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Opinion

Evolutionary Rationale for Phages as Complements of Antibiotics

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Antibiotic-resistant bacterial infections are a major concern to public health. Phage therapy has been proposed as a promising alternative to antibiotics, but an increasing number of studies suggest that both of these antimicrobial agents in combination are more effective in controlling pathogenic bacteria than either alone. We advocate the use of phages in combination with antibiotics and present the evolutionary basis for our claim. In addition, we identify compelling challenges for the realistic application of phage–antibiotic combined therapy.

New Therapeutic Strategies Are Needed

Infections by antibiotic-resistant pathogens are a serious concern. Conventional methods for finding novel antibiotics are inadequate, with the staggering result being that mortality due to antibiotic resistance is estimated to be about 25 000 persons per year in both the USA and Europe according to the Centers for Disease Control and Prevention (CDC) and its European counterpart the ECDC [1,2]. In many developing countries antibiotic resistance is also a major cause of mortality, and many procedures to control resistance are unfeasible [3,4]. Despite this situation, we continue the search for the Ehrlichian 'magic bullet' that will be exempt from the evolution of bacterial resistance and the latter's spread through horizontal gene transfer to other bacteria, be they pathogenic, commensal, or mutually beneficial. Recent research on antibiotic discovery [5] and the isolation and elucidation of potent DNA gyrase inhibitors [6,7] present new opportunities to control the evolution of bacterial resistance and not make the same errors of the past. But can the potency of existing antibiotics be maintained and the pristine status of future discoveries be conserved? Unless the employment of new antibiotics is carefully managed, bacteria will inevitably evolve resistance [8].

Evolutionary-rational approaches are currently lacking in antibiotic management and yet have shown considerable potential in application to other diseases. Specifically, combination therapies have an impressive track record in the treatment of diverse illnesses such as cancer, malaria, or HIV, even if certain aggressive therapies are questionable [9]. For example, combinations of general cellular proliferation inhibitors together with more targeted therapies represent important advances in the treatment of many cancers [10]. Likewise, antiretroviral therapies targeting different steps in the HIV life cycle are remarkably successful in reducing viremia and improving patient health [11]. Optimized therapies such as these are derived from advances in the understanding of the biology and pathogenesis of each disease, and knowledge about mechanisms of sensitivity and resistance to rational drug combinations.

Antibiotic cocktails and combinations with other molecules, such as antimicrobial peptides, are promising alternatives, but may ultimately suffer from some of the same shortcomings as single molecules [12]. By contrast, bacterial viruses (phages) have considerable untapped potential as a complement to antibiotics, not only due to a range of intrinsic differences in their mechanisms

Trends

The efficacy of new and old antibiotics could be preserved if combined with phages.

Positive interactions have been observed between antibiotics and lytic phages in controlling bacterial pathogens both *in vitro* and *in vivo*.

Phage–antibiotic combinations are capable of targeting multidrug-resistant bacteria but their underlying mechanisms remain to be discovered.

Evolutionary biology provides a framework for understanding the interactions between antimicrobial agents and the successful management of bacterial pathogens, their resistance, and their virulence.

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of action, but also because of the virtually infinite diversity of phages, their potential to be rapidly 'trained' (through serial passages on the ancestral bacterial strain), and their ability to evolve *in situ* to overcome bacterial resistance [13]. The treatment of bacterial infections could benefit from our extensive knowledge of the genetics and evolution of antibiotic resistance, coupled with a promising alternative therapeutic agent that could act as a powerful enhancer. In this opinion article we present the multiple advantages of combining these antimicrobials compared to using either independently. We argue that combining phages and antibiotics should be seriously considered as a therapeutic solution to antibiotic-resistant infections, and we provide detailed evolutionary arguments that justify our claim.

Phage Therapy and Hurdles to Its Use

The origins and employment of phage therapy date back at least a century to Felix d'Herelle and others [14]. Though not extensively adopted, phage preparations were produced by the Pasteur Institute in France until 1974 and in the USA until the 1990s [15]. Phage therapeutic products have continuously been used in Eastern European countries, notably in the Republic of Georgia, but solid interest slowed outside of the former Soviet Union with the discovery of antibiotics [14]. With decreasing antibiotic discovery and increasing multidrug-resistant bacteria, phages are being reconsidered as alternative therapies for certain types of bacterial pathogen. Phages have received particular attention as substitutes for antibiotics in food safety, agricultural, and farming settings to contain the spread of antibiotic-resistant 'superbug' bacteria [16]. Poultry, dogs, dairy products, and processed foods are some examples of the current successful employment of phage therapy (e.g., [17]). For example, in the United States, the FDA has approved commercial phage preparations against common bacterial pathogens such as *Listeria monocytogenes* and *Salmonella* to treat ready-to-eat food [18,19].

Leading amongst the factors that explain limited phage employment are the often variable and poorly understood results on their efficacy and their high specificity compared to general-spectrum antibiotics [14,20]. Phage therapy requires an accurate identification of the bacterial pathogen and *in vitro* examination of its sensitivity to the available phages [15,20]. However, perhaps the most important hurdle is the lack of a specific regulatory framework that considers individualized therapies, or uncertainties for the pharmaceutical industry based on the difficulty to register intellectual patents for phage preparations [20,21]. Moreover, despite the promise that phages could replace antibiotics in certain situations, there are very few controlled large-scale clinical studies on their safety and efficacy. Biocontrol Ltd reported the first regulated efficacy trial of phage therapy targeting chronic otitis caused by antibiotic-resistant *Pseudomonas aeruginosa* in 2009 in the UK, showing significant improvement in patients [22]. Current clinical studies like Phagoburn, a European initiative to evaluate the treatment of drug-resistant infections of burn wounds, or funding calls such as those by the Gates Foundation, highlight phage therapy as a central objective for future research [23] (http://gcgh.grandchallenges.org/challenge/addressing-newborn-and-infant-gut-health-through-bacteriophage-mediated-microbiome).

Despite potential in replacing antibiotics with phages for certain types of bacterial pathogen, there is a middle road that recognizes the potential of phages, but also that antibiotics should not be abandoned. We argue below that in many situations the combined use of phages with antibiotics will result in greater success than either separately, both in terms of treatment success and in curtailing the evolution of resistance. Additionally, the development of highly successful specific pairs or cocktails of antibiotics and phages could attract pharmaceutical interest to the field, avoiding some of the extra regulatory problems and polemics of genetically modified phages [21].

Advantages of Combining Phages and Antibiotics

The logic of combining phages and antibiotics stems from an evolutionary understanding that two sufficiently different selective pressures are likely to be more effective than either alone.

Glossary

Biofilm: group of microorganisms that adhere to each other, frequently embedded within a self-produced extracellular matrix.
Competitive release: occurs when one of two or more species competing for the same resource or exposed to the same stressor disappears, thereby allowing the remaining competitor(s) to utilize the resource and repopulate the community.
Lipopolysaccharide (LPS): major component of the outer membrane of Gram-negative bacteria. Large molecules consisting of a lipid and a polysaccharide composed of an O-antigen, expressed as an outer core and inner core joined by a covalent bond.
Phage–antibiotic synergy (PAS): a phenomenon whereby antibiotics stimulate the production of phages by bacterial hosts under certain conditions.
Persisters: dormant variants of regular cells that form stochastically in microbial populations and are highly tolerant to antibiotics.
Porins: proteins that cross a cellular membrane in bacteria and act as a pore through which molecules such as small metabolites or antibiotics can diffuse.
Quorum sensing: the monitoring of the environment for other bacteria resulting in the coordination of gene expression.
SOS response: general bacterial stress response pathway that is induced by DNA damage caused by a wide range of stressors, including antibiotics. Effects of its activation include increased bacterial survival and antibiotic resistance, prophage activation, or the horizontal transfer of virulence factors.

Table 1. Examples of Studies Showing Antibiotic–Phage Positive Effects against Problematic or Model Bacteria

Bacteria	Phage Family	Antibiotic	Effects	Refs
Burkholderia cepacia	Myoviridae	Meropenem, ciprofloxacin, tetracycline	- PAS[a] - Increased survival of larvae	[40]
Escherichia coli	Not described	Enrofloxacin	- Total protection of birds	[17]
Escherichia coli	Myoviridae	Cefotaxime	- PAS - Eradication of bacterial biofilms	[37]
Klebsiella pneumoniae	Podoviridae (T7-like)	Ciprofloxacin	- Eradication of bacteria - Prevention of resistant variants	[27]
Pseudomonas aeruginosa	Podoviridae	Streptomycin	- Decreased bacterial density - Limited antibiotic resistance	[32]
Pseudomonas aeruginosa	Siphoviridae	Ceftriaxone	- Synergistic reduction of bacterial growth	[41]
Pseudomonas fluorescens	Podoviridae	Kanamycin	- Decreased bacterial survival - Limited antibiotic resistance	[29]
Staphylococcus aureus	Myoviridae	Linezolid	- Stopped MRSA hindpaw foot infection - Decreased bacterial density	[28]
Staphylococcus aureus	Myoviridae	Gentamicin	- Decreased bacterial density - Prevention of phage-resistant variants	[30]

[a]Phage–antibiotic synergy.

Interactions between stressors are additive, antagonistic, and synergistic, respectively, when the combined effect is the sum, less than the sum, and greater than the sum, of the individual effects working in the same direction [24] (see [25] for extended definitions). Although treatment success is more likely for synergistic effects, simple additive ones can also obtain it, particularly in *in vivo* contexts where the host's immune system can be integrated as a third line of control [26]. We now have a wealth of examples of positive interactions between antibiotics and lytic phages in controlling bacterial pathogens both *in vitro* and *in vivo* (Table 1). For instance, phages combined with antibiotics can eradicate strains of *Klebsiella pneumoniae* that form **biofilm** (see Glossary) and arrest the emergence of resistant variants *in vitro*; these combinations also stop methicillin-resistant *Staphylococcus aureus* (MRSA) hindpaw foot infection in diabetic mice, and completely protect chickens from *Escherichia coli* infections in broilers [17,27,28]. In these cases and others, the combination treatment of a lytic phage with an antibiotic resulted not only in a better control or eradication of bacteria, but also in the complete prevention of the emergence of resistant variants, compared to single antimicrobial treatments (Table 1).

Darwinian evolution explains these positive outcomes in three non-mutually exclusive ways (Figure 1, Key Figure). First, bacteria may have genetic constraints, whereby the emergence of resistance mutations to one or both control agents are impeded by costs. An experimental example of this is the lower fitness of *Pseudomonas fluorescens* submitted to antibiotic–phage combinations compared to those evolved in single treatments [29]. Second, a direct negative interaction between mechanisms of resistance can be a powerful constraint on their evolution. For example, the antibiotic-induction of an aggregator phenotype in *S. aureus* increases survival to the antibiotic treatment, but predisposes the cells to phage predation (probably by increasing the numbers of phage receptors) [30]. This could also be the case for *Salmonella* bacteria resistant to phages that employ efflux pumps as receptors: phage resistance leading to decreased pump activity would increase sensitivity to antibiotics [31], although experimental evidence is needed to confirm this interaction. Third, synergy can result purely from low bacterial densities reducing the probability of the emergence of resistance mutations, as shown in the

Key Figure

Synergistic Mechanisms between Antibiotics and Phages

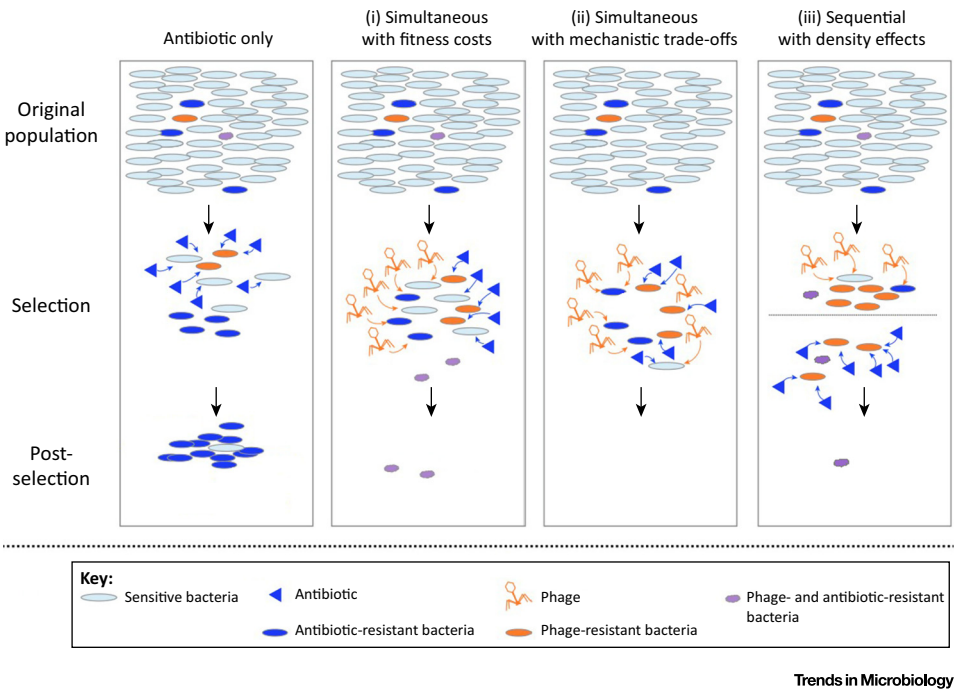

Figure 1. Antibiotic resistance is strongly selected in antibiotic-only therapy (left panel). When phages and antibiotics are combined simultaneously (i) bacteria resistant to both antibiotics and phages may emerge, but grow slowly due to costs and/or are less pathogenic than sensitive bacteria, or (ii) double-resistant bacteria do not emerge due to trade-offs between resistance mechanisms. If phages and antibiotics are applied sequentially then (iii) double mutants are extremely rare or absent in the small populations remaining after the first antimicrobial agent is introduced and do not grow substantially thereafter. In general, bacteria resistant to only one agent are eliminated by the other, and during and following selection the immune system represents a third line of control.

sequential application of antimicrobials to control *P. aeruginosa* [32]. Besides the logic of combining phages and antibiotics, phage therapy benefits from other qualities such as their aforementioned capacities to amplify *in situ* and evolve to overcome potential resistance, restricted host spectrum (e.g., minimizing impacts on the microbiome), the hypothetically unlimited number of phages available as alternatives should certain phages fail, and the assemblage of specific phage cocktails for each bacterial pathogen. Similar to the logic of phage cocktails, cocktails of antibiotics and phages designed and administered following an evolutionary rationale can reduce the development of resistance, especially in multiple bacterial infections, where some pathogenic species or clones could be favored should simple treatments eliminate competitors [33–35]. In summary, evolutionary theory provides a framework for understanding how two antimicrobial agents targeting different cellular processes and involving different resistance mechanisms can act synergistically to decrease bacterial densities and limit the emergence of resistance to either agent.

Targeting Antibiotic-Resistant Bacteria

As described above, combined phage–antibiotic therapies are a promising approach to controlling bacterial pathogens and limiting the evolution of resistance. However, accumulating

research shows that under certain conditions, phage–antibiotic combinations can substantially impact populations of antibiotic-resistant bacteria. A prime example of this is the so-called **phage–antibiotic synergy** (PAS) phenomenon whereby antibiotics may stimulate the bacterial production of phages [36–41]. Of remarkable significance is the finding that sublethal doses of antibiotics produce the PAS effect. This means that regardless of the use of old or new antibiotics targeting multiresistant or naive bacteria, an antibiotic, when combined with phages, can potentially trigger a synergistic effect in reducing bacterial numbers [36,37,39]. The evolutionary reasoning for reducing antibiotic doses in clinical and environmental applications has been discussed elsewhere [42]. Concisely, evidence suggests that aggressive drug strategies maximize the evolutionary advantage of resistant pathogens [9,42,43]. The combination of sublethal doses of antibiotics with phages is a tantalizing alternative that can be both effective at reducing bacterial numbers and contribute to managing antibiotic resistance levels. A potential risk to the use of sublethal antibiotic doses is the activation of bacterial stress-responses, such as the **SOS response**, that could increase survival and resistance to antibiotics (e.g., [44]). Additionally, horizontal dissemination of mobile elements, including virulence factors, and prophage protection against lytic phages are other clinically undesirable side-effects of the activation of the SOS response [45,46]. Further investigation is needed to evaluate this concern, which may be prevented by using only certain types of antibiotics (avoiding, e.g., fluoroquinolones, β-lactams, and aminoglycosides) or combining antibiotics with specifically engineered phages that are able to block stress responses [44,47].

Importantly, Kamal and Dennis recently showed that PAS is not affected by the antibiotic resistance status of the targeted cell [40]. Even for multi-antibiotic-resistant bacteria such as *S. aureus*, *Burkholderia cenocepacia*, or *P. aeruginosa*, several combinations of antibiotic and phages have proven successful [39–41]. Antibiotics belonging to different classes and having different mechanisms of action exhibited PAS when combined with phages (Table 1), compared to the few synergistic combinations reported for antibiotic cocktails. In addition, PAS works not only against bacteria in the planktonic phase but also in protective structures such as biofilms, due to the ability of certain phages to penetrate such structures [35,38]. Some bacterial morphological changes, such as filamentation and clustering, appear to facilitate the process of bacterial lysis resulting in increases in phage plaque size or phage titers [37,39,40]. In short, antibiotics can increase efficacy of phages at eliminating bacterial populations, but the underlying mechanisms of this synergy with antibiotics remain uncertain and should be elucidated for the more refined application of PAS combination therapies (see Outstanding Questions).

Potential Drawbacks of Phage–Antibiotic Combinations

Rational phage–antibiotic combinations would appear impervious to the past failures of antibiotics. Still, the pharmacodynamics of antimicrobial combinations and dosing levels and schedules should also be incorporated into scientific approaches treating bacterial infections [48] (see Outstanding Questions). Evolutionary insights and approaches can help clinical researchers identify both inappropriate antimicrobial combinations and, when two or more are employed sequentially, orderings that amplify selection of highly resistant bacterial populations [49]. Moreover, obviously the use of lysogenic phages, with their limited impacts on bacterial populations and their capacity to transfer antibiotic resistance genes have little potential for phage–antibiotic combined therapies.

We see four possible stumbling blocks to phage–antibiotic combination therapies. First, strong selection for double-resistant variants in combination therapies is a serious concern. Recent work shows this for antibiotic cocktails [43] but, to our knowledge, there is no analogous experimental evidence for combined phage–antibiotic treatments.

Second, phages that preferentially target antibiotic-sensitive variants may promote antibiotic-resistant subpopulations through a phenomenon similar to '**competitive release**', observed in antibiotic combinations [43]. According to this hypothetical scenario, previously antibiotic-treated bacteria can harbor sensitive subpopulations that could be preferentially targeted by phages and thus indirectly favor antibiotic-resistant variants. For example, phages may prefer antibiotic-sensitive active bacteria as hosts rather than **persisters** or bacteria forming antibiotic-resistant biofilms. Extensive work on antibiotic therapies and pathogenesis, and the use of biofilm-degrading phages or phage cocktails, limit this concern [30,35,38].

Third, although screening would eliminate from consideration phage–antibiotic combinations where either agent alone is better than both together, the effect of acceptable combinations may be less than additive, for example, if antibiotics damage hosts in a way that phage infection is aborted or does not produce relevant quantities of offspring or, conversely, if phages block the absorption of the antibiotic by the targeted cell (e.g., *E. coli* phages attaching to **porins** could potentially block the entry of certain antibiotics [50]). On the contrary, the general synergistic phenomenon observed suggests that it is not the case and that phages are able to better infect or produce a more dramatic reduction in bacterial populations when combined with antibiotics. Even in hypothetical combinations where the phage life cycle was partially inhibited by antibiotics or by antibiotic-resistant bacteria, sufficient genetic variation in the phage population could foster adaptation and augmented bacterial control.

Fourth, phages and antibiotics may each independently modulate bacterial virulence, either through plastic responses such as **quorum sensing**, or the competitive release of virulent variants (Box 1). Effects of combined therapy, such as reduced bacterial densities and increased costs, suggest that virulence would likely decrease, although there can be exceptions (Box 1).

Although there is little or no experimental evidence indicating these four phenomena as important obstacles, each will need to be properly evaluated in decisions on whether or not to combine phages and antibiotics in any given treatment.

Box 1. Combined Therapy Effects on Virulence

A potential undesirable consequence of phage–antibiotic therapies is the aggravation of an infection by producing virulent mutant bacterial strains that may worsen patient's outcome if associated with resistance, or be transmitted to other hosts. Although this potential concern is currently unexplored for combined applications, evidence for it from single antimicrobials is conflicting. For example, quorum sensing may coordinate the production of certain virulence factors [51], and exposure to phages or antibiotics individually has been shown to select for cooperative variants with instances of increased virulence [52,53]. However, phages targeting quorum sensing receptors could alleviate this type of interaction and the potential risk of increased virulence [54]. Antibiotic resistance has been often associated with virulence, as in a recent screening associating *Pseudomonas aeruginosa* resistance with a fitness advantage *in vitro* and during *in vivo* infection [55]. But the opposite relationship has also been observed, as in the case of the loss of porins related to both higher antibiotic resistance and attenuated virulence in *Acinetobacter baumannii* [56]. By contrast, a number of studies show that bacteria resistant to lytic phages have attenuated virulence on different hosts, from fish or plants to humans [57–59]. A possible explanation is that the costs of resistance to phages affect the expression of pathogenicity factors or decrease bacterial growth capacity, both resulting in less virulent bacterial variants [57–59].

In combined treatments, antibiotics and phages could doubly target a specific bacterial virulence factor such as components of the cell wall [e.g., combining β-lactam antibiotics and phages that attach to **lipopolysaccharide** (LPS)], reducing the range of potential bacterial variants that are both resistant and virulent. Using antibiotics and phages together could also reduce doses of both antimicrobials, decreasing significantly disease severity, for example, by slowing down the release of bacterial toxins after lysis and suppressing septic shock scenarios. Experimental study is needed to determine how total resistance costs to combined antibiotic–phage therapies and/or density effects result in changes in bacterial virulence. A number of factors specific to bacterial and phage strains are likely to influence this relationship, including molecular mechanisms of virulence and resistance, and environmentally dependent changes in infection and resistance levels or pathways [60].

Concluding Remarks

Antibiotics have been extraordinarily successful at controlling bacterial pathogens. But horizontal gene transfer or *de novo* mutations resulting in increasing numbers of multidrug-resistant bacteria and the diminished discovery of new antimicrobial molecules lead to the unavoidable conclusion that other approaches are now necessary to conserve the action of existing molecules and maintain the high potency of future discoveries. Phages hold considerable potential, but we claim that achieving this will often mean combining them with antibiotics. The synergy observed between antibiotics and phages is a general phenomenon described in many studies, but not yet sufficiently understood and developed to reach actual application. We argue that evolutionary biology provides a framework for understanding control successes and failures of combined therapies, and how and whether we can adapt measures to specific situations. A number of unanswered questions regarding the use of phages as complements to antibiotics provide fertile ground for future research (see Outstanding Questions). Specifically, we need a deeper understanding of the molecular basis for combined antibiotic–phage therapies and in particular the basis for why antibiotic concentrations are so important in mediating outcomes. Our encouraging conclusion is that we have a second chance at controlling bacterial diseases and must avoid making the same mistakes as with the rampant use of antibiotics.

Outstanding Questions

What are the underlying molecular evolutionary mechanisms of the synergistic interaction of antibiotic–phage cocktails?

Can we assess optimum combinations of different types of phages and antibiotics that can then be extrapolated to treat other bacteria?

Which antibiotic doses achieve acceptable or optimal effects when combined with phages?

What is the best timing (sequential, simultaneous) and antimicrobial sequence (first phages then antibiotics or vice versa)?

Can combined antibiotic and phage resistance emerge and what are the consequences for the patient and eventual transmission to other hosts?

What type of scientific evidence is needed on phage therapy to help satisfy safety regulations and legal constraints?

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