Burkholderia cepacia								
K56-2 C6433 AU0728	KS12 KS5 KS4-M DC1	*Myoviridae* *Podoviridae*	MOI 5–120	5-log reduction in lung bacterial load (72 h p.i.)	Jet nebulization and intraperitoneal	1 day	Better protection with aerosol Neutropenic mouse model	[50]
	BcepIL02	*Podoviridae*	MOI 100 (1.10^9–10 pfu)	2-log reduction in lung bacterial load (72 h p.i)	Intraperitoneal and intranasal	1 day	Better protection with intraperitoneal	[39]
Staphylococcus aureus								
SA27	S13′	*Podoviridae*	MOI 100 (1.10^{10} pfu)	70% survival	Intraperitoneal	6 h	Neutropenic mouse model	[51]

[a]Minimal efficient dose depends on the tested phage and the targeted host.

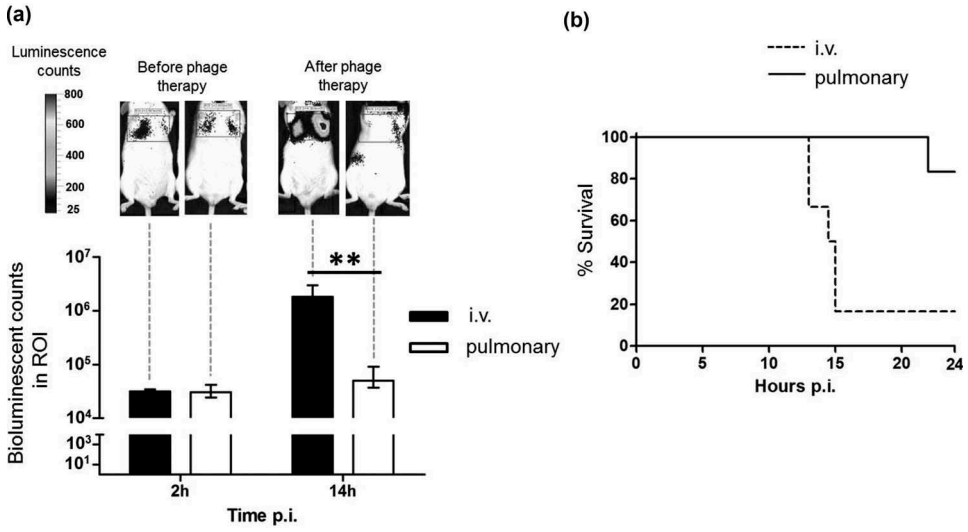

Figure 3. Efficacy of local (pulmonary) vs systemic administration of phage therapy in a murine model of acute *P. aeruginosa* lung infection. Ten-week-old male balb/c mice were infected intranasally with 10^7 cfu of the PAK lux *P. aeruginosa* strain as previously described [37]. A phage cocktail was administered at MOI 10 (i.e. 10^8 pfu) 2 hours post-infection (p.i.), when bacteria where already detected in the lungs, either intravenously (i.v., n = 6) or through the pulmonary route using a Microsprayer™ Aerosolizer (PennCentury, n = 6). The infection was followed by bioluminescent imaging at 2 h and 14 h p.i. (a) Bioluminescent counts in the defined Region Of Interest (ROI), i.e. lungs. For each group, a representative bioluminescence picture illustrates the data shown in the graph. n = 6 in each group, except i.v. group at 14 h p.i. (n = 4, because 2 animals died before 14 hours in the i.v. group). Statistical analysis: Mann-Whitney test. (b) Kaplan-Meier survival curve of infected mice. The experimental endpoint was set at 24 h p.i., where all surviving animals were sacrificed. This study was approved by the ethics committee for animal experiments under Protocol APAFIS#2920-2015113011225044 V3.

5. Challenges for local delivery of inhalable phages

The interest of phage therapy in RTIs and the relevance of the pulmonary route have been previously established, bringing the need for specific pharmaceutical formulations. Aerosol delivery seems well adapted to antibacterial treatments, providing a high drug concentration supply in lungs while limiting systemic exposure, improving comfort to patient and reducing health cost [52]. Such characteristics have led to develop or adapt drugs to treat pulmonary diseases via inhalation [53]. For phage therapy, delivering the product directly into the airways may favor contact between phages and the targeted bacteria, accelerating the onset of the lytic cycle and host destruction. Moreover, their replicative properties may favor phages spreading at the infectious site, even if poorly ventilated and less accessible to aerosol deposition.

Drug aerosols can be generated from either liquid or solid preparations, with the help of specific devices (see Section 4.2). Dry formulations are widely used for small molecules. Their advantages over liquid products are a simple handling and an improved shelf-life due to their dry state [54]. In contrast, liquid formulations allow the delivery of more fragile drugs – which do not tolerate drying – and often in larger amounts [52].

5.1. Challenge no. 1: maintain phages activity within pharmaceutical inhalable formulations

Because lytic phages foreseen for therapy consist of encapsidated DNA in an outer proteinaceous structure, their fate during formulation processes may be considered being similar to proteins. Recent studies about phage formulation confirmed this assumption (Table 2). Mechanisms underlying protein destabilization and denaturation within formulations have been identified. The development of inhalable phage formulations consequently faces potential deleterious stresses, overviewed as follows.

For protein-based products formulation such as phages, the respect of a narrow temperature range is of particular importance. Protein cold denaturation may happen during freezing or freeze-drying, due to crystallization of the aqueous medium [66]. The subsequent unfolding, aggregation and shift in osmotic pressure may be deleterious to phages [57,67]. Heat stresses also lead to protein instability, causing aggregation and irreversible conformation shift [57]. Temperature also plays a crucial role in attachment, penetration and multiplication of phages within target bacteria [68], showing the importance of protein conformation for phages antibacterial effect. pH has to be controlled within formulations, considering its great influence on phages' integrity, aggregation and/or affinity for target bacteria [68]. For inhaled drugs, the European Pharmacopeia recommends pH ranging from 3.5 to 8.0, preferably above 5.0, which meets phages' stability criteria [69,70]. Ionic strength, potently modified during dilution, freeze- or spray-drying, is also of paramount importance for formulation: by influencing osmotic pressure, it may cause an extrusion of phage DNA from the tail or a capsid disruption [67,68]. For inhalable drugs, isotonicity is preferable – even if osmolality is tolerated in a range of 150–549 mOsmol/kg – and may limit the use of osmotically active excipients.

Exposing protein-based products to an interface (air/liquid or hydrophobic/hydrophilic) may change their conformation or folding. Interfacial adsorption, potently generated during formulation (liquid/liquid interface) and/or aerosolization (air/liquid interface), may thus lead to phages aggregation or inactivation. Finally, during their formulation and/or

Table 2. Stresses applied to phages during formulation processes.

Process	Stress(es) Nature	Description	Excipients	Titer loss (log_{10})	Reference
Dilution	Ionic strength	1:100 dilution in a sugar and amino acid mixture	N/A	0.5	[55]
Emulsification	Interfacial adsorption	Water/oil/water double emulsion (dichloromethane)	PLGA + PVA + surfactant	NS (qualitative)	[56]
		Oil/water emulsion (DSPC phospholipid)	Perfluorooctyl bromide, $CaCl_2$	0.2	[57]
	Shearing	Homogenization with high-speed dispersers (14,000 rpm)	PLGA + PVA + surfactant	NS (qualitative)	[56]
Encapsulation	Interfacial adsorption	Water/oil/water double emulsion (dichloromethane)	PLGA + PVA + surfactant	NS (qualitative)	[56]
	Shearing	Homogenization with high-speed dispersers (14,000 rpm)	PLGA + PVA + surfactant	NS (qualitative)	[56]
	Ionic strength	Lyophilization (72 h)	PLGA + PVA + surfactant	NS (qualitative)	[56]
Freeze-drying/ lyophilization	Ionic strength	Unspecified conditions	None	1.3	[58]
	Temperature		Proteins or saccharide	[0.7–1.0]	[58]
	Interfacial adsorption	Primary and secondary drying – Total duration: 24–34 h	None	[1.5–10]	[59]
			Saccharides	[0.5–6.0]	[59,60]
			Others	[0.7–≥8.0]	[60,61]
		Freeze-drying duration: 30 h	Saccharide	[0.5–2.0]	[62]
		Freezing + freeze-drying – Total duration: 120 h	None	[0.8–2.0]	[63]
			Saccharide	[0.6–1.9]	[63]
Spraying	High vibration energy	Ultrasonic nozzle: high vibration frequency (48 kHz)	Saccharide + leucine + mannitol	2.0	[55]
		Two-fluid nozzle: 12 L/min dry air inlet	Saccharide + leucine + mannitol	0.7	[55]
	Shearing	Twin-fluid atomizer: 400 kPa atomizing gas	Oil/water emulsion	0.1	[57]
Spray-drying (two-fluid nozzle)	Ionic strength Shearing forces Temperature Interfacial adsorption	Spraying: Feed flow rate / Atomizing air flow rate; Drying: Air inlet flow rate / Air inlet temperature			
		0.33 mL/min	100 L/min 75°C	Saccharide + leucine + surfactant or casein	[0.3–1.0]	[64]
		2.0 mL/min 6 L/min	300 L/min 85°C	Saccharide	[<0.1–2.6]	[65]
			100°C	Saccharide	[<0.3–4.7]	[65]
		12 L/min	300 L/min 85°C	Saccharide	[0.3–2.8]	[65]
			100°C	Saccharide	[0.7–3.7]	[65]
		1.8 mL/min 12.4 L/min	580 L/min 60°C	Saccharide	[0.5–1.0]	[55]
Spray freeze drying	Ionic strength	Lyophilization (72 h)	Saccharide	<0.5	[55]

N/A: not appropriate; PLGA: poly lactic-co-glycolic acid; PVA: polyvinyl alcohol; NS: not specified.

administration, phages can undergo several mechanical stresses. Shaking and stirring may encourage interfacial adsorption, for instance during emulsification [71]. Shearing is also detrimental to proteinaceous molecules and phages; it occurs during high-speed mixing, filtration, and nebulization [72].

5.1.1. Stresses induced by phages formulation processes

Several laboratories have worked on the formulation of liquid or dry phage preparations, adapted to an administration into the airways. The results of recent phage formulation studies (2004–2016) are summarized in Table 2. For each tested method, the main denaturing stresses are identified. Their consequences on phages are quantified through the decrease of the infectious titer (titer loss). As suggested in Table 2, the most detrimental stresses underwent by phages occur during freezing and/or drying steps, currently used for the manufacturing of dry pharmaceutical products. Indeed, in the absence of protective excipients, phage titers decrease by 1–10 log [58,59,63]. This phenomenon, also observed with proteins, led to use cryo- and dessico-protective excipients, as shown in Table 3.

5.1.2. Excipients to stabilize phages in formulations

Among the tested excipients, sugars seem to protect phages from thermal and dehydration denaturation in a concentration-dependent manner. This is particularly true for sucrose [60,63], trehalose [58–60], mannitol [60], or a matrix composed of lactose and lactoferrin [62]. This protective effect has already been explored for proteins and can be explained by two concepts: (i) the water replacement theory – during the modification of the aqueous environment (freezing or drying), sugars replace water by creating hydrogen bonds with polar amino-acids, preventing the formation of hydrogen bonds between amino-acids, and consequently stabilizing the protein structure; (ii) the vitrification theory – sugars form a vitreous matrix around proteins, thus limiting their mobility, aggregation and denaturation [57,73]. Hydroxypropylmethylcellulose (HPMC) has also proven successful at protecting phages during lyophilization, when associated with mannitol [63]. For the same reasons, trehalose has shown a protective effect during spray-drying, alone [65] or in association with leucine and other excipients [55,64]. The protective effect of trehalose is reinforced when associated with a non-ionic surfactant such as poloxamer, which, in addition, prevents phages' interfacial adsorption at the air–liquid interface [56,57,64].

Such studies provide better insights into phages formulation for inhalation. Nevertheless, several critical points still remain.

First, phages' sensitivity to external factors is highly variable between and within morphological families [68]. For example, Vandenheuvel et al. demonstrated that the titer loss observed after spray-drying was significantly different between a Podoviridae and a Myoviridae [65]. Interestingly, Matinkhoo

Table 3. Effect of cryo- and dessico-protective excipients on phages viability during drying processes.

Process	Stress(es)	Excipients			Titer loss (log_{10})	Reference
		Name(s)	Concentration(s)	Diluent/buffer		
Freeze-drying/ lyophilization	Ionic strength	Standard buffers				
	Temperature	None	–	Salt Magnesium (SM) buffer	[0.8–10]	[58,59,63]
	Interfacial adsorption	None	–	Phosphate Buffer Saline (PBS)	2.0	[63]
		Amino-acids and proteins				
		Glycine	0.1–0.5 M	NaCl 0.9%	≥8.0	[60]
		BSA	5% (w/v)	SM buffer	1.0	[58]
		Dry skimmed milk powder	5% (w/v)	SM buffer	0.7	[58]
		Sugars				
		Sucrose	0.1 M	SM buffer or NaCl 0.9%	[0.6–2.5]	[60,63]
			0.3 M	NaCl 0.9% or SM buffer or PBS	1.2	[60,63]
			0.5–1.0 M	NaCl 0.9% or SM buffer	[0.5–1.6]	[60,63]
		Trehalose	0.1 M	NaCl 0.9%	4.0	[60]
			5% or 0.3–1.0 M	SM buffer or NaCl 0.9%	0.5	[58–60]
		Lactose/lactoferrin	60:40 (w/w)	SM buffer	[0.5–2.0]	[62]
		Mannitol	0.1–0.5 M	NaCl 0.9%	>3.0	[60]
		Other additives				
		HPMC + mannitol	1–2% + 0–1%	SM buffer	[0.7–1.3]	[61]
		PEG 6000	1% or 5% (w/v)	NaCl 0.9%	[1.5–5.0]	[60]
		PVP	1% or 5% (w/v)	NaCl 0.9%	≥8.0	[60]
Spray-drying (two-fluid nozzle)	Ionic strength	Saccharides and derivates				
	Shearing	Dextran 35	4% (w/v)	SM buffer	[7.0–8.2]	[65]
	Temperature	Lactose	4% (w/v)	SM buffer	[4.0–8.0]	[65]
	Interfacial adsorption	Trehalose	4% (w/v)	SM buffer	[<0.1–2.6]	[65]
		Trehalose + leucine + mannitol	0.8% + 0.4% + 0.8% or 1.2% + 0.4% + 0.4% (w/v)	SM buffer	[0.5–1.0]	[55]
		Trehalose + leucine + surfactant or casein	2.1% + 0.5% + 0.05%	NS	[0.3–1.0]	[64]
Spray freeze drying (ultrasonic nozzle)	Ionic strength	Trehalose + leucine + mannitol	0.8% + 0.4% + 0.8% or 1.2% + 0.4% + 0.4% (w/v)	SM buffer	<0.5	[55]

Salt magnesium buffer: contains [10–50] mM Tris–HCl pH 7.4–7.5 [90–150] mM NaCl [8–10] mM $MgSO_4$ ± 0.01% gelatin [55,58,59,62,63,65].
BSA: bovine serum albumin. HPMC: hydroxyl propyl methyl cellulose. PEG 6000: poly ethylene glycol – molecular weight 6 kDa. PVP: polyvinyl pyrrolidone; NS: not specified.

et al. also observed a different titer loss for two Myoviridae phages also undergoing spray-drying, suggesting that morphology is not the only reason for variable sensitivity [64]. Hence, this disparity between phages has to be considered when designing formulation methods, particularly when dealing with phage cocktails containing different morphotypes.

A second hurdle to phage therapy development in human care is the absence of pulmonary toxicity studies for some of the aforementioned excipients. In a clinical translation perspective, complete safety profiles should be established for the chosen excipients, eliciting a putative extended delay for bringing phage therapy to patients.

5.2. Challenge no. 2: successful delivery of phages aerosol in (deep) lungs

Besides offering protection to phages toward preparation and administration stresses, the designed formulations should also be delivered at bacterial infection sites, mainly located in the alveolar area. It has been previously established that particles generated within an aerosol (either liquid or dry powder) should have an aerodynamic diameter comprised between 0.5 and 3 µm to reach deep lungs and achieve a high level of drug deposition at the infectious site [74]. The production of such particles relies on two main parameters. The aerosolization device and particularly the underlying mechanism of particles generation from a drug product plays a fundamental and determining role in the size distribution of released aerosol particles. Particle size is also strongly influenced by the drug formulation, especially for liquid preparations. For example, surface tension or viscosity, which can be modified by adding excipients (e.g. surfactants), can also modify the aerosol's mass median aerodynamic diameter (MMAD) [70,75]. In practice, when developing phage products for inhalation, the combined characteristics related to formulation and the device have to be optimized. The aerosol generators available for such applications are listed and briefly described in the following.

5.2.1. Aerosolization of solid formulations

Dry-powder inhalers (DPIs) have been approved for the administration of COPD, asthma and CF treatments. For patients, their main advantages are their ease to handle, less cleaning requirements after use and quick delivery [52]. These devices can be sorted in two main categories regarding their mode of operation. On the one hand, inactive devices use the energy generated by the patient's inspiration to transform a bulk dry powder into a fine particle mist. On the other hand, active devices, which were developed more recently, have their source of energy enclosed [76].

The pharmaceutical development of a dry powder for phage inhalation implies several steps: generating the powder from a liquid pharmaceutical, demonstrating its stability and optimizing its properties to produce fine particles to enable alveolar deposition. Currently, the research on phages is mainly focused on the first two steps. In the literature, powdering phage suspensions by freeze-drying, spray-drying or spray freeze-drying generated deleterious stresses, possibly hindering the development of dry phage formulations for inhalation (see Table 2).

5.2.2. Aerosolization of liquid formulations

Two main types of devices are used to deliver liquid drugs to the lungs: pressurized metered-dose inhalers (pMDIs) and nebulizers. The Respimat® Soft Mist™ Inhaler also allows delivery of liquid formulations but has not been tested with phages to our knowledge; hence it will not be developed in this review.

pMDIs allow the delivery of a pre-set drug dose through a metering valve. To do so, the drug has to be dispersed in a liquefied propellant gas [77]. Thus, compatibility between drug and gas has to be assessed, which brings a limitation in the use of such devices. Besides, they usually contain organic solvents, the aerosolizable volume is limited (<200 µL) and this delivery method generates interfacial adsorption and drying. Nevertheless, phage delivery through a pMDI has already been tested and led to a limited loss of activity (Table 4).

Nebulizers are an attractive alternative for the administration of liquid aerosols: they allow the delivery of larger volumes (>1 mL) and do not use liquefied propellant gases. Three types of nebulizers can be used: jet, ultrasonic, and mesh nebulizers. All of them bring a risk of shearing and air/liquid interfacial adsorption. Jet nebulizers use a gas flow to atomize the liquid drug into droplets. For phages, the main associated disadvantages are: drug recycling in the reservoir, recirculations leading to repeated stresses, evaporation and about 50% drug loss due to a large dead volume [52]. Ultrasonic nebulizers use the vibration of a piezoelectric crystal to generate droplets from the liquid drug. Their main disadvantages are their incompatibility with suspensions and heating during aerosolization. In mesh nebulizers, droplets are generated while passing through a membrane with calibrated holes. There are two subclasses of mesh nebulizers. In static mesh (SM) nebulizers, a vibration is generated within the liquid drug by an ultrasonic transducer, whereas in vibrating mesh (VM) nebulizers, a piezo element leads to a mesh vibration [52]. Depending on the device, there may be a moderate temperature shift (compared to ultrasonic nebulizers) that can be deleterious to some molecules. Nevertheless, mesh nebulization does not generate drug recycling or evaporation in the reservoir, limiting additional stresses and changes in drug formulation. These nebulizers are thus better adapted for the administration of stress-sensitive drugs, such as biotherapeutics [80].

5.2.3. Generating phages aerosols: state of the art

Several authors have studied the stability of phages after aerosolization, either in liquid or solid formulations. Their results are summarized in Table 4. Overall, aerosolizing phages in liquid rather than solid format (or the reverse) do not seem to affect significantly their ability to reach the lungs. Given the discrepancy in experimental designs, comparing results of these studies is quite difficult and makes impossible to identify a 'most favorable' device to deliver respirable phages. However, such results are still interesting regarding the proportion of 'infectious' phages that are able to reach the lung, showing marked differences in titer loss between devices, but without specific trend among device types. The local dose of active viruses (and multiplicity of infection) is a critical parameter for the success of phage therapy [24]. Titer difference between phages loaded in the device and potently active phages (reaching lungs) might be due to: (i) an eventual destruction of phages during aerosolization (see above) and (ii) a heterogeneous distribution of phages within aerosol particles (which also rely on device's MMAD). The formulation might also play a role in the latter one.

Table 4. Summary of recent *in vitro* phages aerosol performance studies.

Aerosolization device			Total nebulizate		Inhaled fraction (particles with diameter <5.0 µm)		
Category	Brand name	Pharmaceutical formulation	MMAD (µm)	Phage titer loss (log_{10})	Phage titer loss (log_{10})	Total formulation loss	Reference
Dry powder inhaler (DPI)	Aerolizer®	Lyophilized powder	NS	NS	[0.2–1.0]	67%	[62]
		Spray-dried powder	[2.5–2.8]	NS	0.5	30%	[64]
	Osmohaler®	Spray-dried powder	NS	NS	[0.8–1.0]	50%	[55]
		Spray freeze-dried powder	NS	NS	[0.6–0.7]	[50–80]%	[55]
Pressurized metered-dose inhaler (pMDI)	NS	Reverse emulsion	NS	[0.5–0.9]	NS	NS	[57]
Jet nebulizer	Pari LC® Star	Isotonic suspension	4.98	0.7	1.25 (called 'alveolar fraction')	NS	[78]
		Hypotonic suspension	NS	1.15	NS	NS	[78]
	AeroEclipse®	Suspension	NS	[0.8–2.0]	[0.9–2.3]	NS	[79]
Static mesh nebulizer	Omron MicroAir® U22	Suspension	NS	1.9	2.1	NS	[79]
Vibrating mesh nebulizer	Pari eFlow®	Isotonic suspension	5.83	0.7	1.25 (called 'alveolar fraction')	NS	[78]
		Hypotonic suspension	NS	1.15	NS	NS	[78]

NS: not specified.

All of these studies have been conducted *in vitro*, with equipment mimicking human upper airways, size-based particle separators (impactors) and mathematical predictive models. The efficacy and lung deposition of such aerosols should consequently be confirmed *in vivo*.

Although challenging, achieving phage lung deposition via an aerosol is feasible, with in mind defining a proper regimen of administration and optimizing the yield of contact between active viral particles and their bacterial host.

5.3. Challenge no. 3: analyzing phages' viability and their degradation products

The gold-standard method to quantify infectious phages is the plaque assay, which has a limited reproducibility. Actually, Anderson et al. estimated that if the same phage's titer was determined in two different laboratories, one could expect a mean difference of 0.33 log (assuming that both laboratories work with the same bacterial strain and the same titration protocol) [81]. Several optimizations have been proposed in the literature concerning, for example, the titration volume and the composition of the agar layers [81,82]. These parameters may lower the assay's sensitivity threshold and, to a lesser extent, its variability, which is rather inherent in the technique itself. Nevertheless, titer assay remains the only manner to determine the amount of infectious viral particles. This assay is hence irreplaceable but its variability must be kept in mind while interpreting data. Trying to reduce phage destabilization becomes harder when the degradation is inferior to this variability. Indeed, this technique does not permit to quantify minor variations in phage titer, thus limiting optimization. Other analytical techniques have to be developed, adapted both to phages and formulation processes. To our knowledge, only a few complementary methods have been assessed in the analytical field to date, including quantitative real-time PCR [83,84]. High performance liquid chromatography (HPLC) could most likely provide complementary information, by separating the components of phage products and thus enabling the detection and quantification of other species (hollow capsides, protein fragments, or DNA). Size-exclusion HPLC and ion-exchange HPLC have already been successfully applied to phage suspensions, but as purification techniques [84–87]. Adjustments are still needed before using liquid chromatography in analytical purposes. Anyway, full characterization of phage formulations – that means determining both physicochemical and functional features – implies performing at least two different analytical assays, for example, titer assay and HPLC.

6. Conclusion

Alternative approaches to replace or combine with antibiotics are critically required given the rise of antibacterial resistance and the small pipeline of drugs in development, often insufficient because of their conventional mode of action. Nineteen different classes of alternative approaches are currently being considered [6]. Among them, bacteriophages is a unique class of antimicrobials with replicative and evolution properties, which has proven efficient in animal models to treat RTIs. However, there are still major challenges to overcome before the first phage products get a market authorization.

7. Expert opinion

The biology and pharmacology of phages remain subjects of questionings at the basic research level. For RTIs, topical delivery sounds the most relevant and our results clearly highlight the advantage of the pulmonary versus i.v. route. The better bactericidal effect is probably due to higher amounts of phages reaching the site of infection – where their host cells are located – after pulmonary delivery. As for antibiotics, PK/pharmacodynamics studies would be required to support this assumption and describe the fate of pulmonary delivered phages in the presence of the targeted bacteria, after deposition within the respiratory tract. PK studies are important during drug development since they help transposing results to humans, characterizing the best schedule and regimen for phage therapy, and predicting the clinical outcome. To date, only few studies have assessed the fate of phages after local delivery, in the presence or absence of bacteria [39,44] and they are not sufficiently documented to elaborate a mathematical model to determine PK parameters. Thus, further animal studies are required to help developing mathematical models evaluating phage pharmacokinetics in the presence and absence of their host cells *in vivo*. Rodent models are often used for preclinical PK studies because they provide disease models and allow statistical analyses. However, they are usually not predictive of aerosol deposition in human lungs. PK studies in larger animal models, closer to human in terms of aerosol deposition, would be useful to help clinical transposition. Besides, immunogenicity is a major component to take into account in preclinical PK studies, since neutralizing anti-phage antibodies may accelerate the treatment's clearance and hinder its efficacy. Although immunogenicity is rarely investigated, recent results showed that the host's innate immune response to infecting bacteria caused a concomitant removal of phages from the body, with significant effect on the therapeutic response [33]. These findings raise efficacy limits for the treatment of chronic diseases (chronic RTIs) with a defined phage product. They also emphasize the necessity of a manufacturing process conferring reduced endotoxin levels to phage products. This is in line with the evolution of manufacturing and regulatory guidelines, highlighting that the 'new phage therapy era' will have to differ from the seventies Western European/American phage products or those being currently commercialized in Georgia or Russia. Indeed, commercializing phages as a basic bacterial lysate product has now become obsolete considering modern Pharmacopeias constraints. The necessity of reinforced quality assessments will probably make phage therapy a costly, niche market-positioned therapeutic alternative. In addition to the manufacturing process of inhalable phage products, other challenges to overcome for commercialization are their formulation and stability. As reported in this review, a loss of phages titer is often observed during aerosolization, although not very accurately quantified. So far, no one can assume the impact of inactivated or destroyed phages on the PK/

immunogenicity of inhaled phage therapeutics and further investigations are required. Within the few studies on phages aerosolization, all types of devices tested seemed suitable to generate phage aerosols. However, straightforward and extensive comparisons have not been performed so far and further investigations are required. Liquid formulations of inhaled phages are intended to stabilize phages during the aerosolization process to prevent their degradation, and in fine their pharmacological activity. Inhaled phage formulations might also contain excipients and buffers tolerated for pulmonary delivery and should be optimized so that the aerosol fits phages best and achieves drug delivery to the target area within the lungs. As mentioned earlier, developing a formulation for inhalation would require new analytical methods to accurately characterize phages viability and degradation products. So far, this remains a major constraint for the pharmaceutical development of phage products.

Shelf-life of inhaled formulations is also important. Stability of phages is influenced by many factors, including temperature, acidity of their environment, salinity, and ions [68]. In the perspective of clinical use of phages, formulations should ideally be stored either at room temperature (RT, +20°C) or at +4°C. As indicated earlier, data about phage stability within formulations are variable, ranging from 10–20 to more than a hundred days, depending on formats, excipients, etc. [58,59]. Usually, authors considered their preparations as 'stable' if the titer drop was below 1 log. This approach provides extended stability data, given that the titer drop at half-life is around 0.3 log and highlights the necessity of regulatory recommendations for phage products.

As pointed out in this review, randomized controlled clinical trials are required to validate inhaled phage therapy for RTIs. The manufacturing process of inhaled phages preparations becomes a challenging issue since the latter would consequently have to conform to regulatory standards, in line with European pharmaceutical Good Manufacturing Practices (GMP), which is a prerequisite for a properly normalized clinical trial. In particular, concerning endotoxin contaminant content in phage preparations, the European Pharmacopoeia lacks recommendations for pulmonary delivery. Considering lungs' susceptibility to endotoxins, manufacturing phage therapy for RTIs should conform to the intravenous route drugs standards (Ph. Eur. 9th edition, 5.1.10), requiring an efficient purification process.

Besides, trial designs should integrate a standard treatment to compare with phage therapy. The choice of standard treatments is not obvious considering that phage therapy is a unique class of antimicrobial treatment, with replicative properties. Besides, defining a proper primary efficacy endpoint is critical considering the current debate on any new anti-infective agent to treat hospital-acquired pneumonia and VAP [88]. 'Clinical cure rate' is commonly used as primary endpoint in superiority trials for the study of combination therapy with an experimental agent plus currently available antibacterial, versus placebo plus currently available antibacterial. However, this is a subjective criteria (i.e. complete resolution of all signs and symptoms, improvement of chest-X-ray abnormalities, etc.) which might be investigator-dependent. All-cause mortality is the recommended primary efficacy endpoint for non-inferiority trials [89]. It requires a large number of patients with an elevated predicted mortality that may limit the study feasibility. The use of composite mortality and clinical primary endpoints is now encouraged. For phages, the amount or the duration of concomitantly administered antibiotics might be an interesting endpoint to measure [90]. In any case, it is unlikely that phage therapy might be approved in Europe if the primary endpoint is too weak, considering the controversy about phage efficacy in the past and the lack of confidence of the western scientific community toward soviet studies.

Overall, phages have a tremendous opportunity to benefit to patients with RTIs, acting either as a replacement therapeutic option to treat RTIs, or in combination with existing antibiotics to enhance their efficacy and/or reduce arising resistance. Although several hurdles still have to be overcome, the growing interest of both scientific and clinical communities should accelerate the progression of scientific knowledge on phage pharmacology, the elaboration of strategies to develop them as a 'biopharmaceutical product' and the definition of appropriate guidelines. Clearly, randomized controlled clinical trials may help the breakthrough of phage therapy as part of the antimicrobial arsenal for RTIs.

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ORCID

Antoine Guillon http://orcid.org/0000-0002-4884-8620

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Tomasz A. Antibiotic resistance in Streptococcus pneumoniae. Clin Infect Dis. 1997;24(Suppl 1):S85–S88.
2. Podolsky SH. Pneumonia before antibiotics: therapeutic evolution and evaluation in twentieth-century America. Baltimore: JHUP; 2006.
3. Antibiotic resistance threats in the United States. Atlanta (GA): Centers for Disease Control and Prevention; 2013 [cited 2016 Apr 27]. Available from: www.cdc.gov/drugresistance/threat-report-2013/
4. Carlet J, Le Coz P. Tous ensemble, sauvons les antibiotiques - propositions du groupe de travail spécial pour la préservation des

antibiotiques. Ministère des Affaires Sociales, de la Santé et des Droits des Femmes; 2015.
5. O'Neill J. Infection prevention, control and surveillance: limiting the development and spread of drug resistance. Rev Antimicrob Resist. 2016.
6. Czaplewski L, Bax R, Clokie M, et al. Alternatives to antibiotics - a pipeline portfolio review. Lancet Infect Dis. 2016;16(2):239–251.
•• **An overview of the different strategies being currently developed to face microbial antibiotic resistance.**
7. Restrepo M, Jorgensen J, Mortensen E, et al. Severe community-acquired pneumonia: current outcomes, epidemiology, etiology, and therapy. Curr Opin Infect Dis. 2001;14(6):703–709.
8. Jain S, Self WH, Wunderink RG, et al. Community-acquired pneumonia requiring hospitalization among U.S. adults. N Engl J Med. 2015;373(5):415–427.
9. Chastre J, Fagon J-Y. Ventilator-associated pneumonia. Am J Respir Crit Care Med. 2002;165(7):867–903.
10. Bekaert M, Timsit J-F, Vansteelandt S, et al. Attributable mortality of ventilator-associated pneumonia: a reappraisal using causal analysis. Am J Respir Crit Care Med. 2011;184(10):1133–1139.
11. Melsen WG, Rovers MM, Groenwold RH, et al. Attributable mortality of ventilator-associated pneumonia: a meta-analysis of individual patient data from randomised prevention studies. Lancet Infect Dis. 2013;13(8):665–671.
12. Jones RN. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin Infect Dis. 2010;51(Suppl 1):S81–S87.
13. Kollef MH, Shorr A, Tabak YP, et al. Epidemiology and outcomes of health-care–associated pneumonia: results from a large US database of culture-positive pneumonia. Chest. 2005;128(6):3854–3862.
14. Van Der Steen JT, Ooms ME, Adèr HJ, et al. Withholding antibiotic treatment in pneumonia patients with dementia. Arch Intern Med. 2002;162:1753–1760.
15. Rossolini GM, Arena F, Pecile P, et al. Update on the antibiotic resistance crisis. Curr Opin Pharmacol. 2014;18:56–60.
16. Krupovic M, Dutilh BE, Adriaenssens EM, et al. Taxonomy of prokaryotic viruses: update from the ICTV bacterial and archaeal viruses subcommittee. Arch Virol. 2016;161(4):1095–1099.
17. Molineux IJ, Panja D. Popping the cork: mechanisms of phage genome ejection. Nat Rev Microbiol. 2013;11(3):194–204.
18. Loc-Carrillo C, Abedon ST. Pros and cons of phage therapy. Bacteriophage. 2011;1(2):111–114.
19. Abedon ST, Kuhl SJ, Blasdel BG, et al. Phage treatment of human infections. Bacteriophage. 2011;1(2):66–85.
20. Golais F, Hollý J, Vítkovská J. Coevolution of bacteria and their viruses. Folia Microbiol (Praha). 2013;58(3):177–186.
21. Samson JE, Magadán AH, Sabri M, et al. Revenge of the phages: defeating bacterial defences. Nat Rev Microbiol. 2013;11(10):675–687.
22. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. Future Microbiol. 2013;8(6):769–783.
23. Lu TK, Koeris MS. The next generation of bacteriophage therapy. Curr Opin Microbiol. 2011;14(5):524–531.
24. Ryan EM, Gorman SP, Donnelly RF, et al. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration and timing influence the success of phage therapy: bacteriophage delivery routes review. J Pharm Pharmacol. 2011;63(10):1253–1264.
25. Nobrega FL, Costa AR, Kluskens LD, et al. Revisiting phage therapy: new applications for old resources. Trends Microbiol. 2015;23(4):185–191.
26. Monk AB, Rees CD, Barrow P, et al. Bacteriophage applications: where are we now?: bacteriophage applications. Lett Appl Microbiol. 2010;51(4):363–369.
27. Abedon ST. Phage therapy of pulmonary infections. Bacteriophage. 2015;5(1):e1020260.
• **This article summarizes the results of phage therapy attempts in humans.**
28. Rhoads DD, Wolcott RD, Kuskowski MA, et al. Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. J Wound Care. 2009;18(6):237–3.
29. Wright A, Hawkins CH, Änggård EE, et al. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. Clin Otolaryngol. 2009;34(4):349–357.
30. Sansom C. Phage therapy for severe infections tested in the first multicentre trial. Lancet Infect Dis. 2015;15(12):1384–1385.
31. AmpliPhi biosciences provides update on phase I trial: phage therapy well tolerated in first cohort. AmpliPhi Biosciences Corporation; 2016 [cited 2016 Feb 5]. Available from: www.ampliphibio.com/news.html
32. Payne RJ, Jansen VA. Pharmacokinetic principles of bacteriophage therapy. Clin Pharmacokinet. 2003;42(4):315–325.
• **Peculiar pharmacokinetic features of phage therapy.**
33. Hodyra-Stefaniak K, Miernikiewicz P, Drapała J, et al. Mammalian host-versus-phage immune response determines phage fate in vivo. Sci Rep. 2015;5:14802.
34. Górski A, Międzybrodzki R, Borysowski J, et al. Phage as a modulator of immune responses: practical implications for phage therapy. J Adv Virus Res. 2012;83:41–71.
35. Łusiak-Szelachowska M, Zaczek M, Weber-Dąbrowska B, et al. Phage neutralization by sera of patients receiving phage therapy. Viral Immunol. 2014;27(6):295–304.
• **Assessments of phage-induced immunogenicity in human clinical practice.**
36. Semler DD, Goudie AD, Finlay WH, et al. Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. Antimicrob Agents Chemother. 2014;58(7):4005–4013.
37. Debarbieux L, Leduc D, Maura D, et al. Bacteriophages can treat and prevent Pseudomonas aeruginosa lung infections. J Infect Dis. 2010;201(7):1096–1104.
• **Proof of concept of pulmonary phage therapy against *P. aeruginosa* acute pneumonia.**
38. Yang M, Du C, Gong P, et al. Therapeutic effect of the YH6 phage in a murine hemorrhagic pneumonia model. Res Microbiol. 2015;166(8):633–643.
39. Carmody LA, Gill JJ, Summer EJ, et al. Efficacy of bacteriophage therapy in a model of Burkholderia cenocepacia pulmonary infection. J Infect Dis. 2010;201(2):264–271.
40. Kutter E, De Vos D, Gvasalia G, et al. Phage therapy in clinical practice: treatment of human infections. Curr Pharm Biotechnol. 2010;11(1):69–86.
41. Pirnay J-P, De Vos D, Verbeken G, et al. The phage therapy paradigm: prêt-à-porter or sur-mesure? Pharm Res. 2011;28(4):934–937.
42. Reardon S. Phage therapy gets revitalized. Nature. 2014 Jun 3;510(7503):15–16.
43. Henry M, Lavigne R, Debarbieux L. Predicting in vivo efficacy of therapeutic bacteriophages used to treat pulmonary infections. Antimicrob Agents Chemother. 2013;57(12):5961–5968.
44. Morello E, Saussereau E, Maura D, et al. Pulmonary bacteriophage therapy on Pseudomonas aeruginosa cystic fibrosis strains: first steps towards treatment and prevention. PloS One. 2011;6(2):e16963.
45. Cao Z, Zhang J, Niu YD, et al. Isolation and characterization of a "phiKMV-like" bacteriophage and its therapeutic effect on mink hemorrhagic pneumonia. PloS One. 2015;10(1):e0116571.
46. Alemayehu D, Casey PG, McAuliffe O, et al. Bacteriophages φMR299-2 and φNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic fibrosis lung airway cells. mBio. 2012;3(2):e00029–12.
47. Dufour N, Debarbieux L, Fromentin M, et al. Treatment of highly virulent extraintestinal pathogenic Escherichia coli pneumonia with bacteriophages. Crit Care Med. 2015;43(6):e190–e198.
48. Cao F, Wang X, Wang L, et al. Evaluation of the efficacy of a bacteriophage in the treatment of pneumonia induced by multidrug resistance Klebsiella pneumoniae in mice. BioMed Res Int. 2015;2015:752930.

49. Chhibber S, Kaur S, Kumari S. Therapeutic potential of bacteriophage in treating Klebsiella pneumoniae B5055-mediated lobar pneumonia in mice. J Med Microbiol. 2008;57(Pt 12):1508–1513.
50. Semler DD, Goudie AD, Finlay WH, et al. Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. Antimicrob Agents Chemother. 2014;58(7):4005–4013.
51. Takemura-Uchiyama I, Uchiyama J, Osanai M, et al. Experimental phage therapy against lethal lung-derived septicemia caused by Staphylococcus aureus in mice. Microbes Infect Inst Pasteur. 2014;16(6):512–517.
52. Respaud R, Vecellio L, Diot P, et al. Nebulization as a delivery method for mAbs in respiratory diseases. Expert Opin Drug Deliv. 2015;12(6):1027–1039.
53. Hertel SP, Winter G, Friess W. Protein stability in pulmonary drug delivery via nebulization. Adv Drug Deliv Rev. 2015;93:79–94.
54. Shoyele SA, Slowey A. Prospects of formulating proteins/peptides as aerosols for pulmonary drug delivery. Int J Pharm. 2006;314(1):1–8.
55. Leung SSY, Parumasivam T, Gao FG, et al. Production of inhalation phage powders using spray freeze drying and spray drying techniques for treatment of respiratory infections. Pharm Res. 2016;33(6):1486–1496.
56. Puapermpoonsiri U, Spencer J, Van Der Walle CF. A freeze-dried formulation of bacteriophage encapsulated in biodegradable microspheres. Eur J Pharm Biopharm. 2009;72(1):26–33.
57. Hoe S, Boraey MA, Ivey JW, et al. Manufacturing and device options for the delivery of biotherapeutics. J Aerosol Med Pulm Drug Deliv. 2014;27(5):315–328.
58. Jepson CD, March JB. Bacteriophage lambda is a highly stable DNA vaccine delivery vehicle. Vaccine. 2004;22(19):2413–2419.
59. Zuber S, Boissin-Delaporte C, Michot L, et al. Decreasing Enterobacter sakazakii (Cronobacter spp.) food contamination level with bacteriophages: prospects and problems. Microb Biotechnol. 2008;1(6):532–543.
60. Merabishvili M, Vervaet C, Pirnay J-P, et al. Stability of Staphylococcus aureus phage ISP after freeze-drying (lyophilization). PLoS One. 2013;8(7):e68797.
61. Alfadhel M, Puapermpoonsiri U, Ford SJ, et al. Lyophilized inserts for nasal administration harboring bacteriophage selective for Staphylococcus aureus: in vitro evaluation. Int J Pharm. 2011;416(1):280–287.
62. Golshahi L, Lynch KH, Dennis JJ, et al. In vitro lung delivery of bacteriophages KS4-M and ΦKZ using dry powder inhalers for treatment of Burkholderia cepacia complex and Pseudomonas aeruginosa infections in cystic fibrosis: lung delivery of phage powders. J Appl Microbiol. 2011;110(1):106–117.
- **Phages lyophilization for dry powder inhalation.**
63. Dini C, de Urraza PJ. Effect of buffer systems and disaccharides concentration on Podoviridae coliphage stability during freeze drying and storage. Cryobiology. 2013;66(3):339–342.
64. Matinkhoo S, Lynch KH, Dennis JJ, et al. Spray-dried respirable powders containing bacteriophages for the treatment of pulmonary infections. J Pharm Sci. 2011;100(12):5197–5205.
- **Spray-drying phages for aerosolization in dry powder inhalers.**
65. Vandenheuvel D, Singh A, Vandersteegen K, et al. Feasibility of spray drying bacteriophages into respirable powders to combat pulmonary bacterial infections. Eur J Pharm Biopharm. 2013;84(3):578–582.
66. Wang W. Lyophilization and development of solid protein pharmaceuticals. Int J Pharm. 2000;203(1):1–60.
67. Furiga A, Pierre G, Glories M, et al. Effects of ionic strength on bacteriophage MS2 behavior and their implications for the assessment of virus retention by ultrafiltration membranes. Appl Environ Microbiol. 2011;77(1):229–236.
68. Jończyk E, Kłak M, Międzybrodzki R, et al. The influence of external factors on bacteriophages—review. Folia Microbiol (Praha). 2011;56(3):191–200.
69. Beasley R, Rafferty P, Holgate ST. Adverse reactions to the non-drug constituents of nebuliser solutions. Br J Clin Pharmacol. 1988;25(3):283–287.
70. Mc Callion O, Taylor K, Thomas M, et al. The influence of surface tension on aerosols produced by medical nebulisers. Int J Pharm. 1996;129(1–2):123–136.
71. Bekard IB, Asimakis P, Bertolini J, et al. The effects of shear flow on protein structure and function. Biopolymers. 2011;95(11):733–745.
72. Branston S, Stanley E, Ward J, et al. Study of robustness of filamentous bacteriophages for industrial applications. Biotechnol Bioeng. 2011;108(6):1468–1472.
73. Vandenheuvel D, Meeus J, Lavigne R, et al. Instability of bacteriophages in spray-dried trehalose powders is caused by crystallization of the matrix. Int J Pharm. 2014;472(1–2):202–205.
74. Jaafar-Maalej C, Andrieu V, Elaissari A, et al. Assessment methods of inhaled aerosols: technical aspects and applications. Expert Opin Drug Deliv. 2009;6(9):941–959.
75. Saleem IY, Smyth HDC. Tuning aerosol particle size distribution of metered dose inhalers using cosolvents and surfactants. Biomed Res Int. 2013;2013:574310.
76. Atkins PJ. Dry powder inhalers: an overview. Respir Care. 2005;50(10):1304–1312.
77. Terzano C. Pressurized metered dose inhalers and add-on devices. Pulm Pharmacol Ther. 2001;14(5):351–366.
78. Golshahi L, Seed KD, Dennis JJ, et al. Toward modern inhalational bacteriophage therapy: nebulization of bacteriophages of Burkholderia cepacia complex. J Aerosol Med Pulm Drug Deliv. 2008;21(4):351–360.
- **Liquid aerosolization of phages: viability and aerosol characteristics.**
79. Sahota JS, Smith CM, Radhakrishnan P, et al. Bacteriophage delivery by nebulization and efficacy against phenotypically diverse pseudomonas aeruginosa from cystic fibrosis patients. J Aerosol Med Pulm Drug Deliv. 2015;25(5):353–360.
80. Respaud R, Marchand D, Parent C, et al. Effect of formulation on the stability and aerosol performance of a nebulized antibody. mAbs. 2014;6(5):1347–1355.
81. Anderson B, Rashid MH, Carter C, et al. Enumeration of bacteriophage particles: comparative analysis of the traditional plaque assay and real-time QPCR- and NanoSight-based assays. Bacteriophage. 2011;1(2):86–93.
82. Cormier J, Janes M. A double layer plaque assay using spread plate technique for enumeration of bacteriophage MS2. J Virol Methods. 2014;196:86–92.
83. Rekfardt D. Real-time quantitative PCR to discriminate and quantify lambdoid bacteriophages of Escherichia coli K-12. Bacteriophage. 2012;2(2):98–104.
84. Farkas K, Varsani A, Marjoshi D, et al. Size exclusion-based purification and PCR-based quantitation of MS2 bacteriophage particles for environmental application. J Virol Methods. 2015;213:135–138.
85. Zakharova MY, Kozyr AV, Ignatova AN, et al. Purification of filamentous bacteriophage for phage display using size-exclusion chromatography. Biotechniques. 2005;38:194–198.
86. Kramberger P, Honour RC, Herman RE, et al. Purification of the Staphylococcus aureus bacteriophages VDX-10 on methacrylate monoliths. J Virol Methods. 2010;166:60–64.
87. Andriaenssens EM, Lehman SM, Vandersteegen K, et al. CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophages particles. Virology. 2012;434:265–270.
88. Bartlett JG, Barie PS, Niederman MS, et al. Workshop on clinical trials of antibacterial agents for hospital-acquired pneumonia and ventilator-associated pneumonia. Clin Infect Dis. 2010;51(Suppl 1):S1–S3.
89. Spellberg B. Skin and soft-tissue infections: modern evolution of an ancient problem. Clin Infect Dis. 2010;51(8):904–906.
90. Chastre J, Wolff M, Fagon JY, et al. Comparison of 8 vs 15 days of antibiotic therapy for ventilator-associated pneumonia in adults: a randomized trial. JAMA. 2003;290(19):2588–2598.

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