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Review

Phage Therapy: a Reappraisal of Bacteriophages as Antibiotics

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Abstract. The concept of phage therapy to treat bacterial infections was born with the discovery of the bacteriophage almost a century ago. After a chequered history, its current renaissance is fueled by the dangerous appearance of antibiotic-resistant bacteria on a global scale. As a mark of this renewed interest, the unanswered problems of phage therapy are now being addressed, especially for human use. Phage therapy in the agricultural, food-processing and fishery industries is already being successfully applied, and this review, whilst being aware of the potential drawbacks, emphasizes the need for further carefully controlled empirical data on its efficacy and safety in treating human and animal disease, especially in view of its numerous advantages over antibiotics. Finally the potential of phage therapy against bioterrorism and the emergence of second generation phage antibacterials based on phage-derived single-protein lysis systems are addressed.

Key words: phage therapy; bacteriophage; antibiotic resistance.

Bacteriophages and Life Cycle

Bacterial viruses, or bacteriophages, appear to be ubiquitous, there being examples in most bacterial species with sensitivity to one or more phages. Most of these phages have double-stranded DNA; all the known RNA viruses are single-stranded. Except for the filamentous phages, all of the phage groups have a polyhedral capsid which contains the phage genome. This capsid is usually joined to a tail, which is a helical protein structure required for adsorption of the virion to the bacterial cell.

Bacteriophages undergo two possible life cycles. These are the lytic (or virulent) and lysogenic. Lytic phages multiply vegetatively and kill the host cell at the end of the growth cycle. Temperate phages which undergo the lysogenic cycle as well as multiplying vegetatively can also persist in a lysogenic state, whereby the phage genome can exist indefinitely by being inserted in the bacterial chromosome (known as the prophage state). The lysogenic life cycle of phage lambda, for example, ensures the replication of the integrated prophage along with the bacterial genome for many generations. When induction occurs through damage of the DNA, which signifies the imminent death of the host, the phage switches to the lytic cycle, which results in the release of new phage particles.

Early Promise and Misconceptions of Phage Therapy

The discovery of the bacteriophage or phage is somewhat controversial. The consensus is that, 20 years

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after initial observations of unfilterable, heat labile agents with activity against *Vibrio cholerae*[14], it was discovered by Twort in 1915, who made similar observations and who hypothesized this to be due to a virus, and independently by d'HERELLE in 1917[15, 49]. Since it was realized that these bacterial viruses destroy their bacterial host while remaining harmless to humans, it has been the dream of researchers to use phages to treat bacterial infections.

There was much early promise. Using experimental practices common at the time, d'Herelle and co-workers first showed safety by ingesting *Shigella dysenteriae* phage preparations. The Shiga-phage successfully treated patients with dysentery. Others soon afterwards treated staphylococcal skin disease with phages injected in the vicinity of the infection[5]. The commercialization of therapeutic phage preparations to treat bacterial infection in humans was started in France by d'Herelle and in the United States in the 1940s by the pharmaceutical company Eli Lilly. However, because of controversial results[11, 19] and the promise of antibiotics in the 1940s, the commercial pursuit of therapeutic phages in the "West" ceased, although not in Eastern Europe.

Recently, interest in phage therapy, the use of phages to control bacterial infections, has been rekindled[12, 38, 40]. This is mainly to overcome the urgent problem of antibiotic resistance due to multidrug-resistant bacteria. According to recent WHO figures, in the US 14,000 people each year die from drug-resistant infections acquired in hospitals, and worldwide, 60% of such infections are drug-resistant. Bacteriophages are found in all bacteria, so it is hoped to be able to develop control therapies against pathogenic bacteria such as antibiotic-resistant streptococci, *Staphylococcus aureus* and *Streptococcus pneumoniae*.

Limitations of Phage Therapy and Possible Solutions

As an antibacterial therapeutic, phage therapy is not yet a fully established alternative to antibiotics. Some of the problems of using whole phages in therapy include the development of antibodies after repeated treatment with phages or, for example, against phages used to treat enteric pathogens because of prior exposure. Other problems might include the rapid uptake and inactivation of phages by the spleen and the contamination of therapeutic phage preparations with endotoxin from bacterial debris. The problem of antibodies, which could presumably be overcome by the use of cocktails of phages, is not deemed as important as clearance from the bloodstream by the spleen, which is much more rapid. Some of these concerns of phage therapy are now being addressed, with encouraging results. For example, phages prepared from bacteria would need to be purified by cesium chloride centrifugation, as this has been shown to diminish (by greater than 100-fold) contamination of phage preparations with endotoxin and exotoxin often released during lytic growth[28]. Although elimination of phages by the reticuloendothelial system can also be avoided by selecting for phage variants (at least in mice) able to circulate for a long time after intraperitoneal administration, by a natural selection process (the so-called "serial passage technique")[28], this would not be practical and could be overcome by further administration of phages. Despite phages being associated with enteric bacteria in the gut and although we understand so much about the genetics of phages and how effective they are in destroying bacteria *in vitro*, little is known about how they behave *in vivo*, in particular in the human body, and such *in vivo* studies are needed.

A simpler way to overcome many of these problems may be to use products derived from phages, and now, on the studies of RNA phages, two approaches for using bacteriolytic phage peptides have emerged[3]. The first uses the capsid protein A_2 from the RNA phage Qβ which, as for penicillin, interferes with the enzyme involved in the synthesis of the bacterial cell wall, but which is also involved in the binding of the phage to its bacterial receptor. The peptidoglycan structure thus weakened allows the viral progeny to lyse the bacterium. A_2 is unlikely to work *in vivo* because such a large protein, of greater than 40 kDa, when added to growing bacteria would not be able to act on its cytoplasmic target unless smaller molecules based on the protein interaction sites were found. It has been postulated that such molecules targeting specific enzymes within the pathways to generate new cell walls could form the basis of a novel generation of antibacterial agents.

Another peptide having the unique function of blocking the bacterial peptidoglycan synthesis pathway, although by action on a different enzyme, is the E protein of phage ϕX174[4]. DNA phages produce lysins which break bonds in the bacterial cell-wall peptidoglycan structure just before release of phage progeny. Lysins have been shown to kill bacteria *in vitro*[29]. One advantage of these enzymes is their specificity of action, which seems to be down to the subspecies level. The reason for this high level of specificity is that the binding of lysins occurs through carbohydrate structures, which can have a tremendous amount of vari-

ation[24, 26], on the bacterial cell wall. Furthermore, the development of resistance to the lysis is unlikely simply because the kind of mutation that would render the cell wall unable to be bound by lysins would in fact kill the bacterium.

Most recently, lysins have even been use to kill *Bacillus anthracis*[34], although not yet tested in an *in vivo* model of infection with *B. anthracis*. In this case the lysin, an enzyme called PlyG, was extracted from bacteriophage γ and, although the enzyme functions within the cell, it was able to cause lysis when added to the bacteria growing on solid or liquid medium. Another precaution that would need to be taken with certain bacterial infections is that, as with other antibiotics, the lysin would need to be delivered as soon as possible after infection before any lethal levels of toxin could be reached.

Phage Therapy against Bacterial Pathogens in Agriculture and of Fisheries

Antibiotics are widely used throughout the agricultural industry as a prophylactic or as treatment against infectious disease. This widespread use is most likely contributing to the ever increasing levels of antibiotic resistant bacterial pathogens in humans. A likely early use of phage therapy is in the control of bacterial infections of crops for which plants or seeds could be treated. Bacteriophages have been considered, for example, to control *Salmonella* infestation of cut fruit[23]. The most successful use of phage therapy, already in practice, has been in the control of fish pathogens[16, 30]. The phages involved are perfectly suited to the environment as they are usually found in the water. The advantage of using therapeutic phages in a water medium is the close and continuous contact that can be achieved between the host, whether fish, crustacean or even mollusc, and phage.

Recent directives by the US Food and Drug Administration to combat the spread of antibiotic resistance in pathogens involve banning the prophylactic use on livestock of drugs designed for use in humans. To avoid contamination of food products with *Listeria monocytogenes, Salmonella* on cut vegetables and fruits, or the pathogenic *Escherichia coli* O157 : H7, phage therapy is now being advocated for use in the food and livestock market. In the same vein, phages would be able to clean the environment on poultry farms or processing plants, thereby reducing the chance of infecting other flocks.

Advantages of Phage Therapy over Antibiotics

Bacterial resistance to phages, although likely to arise, should not be a major concern, certainly compared with bacterial resistance to antibiotics. This is because phages grow exponentially, essentially shadowing the bacterial growth and thereby mutating at the same rate and, furthermore, due to the plethora of phages, there will certainly be a species that can attack mutated, resistant bacteria. The differences between phage and antibiotic therapy as a control of infection, which includes a much longer time of development of the latter[8], are summarized in Table 1. Studies on *E. coli* 018:K1:H17ColV$^+$, which is pathogenic in calves, showed that when mice were infected with this strain, nine K1 coliphages were found which effectively eliminated the infection after a single dose, compared with several doses of various antibiotics[35]. Based on this study it was suggested that the reason for the greater effectivity of phages over antibiotics is that, whilst both kill bacteria, antibiotics are metabolized and excreted, whereas the phage titers actually increase[27]. Phage multiplication is indeed very rapid, a single phage producing 4×10^3 progeny within an hour, this number increasing exponentially to 4×10^6 an hour later.

Phage Therapy against Bacterial Infections in Animals and Humans

The scale of antibiotic resistance now results in over five million people dying every year from infections not responding to antibiotics. The very dangerous *Staphylococcus* bacterium is only sensitive to one antibiotic, vancomycin, but already in the year 2000 the first case of vancomycin-resistant *Staphylococcus* was found in Japan in a baby undergoing major heart surgery. Some of the reports, especially those which have appeared in English, of phage therapy trials in both experimental animals and humans are listed in Table 2. A successful series of preclinical studies in animals using phage therapy focused initially on *E. coli* infections in mice[35]. Lambs, piglets and calves treated using phages were also cured of the diarrhea-causing *E. coli*[36, 37].

In a study from the Institute for Animal Health, UK, an *E. coli* phage isolated from sewage which was found to infect via the K1 capsular antigen, and which was able to multiply in the blood, was used to protect chickens from septicemia and a meningitis-like infection[2]. Other interesting work in Sweden[7] described the use of a recombinant *Helicobacter pylori*-binding filamentous

Table 1. Comparison of bacteriophage versus antibiotic therapy

Advantages	
bacteriophages	antibiotics
High specificity for particular bacterium, thereby reducing the possibilities of secondary infections developing.	Active against wide range of bacteria, thereby avoiding the need to characterize the infective bacterium.
Repeated administration is unlikely because as long as the target bacterium is present, the phage will be able to reproduce.	
Cheap to produce and to date without any observed side-effects.	
The receptors to which phages are targeted on the bacterial cell surface are virulence factors, so when bacteria develop phage-resistance, they are usually altered, which results in an attenuation of virulence.	
Finding a phage which will be active against a bacteria which has developed phage resistance is rapid, taking only a matter of days.	
Disadvantages	
bacteriophages	antibiotics
Causative agent may need to be identified in order to use appropriate phage, unless a phage cocktail is used.	Repeated administration is needed and antibiotic therapy is often associated with side-effects such as intestinal problems, secondary infections, e.g. with yeast.
	Once a bacteria develops antibiotic resistance, as opposed to phage resistance, it remains pathogenic.
	The development of novel antibiotics (needed for example when bacteria develop resistance) takes on the order of years[8].
	Because of a non-specific mode of action, antibiotics also destroy the commensal microflora especially in the intestine, which may lead to intestinal disorders.
	Expensive to produce.

M13 phage, which when used to pretreat *H. pylori* before oral administration to mice, significantly reduced infection levels.

The finding during years of phage trials in Eastern Europe[1] that it was almost exclusively without side effects on humans should be encouraging. Furthermore, a recent phase I clinical trial in the USA in which phages were injected intravenously to treat Vancomycin-Resistant Enterococcal infections (see http://www.expobio.com) has been completed successfully. The most comprehensive studies described in English on human phage therapy trials were those conducted by the Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences at 10 different hospital departments[41–47]. These studies were conducted on 550 patients in the age range of 1 week old to 86 years old, 518 of whom were suffering from infections with antibiotic-resistant *E. coli*, *Klebsiella*, *Pseudomonas*, *Salmonella*, *Staphylococcus* and *Streptococcus*. Phages, produced in a standardized manner and tested for sterility, were administered either topically 3 times per day, in eye drops, or orally before meals (10 ml) after neutralizing stomach acid with baking soda, gelatin or basic water. Treatment ran for an average of 5 weeks. The success rate was in the range of 75–100%, depending on the bacterium. For the 518 patients for whom antibiotic therapy was ineffective, the success rate was higher, at 94%. The only side effect was liver pain, deemed to be due to endotoxins released on phage-mediated destruction of bacteria. Similarly, the fear of toxic shock due to bacterial debris ruled out intravenous administration, although the aerosol route and rectal infusion were successful.

Other highly effective studies in which phages were administered subcutaneously to patients having antibiotic-resistant infections cured, amongst other ailments, osteomyelitis, against which, interestingly, antibiotics are virtually ineffective due to poor circulation[33]. Other studies are summarized in Table 2[2, 7, 9 10, 20–22, 32, 39, 50, 53].

Regulatory Authorities and Public Awareness

An antipathy to using live viruses, even if specific to bacteria, would not be unexpected. However, the psychological barrier should not be insurmountable, especially once we remember that many of the best modern vaccines consist of live viruses and that phages are

Table 2. Human phage therapy trials and *in vivo* studies in animals

Infection	Causative agent	Summary	References
Suppurative skin infection	*E. coli, Klebsiella, Proteus, Pseudomonas, Stapylococcus*	of 31 patients 23 cases at least had a marked improvement	9
Various	*E. coli, Klebsiella, Proteus, Pseudomonas, Stapylococcus*	phage immunogenicity did not hinder therapy	20
Gastrointestinal, head, neck, skin	*E. coli, Klebsiella, Pseudomonas, Salmonella, Staphylococcus*	506/550 patients (92%) successfully treated	41–47
Suppurative infection	*Staphylococcus*, Gram-negative	oral administration; 56 patients successfully treated, phage in blood (47/56) and in urine (9/56)	50
Brucellosis	Brucella abortus		10
Conjunctivitis, dermatitis, pharyngitis, rhinitis	*Enterococcus, E. coli, P. aeruginosa, Staphylococcus, Streptococcus*	1340 patients treated; 360 with phage (86% clinical improvement), 404 with antibiotics (48% clinical improvement) and 576 with combination (83% clinical improvment)	32
Infective childhood asthma			53
Bovine udder infection			22
Recurrent subphrenic abscess	*E. coli*	patient left hospital after 33 days without any abscesses	21
Cerebrospinal meningitis	*K. pneumoniae*	following unsuccessful antibiotic therapy, newborn successfully treated with orally administered phage	39
Recurrent furunculosis	*Staphylococcus*		6
Diarrhea in calves	*E. coli*	E. coli causing diarrhea eliminated from alimentary tract of piglets, calves and lambs	2, 36, 37
Septicemia, meningitis in chickens and calves	*E. coli*	septicemia and meningitis-like infection in chickens due to *E. coli* K1+ cured with phage which multiplied in the blood	2

ubiquitous, occurring naturally in all the places bacteria are found, such as the food that we eat, unpolluted water, sewage, or in the lower gut, associated with their bacterial hosts. During the many years of use of bacteriophages in Eastern European countries, they were almost exclusively found to be without side effects. In the USA, bacteriophages were injected intravenously, also without side effects, in studies for analyzing the importance of cell surface-associated molecules in modulating the immune response in humans.

Phage Therapy and the Future Bioterrorist Threat

The bioterrorist attacks in the USA last year resulted in 22 people contracting anthrax from *B. anthracis* spores delivered by mail. There were 5 fatalities from inhalational anthrax, although 11 people recovered from cutaneous anthrax. It is now believed that the virulent Ames strain, possessing both the toxin-encoding plasmid pXO1 and the capsule-encoding plasmid pXO2, was used. The ease of preparing *B. anthracis* spores means anthrax is likely to be one of the first-choice weapons of bioterrorists. Fortunately, vegetative-stage cells of the naturally occurring strains of *B. anthracis* are susceptible to penicillin and doxycycline, although they need to be administered for two months due to their ineffectiveness against spores. Although penicillin-resistant *B. anthracis* is rare, this should not preclude the possibility of genetically-engineered, multiple antibiotic-resistant anthrax bacilli being developed and then used for evil purposes. Indeed, antibiotic-resistant *B. anthracis* is easily generated in the lab[31]. Others still have engineered strains to contain non-anthrax genes which, through altered immunopathogenesis, could become resistant to the existing vaccine.

Recent articles such as that of GOTTLIEB[13] have proposed rational (anti-toxin) drug design based on the now complete structural data of the anthrax toxin[25]. As an alternative treatment for anthrax infection (besides the obvious development of antibiotics), phage therapy, in which phages would be used to control the infection, should also be considered, especially now, as the need to tackle the urgent problem of antibiotic resistance has rekindled interest in phage therapy. The paucity of *B. anthracis*-specific phages, means that phage therapy cannot yet be fully embraced to tackle anthrax. Some years ago, however, we observed that lysogenic strains of *B. anthracis* serve as a reservoir for phages that attack certain sensitive strains of anthrax bacillus[17]. Figure 1 illustrates ϕ20 which can lyse the pathogenic LA1 ($pXO1^+/2^+$). As a caveat, many phages specific to *Bacillus* species mediate generalized transduction[18], which during phage therapy could result in the transfer of anthrax toxin genes (so-called phage conversion). This would be the major flaw with using phage CP-51[31], proposed by Walter at the 100th meeting of the American Society for Microbiology (2000), to "destroy stockpiles (of *B. anthracis*) or even as a therapy for the disease".

To overcome the problem of exact diagnosis of the strain of anthrax, as underlined by TEMTE[48], phage cocktails could eventually be used which would lyse the majority of *B. anthracis* strains, thereby also avoiding the possibility of developing resistance to phages. Virulent, non-transducing phages of all the major *B. anthracis* strains should be studied for possible inclusion in such phage cocktails, which could serve as an alternative line of defence against anthrax. For this to be realized, researchers in the burgeoning field of phage therapy must first collaborate with the few anthrax labs and use appropriate phages, such as the ones we described earlier, in preclinical studies to treat *B. anthracis* infection.

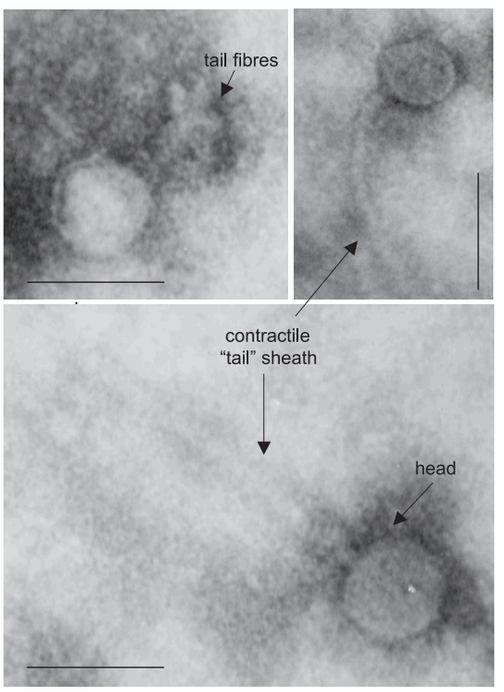

Fig. 1. Anthrax phage, ϕ20. A virulent phage for the pathogenic. LA1 strain of *B. anthracis* ($pXO1^+/2^+$). Bars, 100 nm.

As an alternative to using whole bacteriophage particles to destroy bacteria in therapy, now that the mechanisms by which phages lyse bacteria are becoming elucidated, the latest thinking which should be considered in all applications of phage therapy is to utilize the component parts of the phage needed for bacterial lysis[26, 27, 34]. Afterall, it may even be easier to gain public acceptance of phage therapy, to use one enzyme rather than using a whole virus. Phage-specific lysins should thus be isolated for possible use in therapy, or phage peptides blocking cell-wall synthesis isolated and tested, or even the phages themselves considered as vehicles to deliver antimicrobial peptides.

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