PERSPECTIVES

Risk of Bacteriophage Therapeutics to Transfer Genetic Material and Contain Contaminants Beyond Endotoxins with Clinically Relevant Mitigation Strategies

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Abstract: Bacteriophage therapy is a promising adjuvant therapeutic in the treatment of multidrug-resistant infections and chronic biofilm infections. However, there is limited knowledge about how to best utilize these agents in vivo, leading to a wide range of treatment protocols. Moreover, while bacteriophages are similar to antibiotics in their anti-microbial effects, these are active viruses and are very different from conventional anti-biotics. One main difference that clinicians should be cognizant about is the potential ability of these therapeutics to horizontally transfer genetic material, and the clinical ramifications of such events. In addition, while bacteriophage therapeutics are readily tested for sterility and endotoxins, clinicians should also be aware of other contaminants, such as exotoxins, pathogenicity islands and prophages, that can contaminate bacteriophage therapeutics, and their clinical ramifications. While the perception may be that these are only theoretical issues, regulatory agencies are starting to recommend their evaluation when using bacter-iophage therapy and subsequently these topics are discussed herein, as are ways to test for and mitigate the adverse effects of these issues.

Keywords: bacteriophage therapy, transduction, prophage, horizontal gene transfer, pathogenicity islands, enterotoxins

Introduction

Bacteriophage therapy is a novel therapeutic that is gaining increased interest in the treatment of multidrug-resistant infections and as a potential adjuvant in the treat-ment of chronic biofilm infections.[1,2] Preclinical research suggests that bacterio-phages are virulent against planktonic bacteria and, when combined with antibiotics, can help eradicate biofilms.[3–6] Several case reports support the precli-nical work suggesting that bacteriophage therapy may be effective in treating a wide range of infections, ranging from cystic fibrosis infections to recalcitrant orthopedic infections.[7–10] However, at this nascent stage, clinical trials have yet to show significant benefits of bacteriophages compared to placebos.[11–13] Therefore, it is easy to envision the potential of bacteriophage therapy, but a paucity of knowledge about how to effectively use these therapeutics hinders translational scientists and clinicians in devising reproducible treatment protocols at the present time.[10] While bacteriophage therapeutics are similar to conventional antibiotics in their antimicrobial effects, many differences are apparent. One major difference is

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5629

that bacteriophages reproduce on bacterial hosts, thereby allowing for these therapeutics to potentially horizontally transfer genes.[14] In addition, there is limited knowledge about potential contaminants beyond endotoxins that may be present in bacteriophage therapeutics, which include, but are not limited to, prophages, exotoxins and pathogenicity islands. These topics are important given that regulatory agencies such as the Federal Drug Administration (FDA) are starting to recommend the evaluation of bacteriophage therapeutics for these contaminants, and, if present, to have potential mitigation strategies to reduce the potential consequences. Therefore, this perspective discusses these topics with relation to clinical relevance, in vitro testing of bacteriophage products and mitigation strategies to reduce their potential deleterious effects.

Horizontal Gene Transfer Mechanisms and Contaminants
Transduction and Pathogenicity Islands

Transduction is the horizontal transfer of DNA between bacteria secondary to bacteriophages, which can occur in several ways: generalized, specialized and lateral transduction. These mechanisms are major evolutionary forces that have shaped bacterial adaptation.[15–17] Therefore, when using bacteriophage therapeutics, horizontal gene transfer can occur and clinicians need to be aware of the associated risks. These risks can be divided into 1) generalized transduction potential of the lytic bacteriophage therapeutics themselves, 2) the potential of prophages to horizontally transfer genes or through specialized and lateral transduction, and 3) pathogenicity islands.

Transduction Potential of Lytic Bacteriophage Therapeutics

Lytic bacteriophages are virulent predators of bacteria but some also have generalized transduction capabilities. This form of transduction occurs when bacteriophage DNA is replicated and packaged into virion heads, but packaging of bacteriophage DNA can occur with low fidelity.[15–17] Consequently, given this low fidelity, small pieces of bacterial DNA, which result from the fragmentation of the host chromosome, can be packaged instead of, or in addition to, the bacteriophage genome.[15] These virions, which have bacterial DNA, can then infect other bacteria that have the same or similar bacteriophage attachment receptors. When the bacterial DNA is injected into a new bacterium, three events can occur: 1) it can be degraded, resulting in no horizontal gene transfer; 2) if it was a

plasmid in the former bacterial cell, then it can circularize and form plasmid again; or 3) if there is a homologous region in the recipient bacterial chromosome, then recombination can occur.[15] The rate of generalized transduction for lytic bacteriophages differs based on the fidelity of genome packaging and other parameters, but is estimated to occur in about 1 of 100,000 phage progenies.[15–17] This can be seen in an experiment with *Escherichia coli*, in which generalized transduction delivered an antimicrobial resistance gene to eight *E. coli* bacteria per hour in a population of 1×10^8 *E. coli* bacteria per liter.[18] Therefore, through generalized transduction, resistance genes and other deleterious genes can be transferred among bacteria.

Transduction Potential of Prophage Contaminants

Unlike lytic bacteriophages, prophages are lysogenic bacteriophages that are integrated inertly into the bacterial genome and are controlled by a master repressor.[19] Prophages have no current role in bacteriophage therapy, but when lytic bacteriophage therapeutics are propagated on bacterial hosts prophages can become activated and enter the lytic cycle. Most bacteria harbor several prophages and consequently prophages can contaminate bacteriophage therapeutics. In addition, activation of prophages in vivo can occur. Therefore, prophages are another cause of horizontal gene transfer through specialized transduction and lateral transduction after prophages are activated to enter the lytic cycle.[15,17,19] Specialized transduction occurs when bacterial genes that flank the integrated prophage are incorporated with the prophage's DNA secondary to imprecise excision. These bacterial genes are then packaged into virions and can be horizontally passed to other bacteria.

Lateral transduction is a different type of transduction that has been studied in *Staphylococcus aureus*, which occurs when the integrated prophage starts replicating DNA before prophage excision, thereby resulting in the replication of large segments of adjacent bacterial DNA.[17] These long segments are then packaged into virions and can be horizontally transferred to other bacteria, thereby causing the transfer of genetic material at frequencies 1000 times greater than those seen with generalized or specialized transduction.[20] Therefore, lateral transduction is an evolutionary force that can lead to rapid bacterial evolution.[17,21] Through both mechanisms, prophages that contaminate bacteriophage therapeutics and those that are activated in vivo can horizontally transfer genes.

Pathogenicity Islands

Like prophages, pathogenicity islands reside inertly in the bacterial chromosome under the control of a repressor, but unlike traditional prophages, their repressors are not SOS response inactivated.[22] It is hypothesized that pathogenicity islands are remnants of prophages that have diverged over time.[22] These small mobile genetic elements have been recovered from most bacteria and, with respect to *S. aureus* clinical isolates, are ubiquitous.[22–26] They can also harbor virulence genes associated with adhesions and antimicrobial resistance.[22–26] Unlike prophages, pathogenicity islands need the bacterium to be infected with a helper phage or the induction of a resident prophage to enable an anti-repressor protein to be translated, which inactivates the pathogenicity island repressor.[22] This results in pathogenicity island replication, excision and then reorganization of helper phage or prophage proteins to form small capsids, which can be smaller than normal capsids, that fit the small genome of the pathogenicity island. Pathogenicity island particles are highly infectious and can spread their genetic material to a wide range of bacteria at high frequencies. This is in part secondary to the broad potential host range of temperate phages and the increased burst size.[25–27] These broad host ranges can be seen with staphylococcal pathogenicity islands, which have the potential to infect other bacterial species, such as *Listeria*.[27] Consequently, pathogenicity islands such as lytic bacteriophages and prophages can transfer genes broadly across bacteria.

Potential Bacteriophage Therapeutic Contaminants Beyond Endotoxins

Prophages and pathogenicity islands can contaminate bacteriophage therapeutics because of the activation of these elements when bacteriophages are propagated and amplified on host bacteria. Prophages can be associated with numerous virulence factors that often benefit the bacterium, thereby also giving an evolutionary advantage to the prophage.[28] Consequently, prophages are present in most bacteria and, with respect to *S. aureus*, are associated with such factors as Panton–Valentine leukocidin, staphylokinase and enterotoxins.[15] Likewise, *Pseudomonas* prophages are associated with attachment, formation of biofilms and exotoxin A, while *E. coli* prophages can be associated with Shiga toxins 1 and 2.[29] Therefore, with the activation of prophages, these genes can be translated and thereby contaminate bacteriophage therapeutics. However,

so can other macromolecules, such as lipoteichoic acid, bacterial DNA and cytolysins. Some of these macromolecules are small and water soluble, making their removal from bacteriophage therapeutics arduous.

When creating bacteriophage therapeutics, host bacteria that are used to propagate the bacteriophages can produce macromolecules that can then contaminate the therapeutics. These are different from endotoxins, which are already readily tested for and have set standards. For example, staphylococcal enterotoxins are present in most clinical isolates and have been shown to cause systemic inflammatory responses through the activation of toll-like receptors.[30,31] Recent testing of two bacteriophage therapy products used through FDA-approved expanded access pathways (IND 27250 and 27458), here at the University of Maryland, for recalcitrant *S. aureus* prosthetic joint infections in two patients, showed low levels of staphylococcal enterotoxin A in these therapeutics, with levels at 3 ng/mL. No systemic side effects were seen in either of the patients receiving the therapies, but one patient did have transaminitis, as discussed in a short communication.[32] The other patient, after discussion with the FDA, was treated with repeat intraarticular doses instead of intravenous dosing to mitigate the potential risk associated with the low levels of enterotoxin A. While these are only two examples, this report does show that bacteriophage therapeutics can contain contaminants beyond endotoxins and it supports the need for clinicians to be cognizant that such contaminants may be present.

Testing of Bacteriophage Therapeutics for Transduction Capabilities and Contaminants

At present, testing of bacteriophage therapeutics is limited to ensuring acceptable levels of endotoxins and sterility. However, no formal testing is recommended to evaluate bacteriophage therapeutics for their generalized transduction capabilities and to ensure that there are no other contaminants in the bacteriophage therapeutics themselves, beyond endotoxins. It should be noted that chronic infections that have failed numerous conventional antibiotic regimens are the main type of infections that bacteriophage therapy is currently being used to treat.[33] With these recalcitrant infections, clinicians have ample time to test for these issues and, if needed, to devise mitigation strategies. In this section, testing of bacteriophage

therapeutics for transduction capabilities and contaminants is discussed.

Generalized Transduction

Lytic bacteriophages are not uniform in their ability to undergo generalized transduction. Rather, some have higher rates of generalized transduction than others, likely stemming from the fidelity of packaging of the bacteriophage genome.[15] Consequently, it is important for clinicians to know the theoretical transduction ability of a particular bacteriophage therapeutic, to thereby potentially be able to mitigate the risks of generalized transduction clinically. Testing for generalized transduction ability is not standardized, but two techniques have been proposed.

Testing for Transduction of Ribosomal 16S

Ribosomal 16S are highly conserved bacterial DNA segments and many bacteria have multiple copies, thereby making this a gene that has a high chance of transduction compared to other genes.[34–36] Therefore, testing for bacteriophages having 16S in their virion heads indirectly evaluates the generalized transduction potential for a bacteriophage. This testing includes denaturing the bacteriophage therapeutic capsid to release the phage DNA and then using 16S polymerase chain reaction to amplify 16S genes if these are present.[34–36] While not a perfect testing platform, this is an indirect way of assessing the potential for generalized transduction.

Transduction of Antibiotic Resistance Genes

An alternative technique to assess generalized transduction is based on the ability to transfer antibiotic resistance genes.[37] This method can be used if host bacterial strains have resistance genes such as those seen with methicillin-resistant *S. aureus* or vancomycin-resistant *Enterococcus*.[14,38] Bacteriophages grown on these host strains can then be allowed to grow on bacteria of the same species that do not have these genes but still allow for lytic activity of the same bacteriophage. If general transduction has occurred, there is a chance that these resistance genes will be passed to the non-resistant bacteria, thereby making them resistant to certain antibiotics, which can be easily assessed by exposing those bacteria to specific antibiotics and evaluating their growth.

Testing for Prophages, Pathogenicity Islands and Toxins in Bacteriophage Therapy

As previously stated (in the "Potential Bacteriophage Therapeutic Contaminants Beyond Endotoxins" section),

in order to create bacteriophage therapeutics, propagation and amplification need to be conducted on host bacteria. Given the ubiquitous nature of prophages and pathogenicity islands in clinical bacterial isolates, testing for these elements is important to determine the potential for bacteriophage therapeutics to contain them. This testing can be done with whole genome sequencing of the host bacterium. However, this can be cumbersome and temporally not feasible if using a bacterium that has not been sequenced and emergency use of bacteriophage therapy is needed. Consequently, inducing the bacterium into a stress state with the use of agents such as ultraviolet light and mitomycin C can induce the activation of these agents.[39,40] The presence of these agents can then be assessed using plaque-forming assays, to make clinicians aware that these elements may contaminate the potential therapeutic.[38,39]

Evaluating bacteriophage therapeutics is also important when using a host bacterial strain that may harbor exotoxins and other macromolecules. These can include staphylococcal enterotoxins, enterococcal cytolysins and pseudomonal exotoxins.[40–42] This is especially important when using clinical isolates to propagate bacteriophages, which are known to readily harbor these elements. Whole genomic sequencing is again a means to ensure that no toxins are present in the host bacterium, but, as stated in the previous paragraph, temporal time constraints may not allow for this in certain situations; consequently, there are commercially available quantification assays for some of these toxins, such as staphylococcal enterotoxin A–C. However, it is not feasible to evaluate a therapeutic for all potential harmful macromolecules, given the lack of commercially available testing assays for many of them, such as enterococcal cytolysin. Consequently, given the novelty of this therapeutic, and especially when propagating bacteriophages on clinical isolates, robust discussions with patients about risk versus benefit are warranted to ensure that the appropriate risks, beyond endotoxins, are understood by clinicians and patients.

Clinical Relevance and Mitigation Strategies

At this nascent stage, the clinical ramifications of horizontal gene transfer have not been well studied, but it is easy to envision that it could result in the transfer of resistance genes, making infections more difficult to treat. This is especially of concern with staphylococcal infections, given the high frequency of gene transfer that occurs with lateral

transduction and the wide host range of pathogenicity islands. Beyond the implications of genetic transfer, the potential for bacteriophage therapeutics to contain contaminants such as staphylococcal enterotoxins can also have significant clinical implications, given the highly inflammatory nature of these toxins. It must be noted that most in vivo bacteria have the potential to produce toxins and engage in horizontal gene transfer. However, this does not negate the need to be cognizant about the ramifications of administering large quantities of lytic bacteriophages and potential contaminants that can be present with bacteriophage therapeutics. Rather, the administration of these potential agents into a patient can be a catalyst for horizontal gene transfer and may expose patients to inflammatory macromolecules at levels that far exceed what naturally occurs in vivo. Therefore, clinicians need to be aware of the clinical risks in order to devise potential mitigation strategies. This is especially important since the majority of infections treated with bacteriophage therapy are chronic recalcitrant infections, and therefore ample time is available to test for contaminants and, if needed, to devise mitigation strategies.

Clinical Implications and Mitigation Strategies of Bacteriophage Therapeutic Contaminants

Knowledge of endotoxin levels is essential to prevent patients from being exposed to large amounts of endotoxins, which can cause sepsis-like symptoms.[43] Consequently, strict limits on endotoxin unit (EU) levels are enforced at a maximum of 5 EU/kg/hr.[43] However, no restriction has been recommended for testing or quantification of staphylococcal enterotoxins, enterococcal cytolysins, pseudomonal exotoxin A, lipoteichoic acid and others that have the potential to contaminate bacteriophage therapeutics. This creates potential unrealized risks to patients receiving bacteriophage therapies if the bacteriophages are amplified on clinical isolates that likely harbor these virulence genes. With respect to staphylococcal enterotoxins, these can be associated with significant inflammatory responses given their ability to be superantigens and activate toll-like receptors.[44,45] In fact, staphylococcal enterotoxins can be lethal at certain concentrations.[44,45] Enterotoxin B has been shown to have a 50% lethal dose of approximately 0.02 μg/kg when inhaled.[46] Furthermore, in a past outbreak of food poisoning in the USA with chocolate milk, the mean amount of staphylococcal A toxin recovered from the contaminated products was 0.5 ng/mL.[47] While it is unlikely that large concentrations of enterotoxins will be given with bacteriophage therapy, it is also unknown what the acceptable levels of enterotoxins are, especially when given repeatedly. Moreover, given the ability of these agents to be superantigens, it is unknown whether there are long-term sequelae that are associated with repeated exposures, which have been seen with Shiga toxins.[48,49] As seen with the two compassionate use cases briefly discussed above (in the section "Potential Bacteriophage Therapeutic Contaminants Beyond Endotoxins"), enterotoxins can be present in bacteriophage therapeutics. Consequently, clinicians should realize that bacteriophage therapeutics have the potential to contain contaminants that not only can be superantigens but also may have unknown long-term sequelae.

To mitigate these risks, it is prudent either to sequence the bacterial clinical isolate being used to propagate and amplify bacteriophage therapeutics or to use host bacterial strains that are known not to harbor prophages, toxins or pathogenicity islands. There are well-known bacterial strains that are devoid of these agents that can be used in the creation of therapeutics, such as *Staphylococcus carnosus*, and commercially available strains, such as RN4220.[50,51] Using these strains would allow for products to have acceptable endotoxins as well as reducing potential exposure to other harmful agents. For clinical trials, this would mitigate the risks and should be recommended. However, for emergency use cases, if propagation can only be conducted on clinical isolates, robust discussions of risk with the patient or the next of kin will need to be undertaken beyond the discussion of endotoxins. These discussions would need to focus on other potential contaminants based on the bacteria that the clinician is trying to eradicate, such as cytolysins for enterococcus and enterotoxins for *S. aureus*.

From a regulatory and safety perspective, this may be essential, but it does come with a trade-off. Using bacterial host strains that are not the clinical isolates to propagate and amplify bacteriophages creates pressure to select for bacteriophages that have strong affinities for the host strain's bacteriophage attachment receptor, which may have less affinity for the clinical isolate's receptor. Therefore, given the highly specific nature of bacteriophage binding to bacterial surface receptors, if clinical isolates are not used to propagate bacteriophages, the therapeutic may be less virulent than one wishes when

using it clinically. Further studies are needed to clarify these points, but the risks associated with other contaminants in bacteriophage therapeutics need to be fully discussed with patients to allow them to make informed consent decisions.

Clinical Impacts of Horizontal Gene Transfer In Vivo Bacterial Infections

The clinical ramifications of gene transfer with respect to the passage of antibiotic resistance genes also need to be realized by clinicians who use bacteriophage therapy. It has been shown, in Gram-negative bacteria, that transduction of genes encoding resistance to fluoroquinolones, carbapenems and colistin can occur.[52] *Staphylococcus aureus* has also been shown to use transduction to pass tetracycline resistance to other *S. aureus*. This may seem inconsequential, but tetracyclines are a major oral antibiotic used in suppression therapy for complex biofilm infections.[38] Therefore, if this resistance gene is passed to residual in vivo bacteria, suppression of staphylococcal infections becomes more cumbersome. Other genes can also be passed which can make infections more problematic to treat, such as those implicated in biofilm production, metabolism and toxins. A clinical example of this can be seen in seasonal cholera outbreaks, in which *Vibrio* lysogenic bacteriophages that carry the cholera toxin gene can interact with filamentous bacteriophages, promoting the horizontal transfer of cholera toxin genes.[53,54]

Moreover, recent literature suggests that in vivo infections are not homogeneous collections of a specific single bacterial strain but likely are heterogeneous infections with either several strains of a specific bacterial species or a group of different bacteria.[55–59] Consequently, the potential to transfer genetic material to other bacterial strains or other bacterial species that have similar bacteriophage attachment receptors is apparent.[60] An example of interspecies cross-reactivity of bacteriophages is seen with respect to some *S. aureus* bacteriophages having the ability to infect some coagulase-negative *Staphylococcus* with the use of similar teichoic acid receptors.[60] In addition, bacteriophage–bacteria interactions are not always beneficial but, rather, in some biofilm infections, bacteriophages can cause thickening of biofilms, thereby making them harder to treat. This can be seen with the activation of filamentous bacteriophages in *Pseudomonas* biofilm infections and with low titers of staphylococcal bacteriophages in *S. aureus* biofilm infections.[61,62]

Based on recent clinical trials of bacteriophages, clinicians must acknowledge that at this early stage using bacteriophages as adjuvants and not as stand-alone treatments is prudent. In correlation, mitigating the risk of gene transfer in vivo is also associated with using bacteriophages with approaches that allow for these therapeutics to have the most potentially effective outcomes, which involves using them as adjuvants. While multiplicity of infection (MOI) is a parameter that is impossible to calculate in vivo, it has been shown to have in vitro implications with respect to rates of gene transfer.[18,63] Consequently, it has been theorized that the greatest ability of bacteriophages to transfer genetic material occurs when MOIs are between 1×10^{-2} and 1.[63] This is secondary to the "perfect" parameters for chance encounters of bacteria and bacteriophages to interact and therefore potentially transfer genetic material. At higher MOIs, bacteriophages outnumber bacteria and consequently bacteriophages with horizontal gene transfer potential have a lower chance of interacting and binding to a bacterium, given the lower numbers of bacteria compared to bacteriophages. At very low MOIs, lower levels of genetic transfer also occur secondary to bacteria increasing in numbers more rapidly than bacteriophages can lyse them, creating evolutionary pressures to select for bacteria that are resistant to bacteriophages or outcompeted.

Therefore, there are limited data supporting the effectiveness of using bacteriophages in a similar way to conventional antibiotics, with prolonged intravenous durations.[10] This may be due to the extensive hepatic and urinary clearance, neutralizing antibody production and development of resistance seen with intravenous dosing.[10] In addition, these prolonged intravenous dosing protocols may predispose patients to horizontal gene transfer. However, when using bacteriophage therapeutics after surgical debridement or with direct instilment to sites of infection, this creates theoretically high MOIs, allowing for the potential eradication of clinical infections while also reducing gene transfer in vivo. Consequently, treatment protocols should be devised based on multidisciplinary approaches, as seen with the treatment of prosthetic joint infections, in which direct application of bacteriophage therapy to infected joints has been advocated to be used with surgical debridement surgery in addition to standard-of-care antibiotics.[1,7] These multidisciplinary approaches will not only allow for the best clinical outcomes but also reduce the chance of gene transfer.

Conclusions

Bacteriophage therapy is a promising adjuvant agent in the treatment of numerous infectious syndromes, but we lack robust knowledge to effectively use these therapeutics at the present time. Moreover, current evaluations of bacteriophage therapeutics are limited to assessing sterility and endotoxin levels, but further evaluations and testing are needed, for the reasons discussed here. This is especially the case given the potential of bacteriophage therapeutics not only to transfer genetic material but also to harbor other agents beyond endotoxins. Consequently, clinicians should not be passive providers when administering experimental therapies, but rather should have knowledge of the therapeutics they are administering to thereby have robust discussions about informed consent. This is important given that the testing and mitigation strategies that are discussed here are not arduous to conduct. These mitigation strategies include propagating bacteriophages on bacterial hosts that lack the capacity to transmit exotoxins, prophages and pathogenicity islands, as well as using bacteriophage therapeutics in ways that reduce potential horizontal gene transfer. These are not theoretical issues but, rather, regulatory agencies are starting to advocate for their evaluation. Therefore, clinicians and translational scientists should be aware of these topics to reduce the potential risks when using bacteriophage therapeutics clinically.

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