European Cells and Materials Vol. 39 2020 (pages 193-210) DOI: 10.22203/eCM.v039a13 ISSN 1473-2262

BACTERIOPHAGE THERAPY AS A TREATMENT STRATEGY FOR ORTHOPAEDIC-DEVICE-RELATED INFECTIONS: WHERE DO WE STAND?

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Abstract

Antibiotic resistance represents a key challenge of the 21st century. Since the pipeline of new antibiotics in development is limited, the introduction of alternative antimicrobial strategies is urgently required.

Bacteriophage therapy, the use of bacterial viruses to selectively kill bacterial pathogens, is re-emerging as a potential strategy to tackle difficult-to-treat and multidrug-resistant pathogens. The last decade has seen a surge in scientific investigation into bacteriophage therapy, including targeting orthopaedic-device-related infections (ODRIs) in several successful case studies. However, pharmacological data, knowledge on the interplay with the immune system and, especially in ODRIs, the optimal local application strategy and treatment outcomes remain scarce.

The present review reports the state-of-the-art in bacteriophage therapy in ODRIs and addresses the hurdles in establishing bacteriophage therapy under good clinical practice guidelines. These hurdles include a lack of data concerning bacteriophage production, processing, administration and dosing, as well as follow-up clinical monitoring reports. To overcome these challenges, an integrated clinical approach is required, supported by comprehensive legislature to enable expansive and correctly implemented clinical trials.

Keywords: Bacteriophage therapy, clinical application, orthopaedic-device-related infections, current evidence.

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List of Abbreviations

AMR	antimicrobial resistance
API	active pharmaceutical ingredient
BAL	Belgian approved laboratory
BMP	bone morphogenetic protein
BREX	bacteriophage exclusion
CFU	colony-forming unit
CRISPR	clustered regularly interspaced short palindromic repeats
E. faecalis	*Enterococcus faecalis*
EMA	European Medicines Agency
FAMHP	Federal Agency for Medicines and Health Products
FDA	Food and Drug Administration
FRI	fracture-related infection
GCP	good clinical practices
GMP	good manufacturing practices
HPMC	hydroxypropyl methylcellulose
MDR-PA	multidrug-resistant *P. aeruginosa*
MOI	multiplicity of infection
MRSA	methicillin-resistant *S. aureus*

MSCRAMMs	microbial surface components recognising adhesive matrix molecules
ODRI	orthopaedic-device-related infection
OM	osteomyelitis
P. aeruginosa	*Pseudomonas aeruginosa*
PFU	plaque-forming unit
PJI	prosthetic joint infection
RCT	randomised controlled trial
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
WHO	World Health Organization

Introduction

An infection is one of the major complications that scientists and clinicians face in the orthopaedic field today. Although the rate of infectious complications after elective orthopaedic surgery remains low (~ 3 %) (Cram *et al.*, 2012), the incidence continues to rise. This is due not only to the annual increase in elective joint replacement surgeries (Kurtz *et al.*, 2012) but also to an increased number of operatively treated fractures (Patel *et al.*, 2015). Moreover, the overall infection rate in musculoskeletal trauma remains high and can rise up to 25-30 % after severe open fractures (Papakostidis *et al.*, 2011).

The role of biofilms in ODRIs

Biofilm formation is broadly acknowledged to be the primary challenge in preventing and treating ODRIs (Nishitani *et al.*, 2015). The ability of bacteria to establish such chronic infections, and the human inability to deal with them, is directly associated with biofilm formation. Indeed, in biofilms an environment is created in which bacteria can grow and persist while being protected against the patient's immune response and from any antimicrobial therapy (Brady *et al.*, 2008). The formation of the biofilm's complex structure is a multi-step process that begins with the initial attachment of bacteria to an implant's surface through one or more cell-wall-associated adhesins or MSCRAMMs, which recognise numerous mammalian structures (Patti *et al.*, 1994). Next, surface-bound bacteria enter a robust proliferation phase and secrete matrix components, including carbohydrate polymers and proteins (Nishitani *et al.*, 2015). Several factors are responsible for antibiotic tolerance within biofilms, including a restricted penetration of antimicrobials into the biofilm, a decreased bacterial growth rate and the expression of possible resistance genes (Costerton *et al.*, 1999; Gilbert *et al.*, 1997). Alone or in combination, these factors explain why biofilm formation leads to persistent infections that are resistant to conventional antimicrobial treatment.

One of the major challenges for the treatment of ODRIs is the reimplantation of the device, which in most cases is required for the patient's functionality. During the explantation of the contaminated device and radical debridement, it is very difficult to completely eradicate or resect all biofilm-residing microorganisms from the infected area. In fact, the biofilm may be harboured on tiny fragments of necrotic bone, known as sequesters, and may also reside within the cortical bone itself (de Mesy Bentley *et al.*, 2018). During reimplantation, the biofilm-residing bacteria may be liberated and re-enter their planktonic growth phase, resulting in reinfection (Moriarty *et al.*, 2016).

Treatment difficulties and current challenges

As stated by the WHO, AMR is one of the biggest threats to global health today (WHO, 2015). Pathogens such as MRSA and MDR-PA are emerging as a significant threat in both the hospital and community environment (Boucher and Corey, 2008; van Duin and Paterson, 2016). Within the healthcare setting alone, for example, MRSA infections are estimated to affect more than 150,000 patients annually in the EU, resulting in additional in-hospital costs of €380 million for the EU healthcare systems (Kock *et al.*, 2010). Even with an adequate treatment, MRSA infections are still associated with a higher mortality and increased financial costs relative to sensitive equivalents (Haddadin *et al.*, 2002; Moriarty *et al.*, 2016; Salgado *et al.*, 2007; Teterycz *et al.*, 2010). With respect to ODRIs, the most important resistance mechanisms are those that confer tolerance to anti-biofilm antibiotics (*i.e.* rifampicin for Gram-positive bacteria or fluoroquinolones for Gram-negative bacteria). In cases where no biofilm-active antibiotic is available, amputation or life-long antibiotic suppression therapy is often the only option (Moriarty *et al.*, 2016).

For the above-mentioned reasons, the WHO invests and promotes research and development on AMR (WHO, 2015). While there are some new antibiotics in development, the numbers are insufficient to address the present needs and the financial return on investment discourages further development, particularly considering the potential restrictions on the use of any new antibiotics and the risk of rapid development of resistance. Therefore, scientists are focusing on other antimicrobial strategies and one of these is bacteriophage therapy.

Bacteriophages as novel therapeutics

Advantages of bacteriophage therapy

(Bacterio)phages are viruses that are highly specific, as they often infect only a subset of strains within a bacterial species. They infect bacteria by binding to receptors on the bacterial cell surface and injecting their genetic material into the cell. These receptors are strain-specific and can consist of proteins on the bacterial cell wall, teichoic acids, *etc.*. Then, phages can either hijack the bacterial metabolism, replicate intracellularly and, finally, lyse the host bacterium by which phage progeny are released (known as the

lytic life cycle; Fig. 1) or enter a dormant state within the bacterial cell (known as the lysogenic life cycle). For this reason, with respect to phage therapy, where the goal is to eradicate the pathogenic bacteria, only strictly lytic phages are considered to be adequate. The majority (96 %) of all phages are tailed and belong to the order of the *Caudovirales*. There are three main families of tailed phages: the *Myoviridae* with contractile tails (25 %), the *Siphoviridae* with long, non-contractile tails (61 %) and the *Podoviridae* with short tails (14 %) (Ackermann and Wegrzyn, 2014). Staphylococcal phages used for phage therapy are virulent (strictly lytic) phages, mainly belonging to the family of *Myoviridae* or *Podoviridae* (Deghorain and Van Melderen, 2012). An example of a strictly lytic staphylococcal phage belonging to the *Myoviridae* is phage Remus (Vandersteegen *et al.*, 2013), of which an electron microscopic image is displayed in Fig. 2.

Almost immediately after their discovery in the early 1900s, phages were applied as an antimicrobial therapy to combat bacterial infections (*i.e.* dysentery and cholera) in humans (Summers, 2001). However, due to the discovery of penicillin at the start of World War II, phage therapy was replaced with antibiotic treatment in most parts of the western world. In contrast, phage therapy continued to be applied within the Soviet Union and Eastern Europe (Abedon *et al.*, 2011). To date, in countries including Georgia, Poland and Russia, phage products remain directly available to the general public without prescription as the standard of care for bacterial infections. For instance, the Intesti phage cocktail from the Eliava Institute (Tbilisi, Georgia) targets about twenty different gastrointestinal pathogens (Zschach *et al.*, 2015). Their Pyo bacteriophage cocktail, on the other hand, contains phages targeting *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Proteus* species and *Escherichia coli* (*E. coli*) and is routinely applied for various purulent skin or wound infections (Abedon *et al.*, 2011).

Because of the emergence of antibiotic-resistant strains during the last decades, the therapeutic use of (strictly lytic) phages has seen a renewed interest in western medicine (Cisek *et al.*, 2017). A dramatic, clinical example in which the power of phage therapy is demonstrated is the successful treatment of a 15-year-old post-transplant cystic fibrosis patient who suffered from a drug-resistant *Mycobacterium* infection and was treated palliatively with chronic antibiotics. The *Mycobacterium* infection was eradicated after long-term phage therapy using a cocktail of phages, one of which was engineered to optimise infection and kill bacteria (Dedrick *et al.*, 2019). Indeed, phages have several important properties that contribute to their therapeutic potential. First, phages can self-amplify, which is an asset that contributes to their

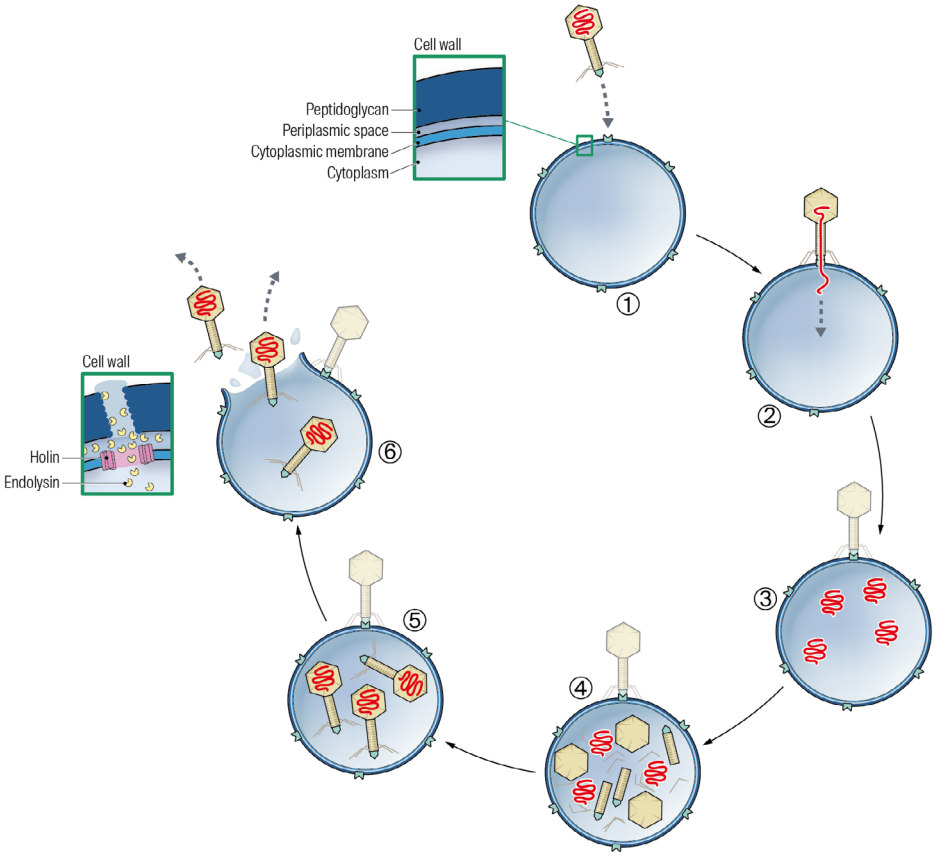

Fig. 1. The lytic infection cycle of bacteriophages. 1) A phage recognises a pattern on the bacterial cell wall and attaches; 2) after attachment to the cell wall, the phage injects its DNA; 3) the replication machinery of the bacteria is hijacked: the viral (phage) DNA is replicated; 4) the bacterial cell begins producing virion proteins; 5) phage proteins are assembled within the cell; 5) bacterial cell lysis is mediated by holin-endolysin interaction and phage progeny is released, being able to infect other bacteria.

efficacy and distinguishes them from conventional antimicrobials (Kutter *et al.*, 2010). Second, some phages display polysaccharide depolymerases on their tail structures, which can act as an adjuvant to phage infection by degrading the extracellular matrix of biofilm-associated bacteria (Pires *et al.*, 2016). Third, phages are considered to be safe as human tissue and normal human bacterial flora are not negatively affected, which can be attributed to their high specificity (they often infect just a subset of strains within a single species) and quick inactivation and clearance once their host is no longer present (Abedon and Thomas-Abedon, 2010; Curtright and Abedon, 2011; Kutter *et al.*, 2010). This also implies that for each new bacterial strain, a specific phage may need to be found (Payne and Jansen, 2003). Luckily, due to their abundant presence in nature, phages can be easily isolated and readily characterised (Chan *et al.*, 2013). Finally, the modes of action of phages differ from those of antibiotics, so they are usually not affected by bacterial antibiotic-resistance mechanisms (Loc-Carrillo and Abedon, 2011), which is the main reason for the increased interest in phage therapy in parallel to the increase in antibiotic resistance seen in recent decades.

Bacteriophage endolysins

Based on the mechanism of action of lytic phages (Fig. 1), another potentially interesting antimicrobial strategy is the use of phage-derived proteins (Czaplewski *et al.*, 2016). Endolysins, or phage lysins, are peptidoglycan hydrolases that are used by lytic phages towards the end of the lytic cycle to break down the peptidoglycan layer of the host bacterium so that the phage progeny can be released. Recombinant purified endolysin can be applied exogenously to eradicate susceptible Gram-positive bacteria, as reported in several *in vitro* and preclinical studies (Haddad Kashani *et al.*, 2018). Currently, there is one endolysin (CF-301) that is being tested in human patients suffering from bacteraemia caused by *S. aureus*. Based on the results from previous trials, the FDA has recently approved a phase 3 RCT to assess the efficacy and safety of this endolysin *versus* standard-of-care antibiotics (Web ref. 1). However, for Gram-negative infections, only few phage endolysins have the ability to pass the outer membrane and break down the peptidoglycan layer (Briers and Lavigne, 2015). In this regard, promising results were obtained with the engineered endolysin-based Artilysins, which are endolysins recombinantly fused to an outer membrane permeabilising peptide (Defraine *et al.*, 2016; Schirmeier *et al.*, 2018). Further progress in the (pre)clinical analysis of endolysins and Artilysins is expected in the upcoming years (Briers and Lavigne, 2015). To the authors' knowledge, these endolysins have not yet been applied in musculoskeletal infection settings. Therefore, the present review will focus on the treatment and prevention of ODRIs using bacteriophages.

Pharmacology of bacteriophages

Pharmacology, the science of drugs, can be subdivided into pharmacodynamics and pharmacokinetics. Both are to be extensively evaluated before an antimicrobial agent or any other drug becomes available for physicians to prescribe. Even though phage therapy is not a novel technique, evidence on its pharmacological profiles is rather limited (Ryan *et al.*, 2011). This section summarises the available knowledge on pharmacodynamics and pharmacokinetics with respect to phages.

Pharmacodynamics is the study of the drug's influence on the organism, body or tissue. It entails the relationship between the drug's efficacy (*e.g.* bacterial eradication, duration of action, *etc.*) and toxicity (*i.e.* side effects). Unlike the metabolic degradation of certain antibiotics, the degradation of phage virions does not lead to toxic by-products, as phages mainly consist of protein and DNA. These phage constituents generally do not elicit immunological adverse reactions, as long as preparations are correctly purified and possible contaminants of the host bacteria such as endotoxins are eradicated

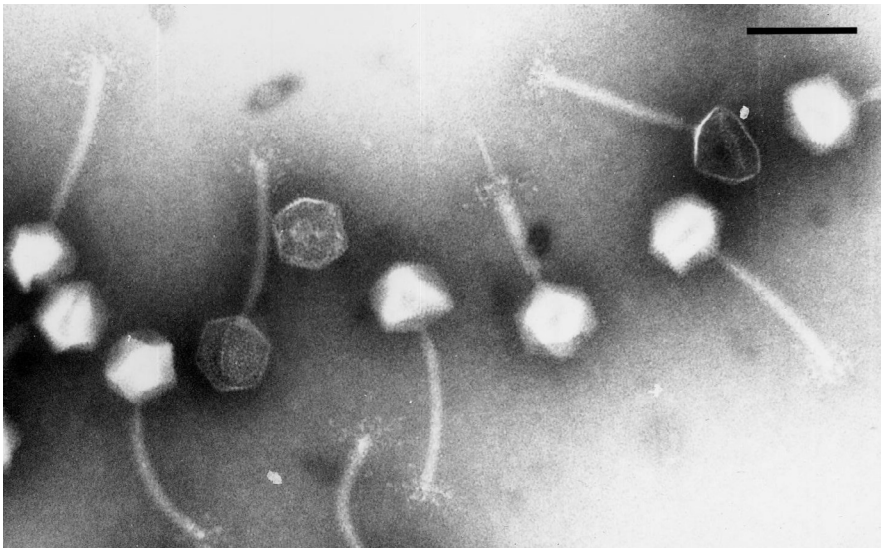

Fig.2. Transmission electron microscopic image of the staphylococcal phage Remus (*Myoviridae*) negatively stained with 2 % (w/v) potassium phosphotungstate (pH 7.0). Scale bar: 100 nm.

(Dufour *et al.*, 2017; Loc-Carrillo and Abedon, 2011; Van Belleghem *et al.*, 2017). The interaction of phages with the human immune system is further discussed below.

Phages display single-hit kinetics which implies that a single adsorption (or infection) of a phage to a bacterium is sufficient to mediate bacterial killing (Abedon and Thomas-Abedon, 2010). However, in clinical practice, where large numbers of bacteria are to be eradicated, a higher ratio of adsorbed phages to bacteria may be required. The exact concentration at which phage therapy shows optimal efficacy depends on several factors. First, it is important to know the difference between active and passive phage therapy. Primary adsorption, infection of bacteria and subsequent bacterial lysis is referred to as passive phage therapy. The secondary adsorption and infection of the phage progeny (*i.e.* the phages that are released after self-replication within the bacterial host) is referred to as active phage therapy (Abedon and Thomas-Abedon, 2010; Curtright and Abedon, 2011; Payne and Jansen, 2003). If the initially applied concentration of phages is high enough, then primary infection can eradicate a sufficient number of bacteria. Of course, this is the most optimal situation so that the therapy success does not depend on the ability of the phages to self-amplify. A general rule-of-thumb for phage therapy is to obtain and maintain a ratio of adsorbed phages to bacteria (or MOI) of 10 (Abedon and Thomas-Abedon, 2010). If for some reason it is expected that phages will have difficulty to penetrate their target bacteria or that they will have a high chance of early decay (*e.g.* by immune-mediated neutralisation), it may be necessary to employ either multiple or continuous dosing or higher phage densities to obtain the same ratio. Active phage therapy can also reach the required ratio, but a sufficient number of bacteria should be present to give rise to a sufficient phage progeny to infect the residual bacteria (Abedon and Thomas-Abedon, 2010).

Pharmacokinetics studies the effects of the organism, body or tissue on the drug's activity and thereby on reaching and sustaining effective drug concentrations at the target sites. Specifically, for phage therapy, the following stages should be considered in the pharmacokinetic profile: absorption, distribution, penetration, adsorption, infection, lysis and phage release (Abedon and Thomas-Abedon, 2010). Absorption and distribution are generally not of importance for local application of phage therapy, which is often required for an ODRI (Onsea *et al.*, 2019; Ryan *et al.*, 2011). For systemic therapy, the persistence of phages depends on the efficacy of the reticuloendothelial system clearance and potentially on the induction of phage-specific antibodies that may cause phage inactivation (Maciejewska *et al.*, 2018). As discussed above, this may be tackled by applying multiple doses and using phage cocktails to prevent cross-linking of anti-phage antibodies (Curtright and Abedon, 2011; Maciejewska *et al.*, 2018). Penetration refers to the ability of phages to penetrate a biofilm (*e.g.* mediated by extracellular polysaccharide depolymerase enzymes). After penetration, the phage can adhere to the surface of the host bacteria and subsequently infect and lyse it, which are referred to as adsorption, infection and lysis, respectively. In the case of an active therapy, phage release follows bacterial lysis (Abedon and Thomas-Abedon, 2010).

In general, it should be clear that the main challenge remains the understanding of the dynamic of phage titers *in vivo*. The involved parameters, including bacterial and phage microbiological traits and individual immunological response, are all interdependent and incompletely understood at the present time.

Interactions with the human immune system

The clearance of phages by the immune system may affect the efficacy of phage therapy (Dabrowska and Abedon, 2019; Maciejewska *et al.*, 2018). Since phages are encountered on a daily basis (*e.g.* through various foods), low titers of phage-specific antibodies are common in patients, but titers may increase during phage therapy. The induction of the innate immune system, that clears phages through phagocytosis (*i.e.* the reticuloendothelial system), as well as of the adaptive immune system by the production of phage-neutralising antibodies, has been associated with early depletion of phages and subsequent impairment of efficacy (Cisek *et al.*, 2017; Malik *et al.*, 2017). For oral and topical applications, this seems to be less of an issue when compared to systemic application. However, it might still be necessary to compensate for this phenomenon by repeating phage administration, increasing phage concentration or using different phages or a phage cocktail (Cisek *et al.*, 2017; Hodyra-Stefaniak *et al.*, 2015; Maciejewska *et al.*, 2018; Malik *et al.*, 2017). Controversially, there are recent studies that do acknowledge the interaction between phages and the human immune system, but state that this does not impact the outcome of phage therapy (Lusiak-Szelachowska *et al.*, 2014; Lusiak-Szelachowska *et al.*, 2017; Zaczek *et al.*, 2016). Furthermore, the stimulation of the immune system by phages may even be required to obtain a good treatment outcome (Roach *et al.*, 2017). Further exploration of this topic is needed to elucidate the role of the human immune response in phage therapy.

Although no serious clinical immunological complications of phage therapy have been reported to date (Maciejewska *et al.*, 2018), an issue that may arise is that of an indirect immunogenicity by means of cell lysis. When looking at the mechanism of action of lytic phages as well as that of some antibiotics such as penicillins, bacteria are lysed and bacterial cell wall components are released. In clinical cases where patients are expected to suffer from high-inoculum Gram-negative infections, this endotoxin release might lead to clinical deterioration and septic shock (Abedon and Thomas-Abedon, 2010; Dufour *et*

al., 2017; Goodridge, 2010). However, the relevance of this phage-related endotoxin-release depends on the clinical situation of the patient. An endotoxin-release can also be caused by antibiotics that target the bacterial cell wall (*i.e.* β-lactam antibiotics) and this is generally due to systemic infections that are already life-threatening (Abedon and Thomas-Abedon, 2010; Dufour *et al.*, 2017). It should be clear that the general interaction between bacteria, phages and the human immune system represents an intertwined triangle which remains difficult to model/predict accurately (Van Belleghem *et al.*, 2018).

Resistance patterns

Bacteria and phages have co-evolved for billions of years. Phage-resistant strains arise and protect the bacterial lineage while on the other side of the spectrum counter-resistant phages arise that can again threaten these strains (Labrie *et al.*, 2010). This co-evolution seems to be an important driver of ecological and evolutionary processes in microbial communities (Labrie *et al.*, 2010; Rohde *et al.*, 2018a; Torres-Barcelo, 2018). The frequency at which phage resistance complicates phage therapy is not clear, although Rohde *et al.* (2018a) reported resistance rates varying from 17 % (to *Staphylococcus* phages) to 85 % (to *E. coli* phages). Bacterial resistance mechanisms can be developed against a phage at almost every stage of its life cycle. Phage resistance mechanisms can be classified into prevention of phage adsorption, prevention of phage DNA entry, cutting of phage nucleic acids and abortive infection systems (Labrie *et al.*, 2010; Maciejewska *et al.*, 2018). The most common form of phage resistance is prevention of phage adsorption by point mutations and/or changes in the expression of receptor-encoding genes, thereby adapting the structure or conformation of bacterial cell surface receptors (Maciejewska *et al.*, 2018). It is interesting that phage resistance through these mechanisms can emerge rapidly, but often goes with a cost of losing bacterial virulence (Levin and Bull, 2004; Maciejewska *et al.*, 2018; Rohde *et al.*, 2018a). That is, the bacterial surface molecules that are involved in phage-bacteria interaction often consist of surface proteins, surface glycans and glycoconjugates such as capsules and lipopolysaccharides. A change in these components could result in reduced virulence and renewed susceptibility to the host's immune system (Levin and Bull, 2004; Maciejewska *et al.*, 2018). Apart from changing the surface receptors, resistance can also be achieved by the production of extracellular matrix, an important constituent of biofilm, which provides a physical barrier between the phages and their receptors. Some phages have evolved to recognise the polymers that comprise the extracellular matrix and degrade them. Other bacteria prevent phage DNA entry by using proteins [*i.e.* superinfection exclusion (Sie) systems] (Labrie *et al.*, 2010; Maciejewska *et al.*, 2018). Recently, a lot of research has been done on resistance mechanisms that interfere with phage DNA introduction in bacteria. Important resistance mechanisms are restriction-modification systems that degrade phage DNA, while the bacterial host DNA is protected (Ofir *et al.*, 2018). Although the molecular mechanisms are still unclear, the CRISPR/Cas system has been found to function as a bacterial acquired immune system that memorises viral genetic material to target future infection attempts (Labrie *et al.*, 2010). The phage exclusion system (BREX) and the prokaryotic Argonaute variants were discovered more recently to act as a barrier for the uptake and replication of foreign DNA (Goldfarb *et al.*, 2015; Swarts *et al.*, 2014). The last resort of bacterial resistance mechanisms is an abortive infection system. This system leads to the death of the infected host bacteria, thereby preventing phage amplification and further infection of other bacteria (Labrie *et al.*, 2010). An important property of the continuous arms race between phages and bacteria is the emergence of phage strategies to counteract or circumvent these resistance mechanisms (Bondy-Denomy *et al.*, 2013). For instance, phages can modify the course of their life cycle (*e.g.* by adjusting burst size, lysis time, *etc.*) (Torres-Barcelo, 2018) and can encode protein inhibitors of CRISPR-Cas systems (*i.e.* anti-CRISPRs) (Stanley *et al.*, 2019). Furthermore, mutations in phage receptor binding proteins can arise and phages can recombine with other viruses (Torres-Barcelo, 2018).

The emergence of phage-resistant strains is a natural consequence of co-evolution. Before setting up a phage therapy regimen, measures should be taken to compensate for resistance. Phage cocktails should be applied that cover a broad host range and target highly conserved structures essential for bacterial survival or virulence (Rohde *et al.*, 2018a).

Pre-clinical evidence

In vitro studies

The most prevalent Gram-positive bacterial species in ODRIs are *S. aureus* (33-43 %), *S. epidermidis* (18-40 %) and *Enterococcus* species (2.5-15 %, mainly *E. faecalis*), while Gram-negative bacilli, including *E. coli* and *P. aeruginosa*, are less frequently isolated (4-7 %) (Arciola *et al.*, 2018; Barros *et al.*, 2019; Cremet *et al.*, 2012; Landraud *et al.*, 2013; Tande and Patel, 2014). *In vitro* studies proving the efficacy of phages against strains isolated from ODRIs are not that numerous.

In 2014, Kaur *et al.* (2014) reported that phage MR-5 could kill *S. aureus in vitro* on Kirschner wires in the presence of linezolid. Coatings that combined both MR-5 and antibiotics (impregnated in hydrogel) prevented implant colonisation with a maximum reduction in bacterial adherence of about 4 $\log_{10}$ units after 48 h. This result was superior when compared to coatings including only MR-5 or linezolid. Moreover, the emergence of resistant mutants was negligible in the presence of both agents, proving the potential of

phage-mediated therapy in these types of infections (Kaur *et al.*, 2014).

Morris *et al*. (2019) screened thirty *S. aureus* strains isolated from patients undergoing total knee or hip arthroplasty for their susceptibility to a set of 31 *S. aureus*-specific phages. Five of these demonstrated activity against more than 90 % of the tested strains and were combined in a StaPhage cocktail. Then, this cocktail was assessed for its bactericidal activity towards both planktonic and biofilm-associated *S. aureus* strains. Planktonic growth was reduced in 8 h-cultures by more than 98 % after treatment with StaPhage, compared to untreated samples. Moreover, viable cells within 48 h-old biofilms, formed on three-dimensional-printed porous titanium scaffolds, were only slightly but significantly reduced for one of the tested strains (from 6.8 $\log_{10}$ to 6.2 $\log_{10}$) after a 48 h treatment with the StaPhage cocktail. On the other hand, the cefazolin control, frequently used as a prophylactic antibiotic, did not show any reduction (100× the minimal inhibitory concentration of cefazolin was applied). The other tested strain did not display any reduction after treatment. These data suggested that, at least for the mentioned phage, biofilms can not only display greater resistance to antibiotic agents but also to phages. The authors concluded that not only the administered concentration, frequency of dosing and administration route, but also the use of customised cocktails to a given patient-specific strain are critical for further *in vivo* studies (Morris *et al.*, 2019).

Barros *et al*. (2019) reported the isolation of nineteen clinical strains, among which six *S. aureus*, seven *E. faecalis* and two *E. coli* strains, all isolated from ODRIs in a Portuguese hospital and resistant to at least one antibiotic in three or more antimicrobial classes. The authors examined the *in vitro* efficacy of several phages, of which phage LM12 was able to lyse 91 % of all tested *S. aureus* strains, phage LM99 was able to lyse 64 % of all tested *E. faecalis* strains and phage JB75 was able to lyse 55 % of all tested *E. coli* strains. *In vitro,* the three lytic phages presented good therapeutic potential, displaying rapid infection cycles with large burst sizes, a high specificity and good tolerance to thermal and pH changes. Based on their data, the authors concluded that phages LM12, LM99 and JB75 could be suitable to treat ODRIs.

While relatively few *in vitro* studies have described phage activity against ODRI-associated strains, a far larger number of studies have investigated the ability of phages to inhibit biofilm formation. For example, phage K was shown to prevent biofilm formation of *S. aureus* grown on silicone disks for 24 h (Lungren *et al.*, 2013). Addition of 2×10^8 PFU phage K for 24 h resulted in a significant reduction of average *S. aureus* counts from 6.3×10^5 CFU to 6.7×10^1 CFU, demonstrating the enormous *in vitro* potential of this phage. Another study by Alves *et al*. (2014) demonstrated that a combination of phage K and DRA88 (a broad host range phage) at an MOI of 10 also effectively reduced *S. aureus* biofilm biomass (48 h old biofilms) within 48 h. A study by Yuan *et al.* (2019) revealed that the lytic phage vB_PaeM_LS1 showed a high potential impact upon the prevention of biofilm formation by *P. aeruginosa*. By scanning electron microscopy, they showed that phage LS1 was able to disrupt a 48 h-old biofilm (grown under static conditions) in 8 h. Counting of bacterial colonies indicated a reduction of about 99.7 % when compared to the control biofilm. At 24 h, regrowth was observed, due to the emergence of phage-resistant colonies.

In vivo studies

S. aureus is the most often applied Gram-positive pathogen in *in vivo* studies (Matsuzaki *et al.*, 2014). *P. aeruginosa* is the most often applied Gram-negative pathogen. Published studies primarily focus on pulmonary infections followed by gastrointestinal infections, septicaemia, urinary tract infections, wound infections and meningitis (Matsuzaki *et al.*, 2014).

Regarding ODRIs, there are few studies available (Table 1). Kaur *et al.* (2016) investigated the benefits of the synergism between antibiotics and phages for the prevention of ODRIs caused by MRSA. A coated Kirschner wire was implanted in the mouse femur and the joint space was inoculated with MRSA. Compared to single coated or naked implants, the use of a dual coated implant was more effective in reducing bacterial adherence and, therefore, protecting the implant from infection (Kaur *et al.*, 2016). Wroe *et al.* (2019) evaluated the application of an injectable phage-loaded hydrogel as a prevention measure for FRIs. The hydrogel was loaded with phages and bacteria for delivery within a perforated polyimide sleeve, which was fitted over the segmental defect in the murine radius. The phage-loaded hydrogel succeeded in significantly reducing the bacterial load recovered after euthanasia. However, as this was a proof-of-concept study, the phage-treated group was only compared to the positive control group, which received no preventive antibacterial treatment (*i.e.* no antibiotic prophylaxis, which is the standard of care in this setting) (Wroe *et al.*, 2019).

Regarding the effect of phages in a therapy setting for ODRIs, few studies are available. In the study by Yilmaz *et al*. (2013), phages were applied locally in a rat model of PJI. The tibiae of the rats were inoculated with MRSA or *P. aeruginosa* and a plastic intravenous catheter with an established biofilm, caused by the respective pathogens, was placed inside the intramedullary canal. Regardless of the pathogen that was applied, the best treatment results were obtained with a combination of antibiotics and phages. In the MRSA group, biofilm dispersion could only be achieved with a combination treatment (Yilmaz *et al.*, 2013). It should be noted that the study set up was not entirely representative of an ODRI as the authors used a plastic catheter sheath with an already established biofilm as an implant rather than metallic fracture fixation materials or prostheses (Moojen, 2013). Kishor *et al.* (2016) investigated the

Table 1. *In vivo* studies investigating the application of bacteriophages for the prevention or treatment of ODRIs. PJI: prosthetic joint infection; FRI: fracture-related infection; HPMC: hydroxypropyl methylcellulose; BMP: bone morphogenetic protein; CRISPR: clustered regularly interspaced short palindromic repeats.

Prevention studies					
Study	**Model**	**Study design**	**Pathogen**	**Phage**	**Outcome**
Kaur *et al.*, 2016	Murine model of PJI	Coated implant 5 groups: - coated with phage in HPMC - coated with antibiotic in HPMC - coated with phage and antibiotic in HPMC - coated with HPMC - negative control (naked implant)	MRSA	MR-5	Dual coating resulted in maximum reduction in bacterial load, associated joint inflammation and faster functional recovery of the limb
Wroe *et al.*, 2019	Murine model of FRI	2 groups: - BMP-2 loaded hydrogel with phage cocktail - positive control (empty hydrogel)	*P. aeruginosa*	ΦPaer4, ΦPaer14, ΦPaer22, ΦW2005A	Significant decrease in bacterial load in phage-treated group
Treatment studies					
Yilmaz *et al.*, 2013	Rat model of implant-associated osteomyelitis	4 groups: - phage - antibiotics - phage + antibiotics - negative control	MRSA/ *P. aeruginosa*	Sb-1/ PAT14 Local injection on three consecutive days	Combination therapy resulted in the highest bacterial killing rate across all groups
Kishor *et al.*, 2016	Rabbit model of osteomyelitis	3 groups: - acute osteomyelitis: phage therapy start after 16 d - chronic osteomyelitis: phage therapy start after 6 weeks - positive control	MRSA	Cocktail with SA-BHU1, SA-BHU2, SA-BHU15, SA-BHU21, SA-BHU37, SA-BHU47	Phage therapy succeeded in eradicating the infection
Cobb *et al.*, 2019	Rat model of implant-associated osteomyelitis and soft tissue infection	- 4 groups: - alginate hydrogel loaded with phage - alginate hydrogel loaded with phosphomycin - alginate hydrogel loaded with phage and phosphomycin - positive control (empty alginate hydrogel)	*S. aureus*	ΦSaBov-CRISPR/Cas9	Phage therapy and the combination of phage therapy and antibiotics resulted in a significant reduction in the bacterial load in the soft tissue, not in bone. A high dose of phosphomycin (3 g) could reduce the bacterial load in bone

efficacy of phage therapy for the treatment of OM (*i.e.* no implant) of the femur caused by MRSA in a rabbit model. A distinction was made between acute and chronic OM. That is, for the acute group, treatment was started 16 d after inoculation with a total of four doses 48 h apart from each other, whereas treatment for the chronic group was only started after 6 weeks. In both phage-treated groups, a total of four doses were given locally, 48 h apart from each other. Phage monotherapy was successful in eradicating MRSA in both the acute and chronic OM setting (Kishor *et al.*, 2016). Finally, Cobb *et al.* (2019) studied the effects of a CRISPR-Cas9-modified *S. aureus* bacteriophage in a rat model of OM of the femur and surrounding soft tissue infection. A contaminated orthopaedic screw was placed in the femur and left for 7 d, after which the screw was removed and the therapeutics injected (*i.e.* antibiotics and/or phages in an alginate hydrogel) into the defect space. After 24 h, all animals were euthanised and treatment effects evaluated.

Both phages and antibiotics, in combination or alone, significantly reduced the bacterial load in the soft tissue but not in bone. Only an extremely high dose of phosphomycin (*i.e.* 3 g) was able to significantly reduce the bacterial load in bone. The authors attributed this to the fact that the phage titer delivered through the alginate hydrogel was limited to 10^7 PFU/mL. Also, no surgical treatment to reduce the bacterial load, which is standard clinical practice, was performed concomitantly (*i.e.* debridement and irrigation) (Cobb *et al.*, 2019).

To date, these are the only preclinical *in vivo* studies that have assessed the efficacy of phage therapy regarding ODRIs. These studies have highlighted the potential of the approach, under controlled conditions. However, there are several areas where these studies may have limitations in relation to eventual clinical implementation. These studies included only monomicrobial infections caused by *S. aureus* (*i.e.* MRSA) or *P. aeruginosa*. As other pathogens such as coagulase-negative Staphylococci (*e.g. S. epidermidis*) and Gram-negative bacteria (*e.g. Enterobacter* species) are also commonly isolated, future preclinical models should focus on these pathogens as well (Vanvelk *et al.*, 2018). Furthermore, it has become a widely held view that most, if not all, biofilms in nature are polymicrobial (Gabrilska and Rumbaugh, 2015; Wolcott *et al.*, 2013). Indeed, a preclinical model assessing the impact of phage therapy upon polymicrobial ODRIs may be more relevant to the human equivalent, but the development of such models is a challenge (Gabrilska and Rumbaugh, 2015; Vanvelk *et al.*, 2018). Therefore, future research on this topic is required to develop more realistic animal models for ODRIs.

Carriers

The administration of local antimicrobial agents for the treatment of musculoskeletal infections offers the prospect of improved therapeutic efficacy over that achievable by systemic delivery alone (Metsemakers *et al.*, 2019). That is, the local application of antimicrobials is not impacted by possible vascular impairment (*i.e.* open fractures), which is a frequently encountered hurdle in the treatment of these infections (Metsemakers *et al.*, 2016). Furthermore, by directly delivering the antimicrobial to the infected site, the pharmacokinetic properties of systemic drug delivery, which can decrease the drug bioavailability, are bypassed (Ryan *et al.*, 2011). If high systemic doses are required to obtain an adequate local concentration, a local approach also reduces the risk of systemic toxicity.

The application of local antibiotics is currently gaining interest and several products are already licensed and approved for human use (Metsemakers *et al.*, 2019; ter Boo *et al.*, 2015). Regarding the local delivery of phages, clinical trials generally use simple phage suspensions directly applied to the wound and without any formulation, which often require repeated dosing. Especially for musculoskeletal infections, this approach is cumbersome and increases the burden on patients as an external draining system is required to apply phages in multiple doses for a period of up to 10 d postoperatively (Fig. 3) (Onsea *et al.*, 2019). Hence, the encapsulation of phages into a sustained release system, that can be applied once intraoperatively, would constitute an ideal situation (Malik *et al.*, 2017). Phages are essentially protein structures, which implies that they are susceptible to all environmental changes that denature protein, such as acidic pH, high temperatures, exposure to organic solvents (*e.g.* disinfectants) and mechanical stresses (Malik *et al.*, 2017; Merabishvili *et al.*, 2017). Therefore, when designing a carrier for phage therapy, these factors should be considered.

Several studies exist on the topical administration of phages using different carriers, such as slow-release biopolymers (Jikia *et al.*, 2005; Markoishvili *et al.*, 2002), bandages (Jault *et al.*, 2018), creams (Brown *et al.*, 2016) or hydrogels (Kumari *et al.*, 2010). Indications for topical administrations range from venous stasis ulcers to burn wounds and other poorly healing wounds that do not respond to conventional therapy. These studies underline the fact that the efficacy of phage therapy is concentration dependent.

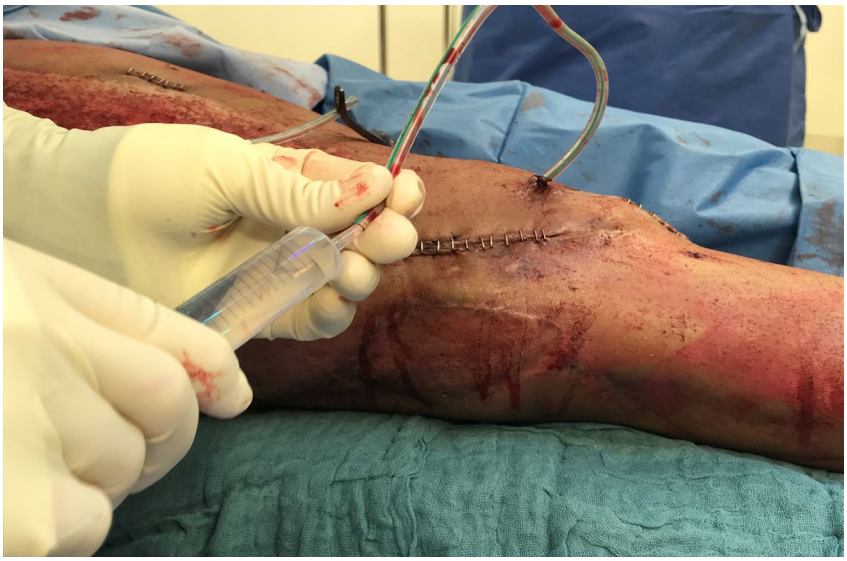

Fig. 3. Local phage application for an ODRI through an external draining system. Courtesy of W-J. Metsemakers and J. Onsea, Department of Trauma Surgery, University Hospitals Leuven, Belgium.

Low-titre phage administration is unlikely to be successful, which again underlines the importance of phage stability in each formulation (Jault *et al.*, 2018; Ryan *et al.*, 2011; Wroe *et al.*, 2019).

In the field of ODRIs, evidence on the optimal carrier for phage therapy is currently scarce. Meurice *et al.* (2012) investigated the application of ceramics (beta-tricalcium phosphate and hydroxyapatite) loaded with phages for the prevention of infection with *E. coli in vitro* (Meurice *et al.*, 2012). The ceramic porosity is an essential factor for the impregnation of antimicrobials, which accelerates the kinetics of their release. Indeed, microporosity increases the surface area of the material so that more phages are retained. The authors concluded that, with the right porosity and composition, phage-loaded ceramics exhibit an antibacterial activity that is close to that of antibiotic-loaded ceramics. Even after 6 d, there were sufficient phages to eliminate bacterial cells (Meurice *et al.*, 2012). Kaur *et al.* (2016) developed a biodegradable drug-delivery system in the form of a coating for the prevention of ODRIs with MRSA. The coating consisted of a polymer (HPMC) that was mixed with linezolid and phages active against the specific strain of MRSA. Compared to naked implants, the coated counterparts with a combination of phage and antibiotic was associated with a maximum reduction in bacterial adherence to the implant. Furthermore, a higher concentration of the polymer was shown to have the most appropriate release kinetics (Kaur *et al.*, 2014). This coating was further investigated in an *in vivo* experiment, which was detailed in the previous section (Table 1) (Kaur *et al.*, 2016). Wroe *et al.* (2019) (Table 1) developed an injectable polyethylene-glycol-based hydrogel loaded with phages directed against *P. aeruginosa*. The authors showed that phages remain active after encapsulation and their release can be regulated by gel degradation *in vivo.* Finally, Cobb *et al.* (2019) studied the effects of an alginate hydrogel loaded with phage and/or antibiotics for the treatment of OM in a rat model. The *in vitro* biofilm experiments performed showed promising results. However, in the *in vivo* rat model, the treatment could reduce the bacterial burden in soft tissue, but not in bone. The authors attributed this to the small volume that could be applied into the small defect size and the thick consistency of the phage solution, which limited the concentration that could be prepared in the phage-loaded alginate hydrogel in the treatment setting (Cobb *et al.*, 2019) (Table 1).

Although these studies show promising results with the carriers that were used, further research is needed in the field of phage formulation, thereby paying extra attention to phage stability.

Clinical evidence

Phage therapy is not a novel concept but has been applied since the start of the 20th century. However, with the advent of antibiotics, it has lost ground in the western world, while research and development of phage therapy continued to thrive within the former Soviet Union and Eastern Europe (Abedon *et al.*, 2011). Phage therapy research of that era was mostly published in languages other than English, which attributed even more to the western scepticism and subsequent fading interest in the application of phages to combat infections. Table 2 presents the clinical studies focusing on musculoskeletal infections, including ODRIs. All the cases included in these studies were diagnosed with therapy-resistant musculoskeletal infections.

The most extensive studies on phage therapy were performed in the former Soviet Union and more specifically in the Eliava Institute in Tbilisi, Georgia, where its clinical use and research remained strong even after the advent of antibiotics (Kutateladze and Adamia, 2008). One of the main indications for phage therapy in the Eliava Institute is OM (Kutateladze and Adamia, 2010). In the 1970's, phage therapy trials were conducted assessing the therapeutic effectiveness of their custom-made staphylococcal phage preparation against different infectious diseases, including OM and septic arthritis. In one of these trials 120 patients were included. Regardless of the therapy applied (antibiotics only, phage monotherapy or combination therapy), a 100 % healing rate was reported (Kutateladze and Adamia, 2010). The added value of this trial is difficult to interpret as there are no (English) data available on population characteristics and patients in the antibiotics-only group recovered fully as well. Furthermore, the phage monotherapy group only consisted of nine patients and in the combination group the antibiotic therapy itself could have influenced the results.

Regarding musculoskeletal infections, one case series was reported by a French group in 1979. They treated seven patients with chronic infections unresponsive to conventional antimicrobial therapy. Five patients were treated successfully with phage therapy, with the follow-up period ranging between 13 and 18 months. Phages were applied locally (through a draining system or topically) after debridement of the wound. Although one patient with a spinal infection showed improvement as one of the causative pathogens (*i.e. S. aureus*) could be eradicated, the *P. aeruginosa* infection persisted. The authors reported a single treatment failure (Lang *et al.*, 1979).

In Poland, Slopek *et al.* (1987) provided an overview of 550 cases treated with phages between 1981 and 1986, with success rates of 90 % in patients with "pyogenic arthritis and myositis", "osteomyelitis of long bones" and "osteitis of long bones after fracture". Phages were administered locally and orally in these cases, no further treatment details were reported (*e.g.* treatment duration, dosage, formulation, *etc.*) (Slopek *et al.*, 1987). In a subsequent case series from the same centre, similar success rates were reported (Weber-Dabrowska *et al.*, 2000).

Table 2. Human clinical studies on phage therapy for musculoskeletal infections. PJI: prosthetic joint infection; OM: osteomyelitis; FRI: fracture-related infection.

Reference	Sample size	Patient characteristics	Intervention	Outcome
Lang *et al.*, 1979	7	PJI (n = 2) OM (n = 1) Septic arthritis (n = 1) Spinal infection (n = 1) FRI (n = 2)	Phages adapted to isolated strains Administration either topical or by injection through a draining system. Some cases received combination treatment with antibiotics	5/7 treated Recurrence of spinal infection and one FRI
Kutateladze and Adamia, 2010	120	Patients with staphylococcal OM or arthritis	Three groups: - antibiotics (n = 60) - phage monotherapy (n = 9) - phage + antibiotics (n = 51) Administration of Eliava staphylococcal phage preparation topically or intravenously	100 % success rate in all groups
Slopek *et al.*, 1987	100	Purulent arthritis and myositis (n = 19) OM of the long bones (n = 40) FRI (n = 41)	Administration locally and/or orally Some cases received combination treatment with antibiotics	Success rates: - purulent arthritis and myositis: 89.5 % - OM of the long bones: 95 % - FRI: 90.2 %
Weber-Dabrowska *et al.*, 2000	81	OM of the long bones (n = 40) FRI (n = 41)	Administration locally and/or orally Unclear if some patients received combination treatment with antibiotics	Success rates: OM of the long bones: 95 % FRI 60%
Vogt *et al.*, 2017	1	OM	Repeated dosing of phage cocktail Pyo bacteriophage through draining system, in combination with antibiotic therapy	Eradication of the infection
Ferry *et al.*, 2018a	1	OM (post-radiation)	Application of customised phage cocktail every 3 d, in combination with intravenous antibiotic therapy	Patient died 45 d after treatment due to cancer progression
Ferry *et al.*, 2018b	1	PJI	Single intraoperative injection of a customised phage cocktail in combination with intravenous antibiotic therapy	Eradication of the infection
Nir-Paz *et al.*, 2019	1	FRI	Intravenous repeated administration of customised phage cocktail, in combination with intravenous antibiotic therapy	Eradication of the infection (after two phage therapy regimens)
Tkhilaishvili *et al.*, 2019	1	PJI	Repeated dosing of customised phage cocktail, in combination with intravenous antibiotic therapy	Eradication of the infection
Onsea *et al.*, 2019	4	OM	Repeated dosing of BFC1 cocktail or Pyo bacteriophage cocktail in combination with intravenous antibiotic therapy	Eradication of the infection in all cases

Although the above-mentioned studies showed promising results regarding the implementation of phage therapy in musculoskeletal infections, these results should be interpreted with caution. The methodology of these earlier trials was not in accordance to GCP guidelines as known today. They included reports on heterogeneous patient populations [different causative pathogens, locations of infection, type of infection (prosthetic joint infection, FRI, OM, *etc.*)] and details on production, formulation, administration, dosing and follow-up time are often missing.

Recently, with the rise of AMR, phage therapy has gained interest in Western Europe, which is displayed by the number of case reports on phage therapy for treatment-resistant musculoskeletal infections (Ferry *et al.*, 2018; Nir-Paz *et al.*, 2019; Onsea *et al.*, 2019; Tkhilaishvili *et al.*, 2019; Vogt *et al.*, 2017). Although these reports described successful outcomes for the combination of phage therapy with antibiotics, they included a multitude of treatment schedules (*e.g.* routes of administration, applied phage cocktail) (Onsea *et al.*, 2019).

The gold standard for clinical trials is the RCT. According to the authors' knowledge, only one RCT (PhagoBurn trial) was published using topical administration of a phage cocktail in line with GMP guidelines against *E.coli* and *P. aeruginosa* in burn-wound patients (Jault *et al.*, 2018). The PhagoBurn trial was conducted in burn-wound centres in France and Belgium. This study underlines the importance of phage stability and formulations since phage titres decreased after manufacturing and patients in the experimental group were treated with a lower titer than intended. Although clinical evolution was slower, a relevant reduction in bacterial load with fewer adverse events was still seen in the group that was treated with phage therapy *versus* the group treated with standard treatment (*i.e.* sulphadiazine silver cream) for extensive burn wounds (Jault *et al.*, 2018). Recently, further efforts have been made to establish clinical trials using phage therapy, in line with GCP and GMP guidelines, for various infectious conditions (*e.g.* chronic otitis, infected burn wounds, diabetic foot ulcers, bone and joint infections, chronically infected cystic fibrosis, *etc.*) (Rohde *et al.*, 2018b). Regarding ODRIs, a research project (PHOSA, Web ref. 2) on phage therapy in multi-drug resistant *S. aureus* musculoskeletal infections and diabetic foot ulcers was commenced. This project aims at producing a potent phage cocktail that is in accordance with GMP guidelines.

Bacteriophage regulations

Upon their rediscovery, phage preparations were classified as medicinal products in the EU and as drugs in the US, without formal phage-specific requirements or concessions (Verbeken *et al.*, 2012).

Technically speaking, predefined phage products or drugs, which would be produced on an industrial scale, could pass through the classical drug development and marketing pipeline, provided specific adaptations are made. However, phage specificity is bound to make it unlikely that such preparations will be able to timely handle i) the large variety of bacterial pathogens involved in most indications, ii) the time- and geography-related changes in the incidence of infectious species and clones, iii) the inevitable emergence of phage-resistant bacterial clones (Pirnay *et al.*, 2011).

According to some academic stakeholders, therapeutic phages should be prepared individually (a phage bank) and tested for effectiveness against the pathogens derived from a single patient (a "phagogram"). Intermediary or combined (industrially prepared and personalised phage preparations) approaches may be possible (Pirnay *et al.*, 2011). However, the pharmaceutical legislation was developed to regulate industrially manufactured medicines intended for large-scale distribution, not personalised therapeutic approaches. Accordingly, the currently implemented regulatory framework is not compatible with personalised and sustainable (phage therapy) concepts (Verbeken *et al.*, 2012).

The repeated calls for a specific regulatory framework for phage therapy have not been heard by the European legislator (*i.e.* EMA), which shows strong resistance to change in this regard (Fauconnier, 2019). Meanwhile, some clinicians and academics are exploring national solutions to accelerate the availability of phages for the treatment of an increasing number of desperate patients. Phage therapy has been performed sporadically in Europe under the umbrella of the Article 37 (Unproven Clinical Practice Interventions) of the Helsinki Declaration. Even though no safety issues were reported and most targeted infections seemed to have been resolved, the small number and diversity of these "Helsinki" phage therapy cases so far did not allow for the unambiguous demonstration that the positive clinical outcome was related to the phage use. In Poland, a member of the EU, phage therapy is considered to be an "Experimental Treatment", covered by the adapted Act of December 5th 1996 on the Medical Profession (Polish Law Gazette, 2011, No. 277 item 1634) and the Article 37 of the Declaration of Helsinki (Gorski *et al.*, 2009).

Other phage applications were performed under the umbrella of the "compassionate use", which is a treatment option allowing the use a not yet authorised medicine. Under strict conditions, products under development can be made available to groups of patients who have a disease for which satisfactory authorised therapies are lacking and who cannot participate in clinical trials. In principle, the compassionate approach can only apply to drugs that are being tested or have entered the marketing authorisation application process after the early

study results have demonstrated efficacy and safety but have not yet been approved. Like the Article 37 of the Helsinki Declaration, the compassionate use treatment option can only be introduced if the drugs – phages in this case – are expected to help in life-threatening or long-term (chronic) and/or seriously debilitating diseases that are not treatable with the currently available therapies. In France, the competent authority has set up a specific committee for phage therapy consisting of (external) experts in various fields. Their task is to specifically evaluate and guide phage therapy requests, discuss them in dialogue with the treating doctors and pass on a consensus advice to the competent authority, which will then authorise the request or not. From 2006 to 2018, 15 patients with osteo-articular, otitis and abdominal infections were treated compassionately with phages in France. Eleven were healed after one treatment with phages (Patey *et al.*, 2018).

On July 5th 2016, in response to parliamentary questions regarding the implementation of phage therapy, the Belgian Minister of Social Affairs and Health acknowledged that it is indeed not sensible to treat phages as industrially prepared drugs and, therefore, proposed to investigate the option of magistral phage preparations (Pirnay *et al.*, 2018). The "magistral preparation framework" provides physicians with a practical way of adapting patient treatments to specific needs and making drugs available that are not (yet) available on the market. European and Belgian legislation define a magistral preparation (compound prescription drugs in the US) as "any drug prepared in a pharmacy in accordance with a medical prescription for an individual patient" (Article 3 of Directive 2001/83 of the European Parliament and Article 6 quarter 3§ of the Belgian Medicines Law of March 25th 1964). Magistral preparations are made by a pharmacist (or under his/her supervision) from their constituent ingredients, according to the technical and scientific standards of pharmaceutical technology, for a particular patient, upon a doctor's prescription. Phage APIs to be included in magistral preparations must meet the requirements of a monograph (describing their preparation and quality control testing). In addition, phage APIs must be accompanied by a certificate of analysis, which must be issued by a BAL, quality control laboratories that have been granted an accreditation by the Belgian regulatory authorities to perform batch release testing of medicinal products. On January 10th 2018, the phage API monograph received a formal positive advice by the FAMHP and from that date, in Belgium, phages have been delivered in the form of magistral preparations to a selection of patients under the direct responsibility of medical doctors and pharmacists. However, with increasing demand and increasing need, it is time to find a broader solution for the phage therapy regulatory issues. As a next step, supranational competent authorities are urged to adopt the initiatives originally launched by some national regulatory authorities (Fauconnier, 2019).

Conclusion and future perspectives

The global rise in AMR has heightened the need for alternative antimicrobial strategies. One such strategy that seems very promising is phage therapy. The present review has focused on the current evidence for the application of lytic phages in ODRIs. Not many *in vitro* phage studies have been performed using ODRI-associated strains, but many studies have proven the ability of phages to inhibit biofilm formation. Furthermore, the available *in vivo* studies show a high efficacy concerning the eradication of the infection, but the methodology is not comparable between studies. Also, these studies include only monomicrobial infections caused by MRSA or *P. aeruginosa*. Future research with preclinical models of ODRIs caused by other frequently isolated pathogens is required. Clinical studies have shown promising results in patients with severe musculoskeletal infections, including ODRIs. Most of these studies have shown the added value of concomitant antibiotics. Important to note is that the earlier clinical trials conducted in Eastern Europe did not conform to GCP guidelines as known today. More recently, sporadic phage applications have been performed in Western Europe under the umbrella of the Article 37 of the Helsinki Declaration. Even though no safety issues were reported and most targeted infections seemed to have been resolved, the small number and diversity of these "Helsinki" phage therapy cases so far did not allow for the unambiguous demonstration that the positive clinical outcome was related to the phage use. More research including reports on larger patient populations treated with phage therapy are required to fully implement phage therapy in the treatment of ODRIs. Furthermore, national and international regulatory authorities should urgently optimise the regulatory framework for phage therapy.

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Discussion with Reviewer

Lauren Priddy: How might it be possible to maintain or even predict changes in an MOI over time?
Authors: Modelling/predicting the presence of phage *in vivo* is extremely difficult, as it is impacted by various parameters, including dose and growth of the bacteria, phage specific traits (basic microbiological traits including burst size and latent period), possible emergence of bacterial phage resistance, potential immunological response by the individual patient, *etc.*. All these parameters are co-dependent, preventing this modelling with any sense of accuracy. In general, it should be clear that major challenges remain in terms of understanding the dynamics of phage titers *in vivo*. The involved parameters, including bacterial and phage microbiological traits and individual immunological response, are all interdependent and incompletely understood at the present time.

Editor's note: The Scientific Editor responsible for this paper was Chris Evans.