Bacterial Resistance to Phage and Its Impact on Clinical Therapy

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1 Introduction

The faltering of antibiotics is projected to cause an unprecedented public health crisis by 2050, with estimates that more than 10 million deaths will be due to resistant infections each year (O'Neil 2016). Since their discovery in 1928, antibiotics have revolutionized medicine, and the consequences of returning to a pre-antibiotic era are understandably a cause for alarm. The first antibiotic, penicillin, was discovered from a natural encounter between staphylococci and the mold *Penicillium*, with the latter releasing the antibiotic substance that killed the former (Ligon 2004). Initial efforts at purifying penicillin delayed its introduction into human medicine, and by the time of the publication of the first clinical applications in 1943, resistance to the antibiotic had already been documented (Rammelkamp and Maxon 1942; Abraham and Chain 1988). A. Fleming himself warned that misuse of antibiotics would encourage the development of resistant strains during his Nobel Prize speech in 1945. It would have been difficult to fathom at that time the degree to which antibiotic resistance has escalated to in our current-day situation.

The surest option to curtail the gravity of the antibiotic resistance crisis is to develop alternative treatment strategies, with one of the most obvious being phage therapy. The obviousness of phage therapy stems from the fact that it was discovered prior to antibiotics, has been used in clinical medicine in some countries for nearly a century, and has shown promise in in vivo animal studies and compassionate use cases (albeit with poor performance in structured clinical trials to date). While such considerations do not deter the development of other antimicrobial strategies, the

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empirical evidence and historical experience with phage that already exists may contribute to a more rapid clinical implementation in order to respond to current medical needs. Much effort is ongoing to evaluate the therapeutic potential of phage therapy for the treatment of antibiotic-resistant infections, which in part requires evaluating the risk of bacterial resistance to phage and its impact on clinical outcomes.

Phages are natural environmental predators of bacteria, and, as it follows Darwinian theory of evolution, such pressure will result in the selection of resistant bacteria able to survive those conditions. Bacteria are exposed to both chemical (antibiotic) and viral (phage) environmental pressures in nature, and antibiotic resistance has been documented in isolated microbial ecosystems millions of years old (Pawlowski et al. 2016). Unlike static antibiotics, phages have the propensity to coevolve with their bacterial hosts. This coevolutionary mechanism between phage and bacteria is a perpetual arms race, with altering peaks in populations of phage or bacteria. The natural occurrence of this arms race is well documented for seasonal patterns of *Vibrio cholerae* and its phages in Bangladesh, where an environmental bacterial outgrowth is followed shortly after by a flourish of phage until a phage-resistant variant (PRV) appears (Faruque et al. 2005).

Alternating patterns of coevolution have also been repeatedly documented in vitro. Luria and Delbruck noted that phage lysis of bacterial populations was selected for PRVs to allow for bacterial regrowth (Luria and Delbruck 1943). The underlying genetic changes that give rise to coevolutionary events often involve recognition elements (receptor and tail fibers) and have also been documented for *Escherichia coli* and phage T3 (Perry et al. 2015), PP01 (Mizoguchi et al. 2003), or lambda phages (Spanakis and Horne 1987; Meyer et al. 2012). Depending on the nature of mutations in PRVs, phage may or may not have the ability to coevolve, and many studies have supported the occurrence of such evolutionary dead ends where coevolution does not occur (Mizoguchi et al. 2003; Oechslin et al. 2016).

Despite the wealth of studies detailing the development of phage resistance in vitro, it is not possible to extrapolate their conclusions to therapeutic application. Even within these studies, factors such as nutrient availability or growth conditions (shaking or static incubation, carbon sources) have been shown to influence the apparition of resistance (Schade et al. 1967; Bradley 1972; Barrangou et al. 2007; Filippov et al. 2011; Jackson et al. 2017; Koonin et al. 2017). This chapter aims at first presenting the concepts necessary for detecting and understanding phage resistance mechanisms and then at analyzing its documentation in therapeutic literature.

2 Detecting Resistance

The development of resistance during treatment entails that bacteria were sensitive to the phage upon initial exposure, and this initial sensitivity, as detailed later, is an essential condition for successful clinical applications. Several methods can be used to determine phage sensitivity or resistance, which indicate the extent of bacterial lysis and/or phage replication. The most widely used methods are the spot or drop

test, efficiency of plating (EOP), viable counts of phage and/or bacteria, and turbidity reduction as a visual indicator of bacterial lysis by phage, all methods which have been used since the time of phage discovery in the 1920s (Table 1). Alternatively, colorimetric indications of bacterial growth can also be used to determine phage sensitivity and are being revived in recent studies (Tengerdy et al. 1967; Estrella

Table 1 Standard methods used to determine phage sensitivity of bacterial strains

Method	Description	Materials	Advantages	Disadvantages
Drop test (spot test)	Drops of phage are placed onto a bacterial overlay and observed for lysis	Soft agar; agar; Petri dishes	Inexpensive; highly used	Qualitative, read by eye
EOPs	Phage titration on strains compared to a standard; expressed as percent	Soft agar; agar; Petri dishes	Inexpensive; more quantitative than drop tests	Requires more Petri dishes; time-consuming; human error in counting
Viable counts	Bacteria and phage are enumerated using standard techniques	Soft agar; agar; Petri dishes	Inexpensive; quantitative	Must stop infection dynamics for proper counting; time-consuming
Turbidity reduction	Bacterial growth in liquid culture; can be read by eye or plate reader	Spectrophotometer; tubes or 96-wells plates	High throughput	Expensive infrastructure for plate reader; influenced by growth conditions
Metabolic activity	Bacterial growth in liquid culture; can be read by eye or plate reader	Plate reader; tubes or 96-well plates	Easy to interpret; high throughput	Expensive infrastructure for plate reader; influenced by growth conditions
In silico prediction[a]	Genomic sequences of patient isolate and phages are compared for matches	Genomic sequencer; extracted DNA; sequenced phages; computing capacity	Intelligent design; high throughput	Not yet developed; computational burden; repetitive in the case of resistance
Potassium release[a]	Electric signals generated by potassium levels are detected to indicate phage infectivity and lysis	Microchips; software; plate reader	Fast	Not yet developed; not able to detect resistance

[a]These methods are currently under development and have not yet been used for phage therapy applications

et al. 2016). Specifically, the method entails measuring the reduction of tetrazolium salts as an indicator of the metabolic activity of bacteria. Some methods, such as turbidity reduction and colorimetric tests, have been automatized by the use of plate readers, which increase the throughput of screening and render the inference of phage sensitivity more quantitative than visual observation.

These same tests are used both to select phages to be combined into a product or be used for treatment and then to monitor the sensitivity of a bacterial isolate over time during the course of treatment. Ultimately, it is desirable that the method requires little infrastructure, is easily interpretable, and produces consistent results. Each of the different methods mentioned provides certain advantages and constraints in terms of time, material, and expense, which are detailed for each method in Table 1. It is notable that current phage sensitivity methods are conducted in rich growth media, which may, or may not, reflect the real propensity to develop resistance during treatment. Further methods are available to characterize more detailed growth parameters of bacteria-phage interactions, such as one-step growth or burst size estimations, but they are not performed on a routine basis even for phage characterization and certainly not for monitoring resistance in therapy (Kropinski 2018).

The development of a reliable system for phage sensitivity testing is an area of ongoing innovation, where researchers are trying to develop faster and more easily interpreted methods. One area of future diagnostics is the use of in silico predictions of bacteria-phage interactions with genomic data, where the genome of patient isolates is sequenced and screened against an existing phage genomic database to identify matches (Leite et al. 2018). The detection of intracellular components from host bacteria released due to phage lysis, such as potassium, is another method that could be measured, as by changes in electrical current. Methods that are currently used usually follow the course of phage infection for 16–24 h or more in order to determine phage sensitivity or resistance: many strains that show initial sensitivity to the phage may develop resistance after hours of incubation. While this lag time could delay treatment for urgent infections, it is difficult to develop a method that would balance the need for speed with the physiological processes of bacteria and phage resistance.

3 Mechanisms of Bacterial Phage Resistance: Mutations, Evolution, and Costs

Current knowledge on the mechanisms of phage resistance comes primarily from laboratory studies. Bacteria can resist phages mainly through the following mechanisms: (1) spontaneous mutations or phase variation of surface receptors, therefore preventing adsorption of phages to their bacterial host, (2) specific cleavage of incoming phage DNA by bacterial restriction-modification systems (RMS), or (3) by bacterial adaptive immunity such as the CRISPR-Cas system (clustered regularly interspaced short palindromic repeats loci, coupled to CRISPR-associated genes) (Labrie et al. 2010). The potential impact that these mechanisms may have on

therapy depends on the time to development of resistance and its specificity to certain phages, the toll resistance takes on bacterial fitness, and the ability of the phage to counteract resistance.

3.1 Mutations

Spontaneous mutations of surface molecules can prevent phages from adsorbing and subsequently injecting their DNA into their host bacterium. It is also the most frequent mechanism driving both resistance to phage and phage-bacteria coevolution (Koskella and Brockhurst 2014). Indeed, the first step of viral infection is adsorption of the viral particle via a lock-key mechanism between the receptors present on the phage tail fibers that interact with receptors present on the bacterial surface. Completion of the phage life cycle results in the killing and lysis of the host bacterium, resulting in a selection pressure that evolves both phage to increased infectivity and bacteria to phage resistance (Buckling and Rainey 2002). Spontaneous mutation of bacterial surface components that act as phage receptors, such as lipopolysaccharides (LPS), outer membrane proteins, cell-wall teichoic acids (WTA), capsules, and other bacterial appendices, such as flagella, many of which may also be virulence factors (e.g., LPS), can result in phage resistance (Bertozzi et al. 2016). The ultimate effects of these mutations are dependent upon the function of the structure to the host and the extent of the modification.

Phage resistance acquired through mutation of surface LPS has been observed in multiple bacterial species. In *E. coli*, PRVs were observed to have altered LPS composition after phage PP01 infection with reduced production of high- and low-mass LPS (Filippov et al. 2011). Resistance conferred by LPS modification was also observed for *Pseudomonas aeruginosa* (Le et al. 2014; Oechslin et al. 2016). The loss of the O-antigen that is required for phage adsorption was observed to be due to large chromosomal deletions encompassing the *galU* gene, which is involved in LPS synthesis. In addition, Filippov et al. used site-directed mutagenesis to demonstrate that *Yersinia pestis* can resist phage through alteration of different parts of the LPS (Filippov et al. 2011). LPS surface alteration was also observed to be phase variable in *V. cholerae* and used by the bacteria to escape O1 antigen-specific phages in nature (Seed et al. 2012). In this case, resistance was the result of single-nucleotide deletions in two genes critical for O1 antigenic variation.

Outer membrane proteins have also been observed to be involved in bacterial resistance toward phage, for example, in the case of the *E. coli* OmpC protein. It was observed that during the interaction between *E. coli* O157:H7 and bacteriophage PP01, OmpC silencing, in addition to LPS alteration, enabled the bacteria to escape phage infection (Mizoguchi et al. 2003). Similar observations were reported for *V. cholerae*-resistant variants that had decreased expression of the membrane protein OmpU (Seed et al. 2014).

During gram-positive bacterial infection, phages often use teichoic acids in order to adsorb on the bacterial surface to initiate infection. This is, for example, the case for phages that use the glucosylated teichoic acids of *Bacillus subtilis* or the *N*-

acetylglucosamine (GlcNAc) side chains of *Staphylococcus aureus* (Tipper et al. 1965; Young 1967; Yasbin et al. 1976). Spontaneous mutants of *Bacillus anthracis* to phage AP50c repeatedly had altered forms of the cell-anchoring protein, CsaB, which, while not the receptor protein itself, is thought to be responsible for linking receptor proteins to the cell surface (Bishop-Lilly et al. 2012). Phages of *S. aureus* largely target structures within the WTA on the host surface, and both the inactivation of the TagO protein that is responsible for WTA biosynthesis and altered glycosylation patterns have resulted in phage resistance in vitro (Xia et al. 2011; Chang et al. 2015; Uchiyama et al. 2017). A role of cell-wall components for phage adsorption has also been determined for *Enterococcus faecalis* and phage NPV1, where phage resistance is conferred by mutations in the *epa* gene cluster that is responsible for rhamnose cell-wall polysaccharides (Ho et al. 2018). Much of the information on resistance to these phages comes from mutational studies to identify host receptors rather than arising through infection dynamics, which is likely due to the importance of WTA structures to host viability, although in vitro resistance has been detected (Bishop-Lilly et al. 2012; Estrella et al. 2016; Jo et al. 2016).

Capsular polysaccharides can be involved in both receptor function and adsorption prevention. The capsular layer can act as an important mechanism of defense against phages, such as for the K1-capsule in *E. coli* that physically blocks recognition of LPS by phage T7 (Scholl et al. 2005). However, the capsular layer can conversely also act as a phage receptor, like in the case of phage K1–K5 that can recognize K1 and K5 antigens (Scholl et al. 2001). In addition, capsules are important virulence factors that help pathogenic bacteria to evade or counteract host defense during the infection (Jann and Jann 1992).

Other structures, like bacterial flagella or pili, can act as phage receptors. The flagellum can act as a primary receptor that helps the phage to adsorb to its secondary receptor located on the surface of the bacteria. Adsorption to the flagellum is generally reversible and helps the phage to move alongside its base where a second adsorption event takes place (Schade et al. 1967; Guerrero-Ferreira et al. 2011). In a similar way, the pilus can also be used as primary receptors, and its contraction is believed to bring the phages closer to the bacterial envelope (Bradley 1972).

In addition to spontaneous mutations of bacterial surface proteins, other bacterial resistance mechanisms are known to target virtually all infection steps of the phage life cycle. Innate (non-specific) resistance mechanisms, such as abortive infection or restriction-modification systems, are examples of such downstream mechanisms (Labrie et al. 2010). However, none of these systems have the ability to react to or evolve resistance to phage during the course of an infection and thus are less important in the context of resistance acquisition during phage therapy. Their implication could be confined to impact the host ranges of the phage rather the time course of resistance development, therefore making phage sensitivity testing an important prerequisite for phage therapy.

Adaptive systems such as the CRISPR-Cas system could, however, have a larger impact in the development of resistance during therapy. This system was reported in 45% of bacterial genomes and can cleave incoming phage DNA to provide adaptive resistance during infection (Barrangou et al. 2007; Koonin et al. 2017). Foreign

nucleic sequences, known as spacers, will first be integrated into the CRISPR locus and then will further serve as a guide for Cas-nuclease cleavage of subsequent foreign DNA that matches the spacer sequence. Spacers that are incorporated in the host genome define the specificity of the immune response of the host and its progeny (Jackson et al. 2017). It should be emphasized that a large number of anti-infective mechanisms are still to be discovered, as recently reported by Doron et al. (2018).

Deletion of bacterial surface receptors usually results in total resistance toward the phage or at least partial resistance in the case of primary receptor mutation and/or production of extracellular matrix or capsules that results in interference with phage adsorption (Labrie et al. 2010). For these reasons, the loss of the phage receptor can lead to an evolutionary dead end if the phage does not have the possibility to develop a counter resistance during the process of infection (reviewed in Dennehy (2012)). However, experiments using continuous chemostat culture have demonstrated the possible coexistence of phages with their respective PRVs, like for the case of *E. coli* O157:H7 and phage PP01 (Mizoguchi et al. 2003). In this specific case, phage resistance was observed to be associated with two resistant variant populations having an alteration in their LPS structure or decreased expression of their OmpC surface protein in addition to mucoid-type colonies. In parallel, phages were observed to evolve different host ranges for bacterial PRVs, which suggest multiple coevolutionary cycles, permitting parallel phage and bacterial expansion and mutual counterselections.

Antagonistic coevolution was also observed during many bacterial generations using *Pseudomonas fluorescens* and phage SBW25φ2. In this case, the bacterial host became resistant to a wider range of phage genotypes as phages infected a wider range of host genotypes, producing reciprocal increases in host resistance and phage infectivity (Buckling and Rainey 2002; Hall et al. 2011a, b). In addition, such an arms race was also observed to become weaker with subsequent generations due to the fitness costs associated with generalist adaptive mutations (Hall et al. 2011a, b). Indeed, a side effect of phage resistance is the fitness cost that may be associated with the specific mutation conferring resistance. For example, in *E. coli* and lambda phage, infection that will select for bacteria with reduced LamB porin expression also alters maltose uptake and can be detrimental in a maltose poor media (Spanakis and Horne 1987).

Resistance derived from de novo mutations that result in modification of the target phage surface receptors and prevents its adsorption also usually results in phenotypic effects. For example, PRVs resulting in loss or modification of their LPS structure can lead to so-called rough phenotypes (Kim et al. 2014); resistance through defective pili results in bacteria with altered twitching motility phenotype (Oechslin et al. 2016); production of alginate can result in PRVs having a mucoid aspect (Scanlan and Buckling 2012); production of capsular polysaccharides promotes aggregation at the bottom of the culture tubes (Capparelli et al. 2010); and phage resistance in *P. aeruginosa* is often associated with melanized phenotypes (Le et al. 2014; Oechslin et al. 2016).

However, the phage receptors that are present on the bacterial surface often act as virulence factors. For this reason, strains with receptor modification will be resistant to phage, but may also exhibit reduced virulence as discussed in the following chapter (León and Bastías 2015; Oechslin 2018).

4 Resistance Development During Therapeutic Application

4.1 Animal Studies

The need for alternatives to antibiotics extends beyond human medicine to animals and livestock, which have been recently targeted for their overuse of antibiotics and thus contribution to the resistance crisis (Martin et al. 2015). Most of the work done in phage therapy for animals deals primarily with gastrointestinal infections, as well as the control of pathogens rather than therapeutic treatment. Field studies or preclinical experiments in animals have the morbid advantage of being able to sacrifice study animals and deeply explore bacteria-phage interactions within the body at specific anatomical sites, which are obviously not fathomable for clinical applications. These studies therefore offer more investigation into the development of phage resistance than what can be learned through therapeutic application. The field and in vivo studies discussed here do not fully cover the vast literature on animal models conducted in controlled environments, but the several studies included here have yielded pertinent information on the development of phage resistance in vivo (also reviewed in Oechslin (2018)).

4.1.1 Livestock Gut Decolonization

Many well-documented studies on phage therapy and the emergence of resistance started with the control of gut-colonizing pathogenic bacteria in livestock animals including cattle, pigs, and poultry. This was the case with a series of studies done by Smith and Huggins on oral phage administration to prevent *E. coli*-induced diarrhea in colostrum-fed calves (Smith and Huggins 1983). Phage therapy could prevent diarrhea when given 8 h after bacterial inoculation, although it was able to resolve intestinal symptoms in only half of the animals when administered at the onset of the diarrheal symptoms. Interestingly, resistant bacteria were recovered from the small intestine in the case of calves failing to show a clinical response to phage application; yet the resistant strains did not cause diarrhea when reinoculated to healthy colostrum-fed calves. The decreased virulence was explained by the loss of the K-antigen, which is a known virulence factor for enteropathogenic strains and can act at the same time as a phage receptor (Taylor and Roberts 2005). Of note, similar results for the treatment and prevention of *E. coli* diarrhea in calves were described in a second study by Smith et al. (1987). However, K-positive resistant variants were also isolated in addition to K-negative variants and were observed to be as virulent as the parent strain.

Phage therapy has also been used to control *Salmonella* spp. and *Campylobacter* spp. gut colonization and infection in poultry (Sklar and Joerger 2001). It was able to

only reduce, but not eliminate, bacterial colonization in the case of *Salmonella enterica*. Treatment failure was not only attributed to phage resistance observed posttreatment, but also possibly linked to other factors, including the intracellular lifestyle of the bacteria. Similar observations were made by Atterbury et al., who observed a benefit by increasing the phage titer of the preparations without, however, achieving bacterial eradication (Atterbury et al. 2007). Interestingly, a positive correlation was observed between phage concentration and the emergence of phage resistance, with higher resistance rates following application of higher phage titers. In addition, PRVs were still able to colonize the gut. Similar observations were also reported by Carvalho et al., including a quick reversion to the original sensitive phenotype after resistance appeared, which could possibly explain why resistant variants can still colonize the gut (Carvalho et al. 2010). Similar conclusions were also reported for the treatment of *Campylobacter jejuni*, for which the observed decrease in bacterial load was dependent on the amount and time of phage administration (Loc Carrillo et al. 2005). As for *S. enterica*, resistance reversion was observed due to phase variation by inversion of a large genomic sequence that restored gut colonization capability (Scott et al. 2007). Phase variation was also observed to be associated with capsular polysaccharide production during *C. jejuni* and phage F336 application, although resistance was not associated with decreased gut colonization capabilities (Sørensen et al. 2012).

Taken together, these studies raise important questions about the selection of phage resistance in the intestine and its possible implication for phage therapy. Indeed, the complexity of the intestinal environment and its physiochemical conditions, including viscosity, the concentration dependency of phage resistance development, and phenotypic reversion, must be considered for future phage therapy applications.

4.1.2 Experimental Therapy in the Intestine

The efficacy of phage therapy and the emergence of resistance have also been investigated in different mouse models of intestinal colonization, which have produced interesting findings in terms of phage-bacterium dynamics in the gut. In a first study employing a 21-day oral administration of a cocktail composed of three different bacteriophages to mice colonized with enteroaggregative *E. coli*, the bacterial titer was not observed to decrease even though phage amplification was observed over the course of the experiment (Maura et al. 2012). Interestingly, bacteria recovered on day 21 were still susceptible to the phages present in the cocktail. Another study using phage T4 oral administration during a long-term period of 240 days reported that phage-resistant bacteria emerged after only 92 days and constituted 100% of the isolated colonies. In addition, PRVs were observed to persist over the 240 days of the experiment even when phage therapy was stopped after 92 days. In a very interesting study done by Duerkop et al. using germ-free mice colonized with *E. faecalis* V583, phage therapy was observed to decrease the fecal bacterial load after 24 h by threefold, and the level of colonization remained stable after 48 h (Duerkop et al. 2016). Phage resistance was observed to increase during the time of phage therapy: while 15% of the colonies were

susceptible after 24 h, 100% were resistant after 48 h. After sequencing the PRVs, resistance was revealed to be associated with multiple mutations in the integral membrane protein PIPef that promotes phage infection. In an attempt to prevent intestinal colonization or cholera-like diarrhea in infant mice and rabbit models, Yen et al. used a phage cocktail composed of three different phages (Yen et al. 2017). Oral administration up to 24 h before cholera infection reduced intestinal colonization and prevented cholera-like diarrhea even though PRVs could be observed. Resistance was associated with mutations in the O-antigen gene and outer membrane protein OmpU, although none of the isolates were resistant to all three phages.

4.1.3 Acute Infections

Besides models dealing with the gastrointestinal tract, several studies have also evaluated phage therapy in several models of acute infection. In an early study on phage therapy done by Smith and Huggins using a mouse model of meningitis, mortality was significantly lower when administering phage treatment 16 h after infection rather than antibiotics (Smith and Huggins 1982). Importantly, although no colonies isolated from mice brain were observed to be antibiotic resistant, PRVs were observed in 5 out of the 36 mice tested. Phage variants were K1-antigen negative, which suggests decreased infectivity, as described before (Smith and Huggins 1983).

In a study done by Pouillot et al., a model of murine neonatal sepsis was used to evaluate phage subcutaneous injection after rat pups were intraperitoneally infected with the virulent *E. coli* O25b:H4-ST131 strain (Pouillot et al. 2012). Interestingly, phage resistance was observed when the treatment was delayed 24 h post-infection, although their virulence was reduced in a sepsis model. In a model mouse liver abscess, Hung et al. did not observe the emergence of phage resistance after single-dose administration that could efficiently protect mice in a dose-dependent manner (Hung et al. 2011). Of note, PRVs could be selected in vitro during time-kill curve experiments, but their virulence was significantly attenuated in vivo.

Similar observations were done by Oechslin et al., where the efficacy of an antipseudomonal phage cocktail was evaluated in a model of rat endocarditis (Oechslin et al. 2016). Indeed, bacterial regrowth due to phage resistance could be observed after 24 h in vitro due to the selection of PRVs having acquired mutations either in the *galU* gene coding for LPS synthesis or in the PilT ATPase involved in pilus retraction. Interestingly, both resistant variants were less able to infect sterile rat valves, indicating that phage resistance comes at a high fitness cost. PRVs were not observed in vivo either before or after phage therapy treatment, which decreased the bacterial load by 2.3–3 log colony-forming units (CFU) depending on the mode of phage administration. Finally, the emergence of PRVs in vitro with reduced virulence that were not observed in vivo during phage treatment using two different phages (PPpW-3 and PPpW-4) was also confirmed with ayu fish orally infected by *Pseudomonas plecoglossicida* (Park et al. 2000). PRVs selected in vitro were less virulent when injected intramuscularly in the fish. Moreover, bacteria could be eliminated in fish receiving phage therapy, and the isolates recovered from control fishes were still susceptible to the two phages used in the experimental treatment.

4.2 Clinical Therapy

The first administration of bacteriophage for the treatment of a bacterial infection in humans dates back to 1917 (d'Hérelle 1917, 1931). The use of phage became more widespread at this time, prior to the introduction of antibiotics, after which point it was further developed by the Soviets, where it is practiced to this current day in countries of the former Soviet Union (Parfitt 2005; Summers 2012; Kutateladze 2015). The documentation of early phage therapy investigations is intermittent, with difficulties in original source and language availability. However, the ability of bacteria to develop resistance to phage and the importance of phage sensitivity to treatment outcomes have been documented since the 1930s (Eaton and Bayne-Jones 1934). Despite the wealth of historical literature, this chapter focuses on recent experiences with clinical phage therapy since 2000.

Phage therapy has experienced a revival of sorts due the increasing resistance to antibiotics, with a surge in activity over the past several years. However, no phage products have yet received a marketing authorization in Western countries to permit their use in clinical medicine, and only three formal clinical trials have been completed, although several phase ll studies have been announced for planned start dates in 2019. This limits the current use of phage therapy beyond countries where it has been historically approved, therefore causing a scarcity in available data on phage or the development of phage resistance in human medicine. However, phage therapy is increasingly being used as compassionate means to experimentally treat patients with antibiotic-resistant infections, particularly in Poland, Belgium, France, Australia, and the United States (Leszczynski et al. 2006; Letkiewicz et al. 2010; Khawaldeh et al. 2011; Jennes et al. 2017; Schooley et al. 2017; Lyon et al. 2018). These reports do little to contribute to a greater understanding of efficacy, but occasionally provide more details on each treatment than clinical trials, such as the need for phage modification due the apparition of PRVs or reverting antibiotic sensitivity.

4.2.1 Resistance Detected in Modern Phase ll Clinical Trials

Three modern clinical trials have been completed for phage products since 2009, covering burn wound and chronic otitis infections of *P. aeruginosa* and *E. coli* diarrhea (Table 2; Wright et al. 2009; Sarker et al. 2016; Jault et al. 2019). In formal trials, the product composition, application and dosage regimens, and analyses, such as phage sensitivity testing, are predetermined as part of the clinical trial protocol prior to patient enrollment. Two of the three studies did not include phage sensitivity testing as an enrollment criterion, therefore making it difficult to ascertain if phage resistance of patient isolates, when detected, was present prior to or developed as a result of phage administration. However, microbiological analysis of bacterial isolates after phage administration revealed clinical insensitivity to phage in some cases, which both supports the importance of sensitivity testing a priori and hints at some limitations of employing fixed-composition phage products designed to maximize host range.

Table 2 Summary of published clinical reports of phage therapy with relevant investigations into the development of phage resistance

Study	Pathogen	Phage product	Sensitivity testing		PRV analysis	References
			Pre-	Post-		
RCT, phase ll	*P. aeruginosa*	Fixed 6 phages	Yes	No	No	Wright et al. (2009)
RCT, phase ll	*E. coli*	Fixed 10 phages	No	Yes	No	Sarker et al. (2016)
RCT, phase ll	*P. aeruginosa*	Fixed 12 phages	No	Yes	No	Jault et al. (2019)
Summary reports	*S. aureus*, *E. coli*, *E. faecalis*, *P. aeruginosa*	Personalized	Yes	Yes, partial	na	Międzybrodzki et al. (2012), Górski et al. (2016)
Pilot study	*E. coli*, *Enterococcus*, *Streptococcus*, *P. aeruginosa*, *Staphylococcus*, *Proteus*	Commercial Pyobacteriophage	Yes	No	No	Ujmajuridze et al. (2018)
Case report	*P. aeruginosa*	Personalized 6 phages	Yes	Yes	na	Khawaldeh et al. (2011)
Case report	*P. aeruginosa*	Personalized 2 phages	Yes	Possibly	No	Duplessis et al. (2018)
Case report	*S. aureus*	Commercial staphylococcal monophage, Fersis, Pyobacteriophage	Yes	Yes	No	Zhvania et al. (2017)
Case report	*A. baumannii*	Personalized 4, 4, and 2 phages	Yes	Yes	No	Schooley et al. (2017)

PhagoBurn

The PhagoBurn trial was the first, multicentric European clinical trial for phage therapy (Jault et al. 2019). The trial investigated the efficacy of topical application of a 12-phage cocktail to reduce *P. aeruginosa* in burn wounds compared to a standard-of-care (SOC) antimicrobial cream of silver sulfadiazine. The study suffered production setbacks, could not reach enrolment populations, and did not report evidence of efficacy, among others. Authors did note that the lack of susceptibility testing prior to phage treatment decreased the number of patients who achieved the primary endpoint of the trial (Jault et al. 2019). The reason for not including phage sensitivity as an enrollment criterion is likely due to the fact that the cocktail was expected to have a broad epidemiological coverage of *P. aeruginosa* strains. Authors also noted that pre-sensitivity testing would have complicated the clinical protocol. However, in terms of phage resistance, 50% of 73 bacterial colonies from 10 patients in the phage treatment group were fully or intermediately resistant to the test product. Interestingly, four of the ten patients harbored colonies with different phage susceptibility profiles. As these colonies were isolated at day 0, it is likely that the patient isolates were phage resistant prior to phage administration.

Acute Pediatric *E. coli* Diarrhea

The other trial that did not include phage pre-sensitivity testing investigated the utility of phage for the treatment of pediatric *E. coli* diarrhea in Bangladesh (Sarker et al. 2016). This trial administered either a commercial phage cocktail targeted against *E. coli* and *Proteus* spp., an in-house T4-phage cocktail, or placebo to children with microbiologically diagnosed, acute *E. coli* diarrhea. The test product was applied orally, three times daily for 4 days without gastric neutralization. The trial was terminated early, as no indication of efficacy of phage application was observed on diarrhea parameters, such as disease severity or resolution, at an interim review. When *E. coli* colonies were isolated from patient stool after phage administration, only 50% were sensitive to phage, although harboring sensitive isolates did not correlate with higher stool titers. Both this study and PhagoBurn did not report data to support a therapeutic effect of phage therapy (albeit with plausible explanations), and both cited issues with phage-resistant bacteria, thus highlighting the importance of phage sensitivity testing for trial inclusion. The fact that isolates varied in phage sensitivity within some patients also indicates that multiple colonies should be included for this testing.

Chronic *P. aeruginosa* Otitis

The only formally structured completed trial that did include phage sensitivity testing as an inclusion criteria dates back from 2009, where a phage product, Biophage-PA, consisting of *P. aeruginosa* phages, was tested in a small phase I/II for its efficacy to treat chronic otitis (Wright et al. 2009). A total of six phages were tested individually against the patient isolate to ensure sensitivity, and then 1×10^5 plaque-forming units (PFU) per phage was administered together as a phage cocktail in a single dose to the ear. Patient samples were analyzed microbiologically 7, 21, and 42 days after phage application. A significant difference was observed between

the 12 patients receiving the phage product and the 12 receiving placebo in terms of clinical improvement scores and average bacterial counts.

The use of mean counts across patients may mask individual clinical responses. Indeed, close inspection of the individual patient bacterial counts over time in phage recipients revealed that two patients displayed an increase in bacterial load from day 0 to day 7 and the number of bacteria detected increased for six patients between days 7 and 21, despite initial sensitivity to phage. Unfortunately, no phage sensitivity testing was performed on the bacterial isolates at these different time points to test for the apparition of PRVs. Authors also noted an average 200× amplification of the test product phage components, although this was not reported per patient. Endogenous phage was detected in five of both placebo and phage recipients.

Collectively, there is little data to analyze about the development of phage resistance from recent clinical trials of phage therapy. This is largely due to the lack of pre-sensitivity testing to phages, as well as a lack of detailed microbiological analysis throughout the course of treatment. As mentioned previously, phage sensitivity is an essential requirement for phage therapy to even have a chance at providing a therapeutic effect and, therefore, should be required for all future clinical investigations. Even if phage sensitivity is included as an enrolment criterion, continual testing is required throughout treatment to investigate for the development of PRVs. If phage therapy is ever to be fully understood, thorough microbiological analysis of PRV strains should be done to shed light on how frequently resistance develops, what mechanisms are responsible for it, and how these changes might influence the pathogenicity or virulence of infecting bacterial strains.

4.2.2 Phage Resistance in Pilot Studies and Case Reports

Much more numerous than clinical trials are case reports, pilot studies, or summary reports of phage use (Table 2). Phage sensitivity testing preceded clinical application in these instances, usually for the formulation of personalized preparations as few preformulated products are currently available. However, reporting on compassionate use and smaller studies is often inconsistent or incomplete, making a comparative analysis difficult. Several reported cases that help to illustrate examples of phage resistance in human therapy are detailed below in chronological order of publication.

P. aeruginosa Urinary Tract Infection (UTI)

A six-phage personalized bacteriophage cocktail was used for the treatment of one patient in Australia in 2011 with a refractory UTI caused by *P. aeruginosa* (Khawaldeh et al. 2011). The phages were selected and prepared by the Eliava Institute at a titer of 1×10^6 PFU/mL. The preparation was administered directly into the bladder in doses of 20 mL every 12 h for a total of 10 days. Urine samples were collected frequently and elaborated for the detection of viable bacterial and phage counts, as well for bacterial DNA. The sensitivity of the patient isolate to the phage cocktail was confirmed three times, at days 1, 3, and 7, and concomitant antibiotic therapy (colistin and meropenem) was applied from the 6th day after the onset of phage therapy. No resistance to phage was detected, and bacterial titers continued to decrease over the 1st week of treatment until day 8 when no viable titers of

P. aeruginosa were cultivable. Phage titers increased after administration until they were no longer detectable, which occurred shortly after the sterilization of *P. aeruginosa* from the urine. Additionally, the authors investigated the clonality of the infection with DNA fingerprinting to show that all isolates were identical in their banding pattern, as well as in their sensitivity to the phage cocktail and antibiotics over time. Lastly, the presence of a secondary pathogen, *E. faecalis*, was monitored by PCR: its concentration did not vary with phage administration, but finally decreased after *P. aeruginosa* was eradicated and meropenem exposure was prolonged.

This is one of the few studies, despite being one of the earliest reported, which duly documented many aspects critical for understanding phage therapy and the development of resistance, with both bacterial and phage titers and clear testing for continued sensitivity over the course of treatment. Therefore, although it does not provide resistance data due to its absence, it represents a well-documented instance of the compassionate use of phage therapy that helps to clearly indicate that resistance did not occur. The types of information reported within this case report would be useful for all future cases of clinical applications.

S. aureus Skin Infection

A 16-year-old patient from France with Netherton syndrome, a complex skin condition, was treated with phage therapy at the Eliava Phage Therapy Center in 2016 (Zhvania et al. 2017). The skin condition caused the patient to suffer from chronic skin infections from antibiotic-resistant *S. aureus*, as well as allergies to most standard dermatologic products and antibiotics. Both the *Staphylococcus* bacteriophage and Pyobacteriophage commercial products from Eliava were well tolarated and applied locally via soaked bandages and impregnated creams or orally (10 mL each daily) after stomach acid alkinization. The treatment regimen was long and divided into two phases: first two, 20-day treatments separated by an interim 2-week break and second with alternating 2-week periods of phage administration with rest over 3 months. The authors tested the sensitivity profile of the infecting strain and indicated that while no resistance was detected after 1 month, a change in sensitivity at 3 months led them to exchange the Pyobacteriophage product for another commercial preparation, Fersis. The difference in activity of these two products was surprising, considering that a recent metagenomic comparison of these therapeutic preparations revealed that they contain highly similar phages against *S. aureus* (McCallin et al. 2018). Closely related members of these *S. aureus* phages, the *Spounavirinae*, are also the sole component of a clinical-grade product being developed by AmpliPhi Biosciences, AB-SA01 (Lehman et al. 2019). This preparation, which contains three phages sharing between 94 and 97% genetic identity, was also selected for their difference in host range and therefore indicates that these small genetic differences can lead to different clinical efficacies, as shown by this case report (Zhvania et al. 2017; Lehman et al. 2019). Ultimately, the overall bacterial load in different sites of the body was decreased by phage treatment, and the patient's severity of symptoms was greatly reduced, leading to an

improvement in quality of life. The authors note that chronic cases may benefit from periodic treatments with phage therapy to manage the underlying condition.

P. aeruginosa Bacteremia

Duplessis et al. reported the intravenous use of a two-phage cocktail against multidrug-resistant (MDR) *Pseudomonas* bacteremia for the treatment of a hospitalized 2-year-old patient (Duplessis et al. 2018). Phage sensitivity was performed prior to treatment, and the phages were selected from 25 active phages due to their lytic activity and targeting of different bacterial receptors. In a first course, phages were administered every 6 h at a dose of 3.5×10^5 PFU for 36 h for a total of 6 doses, several days after which blood cultures became and remained sterile for *P. aeruginosa* for 2 days. The presence of *Pseudomonas* reappeared after phage administration ceased, and phage therapy was recommenced, which again caused blood cultures to revert to negative. This continued for 5 days until confounding health problems led to a worsening condition with bacteria again being detected, and care was withdrawn, after which the patient died. It is not clear from the reported information if the bacterial outgrowth observed at the stage of clinical worsening appeared before or after the cessation of phage therapy, although authors noted that bacterial isolates from this time point were resistant to additional interventions (not specified).

A risk of performing compassionate treatment is an increased risk of treatment failure due to confounding medical conditions, which included DiGeorge syndrome, severe heart failure conditions, and the development of the flu for this particular patient. Despite the strain being resistant to antibiotics, concomitant therapy was continued throughout the course of treatment (meropenem, tobramycin, and polymyxin B) in order to maximize the possibility that the two antimicrobial strategies would have an additive effect for treatment. Authors reported on the time to positivity (TTP) as the measure for bacteria detected, which is commonly used for the diagnosis of bacteremia (Ning et al. 2016; Tang et al. 2017). Interestingly, the blood culture taken at the time when phage therapy was discontinued for the second time had a TTP nearly double of cultures isolated on 14 other days, which may indicate residual phage activity or the apparition of slow-growing PVRs, although it is not possible to verify such conclusions without microbiological analysis of the bacterial isolates.

Case Reports From IPATH

Several case studies have been communicated from the University of California, San Diego School of Medicine, which has led them to open an experimental therapy center, the Center for Innovative Phage Applications and Therapeutics (IPATH), as a result of their experiences with these compassionate cases (Schooley et al. 2017; Aslam et al. 2018; Furr et al. 2018; Wooten et al. 2018). In three instances, the reports of treatment performed there have mentioned the apparition of PRVs, although the full documentation of two cases is only currently available in short format. During the treatment of a lung infection in a cystic fibrosis patient, it was reported that a change in microbiological susceptibility to phage was noted for some

isolates of *P. aeruginosa*; however, no more details were available from this short-record format (Furr et al. 2018). Another record of compassionate use was for the treatment of a lung transplant recipient with a MDR *P. aeruginosa* infection (Aslam et al. 2018). Treatment was ultimately successful, but the cocktail composition was changed several times due to the apparition of PRVs.

A published case report from the same authors on the use of phage therapy for the treatment of a MDR *A. baumannii* abdominal infection provided quite possibly the most detailed analysis of aspects related to phage resistance to date (Schooley et al. 2017). Four phages were initially selected for treatment based on a large screening of phage collections for activity against the patient isolate and their previously determined host range spectrum. Resistance was detected at already 8 days after the initiation of treatment, and the phage composition was changed accordingly. In total, the patient was administered three different phage cocktails at different times and administration routes, which consisted of four, four, and two phages, respectively (one phage was retained from the second composition to the third). The first cocktail was applied intracavitary, while the subsequent preparations were applied intravenously. The changes to the phages used were due to the detection of phage resistance in vitro by monitoring bacterial growth of bacteria isolated at different time points in liquid culture; the therapeutic administration of the first cocktail shown to be inactive in vitro was continued. It was not reported if multiple isolates were tested or if pathogen clonality was investigated, therefore making it difficult to ascertain if resistance to the phage cocktails occurred in the parent strain background or if it merely selected for different isolates in a mixed infection.

The complexity of this case highlights the highly empirical nature of compassionate phage therapy. Publications of case reports have provided more information on phage resistance than formal clinical trials, yet multiple phage modifications, concomitant antibiotics, and underlying medical conditions make it difficult to compare cases or provide advice beyond the necessity to test for PRVs and modify phage compositions accordingly. Additionally, none of the abovementioned studies that did detect phage resistance during clinical treatment have gone as far as to follow-up with molecular characterization of the isolated PRV strains. Such analyses would provide information on lingering questions, such as if resistance develops via certain mechanisms or affects certain targets that would influence bacterial fitness. PRVs detected in vitro have been shown to have reduced virulence in vivo in several animal studies (Oechslin 2018). For this reason, the detection of PRVs by drop tests or liquid assays in rich media might not reflect their true clinical viability. While the purpose of compassionate use is to maximize therapeutic benefit for the patient and interventions should be empirically designed to do so, the opportunity to analyze clinical isolates and their PRVs should be exploited to also maximize future therapeutic benefits.

Pilot Study for UTI Treatment

A recently pilot study using phage for the treatment of UTIs caused by different bacterial pathogens was conducted in the prospect of designing a future formal clinical trial in Tbilisi, Georgia (Ujmajuridze et al. 2018). It is the only study to date which incorporates the adaption of phages to a set of clinical strains in order to

increase pathogen coverage prior to treatment. A commercial phage preparation, Pyobacteriophage (Eliava BioPreparations Ltd., Tbilisi, Georgia), underwent adaptation to clinical strains of *S. aureus*, *E. coli*, *P. aeruginosa*, *Streptococcus*, and *P. mirabilis* isolated from 130 patients undergoing transurethral resection of the prostate (TURP). The adaptation process increased the overall coverage of these isolates from 41 to 75%. The adapted preparation was then used to treat nine patients with sensitive bacterial isolates, and the primary outcome, pre- and post-bacterial viable counts, was recorded for eight. It is not clear if these patients' strains were included during the adaptation process or if they were nine new patients after the adaptation was complete.

The adapted Pyobacteriophage contained phages against the different pathogens in concentrations ranging from 10^7 to 10^9 PFU/mL, and treatment consisted of 20 mL applied directly to the bladder via a suprapubic catheter for 7 days, two times per day for 30–60 min. The results of phage application on pathogen load varied between the eight patients for which data was recorded: cultures became sterile for at least three patients, a decrease in original pathogen concentration was observed in four cases (although one patient developed an infection with a secondary pathogen), one patient's isolate showed no effect from PT, and no data was recorded posttreatment for one patient. Unfortunately, no data was reported on the phage sensitivity of the bacteria enumerated posttreatment nor for phage titers to indicate phage amplification, therefore making it again difficult to determine the development of phage resistance or, if it did, to understand how and what effect it may have had on treatment. The different results obtained between patients raise questions for phage therapy and the development of resistance. Considering that phage sensitivity was an inclusion criterion for the treatment population, the patient whose *E. coli* pathogen load remained the same throughout treatment is interesting in terms of resistance. Another case where the primary pathogen disappeared, but *E. coli* appeared is surprising because *E. coli* was a target of the adapted Pyophage prep.

Phage adaption was used in this pilot study to increase the activity of the preparation against a set of strains from a specific location, at a restricted time, and in a certain pathology. The principle of updating commercial phage preparations against relevant strains is common practice in countries with a history of phage therapy. This same concept could, in theory, be applied for adjusting a phage preparation for a single patient, time permitting, as a mechanism to counteract the development of phage resistance. However, multiple phages per pathogen are included in the commercial Pyobacteriophage preparation (Villarroel et al. 2017; McCallin et al. 2018). As this pilot study entails the adaptation of a cocktail, and not necessarily individual phages, the increased host range of the adapted preparation could be due to the selection of certain phages or population variants.

Summaries from the Polish Phage Therapy Unit (PTU)

One of the few institutes with a long-standing experience in phage therapy is the PTU in Poland, which has been treating patients compassionately with phages since the 1970s. They have published summaries of their experiences, with reports covering >1300 patients (Weber-Dabrowska et al. 2000, 2001, 2003; Międzybrodzki et al. 2012; Górski et al. 2016). The authors underline the initial sensitivity of the bacteria

to the applied phage as a requirement, with sensitivity to at least one phage from their collection being a prerequisite for receiving phage therapy at their establishment. In a study of the effectiveness of monophage therapy (the use of one phage per pathogen), a response to treatment was identified for 40% of 153 patients, although the rate of success was significantly associated with pathogen target and route of administration (Międzybrodzki et al. 2012). In a subset of 92 patients, authors investigated the development of resistance to phage during treatment in terms of phage typing profile, resistance to the applied monophage, and resistance to all phages against that pathogen in their collection. A change in phage profile was observed in 70, 100, 100, and 91% of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* isolates, respectively, therefore indicating changes in the pathogen clonality as a result of phage application. Resistance to applied phage was noted in 17, 43, 86, and 36% of strains of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* isolates, respectively, although these values were lower in terms of resistance to all phages to 8, 21, 29, and 27% for the same pathogens, respectively. The development of resistance varied by pathogen, with the high level of resistance observed for *E. coli*, due to frequent changes to phages used for treatment. The difficulty in targeting *E. coli* with phages is reflected in the composition of their phage collection that targets 15 bacteria species, with 22% of all phages targeting *E. coli*. Despite the development of resistance observed for some patients, this has not deterred the continued use of phages for the treatment of antibiotic-resistant infections at this institution.

5 Ways to Overcome Resistance

As observed with the abovementioned cases in both humans and animals, the development of resistance to phage is a possibility, to varying extents, within the context of phage treatment. There are several strategies available to avoid or counteract resistance in order to reduce a negative impact on therapeutic outcomes. In terms of phage-only strategies, cocktail formation, phage substitution, and phage training are all strategies that have been employed to counteract phage resistance. By combining certain phages together or with other antimicrobial strategies, bacteria are less likely to be able to develop resistance, and thus there are ways to design treatments to maximize therapeutic effects. Should resistance develop, it is possible to substitute new phages with activity against the bacterial isolate or to adapt phages in vitro to increase their activity. Bacterial resistance to phage may have additional benefits for treatment that could render the development of resistance an intended effect of future phage therapy efforts.

5.1 Cocktail Formulation

The use of multiple phages together as a phage cocktail is commonly employed for phage products. Many commercial phage preparations from Eastern European countries are indeed cocktails and contain phages against different bacterial hosts,

as well as multiple phages against a single host species (McCallin et al. 2013, 2018; Villarroel et al. 2017). The number and diversity of phages to make a sufficient cocktail vary between bacterial hosts and indications, with certain species requiring high diversity, such as *E. coli*, while other pathogens, such as *S. aureus*, can be targeted with one or relatively few, genetically similar phages (McCallin et al. 2018; Lehman et al. 2019). On the other hand, 14 phage types with homology to *E. coli* phages were detected in a recent metagenomic sequencing of a commercial phage product, therefore indicating a high number of phages to target this pathogen (McCallin et al. 2018).

Cocktail composition can be formulated with the intention of having a broad spectrum of activity, such as in the PhagoBurn study, against a particular species or type of infection (Jault et al. 2019). However, selecting phages with different host ranges might not be sufficient to meet clinical needs. The aforementioned PhagoBurn study used a cocktail of 12 phages to cover a large panel of *P. aeruginosa* isolates, and yet many patients during the trial harbored insensitive strains to the phage cocktail, to the point that it was identified as a factor for patient withdraw (Jault et al. 2019).

Cocktail composition can also be guided by selecting phages that would decrease the likelihood of resistance developing. The preclinical development of a four-phage cocktail to target *S. aureus* took into consideration the ability of each component phages to mitigate the development of resistance to other components (Lehman et al. 2019). For this product, phages that could complement resistance were selected, and the overall mean apparent frequency of resistance was reduced in vitro, although not significantly.

Cocktail composition should be updated periodically in order to retain activity against epidemiological strains. A finding from the Polish experience with PT is that phage susceptibility of epidemiological strains does indeed vary over time (Międzybrodzki et al. 2012). This concept represents a major contradiction to current approval pathways for medicinal products, where phages are intended to have fixed, stable compositions. It is indeed a possibility that the therapeutic potential of phage therapy, and the associated risk of developing phage resistance, could be constrained by man-made regulations.

5.2 Phage Substitution

A common strategy to counter phage resistance during phage therapy is simply to replace the phage(s) to which the patient isolate has developed resistance against with an active one. Long-established phage therapy treatment centers, such as Eliava or the PTU, have large phage collections from which phages can be selected to formulate personalized therapies and adapt them accordingly. This type of modification requires the periodic sensitivity testing of the causative pathogen against the applied phage(s) and additional available phages that can be rapidly applied when needed. Phage substitution during active treatment therefore represents a personalized or tailored approach to phage therapy.

Changing the phages used in treatment has been observed in a number of clinical case reports in response to detected resistance (Schooley et al. 2017; Zhvania et al. 2017). The treatment of the MDR *A. baumannii* abdominal infection required three changes to the phages used throughout treatment, with resistance being detected after 8 days (Schooley et al. 2017). The added value that phage substitution could have in phage clinical trials, however, remains unexplored, as previous trials have used fixed-composition products. In any case, the permission to do trials with a personalized approach is currently unclear within the current regulatory framework that permits little modification of clinical protocols, especially not to the active product.

One may argue that if the original cocktail formulation is designed correctly, phage substitution would not be necessary. Indeed, the rationale behind the preclinical selection of phages to be included in the BA-SA01 from AmpliPhi Biosciences was that the four separate components would kill PRVs should they develop (Lehman et al. 2019). However, there is still not enough evidence to make generalizations of resistance developing during clinical treatment at this time. The lack of this information highlights a knowledge gap in phage research, where publications on the discovery and basic characterization of phages are numerous, and yet the intricate interactions between phage and host remain largely unknown for most species of bacteria.

5.3 Phage Training

Another approach that was proposed to overcome or minimize bacterial resistance is the use of "phage training" (Pirnay et al. 2012). Training or adaptation of a phage to its bacterial host can be achieved in vitro by serial rounds of coinfection using a continuous bacteriophage culture with the same original non-evolving host at each passage. Phage adaptation is also referred to as Appelmans' protocol since it is generally recognized that phage training protocols are based on Appelmans' experiments for the titration of bacteriophages developed in the 1920s (Appelmans 1921). Different studies reported that evolving the lytic phage ?2 toward its *Pseudomonas fluorescens* SBW25 host led to an increased phage growth rate, but not increased infectivity range (Poullain et al. 2008; Hall et al. 2011a, b). It is however observed that coevolution passages, where both phage and host are transferred, can result in the evolution of broader infectivity range (Poullain et al. 2008). Interestingly, Morello et al. reported that phage optimization toward a clinical strain after five consecutive passages in liquid culture improved both in vivo treatment efficacy and host infectivity on a panel of 20 *P. aeruginosa* cystic fibrosis strains (Morello et al. 2011).

Phage adaptation can also increase pathogen clearance in addition to tempered bacterial resistance evolution (Friman et al. 2016). The phage infection capacity against *P. aeruginosa* PAO1 of four different phages could be increased after six serial passages so that virtually all the original PAO1 population was susceptible to phage infection (Betts et al. 2013). This was the case even if the bacteria had evolved

in the presence of the phage for one transfer, indicating that phage training is a useful tool to overcome the initial step of bacterial resistance. Finally, it is important to notice that Betts et al. reported that when phages and bacteria were subcultured for a total of ten serial transfers, variable outcomes regarding infectivity and resistance were observed for these coevolutionary passages (Betts et al. 2014). Indeed, some phage infecting *P. aeruginosa* PAO1 became less infective against bacteria from previous time points, therefore suggesting that phage training can be a phage-specific process that has to be considered for further therapeutic applications.

This was exemplified by the study of Ujmajuridze et al. in which a commercial preparation called Pyobacteriophage was first adapted and then tested in nine patients having UTIs (Ujmajuridze et al. 2018). After testing the sensitivity of the cocktail regarding 118 patient strains, resistant or intermediate resistant strains were used in 4 adaptation cycles, which could increase the total sensitivity of the phage cocktail from 41 to 75%. The implementation of phage training in the therapy could be advantageous since it could also increase phage coverage for bacterial clones present within a population, like in the case of patients with cystic fibrosis that are infected by highly phenotypically diverse *P. aeruginosa* (Rohde et al. 2018). Phage training could also allow to decrease the number of phages used during therapy due to increased coverage of the circulating strain and prevention of phage resistance emergence, thus simplifying the production process (Rohde et al. 2018). However, genomic sequencing of adapted phages should be done in order to show that the adaptation process is truly selecting for phage variants and where such mutations are located.

5.4 Combined Activity with Antibiotics

The development and use of phage therapy are not intended to be a direct replacement of antibiotics and likely never will be. Currently, most indications being developed and all compassionate use cases only target antibiotic-resistant bacteria, although phages are equally active against antibiotic-sensitive strains. More likely is that phages and antibiotics will be employed for future treatments in tandem strategies, where they are either combined together or in subsequent administrations, in order to provide combinatory therapeutic effects of employing different antimicrobial strategies. The possible benefits of using the two strategies have been documented both in vitro, in animal models, and in some clinical case reports.

5.4.1 Phage-Antibiotic Synergy

Combining phage with antibiotics can result in a synergistic antimicrobial activity to improve therapeutic efficacy and prevent the emergence of phage resistance. It has been reported that some types of phages produce bigger lytic plaques when amplified with sublethal concentrations of antibiotics (Comeau et al. 2007). Synergism is defined as a combination of phage and antibiotic that produces at least a 2-log greater reduction in bacterial load than either strategy alone. This phage-antibiotic synergism, termed PAS (for review on the topic, see Torres-Barcelo and Hochberg

(2016)), was observed to be an efficient alternative for the treatment of *P. aeruginosa* infections, where different in vitro studies have demonstrated that combining both phage and antibiotics results in lower bacterial density than during single treatment alone due to limiting mutations that lead to resistance (Knezevic et al. 2013; Torres-Barceló et al. 2014). This positive synergism is proposed to result from a reduction in the bacterial population size due to phage predation, which could limit the ability of bacteria to resist antibiotic pressure (Torres-Barceló et al. 2014). Morphological alterations, including antibiotic-induced filamentation, are also suggested to facilitate phage access to its target or increase phage assembly and maturation, as also observed for *E. coli* (Comeau et al. 2007; Knezevic et al. 2013).

PAS has also been observed to be useful for the eradication of *Pseudomonas* biofilms (Nouraldin et al. 2016) and was recently confirmed for the first time in vivo in a rat model of *Pseudomonas*-induced endocarditis (Oechslin et al. 2016). In this study by Oechslin et al., the combination of phages and ciprofloxacin was highly synergistic in vivo with a >6-log reduction of CFU in treated animals compared to a 2.5-log reduction of CFU using phage alone. In addition, the combination of phages and ciprofloxacin was also observed to efficiently prevent the emergence of phage resistance in vitro. Synergistic effects for ciprofloxacin with phages have also been documented in *S. aureus* both for CFU reduction and the suppression of resistance development (Jo et al. 2016).

5.4.2 Resistance Reversion

The synergism expected with antibiotics and phage combinations may not be limited to a direct increased killing of the two combined antimicrobial strategies, but also to alternating patterns of resistance and resistance reversion to either phage or antibiotics. Chan et al. observed that the selection of phage resistance can restore antibiotic susceptibility of MDR *Pseudomonas* (Chan et al. 2016). By selecting a specific phage that uses the outer membrane porin M of the multidrug efflux systems, MexAB and MexXY, as a receptor, it can result in PRVs with altered efflux pump function and thus increased sensitivity to many different drug types. This phage has since been used successfully in the compassionate treatment of an aortic valve graft infection in combination with ceftazidime (Chan et al. 2018). Phage-induced mutations in the *epa* operon responsible for cell-wall components in *E. faecalis* simultaneously create an increased sensitivity to daptomycin, a lipopeptide antibiotic (Teng et al. 2009; Dale et al. 2015; Ho et al. 2018).

In terms of therapeutic applications in humans, phage has often been administered with antibiotics in order to maximize the chances of therapeutic benefit for the patient. The choice of antibiotic and dosage combinations is largely experimental, with decisions being logically based off results of phage sensitivity tests and antibiograms. After the administration of the first phage cocktail in the case report for *A. baumannii* infections, authors noted a change in antibiotic susceptibility of the patient isolate that had become sensitive to minocycline and resistant, to some extent, to the applied phages. The antibiotic was then added to the treatment regimen, and phage was continued (Schooley et al. 2017). Minocycline binds to bacterial ribosomal units to inhibit protein synthesis, and therefore phage resistance

mechanisms may have triggered a reversion of antibiotic resistance mechanisms, which can occur through efflux pumps, drug modifications, or ribosomal protection proteins (Garrido-Mesa et al. 2013; Nguyen et al. 2014). However, no information concerning the biological mechanisms responsible for the switch in resistance of the clinical isolates is available from this case report or any other. Such fundamental investigations into clinical isolates and the development of PRVs coupled with antibiotic susceptibility would provide a greater understanding of how to maximize PAS in future treatment strategies.

The use of antibiotics in compassionate cases on one hand stymies a clear causality between phage and infection resolution; on the other hand, it hints at the value of phage-antibiotic combinations in providing therapeutic benefit. Indeed, clinical outcomes have been more positive for case reports, which can choose phages and antibiotics ad libitum, than results observed with clinical trials. It has recently been shown that even the order in which antibiotics and phage are applied may have consequences for its combined therapeutic potential (Kumaran et al. 2018).

It was also observed that the use of subinhibitory concentrations of streptomycin can also increase the phage resistance mutation rate in *P. fluorescens* and, conversely, phage exposure could also increase the rate of mutation to streptomycin resistance. However, it is important to notice that no positive correlation between drug and phage resistance was observed in a large collection of laboratory or clinical *E. coli* isolates (Allen et al. 2017). These results hopefully suggest that the use of antibiotics in medicine or agriculture is unlikely to induce changes in phage resistance or phage-antibiotic cross-resistance in the environment.

6 Conclusions and Perspectives

Resistance to penicillin was detected prior to its incorporation into clinical medicine, a fact which has not negated the innumerable bacterial infections it has resolved over the past 80 years. The capacity of bacteria to resist phage during treatment has been documented, but is not yet generalizable to clearly determine to what extent resistance could affect clinical outcomes. Additionally, the high specificity of interactions between phage and bacterial host has raised previously unrecognized subtitles of infectious pathologies that could be previously ignored with broad-spectrum antibiotics. Resistance to phage has been shown to vary widely for different pathogens and may be influenced by product design, application method, and dosage.

The most informative data that can indicate appropriate use of phages comes from actual clinical applications, of which there are relatively few at this time. Even so, unstandardized reporting of phage therapy in humans or animals has limited our ability to understand both the likelihood of developing resistance to phage during treatment and the impact this resistance has on clinical outcomes. Without both pre- and post-phage sensitivity testing, it is not possible to ascertain if resistance develops throughout the course of treatment, and such analyses should be included in future studies. For studies that do detect resistance, it would be beneficial to characterize the molecular mechanisms that give rise to its occurrence.

Phages have the advantage of being strongly supported by fundamental research, including resistance mechanisms, and therapeutic development is now strongly supported by technological ability and innovation, compared to the time of phage discovery. Strategies such as cocktail formulation, phage substitution, phage training, or combination with antibiotics can be used to maximize therapeutic benefits of phage treatment. While this review covers only relationships between natural phage and their hosts, genetically engineered phages or phage lysins may hold even more potential to reduce risks of resistance. The importance of phage resistance on clinical outcomes will reflect the developmental pathway of phage therapy in terms of regulatory frameworks and logistics that stretch beyond biological mechanisms.

The underlying reason for the current search for novel antimicrobials is rooted in the ability of bacteria to develop resistance to past ones, therefore making the exploration of resistance to future strategies a logical investigation. However, not all resistance is created equal. While experience has shown that there is a veritable possibility of resistance to phages developing as a result of therapeutic application, the likelihood of resistance occurring can be counteracted—or even harnessed—to mitigate negative effects on treatment outcomes. Resistance should therefore not be a deterrent to phage therapy, but needs to be better understood and taken into consideration for designing future phage strategies.

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