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► **To cite this version:**

Baptiste Gaborieau, Laurent Debarbieux. The role of the animal host in the management of bacteriophage resistance during phage therapy. *Current Opinion in Virology*, 2023, 58, pp.101290. 10.1016/j.coviro.2022.101290 . pasteur-04158880

**HAL Id: pasteur-04158880**

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Submitted on 11 Jul 2023

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ELSEVIER

# The role of the animal host in the management of bacteriophage resistance during phage therapy

Baptiste Gaborieau<sup>1,2,3</sup> and Laurent Debarbieux<sup>1</sup>

Multi-drug-resistant bacteria are associated with significantly higher morbidity and mortality. The possibilities for discovering new antibiotics are limited, but phage therapy — the use of bacteriophages (viruses infecting bacteria) to cure infections — is now being investigated as an alternative or complementary treatment to antibiotics. However, one of the major limitations of this approach lies in the antagonistic coevolution between bacteria and bacteriophages, which determines the ultimate success or failure of phage therapy. Here, we review the possible influence of the animal host on phage resistance and its consequences for the efficacy of phage therapy. We also discuss the value of *in vitro* assays for anticipating the dynamics of phage resistance observed *in vivo*.

## Addresses

<sup>1</sup> Institut Pasteur, Université Paris Cité, CNRS UMR6047, Bacteriophage Bacterium Host, Paris, France

<sup>2</sup> Université Paris Cité, INSERM UMR1137, IAME, Paris, France

<sup>3</sup> APHP, Hôpital Louis Mourier, DMU ESPRIT, Service de Médecine Intensive Réanimation, Colombes, France

Corresponding author: Debarbieux, Laurent  
([laurent.debarbieux@pasteur.fr](mailto:laurent.debarbieux@pasteur.fr))

**Abbreviations:** LPS, Lipopolysaccharide; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; MOI, Multiplicity of Infection

Current Opinion in Virology 2023, 58:101290

This review comes from a themed issue on **Bacteriophage therapy**

Edited by **Joana Azeredo** and **Jean-Paul Pirnay**

For complete overview about the section, refer "[Bacteriophage therapy \(2022\)](#)"

Available online 10 December 2022

<https://doi.org/10.1016/j.coviro.2022.101290>

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## Introduction

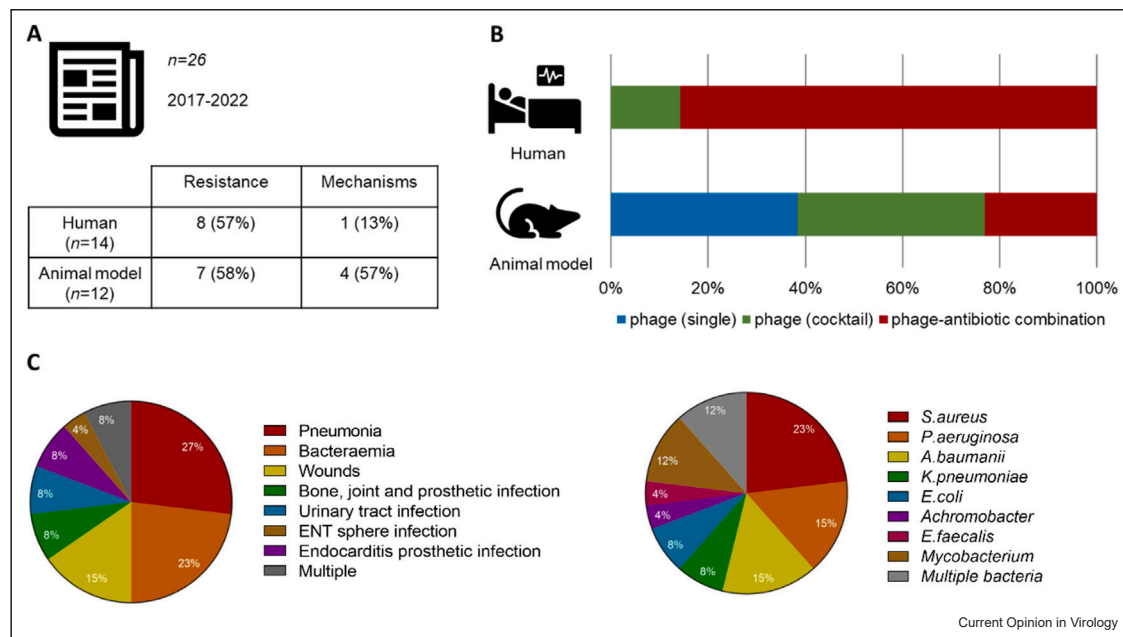
Antibiotic resistance is a major threat to public health worldwide, exposing human populations to an increase in the risk of treatment failure [1]. The number of deaths due to antibiotic resistance is expected to increase to 10 million per year worldwide by 2050 in the absence of a major new treatment [2]. In the current context of a low discovery rate for new antibiotics, phage

therapy is increasingly attracting attention as a solution for treating infections caused by multi-drug-resistant bacteria [3]. However, aside from a few case series and randomised controlled trials, there is very little recent data on the clinical efficacy of bacteriophages (referred to here as ‘phage’) [4].

Bacteria and phage coexist and coevolve in the environment, including within animals. The antagonistic nature of their relationship has long been studied, revealing a large array of molecular mechanisms underlying bacterial defence and phage counterdefence systems [5]. Mutations and specific genes enable bacteria to disrupt every step in the phage infection cycle, from the adsorption of the viral particle to the release of viral progeny [6]. The ‘golden age’ of phage research, extending from the 1950s to the 1980s, led to a first wave of discovery of bacterial defence mechanisms, such as restriction-modification systems. We have now entered a second wave of discovery, which began with the identification of CRISPR/Cas systems and now extends to *in silico* searches for new defence systems, some of which have already been validated experimentally [6–8].

Despite the existence of such a large number of diverse bacterial defence systems, many studies on experimental infections have reported efficacy for phage therapy [9]. Interestingly, many of these studies, which were designed to demonstrate the efficacy of phage therapy, ignored the inevitable issue of the selection of phage-resistant bacteria. It seems likely that in these studies, the immune system of the animal host could have controlled the growth of the phage-resistant bacterial population, thereby ensuring that treatment was successful [10]. However, it has also been shown that the fitness cost (i.e. a reduction/loss of bacterial virulence) often associated with phage resistance does not jeopardise the overall efficacy of treatment [11,12]. In a retrospective analysis of human treatments over a period of several years in Poland, the frequency of phage resistance ranged from 17% to 85% for phages used against *Staphylococcus aureus* and *Escherichia coli*, respectively [13]. In a study in the context of compassionate treatment, Lebeaux et al. reported that phage resistance resulted from mutations in the bacterial genome [14]. Moreover, in a large review of experimental models, Oeschlin also found that phage resistance was not a rare event [15]. In the face of these findings, possibilities for monitoring, analysing and exploiting phage resistance to improve the success of phage therapy have been discussed and reviewed [16–18].

Figure 1



Main characteristics of the 26 phage therapy studies reporting phage resistance information since 2017. **(a)** Number of studies, including phage resistance information, the frequency of resistance and the mechanisms identified; **(b)** proportion of combined treatments; **(c)** bacterial infections and the species involved.

Here, we review recent clinical case reports of phage therapy ( $n = 14$ ), including assessments of phage resistance [14,19–31]. We also consider 12 recent pre-clinical studies of phage therapy targeting acute extra-intestinal infections in which the emergence of phage resistance was monitored [32–43] (Fig. 1). We highlight the potential role of the animal host in selecting phage-resistant clones and consider the consequences of phage resistance for treatment outcome. Based on the mechanisms of phage resistance identified, we then discuss the value of *in vitro* tests for determining which mechanisms are at work *in vivo* and the potential use of this information for improving phage therapy.

### Immunophage synergy underlies the success of phage therapy despite phage resistance

The success of phage treatment is dependent on the intrinsic lytic properties of therapeutic phages, the probability of these phages encountering the targeted bacteria and antibacterial action in synergy with the immune system. The importance of this immunophage synergy was demonstrated by the failure of phage treatment for acute pneumonia caused by *Pseudomonas aeruginosa* in immunocompromised mice [10]. This failure resulted from the uncontrolled growth of phage-resistant bacteria that would normally have been eliminated by the immune system [44]. In these experiments, an *in vivo* imaging tool was used to measure and quantify the real-time kinetics of phage therapy treatments [45].

A recent pharmacometrics study assessing the dose and route of administration for curing otherwise lethal murine pneumonia induced by a pathogenic strain of *Escherichia coli* also made use of the same imaging tool to develop a mathematical model that highlighted the crucial role of a refractory bacterial population, including inaccessible bacteria and/or bacteria resistant to phage, in determining treatment outcome in immunocompetent animals [46]. These studies have clearly shown that tripartite interactions between phage, bacteria and the host take place during phage therapy. However, the relative roles of the two antibacterial components, phage and host response, remain unknown.

### The type of phage resistance affects bacterial clearance by the host

During the course of the experimental phage treatment of *P. aeruginosa* pneumonia in mice, Chang et al. reported that the frequency of phage resistance increased with phage dose (30% for a MOI of 4, 74% for a MOI of 250 and 90% for a MOI of 25 000). Surprisingly, bacterial load in the lungs 24 h post treatment was similar in all groups (1.3–1.9  $\log_{10}$ ), suggesting that phage-resistant clones grew as well as the wild-type strain in the lungs [38]. The phenotypes associated with these clones included a greater susceptibility to ciprofloxacin, impaired twitching motility and impaired blue-green pigment production, suggesting the involvement of several different resistance mechanisms. Moreover, cytokine levels

in the lungs 24 h post treatment decreased steadily with increasing phage dose, suggesting that phage-resistant clones are less pro-inflammatory than the wild-type strain. These clones may, therefore, persist for longer in the lungs due to a slower elimination by the immune system than that for the wild-type strain. These findings contrast with those of several previous studies showing that phage-resistant clones have a lower virulence and are less fit to persist at the site of infection [15,32,34,35,47]. Indeed, many receptors used by phages (e.g. LPS, pili, flagella and capsules) are also major virulence factors [48]. However, the diversity of phage resistance traits has also been linked to the conditions of selection [49,50]. A recent study by Gaborieau et al. [51] supported the above interpretation of the study by Chang et al., and added an additional layer of complexity. Indeed, in an experimental phage therapy study, these authors found that an *E. coli* strain became resistant to a single phage through mutations affecting either LPS or the K15 capsule. Mutants with a truncated LPS (alteration of the phage receptor) lost their virulence, whereas other mutants had higher levels of capsule production (masking the receptor) and intact virulence (assessed in mice). Thus, during the course of treatment, phage-resistant clones with opposite fitness costs can be selected. The extent to which the selection of phage-resistant clones temporally coincides with immunophage synergy is therefore key to the overall success or failure of treatment.

#### Host responses to phages during treatment

A single dose of phage is most commonly used in the treatment of experimental infections. By contrast, repeated administrations are frequent in the treatment of humans. For example, the twice-daily intravenous administration of phages over several months has been reported for the management of *Mycobacterium abscessus* infection in a cystic fibrosis patient who had undergone lung transplantation [29]. Interestingly, no emergence of phage resistance was detected during this repeated treatment. In another non-immunocompromised patient receiving the same phage treatment, the authors reported the production of neutralising antibodies after the first month of treatment. This antibody response decreased phage infectivity and ultimately led to phage administration being stopped [52]. Studies of phage treatment for mycobacterial infections in another 20 patients showed that neutralising antibodies were not systematically produced and were not significantly associated with treatment failure [53]. The observation that the production of neutralising antibodies against phages does not necessarily jeopardise treatment outcome is an encouraging finding, because treatment periods are often sufficiently long for such a response to develop. However, it raises the broader question of how the host immune system deals with phage in general, and during treatment, in particular. In the general case, the immune system is confronted

with relatively small numbers of highly diverse phages present in the human-associated microbiota, whereas, during treatment, it is faced with large numbers of one or a few repeatedly administered phages.

Direct interactions between phage and immune cells, in the absence of bacteria, have been studied *in vitro* and *in vivo* and reviewed, but no general conclusions have yet been drawn, probably due to the broad diversity of the structural proteins that constitute the viral particles [54,55]. A recent case report provided a broad overview of the immune response during a phage treatment targeting *P. aeruginosa* (chronic osteoarticular infection) based on transcriptomic analyses of whole-blood samples at various time points [56]. This study revealed that most of the affected genes related to the immune response to bacteria, including those involved in autophagy, whereas a few were involved in intracellular viral trafficking. Based on these findings, the authors suggested that phage drives a globally positive immunomodulatory effect, killing bacteria over time. Together, the findings of recent case reports support deeper investigations of the mechanistic role of the immune response during phage treatment and call for the monitoring of this response in future randomised clinical trials. Within the more limited scope of this review dealing with phage resistance, it should be pointed out that any significant impact of the immune response on the density of either bacteria or phage would potentially affect the rate of emergence of phage resistance by mutation. Indeed, it is anticipated that, during phage treatment in an immunodeficient host, the bacterial population will be controlled principally by the phages. If the phages do not control the infection fast enough, bacterial growth will occur, increasing the chances of selecting phage-resistant mutants with a competitive advantage over the initial phage-susceptible population. Conversely, if antibodies neutralise the phages, the immune response to the bacteria may be saturated, leading to bacterial proliferation that may ultimately increase the frequency at which phage-resistant clones are selected.

#### Phage resistance mutations *in vitro* and *in vivo* converge on the same pathways

Environmental conditions shape the dynamics of interactions between phages and bacteria. The conditions for the selection of phage-resistant clones during phage therapy are almost certainly different from the well-controlled and less restrictive (e.g. no immune reaction, no competition for iron) conditions *in vitro* in the laboratory. Salazar et al. explored the development of an arms race in an *E. coli* phage/bacterium system in a murine model of bacteraemia and on solid medium *in vitro*. Phage-resistant clones emerged in both sets of conditions, and the genome sequences of the 21 resistant clones revealed the presence of a mutation affecting the LPS biosynthesis pathway, with 10% of the resistant clones also having mutations in the

gene encoding the porin OmpA [32]. Similarly, phage-resistant clones of *A. baumannii* strain AB900 challenged with the phage  $\phi$ FG02 in a model of bacteraemia or in co-culture *in vitro* displayed mutations exclusively of the *K* genes encoding the capsule. Interestingly, a strong convergence on a single gene was also observed, as five of the eight resistant clones isolated from eight mice targeted *gtr29*, which was also previously shown to be targeted in *in vitro* conditions [34]. An original study followed the parallel evolution of *P. aeruginosa* subjected to phage treatments in a single patient (intranasal decolonisation over a period of five days) and in rich medium *in vitro* over a period of 12 days [27]. Phage treatments were similar in both conditions, with phages applied daily. Phage resistance followed similar dynamics *in vitro* and *in vivo*, with similar rates of resistance associated with trade-offs against bacterial virulence (assessed in a wax moth larval model) or growth rate. An analysis of the genomes of phage-resistant clones revealed that 62% of mutations *in vitro* were also found *in vivo*, and that 77% of these mutations were *de novo* mutations. These mutations occurred at a high frequency within the same genes *in vitro* and *in vivo* (30% for *flgL*, 45% for *mutS*, 50% for *mreC*, 30% for *mreD* and 40% for *pilB*) [27]. However, the authors did not directly associate the observed mutations with a virulence trait, perhaps because the *in vivo* clones were isolated from colonised patients in which the bacteria were adapted to the immune response. Finally, a study of more than 50 phage-resistant clones isolated from animals 24 h post phage treatment (murine pneumonia) and from *in vitro* liquid medium confirmed the convergence reported above, as the mutations detected affected only the LPS and K15 capsule biosynthetic pathways, regardless of the conditions in which they occurred [51]. The rate of resistant clone emergence was faster *in vitro* than *in vivo*, and it was not possible to distinguish between the origins (*in vitro* vs. *in vivo*) of the resistant clones, or the pathway mutated (LPS vs. K15 capsule) on the basis of fitness costs, which were evaluated by measuring growth rates and resistance to phagocytosis (amoeba model). Interestingly, some, but not all, phage-resistant clones in which the same pathway was affected, were susceptible to other phages that were unable to infect the ancestral bacteria. This observation, also reported by Gordillo Altamirano et al. [34], opens up possibilities for developing phage cocktails based on the information collected from phage-resistant clones. Overall, these studies suggest that the phage resistance mechanisms operating during phage treatment could be identified on the basis of assays *in vitro*, paving the way for systematic analyses of phage resistance before the implementation of phage treatments in clinical practice.

## Perspectives

An increasing number of recent clinical reports of compassionate phage treatment have included the monitoring

of phage resistance. Such monitoring is essential and should be considered systematically in future randomised controlled trials, as it provides information about the coevolutionary dynamics occurring *in vivo*. Based on the vast array of publications characterising phage resistance in laboratory conditions, this naturally occurring phenomenon undoubtedly occurs during phage therapy. However, the emergence of phage resistance does not systematically preclude a positive outcome of treatment. The nature of the mutations has a major impact on the behaviour of phage-resistant clones, and these clones may replace the susceptible population, thereby worsening clinical outcome, or may be rapidly eliminated, to the benefit of the patient. These paradoxical outcomes depend, to some extent, on the ability of the host immune response to support the management of pathogenic bacteria through immunophage synergy.

The recent reports reviewed here have shown that *in vitro*-selected phage-resistant clones display mutational convergence with *in vivo*-selected clones. We can, therefore, anticipate that the characterisation of each putative therapeutic phage will, in the future, include the sequencing of phage-resistant clones selected *in vitro*. Moreover, these resistant clones should also be further exploited to determine their susceptibility to other phages. The incorporation of these data into public databases will help guide the choice of the phages to be assembled into therapeutic cocktails [57]. The mutational convergence between *in vitro* and *in vivo* conditions argues against a direct role of the immune system in the type of selection acting on phage-resistant clones. However, the impact of the immune system on the density of bacterial and phage populations certainly affects the rate of selection of phage-resistant clones. To date, far fewer patients have been treated with phages than with antibiotics. Therefore, other resistance mechanisms, such as CRISPR–Cas systems or any novel phage defence system identified from recent *in silico* studies, may become more relevant *in vivo* in the near future. Some of them being less costly may be selected during the long-term use of phages. Possible extensive use of phages in the agrofood industry may also be a game-changer for the selection of phage-resistant bacteria.

Finally, most clinical treatments are currently provided in combination with antibiotics. When the bacterial targets of antibiotics are involved in phage infection, the phage-resistant clones have been found to display either a higher or lower susceptibility to antibiotics [58,59]. The possibility of using phages to re-sensitise bacteria to antibiotics has recently been discussed [18]. The expected benefits of combining phages with other antibacterial agents should make it possible to decrease bacterial load more rapidly, ultimately accelerating the control of infection with the help of the immune response [56].



## Funding

This work was partly supported by Agence Nationale de la Recherche, France [ANR-19-AMRB-0002], the National Institutes of Health, USA [1R01AI46592-01] and the Amicale des Anciens Internes des Hôpitaux de Paris, France.

## Conflict of interest statement

The authors declare no conflict of interest.

## Data availability

No data were used for the research described in the article.

## Acknowledgements

We thank Devon Conti for discussions and suggestions.

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- of special interest
- of outstanding interest

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