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# An overview of the current state of phage therapy for the treatment of biofilm-related infections

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Bacterial biofilms are involved in many chronic and difficult-to-treat infections. Phage therapy against infectious biofilms is becoming a promising strategy, as suggested by the increasing number of publications demonstrating the efficacy of phages against *in vitro* formed biofilms. However, the translation between *in vitro* results to *in vivo* phage therapy outcome is not straightforward due to the complexity of phage-biofilm interactions in clinical contexts. Here, we provide a critical overview of the *in vitro* studies of phages for biofilm control of clinical pathogens, followed by the major outcomes and lessons learned from the recently reported case studies (between 2018 and 2021) of phage therapy against biofilm-related infections.

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## Introduction

Biofilms can be defined as complex structures of microbial cells attached to biotic or abiotic surfaces and embedded in a self-producing matrix of extracellular polymeric substances (EPSs) [1•]. The biofilm mode of growth of bacteria contrasts with the free-living planktonic growth mode, and results from differential gene expression and metabolism, which leads to the partial protection of bacteria from adverse environmental conditions. Consequently, bacteria growing in biofilms can tolerate up to 1000 times higher concentrations of antibiotics than planktonic cells [2], making the biofilm eradication a huge challenge, namely in clinical settings where biofilms are responsible for high rates of chronic and recurrent bacterial infections [3]. Biofilm-related infections include device-related infections, when biofilms are developed in medical devices such as catheters, implants, or contact lenses, and tissue-related infections such as chronic lung infections, chronic wounds, osteomyelitis, endocarditis, among others [3]. There are multiple factors linked to the high tolerance of biofilms to antibiotics or other antimicrobial compounds namely, the difficult penetration of antimicrobials through the biofilm matrix, the high genetic diversity of biofilm cells (hypermutability), the presence of persister cells and the polymicrobial nature of most of the biofilms found in clinical contexts [3]. The need for new and effective treatments against biofilm-related infections has triggered the interest in (bacterio)phages as antibacterial agents [4]. Consequently, an increasing number of case studies about the safety and efficacy of phage therapy in patients with biofilm infections have been published over the last years and will be discussed here.

## *In vitro* studies of phages for biofilm control of clinical pathogens

The *in vitro* evaluation of phage efficacy against biofilm-related infections has been mostly performed on monospecies biofilms formed on polystyrene microtiter plates using single phage preparations, phage cocktails, or phages combined with antibiotics. Typically, a simple set up is prepared, using a specific reference or clinically relevant strain to form biofilm and study phage efficacy. However, the *in vivo* complexity of biofilms in human infections is not well mimicked in these assays, since clinical biofilms harbor a complex and heterogeneous community with multiple bacterial species and sometimes even fungi [3]. Currently, *in vitro* methods based on biofilm formation in microtiter plates still represent the most used approach to evaluate phage efficacy against biofilm-associated infections because of being non-time, non-cost, and non-labor-intensive [5]. The polystyrene microtiter plates allow the adhesion of bacterial cultures to the surface of the wells for a certain period of time and then, the phage efficacy can be assessed by cell viability, biomass quantification, metabolic activity, or microscopic visualization [5]. Nevertheless, to better simulate biofilms in real infection conditions or clinical settings, other types of materials/devices have been used instead of the microtiter plates including, stainless steel coupons [6], catheter

sections [7,8], collagen [9•] or human cells monolayers [10]. *In vitro* models are not entirely capable of reproducing real infection conditions and the translation of *in vitro* results into clinical outcomes is not straightforward. Nonetheless, all possible knowledge acquired from the *in vitro* studies can be a major advantage to investigate the variables that rule biofilm control by phages and to understand the potential of phage therapy. In brief, *in vitro* biofilm-forming methods are useful to assess the biofilm-killing efficacy of phages alone or combined with co-adjuvants; to test phage formulations *in vitro* (to understand its release, dose, and stability) before doing *in vivo* tests; to predict the emergence of phage-resistant variants and its implication in the therapeutic context; and to study phage-antibiotics synergy. For instance, the evaluation of a bacterial population after biofilm challenge with phages has demonstrated that the bacterial survivors usually have reduced fitness compared with ancient bacteria before phage treatment. In order to survive phage predation, bacterial biofilms undergo fitness costs that might lead to modifications on phage receptors [11•,12], defective growth, and reduced virulence both *in vitro* and *in vivo* [11•,13]. Additionally, due to the selective pressure exerted during phage treatment, these bacterial variants can acquire mutations that lead to re-sensitization to antibiotics [14,15] and the immune system [16]. The fact that bacterial resistance against either phages or antibiotics usually represents a fitness cost, makes the combined therapy an interesting approach; therefore, it has been extensively demonstrated, *in vitro*, that some combinations of antibiotics with phages are synergic and can lead to a better biofilm control [17–20], with exception of antibiotics that directly impair the phage life cycle progression, such as protein syntheses inhibitors [21,22]. The administration strategy (simultaneously versus sequential) can highly influence the phage-antibiotic synergy, being usually the sequential treatments better than simultaneous to obtain higher biofilm reductions [19,23]. However, extrapolations of the outcomes for phage-antibiotic combinations should be carefully addressed because there are many factors that may influence the phage-antibiotic synergy such as, the antibiotic class, the *in vivo* biological environment (for example, the presence of human serum), phage species as well as phage and antibiotic concentrations [24•]. Moreover, it is important to take also into consideration the fact that both antibiotics and phages can have a positive influence on biofilm formation. For instance, low levels of phage predation can lead to the accumulation of extracellular DNA leading to thicker biofilms [25] and antibiotics can function as communication molecules to modulate microbial communities [26].

An important aspect is that the *in vitro* assays do not always mimic the biofilm-host conditions, where the complexity of existing polymicrobial communities, as above mentioned (including viable but non-culturable cells-VBNC), *in vivo* stressors/inhibitory compounds, bacteria under different metabolic states, and host immune system, may have a high influence in the outcome of phage efficacy. Therefore, improved *in vitro* methods should be developed to better assess phage formulations, such as biofilm formation systems that allow to mimic long-term infections using dynamic conditions or surfaces that simulate human tissue/device surfaces like, for example, the implementation of 3D printed organs or tissues [27].

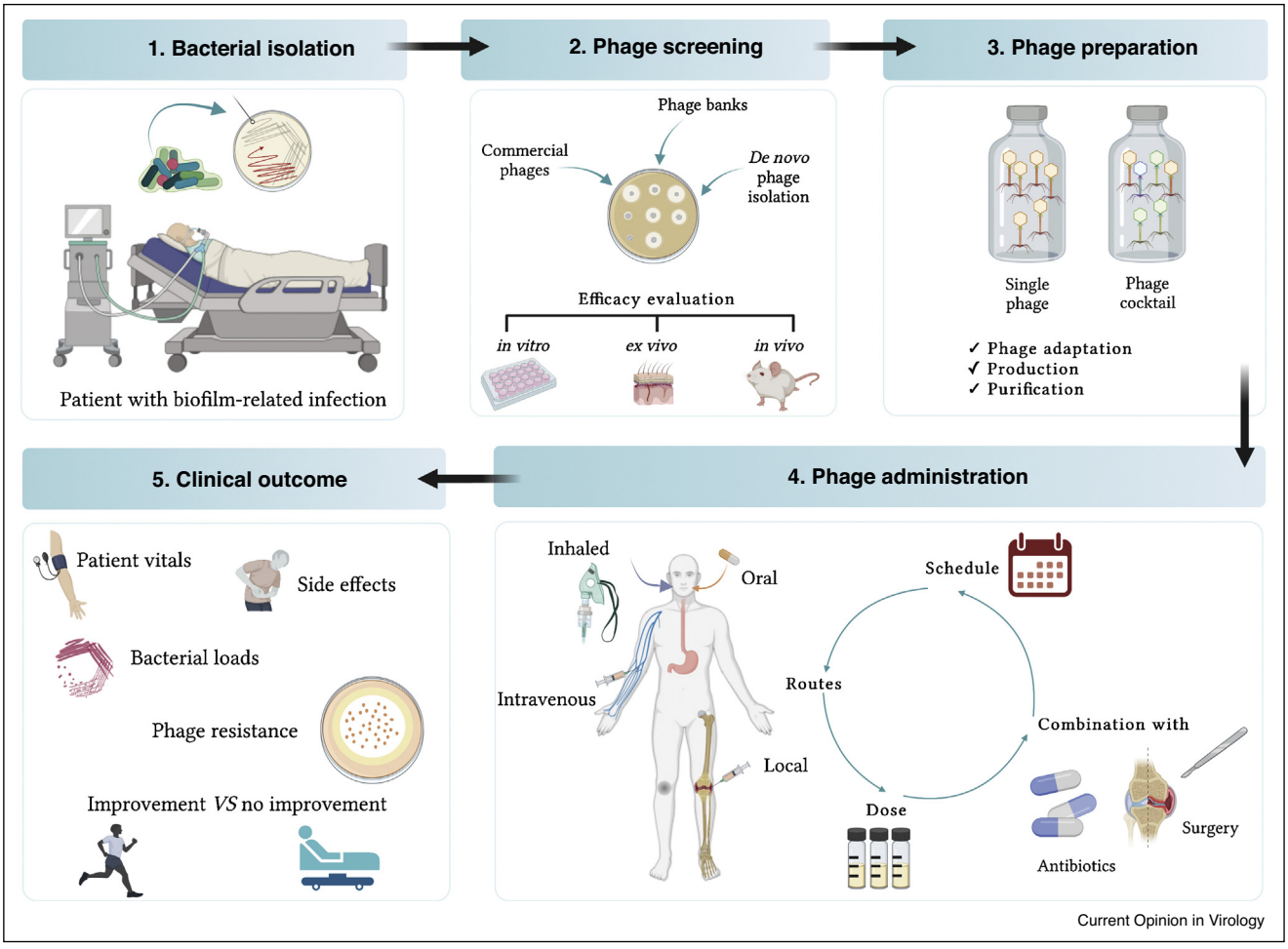
## Outcomes and lessons learned from the recently reported case studies

An increasing number of clinical case studies about the use of phages to treat a wide range of infectious diseases have been reported over the last years. In this section, we discuss the most recent phage therapy clinical cases (2018–2021) associated with biofilm infections. A detailed analysis of the cases reported herein is presented in supplementary material (Table S1).

### Phage formulation and stability

The pipeline to develop a phage therapy formulation for a biofilm infection usually starts with the isolation of the bacterial pathogen causing infection followed by the screening for phage(s) with lytic activity against the isolated strain, which is typically done in phage banks or large phage collections from phage therapy centers or pharmaceutical companies (Figure 1). This screening allows the identification of the best phage candidates for therapy and usually results in the formulation of a phage cocktail — multiple phages in a single preparation. In some cases, this initial screening step to develop a personalized phage formulation for the target bacteria can be skipped and phage cocktails already developed or commercially available can be used instead. However, these pre-developed phage cocktails will possibly result in a less effective treatment comparatively with the personalized phage formulations that are specifically designed for the bacterial pathogen causing the disease. According to our search, the leading bacterial pathogen of the reported cases was *Pseudomonas aeruginosa* (40%) followed by *Staphylococcus aureus* (24%) and, regarding phage formulation, cocktails were used in 68% of the clinical cases, while only 31% have used single phage preparations (Figure S1). In fact, the use of phage cocktails targeting different bacterial receptors is an interesting approach to delay and reduce the emergence of bacteriophage-insensitive mutants (BIMs) during phage treatment, which was already proved both *in vitro* and *in vivo* [12,28,29]. Another strategy that can be considered to delay the emergence of BIMs is the phage training. In a coevolutionary *in vitro* experiment, Borin *et al.* observed that trained phages were able to suppress *Escherichia coli* more strongly and delay the evolution of resistance than untrained phages [30••]. In addition, although it was not observed in the reported clinical cases, it might be interesting to perform a phage adaptation to the biofilm

**Figure 1**




Phage therapy pipeline for the treatment of biofilm-related infections. Created with Biorender.com.

phenotype before phage treatment in order to improve the outcome of the therapy. This strategy might be useful because biofilms have particular characteristics that can impair phage predation and the success of the treatment [1•]. However, in a clinical context, the race against time might not allow to implement such phage adaptation/training strategies that require several days to perform and therefore, a possible alternative could be an initial phage screening in biofilms instead of the commonly used spot test or screening in suspended cultures. This would enable the selection of phages with better anti-biofilm properties against the target strain, which may highly contribute to the therapy improvement. In a case study reported by Chan *et al.*, the authors assessed, *in vitro*, the potential synergy of a single phage with antibiotics before its *in vivo* application, which contributed to a better design of the therapeutic approach to treat a chronic *P. aeruginosa* infection of an aortic graft that resulted in infection resolution [31].

An important aspect that should be taken into account during phage formulation is the possible incorporation of phages in hydrogels or other matrices for an efficient delivery. Such strategies might be useful for topical applications in chronic wounds or as a coating for implanted materials [32]. For instance, in a case reported by Ferry *et al.*, the incorporation of phages in the commercially available DAC® (Defensive Antibacterial Coating) hydrogel revealed to be a promising approach to treat biofilms in patients with knee megaprosthesis infection [33••]. Nonetheless, it is important to highlight that the stability of the phage formulations should be ensured as some products may affect phages' activity and reduce its concentration [34].

## Treatment protocols

The phage therapy protocol adopted to treat biofilm-related infections should be adjusted according to the type of infection. For instance, the preferential routes of

phage administration for chronic lung infections are the intravenous, the inhalation or the combination of both routes during treatment [35,36] (Table S1). Concerning chronic wounds, the treatment is usually done by topical application [37•], while osteomyelitis usually require the combination of local injection and debridement surgery [38] (Table S1). The mechanical debridement can indeed be a useful strategy against biofilm-related infections since by disrupting the biofilm architecture, phages will have an easier access to the cells that are usually protected by the matrix, which was also shown *in vitro* by Melo *et al.* for *Staphylococcus epidermidis* species [39]. In addition to debridement, other combined treatments can be used to improve the efficacy of the phage therapy, including the co-administration with antibiotics that was used in the majority of the clinical cases that were analyzed here (Figure S1). However, as already mentioned, it is important to highlight that phages may not always have a synergistic effect with antibiotics as some of them can potentially interfere with phage replication and so, the combined treatment should be carefully studied prior to *in vivo* application [4,19].

Concerning the phage dose required for a biofilm treatment, according to our literature review of clinical cases, it ranges between $10^6$ to $10^{10}$ PFU/mL and the phage preparation is usually administered multiple times during the treatment period, depending on the infection type (Table S1). A single phage dose was only applied in approximately 14% of the clinical cases (Figure S1).

### Clinical outcomes and safety

During and after a biofilm infection treatment with phage therapy, a close follow-up of the patient to identify possible adverse reactions, to understand the evolution of the clinical condition, and to identify possible recurrence of bacterial infection, should be made (Figure 1). According to our literature review, the clinical outcome is typically evaluated by the analysis of bacterial cultures combined with monitoring of specific symptoms that can vary according to the type of infection. For instance, in patients with chronic lung infection, variants such as levels of forced expiratory volume, need for oxygen support, levels of cough and sputum production, and recurrence of exacerbations, are of high importance to understand the efficacy of phage therapy in this biofilm-related condition [40,41]. On the other hand, for patients with chronic wounds or prosthetic joint infections, observation of tissue healing, release of secretions, and levels of pain, are important indicators of the clinical condition after phage therapy [42–44]. The evaluation of the development of bacterial resistance to phages and the assessment of changes in the antibiotic resistance profile are limited to a small part of the reviewed clinical cases [40,45–49], and it is usually only performed at the end of phage treatment. However, the screening of these parameters during the treatment period would also be important to enable the adaptation of the treatment according to the collected microbiological data, thus increasing the chances of a positive clinical outcome. Also, given the high complexity of biofilm systems, these studies could be complemented by metagenomic or transcriptomic analysis of the samples collected during phage therapy, in order to better understand the population dynamics during the phage treatment process.

From the 78 cases of patients treated with phage therapy that were identified through our literature analysis, a positive outcome was reported for 96 % of them, meaning that only 3 patients (4 %) showed no clinical improvement after phage treatment (Figure S1). Complete resolution of the bacterial infection, demonstrated by bacterial eradication and/or complete clearance of clinical symptoms, was documented for 52 patients (67 %) (Figure S1). The cases in which the infection was not completely resolved but the clinical condition improved and/or the bacterial loads reduced, were classified as clinical improvement and are the case of 23 patients (30 %) (Figure S1).

Based on our literature review, a total of 9 different clinical conditions associated with biofilm infections have been reported and, the most common among the patients were lung infections and chronic wounds (Figure S1). Because of the high tolerance of biofilms to antibiotics, the treatment of such infections is very challenging and typically fails, resulting in the usual need for limb amputation in patients with chronic wounds [50], and high levels of morbidity and mortality in patients with chronic lung infections [51]. From the cases identified in our search, phage therapy was able to contribute to complete infection resolution of all the 21 patients with chronic wounds, and a positive outcome was observed for 24 out of the 25 patients with lung infections, from which 6 achieved complete infection resolution (Table S1). Although further studies are needed, especially clinical trials involving large number of patients, these results suggest the potential of phage therapy to manage complex biofilm-related infections.

Besides the clinical outcome, it is also important to understand if phage therapy is a safe strategy to treat patients with biofilm-related infections. In our search, information about phage safety was provided for 69 patients. Overall, the phage preparations used can be considered safe, since the occurrence of adverse events that seemed to be associated with phage therapy was only reported for two patients (2.6 %) (Figure S1). For instance, a patient with osteomyelitis of the femur caused by *Enterococcus faecalis* and resolved by the local application of a commercial preparation of phages, developed local redness and experienced pain during the treatment [52]. Although these symptoms have not been directly linked to phage therapy, the presence of endotoxins in the phage cocktail could not be excluded as a contributing

factor for this adverse reaction [52]. In a different case study, a patient with a prosthetic joint infection caused by methicillin-resistant *S. aureus* and eradicated by the local and intravenous application of a single phage, developed a transaminitis after the third intravenous phage dose and consequently, the treatment was stopped [53]. In this case, the authors affirmed that this adverse event seemed to be caused by phage therapy, although it was reversible and non-life-threatening.

Although phages are generally considered as safe, due to their low inherent toxicity and high specificity for their target bacteria, limiting collateral damage to normal microflora [54], the step of phage production and purification is crucial to ensure a safe outcome for the patients. An important aspect that needs to be considered and reduced to the minimum possible in phage preparations is the level of contamination with endotoxins of gram-negative bacteria and protein toxins produced by other pathogenic bacteria. These molecules have high levels of toxicity and can cause a large variety of reactions in humans, which makes essential the use of appropriate methods for their removal, to ensure the safety of phage therapy in humans [55].

## Conclusions and final remarks

Phage therapy to combat difficult-to-treat biofilm infections is gaining an increasing popularity due to growing number of successful clinical cases. The chronic nature of biofilm-related infections is ideal for a personalized phage therapy modality, providing a treatment time buffer that allows the proper development and preparation of personalized therapeutic phage cocktails. However, biofilms are challenging for therapeutic phages, mainly due to the protection conferred by the biofilm matrix and resistance mechanisms. Therefore, *in vitro* data and clinical reports suggest that the association of phages with other chemical/mechanical treatments may be beneficial to increase the efficacy of phage therapy against biofilm infections.

It is important to perceive that the knowledge on phage-biofilm interactions has been mainly generated by *in vitro* studies that most of the time fail to mimic *in vivo* conditions. On the other hand, *in vivo* studies and clinical trials on biofilm-related infections are limited. Therefore, the clinical cases summarized herein are of great importance to understand the safety and efficacy of personalized treatments of biofilm-associated diseases. Nonetheless, these studies do not provide a thorough evaluation of the healing process, particularly what concerns the analysis of the bacterial and phage populations during phage treatment. This data would be particularly useful to unveil the real impact of phages in clinical biofilms and to understand the pharmacokinetics of local administration of phages. Moreover, there is a lack of standard methods for assessing phage efficacy both *in vitro* and *in vivo*, as the treatment protocols vary greatly according to the clinical studies, even among the ones dealing with similar pathologies. Therefore, there is a need to standardize the methods used to characterize the efficacy of phages against biofilms in order to formulate reproducible protocols for a rigorous assessment of phage therapy against infectious biofilms.

## Conflict of interest statement

Nothing declared.

## Editorial disclosure

Given her role as Guest Editor, Joana Azeredo had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Jean Paul-Pirnay.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.coviro.2022.101209.

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