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REVIEW ARTICLE

Phage therapy efficacy: a review of the last 10 years of preclinical studies

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ABSTRACT

Due to the rise of multidrug-resistant infections in humans, phage therapy is gaining renewed attention in Western medicine. Despite the increasing number of publications focussed on the isolation, characterization and *in vitro* performance of different phages, there is still a lack of concise pre-clinical information to guide the application of phage therapy in clinical practice. Nevertheless, over the last decade, efforts have been made to conduct more detailed studies of the *in vivo* efficacy of phages. Here, we review the most relevant *in vivo* studies performed in the last decade covering phage efficacy in both preclinical and clinical trials. We compare different routes of administration, dosage effect and different animal models of distinct types of infections. Moreover, insights into case studies and results from clinical trials are presented. Challenges and limitations of phage use as evidenced by the current state of research are also discussed in order to improve both the trustworthiness and success of the implementation of phage therapy.

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Introduction

Multidrug-resistant (MDR) bacteria are recognized as one of the most critical public health problems. The Infectious Diseases Society of America recognized the worldwide dissemination of antimicrobial resistance as one of the greatest threats to human health (Spellberg et al. 2011). Taking into account that MDR-related infections are responsible for a relatively high number of hospital mortalities and long hospitalization periods, the rise of antimicrobial resistance has a huge impact on society and the economy (Vardakas et al. 2013). Indeed, it is estimated that 48,000 people die yearly in Europe and the USA as a consequence of MDR infections (Davies 2013; Executive Office of the President President's Council of Advisors on Science and Technology 2014). The World Health Organisation recently published a list of MDR priority pathogens, encouraging both the scientific community and the pharmaceutical industry to develop new antimicrobials to combat these dangerous pathogens (World Health Organization 2017).

Today, a century after their discovery, bacteriophages (phages) are proposed as a therapeutic solution to combat MDR infections. Phages are viruses and, as obligatory cell parasites, they only propagate on bacterial hosts. Phages can either be strictly lytic or temperate depending on their propagation strategies. During a bacterial cell infection, a phage adsorbs to its host and inserts its genetic material into the cell. Phages can further follow one of two life cycles: lytic or lysogenic. All phages can follow the lytic cycle, which ends up with the death of the bacterial cell. In addition, some phages can follow the lysogenic cycle in which the viral genome is integrated into the bacterial genome and is further replicated along with bacterial cell division. Under certain conditions, lysogenic phages can be induced to follow a lytic cycle. Temperate phages are described to be involved in horizontal gene transfer phenomena, namely on the spread of virulence factors. Consequently, this type of phage is not recommended for phage therapy. In opposition, phages that are strictly lytic, also known as virulent phages, are the ideal weapons to combat bacterial cells, as their multiplication always results in lysis of their hosts (Loc-Carrillo and Abedon 2011). Similar to treatment with antibiotics, phage therapy also possesses specific problems, weaknesses and benefits (Table 1). However, this type of therapy gives the possibility of applying targeted, tailor-made antibacterial agents, providing

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Table 1. Pros and cons of phage therapy.

Pros	Cons
Highly specific By targeting specific bacterial cell wall receptors, leaving untouched the remaining microbiota. This property favours phages over other antimicrobials that can lead to dysbiosis (imbalance) of the microbiota	Pre-diagnosis It is compulsory to identify bacterial species and/or strains present in the infection site, to allow for a rational and personalized phage therapy
Unique mode of action As they are self-replicating and self-limiting "intelligent drugs". They multiply and persist till a minimum number of host cells are still present. After dealing with the infections, phages are cleared from the system	Neutralization As some studies have shown that phages can be removed by the mononuclear phagocyte system and inactivated by the development of neutralising antibodies
Synergy Offering the possibility to be co-administrated with antibiotics or other phages (cocktail) to limit the appearance of resistant phenotypes. Even if bacteria develop resistance to phages, they usually are less virulent	Intracellular pathogens As some infections caused by obligatory or facultative intracellular bacteria (e.g. *Mycobacterium leprae*, *Chlamydia* sp.) can invade and thrive inside human cells, becoming inaccessible for phage therapy
Modification of phages By genome engineering to make up for some disadvantages such as host range specificity (limited to few strains), limited amplification and inaccessibility to target intracellular pathogens	Consumer's acceptance Being viruses, the public opinion will ultimately dictate the investment of pharmaceutical companies and the commercialization of phage products
Safety By being constantly present in human microbiome, supplied with food and as demonstrated in human tests, showing no adverse effect using different types of administration	
Low cost As they are relatively cheap to isolate and propagate to high titres	

higher specificity than most antibiotics. Furthermore, to extend the spectrum of activity or to counteract the emergence of phage-resistant phenotypes, phages may be delivered as cocktails that extend therapeutic possibilities offered by individual phage strains (Chan et al. 2013).

Although during recent decades efforts have been made to isolate and *in vitro* characterize phages with therapeutic potential to target different MDR pathogens, there is still no consistent and unified account of phage action *in vivo*. This review covers the last 10 years of phage therapy outcomes demonstrated in preclinical and in clinical trials (Table 2). Major conclusions regarding safety and efficacy assessment of phages against different *in vivo* infection models are presented, and the most important variables that influence the outcome of phage therapy are discussed.

Pre-clinical trials

Skin and soft tissue infections

Skin infections are a consequence of an integrity breakage of the skin and they occur frequently. These infections comprise erythrasma, impetigo, ecthyma, folliculitis, erysipelas and cellulitis, ranging from harmless to life-threatening (O'Dell 1998; Ki and Rotstein 2008). Skin and soft tissue infections (SSTIs) are common reasons for emergency care settings and hospitalisations. Indeed, in the USA the number of hospital admissions due to SSTIs dramatically increased by 65% from 1997 to 2005 (Hersh et al. 2008). The number of SSTIs was reported to be stabilised during 2005–2010 with an average prevalence of 48 cases per 1000 person-years (Miller et al. 2015). The skin is colonized naturally by a diverse population of microorganisms, where the majority are bacteria (Byrd et al. 2018). The most humid areas of the body, namely the axilla, groyne, nares and intertriginous area, are the ones where larger bacterial populations are found (Grice and Segre 2011). Although some specific bacteria can colonize specific parts, the composition of the skin microbiota depends on climate, age, gender, hygiene conditions, stress, nutrition habits and site-specific biochemical interactions (SanMiguel and Grice 2015).

Usually, the major cause of SSTIs is wounds, including those developed in burn wounds (Church et al. 2006). Also, diabetic foot infections are a major world epidemic, being a consequence of diabetes mellitus (Lavery et al. 2006). Patients who develop this type of infection have a high risk of amputation due to poor blood circulation, biofilm development on chronic wounds and also because the bacterial pathogens that colonize the wound often exhibit antimicrobial resistance to conventional antibiotics (Trivedi et al. 2014). Antibiotic-resistant bacteria causing complicated SSTIs are increasingly reported (Eckmann and Dryden 2010): *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium* spp., *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Enterococcus* spp., and *Escherichia coli* are among the most important causes of SSTIs in the USA (Moet et al. 2007; Ki and Rotstein 2008; Zilberberg et al. 2009; Jenkins et al. 2010; SanMiguel and Grice 2015) (Figure 1).

The use of phages for the treatment of SSTIs has been widely studied and tested in different animal models (Table 2). The most common *in vivo* model is

Table 2. Relevant studies about the *in vivo* application of phages on pre-clinical trials since 2008.

Species (strain)	Route	Pathogen	Main outcome	References
Skin and soft tissue infections				
Mice (BALB/c)	Topical	*K. pneumoniae*	Burn model. Application of a phage-containing hydrogel with a MOI of 200 rescued more mice than antibiotics or chemicals.	Kumari et al. (2011)
Mice (BALB/c)	Topical	*K. pneumoniae*	Burn model. A comparison of the effect of single phage versus phage cocktail consisting of five phages was performed. Despite the good results of single phage therapy, the phage cocktail demonstrated better results.	Chadha et al. (2016)
Rats (Wistar)	Topical	*A. baumannii*	Wound infection in uncontrolled diabetic rats. Using a MOI of 10, phages were locally sprayed and results were compared with the antibiotic colistin. Phage treatment was more efficient than antibiotic treatment.	Shivaswamy et al. (2015)
Mice (BALB /c)	Subcutaneous injection or topical	*A. baumannii*	Phage Abp1 topical application resulted in smaller wound sizes than in injected wounds.	Yin et al. (2017)
Rats (Wistar)	Topical	*S. aureus*, *P. aeruginosa*, and *A. baumannii*	A phage cocktail comprising six phages was used on chemically induced diabetic rats. Although phage treatment decreased significantly bacterial cell counts and improved wound healing, the effect was shown to be pathogen-dependent. The best results were obtained when phage was administrated after wound débridement.	Mendes et al. (2013)
Pigs (Yorkshire)	Topical	*S. aureus*, *P. aeruginosa*, and *A. baumannii*	A phage cocktail comprising six phages was used on pig wound infection model. The results were species-dependent, but in general there was a synergism with mechanical debridement. Improvements were more significant in the rodent model than in the porcine model.	Mendes et al. (2013)
Mice (Male albino)	Injection	*P. aeruginosa*	Wound model. A phage cocktail comprising 11 phages was added into subcutaneous pockets containing two biofilm-laden catheter sections, demonstrating a significant decrease on the number of bacterial cells and a rise on the number of phages.	(Basu et al. 2015)
Mice (BALB/c)	Local injection	*S. aureus*	Phage MR-10 was administered locally on alloxan induced diabetic mice with diabetic foot infection. Using a MOI of 100 results were similar with linezolid. Combined treatment was more effective.	(Chhibber et al. 2013)
Mice (BALB/c)	i.p.	*E. coli*	Mice bearing tumours had been subjected to tumour surgery before the experiment. Surgical wounds were infected with *E. coli* and engineered T4 phage displaying tumour-specific peptides was used for combined treatment. The acuteness of wounds, bacterial load and inflammatory markers in phages-treated mice were markedly decreased.	Dabrowska et al. (2014)
Mice (BALB/c)	i.p.	*K. pneumoniae*	Burn model. A single injection of phage Kpn5 at a MOI of 1 rescued significantly mice when administrated immediately and even after 18 h of bacterial challenge.	Kumari et al. (2010)
Mice (BALB/c)	s.c.	*M. ulcerans*	Mice treated with a single injection of the mycobacteriophage D29 presented decreased footpad pathology associated with a reduction of the bacterial burden.	Trigo et al. (2013)
Rat (Wistar)	i.m.	*S. aureus*	Transfersome-entrapped phage cocktail healed thigh infections after 7 days, being infections of untreated animals resoled only after 20 days.	Chhibber et al. (2017)
Eye and ear infections				
Mice (C57BL/6)	Topical	*P. aeruginosa*	A single dose of phage eye-drops significantly improved keratitis, suppressing neutrophil infiltration and bacterial clearance in the cornea.	Fukuda et al. (2012)
Mice (C57BL/6)	Topical	*P. aeruginosa*	A single phage cocktail successfully controlled and prevented bacterial-induced keratitis, being more effective than antibiotics.	Furusawa et al. (2016)
Dog	Topical	*P. aeruginosa*	A single phage cocktail dose (10^5 PFU/ear) applied directly to the auditory canal significantly reduced clinical scores after 48 h, without inflammation or other adverse events detected.	Hawkins et al. (2010)
Dog	Topical	*P. aeruginosa*	A small dose of phage (400 PFU) was enough to cure a chronic otitis.	Marza et al. (2006)

(*continued*)

Table 2. Continued.

Species (strain)	Route	Pathogen	Main outcome	References
Respiratory tract infections				
Upper respiratory infections				
Mice (C57BL/6)	i.n.	*S. aureus*	A single phage (MOI 1 or 10) completely decolonised *S. aureus* from nares at day 7 after treatment, as compared to untreated group. Mupirocin treatment had a similar effect. Phage with mupirocin achieved complete clearance at day 5.	Chhibber et al. (2014)
Sheep (Marino-cross)	i.n.	*S. aureus*	A phage cocktail (10^6 PFU/ml) used to flush frontal sinusitis once daily for 3 days were proved to decolonise nares and to be safe for short-term applications. EDTA treatment has a similar effect. Phage and EDTA did not synergized.	Drilling et al. (2014)
Sheep	i.n.	*S. aureus*	A phage cocktail was proved to be safe for long-term applications. Flushing frontal sinusitis for 20 days with phages revealed not to cause side effects, inflammatory responses or tissue damage.	Drilling et al. (2017)
Sheep (Merino)	i.n.	*P. aeruginosa*	Phage cocktail applied twice daily (10^8 to 10^{10} PFU/ml) significantly reduced a 7-day old biofilm in sheep frontal sinuses, without safety concerns observed.	Fong et al. (2019)
Lower respiratory infections				
Mice (Swiss-Webster)	i.n.	*K. pneumoniae*	Phages (10^9 PFU/mouse) added 2 h after infection reduce the bacterial load in the lungs and increase survival rate of mice (80%) compared with mocked-treated group (30%). Reduced loss weight and inflammatory cytokine levels were observed.	Cao, Wang, et al. (2015)
Mice (BALB/c)	i.n.	*P. aeruginosa*	Curative treatments rescued 95% of immunocompent mice when applying phages with 10^8 PFU/ml, 2 h after infection. A 4-day preventive treatment resulted in a 100% survival.	Morello et al. (2011)
Mink (Jinzhou Black)	i.n.	*P. aeruginosa*	Alternative model to study haemorrhagic pneumonia. Challenged mink received phage treatment 2-h post-infection. At 12 days, 80% of mink survived with phages at MOI of 10.	(Cao, Zhang, et al. 2015)
Mice (BALB/c)	i.n.	*P. aeruginosa*	Phages added at MOI of 10 could complete clear lung from infection either added at 24, 36, and 48 h post infection. UV-inactivated phages did not have protective effect.	Waters et al. (2017)
Mice (BALB/c)	i.n.	*P. aeruginosa*	Phage cocktail reduced biofilms in a cystic fibrosis bronchial epithelial CFBE41o-cells. Tests in infected mice with a lux tagged strain, showed a reduction of 4 logs in the lungs, after 6 h of treatment.	Alemayehu et al. (2012)
Mice (BALB/c)	i.n. vs. i.p.	*B. cenocepacia*	Comparison of different phage administration routes. One day after infection, i.n. delivered phage significantly decreased bacterial load in the lungs and was more efficient than i.p. treatment.	(Semler et al. 2014)
Mice (BALB/c)	i.p.	*K. pneumoniae*	Liposome-entrapped phages could treat pneumonia at 3 days or prevent the disease 1 day prior infection. Nonliposomal could only treat mice at 1 day or prevent pneumonia when added 6 h before challenge.	Singla et al. (2015)
Mice (BALB/c)	i.p.	*K. pneumoniae*	A single phage dose (10^{10} PFU/ml) administered immediately rescued 100% of animals from *K. pneumoniae*-mediated respiratory infections, but a 6 h delay in treatment was ineffective.	Chhibber et al. (2008)
Mice (BALB/c)	i.v.	*S. aureus*	Phage (10^8 PFU/ml), dindamyxin (8 mg/kg of body weight) or combined treatments had similar mice survival rates. In lung tissues, it resulted in lower 0.5, 4.4, and 4.0 CFU/gm bacterial load, respectively, compared with non-treated group (8 CFU/g).	Oduor et al. (2016)
Sheep	Extension cannula	*S. aureus*	Long-Term administration of NOVO12, (a cocktail composed of two phages) locally applied to the frontal sinus for 20 days was regarded as safe, without inducing inflammatory responses or tissue damaged.	Drilling et al. (2017)
Mice (Swiss Mice)	endotracheal route	*P. aeruginosa*	A phage dry-powder formulation (2 mg/mice) applied 2 h after the bacterial challenge, decreased the bacterial load in the lungs by 5.3 log, compared with non-treated group.	Chang et al. (2018)
Mice (C57BL/6)	i.p.	*P. aeruginosa*	Single phage treatments (10^9 PFU/ml) significantly decreased the infection in the lungs (>4 logs) and protected mice (>65%) from an otherwise deadly infection, when applied 4 h post-infection.	Jeon and Yong (2019)
Mice (BALB/cJRj)	i.v.	*E. coli*	Phages cleared lung infection faster than antibiotics, but both displayed similar endpoint. The rapid lysis of	Dufour et al. (2019)

(*continued*)

Table 2. Continued.

Species (strain)	Route	Pathogen	Main outcome	References
			bacteria did not increase the innate inflammatory response compared to the antibiotic treatments.	
Gastrointestinal tract infections				
Mice (C3H)	Oral	*E. coli*	Phages were able to specifically kill *E. coli* added to mice without having impact on the commensal flora. Doses as low as 10^3 PFU/ml of drinking water were detected on the stools.	Chibani-Chennoufi et al. (2004)
Mice (BALB/c)	Oral	*E. coli* O104:H4	Demonstration of the different *E. coli* susceptibility profiles to phage in mice gut. Strains collected from the ileum were sensitive to all three phages, while those from faeces were infected by only one.	Maura et al. (2012)
Mice (BALB/c)	Oral	*E. coli*	Phage orally given (10^9 PFU/ml) protected mice from salmonellosis and prevented weight loss after 10 days of treatments.	(Nikkhahi et al. 2017)
Mice (BALB/c)	Oral	*E .coli*	A single dose of phage (10^9 PFU/ml) protected mice from enteropathogenic *E. coli* during the 10 days of the study.	(Vahedi et al. 2018)
Mice (Swiss Albino)	Oral	*V. cholerae*	Phage cocktail (10^8 PFU/ml and ciprofloxacin (40 mg/Kg) given once daily could reduce the bacterial load by 3 and 5 logs/g of tissue homogenates, respectively. Rehydration treatment supplied in the drinking water did not reduce the bacterial counts but could diminish the cholera infection in a higher extent.	Jaiswal et al. (2014)
Hamster (Syrian golden)	Oral	*C. difficile*	Phage cocktail (10^8 PFU/ml) significantly reduced bacterial colonisation at 36 h post-infection. Phage treatments also delayed the onset of symptoms by 33 h compared to the time of onset of symptoms of control groups.	Nale et al. (2016)
Mice (BALB/c)	Oral	*V. parahaemolyticus*	Mice treated with phage 1 h after inoculation with bacteria were protected from infection and death caused by i.p. administration of bacteria (MOI 10).	Jun et al. (2014)
Rabbit	Oral (gastric tube)	*V. cholerae*	Phage cocktail (10^8 PFU) could significantly reduce shedding of bacteria when administrated 6 and 12 h after challenge. No effects were obtained using profilatic treatment, 6 and 12 h prior-infection.	Jaiswal et al. (2013)
Mice (BALB/cYJ)	Drinking water	*E. coli*	A single dose of phage cocktail decreased bacterial throughout the gut with a lower impact on microbiota composition comparatively to antibiotic treatment.	Galtier et al. (2016)
Urinary tract infections				
Mice (BALB/c)	i.p.	*E. coli*	Administration of T4 and KEP10 phages caused a marked decrease in the mortality of mice inoculated transurethrally with a UPEC strain. Phages were administered in MOIs 0.01–60, they were effective in MOIs 0.5–60 with dose and phage dependency.	Nishikawa et al. (2008)
Mice (C57BL/6NCrl)	i.p.	*C. turicensis*	Administration of phage (10^{11} PFU/ml) immediately after challenge reduced the bacterial burden in the kidney by 70%, rescued the levels of meondialdehude and did not affected the antioxidant status.	Tothova et al. (2011)
Mice (Kunming)	i.p.	*S. enteritidis*	Phage single dose (10^{10} PFU/mouse) 1 h after bacterial challenge protected 40% of mice from a lethal infection.	Tang et al. (2019)
Bacteraemia				
Mice (BALB/c)	i.p.	*K. pneumoniae*	A phage cocktail of three phages was applied in a step-by-step approach reducing the emergence of phage-insensitive mutants. Furthermore, phage cocktail rescued more mice than single phage approach.	Gu et al. (2012)
Mice (BALB/c)	i.p.	*P. aeruginosa*	A single phage administration (MOI of 100) rescued 100% when phage was injected 45 min after bacterial challenge. Even when mice was moribund a single injection rescued 50% of mice.	Vinodkumar et al. (2008)
Mice (ICR)	i.p.	*P. aeruginosa*	A single phage was administered on immunocompetent and on neutropenic mice using different MOIs (1, 10, and 100). Normal mice were rescued by 80–100% with all MOIs tested. Infected neutropenic mice were not protected by the phage.	(Tiwari et al. 2011)
Mice (ICR)	i.p.	*E. faecalis*	A single injection of a phage cocktail consisting of two different phages at a MOI of 0.02 rescued all mice when administered 1 h after bacterial challenging.	Gelman et al. (2018)
Mice (ICR)	i.p.	*S. aureus*	Injection of phage S13'after 6 h of infection rescued mice from lung-derived septicaemia.	Takemura-Uchiyama et al. (2014)
	i.p.	*S. aureus*		Sunagar et al. (2010)

(*continued*)

Table 2. Continued.

Species (strain)	Route	Pathogen	Main outcome	References
Mice (BALB/c)			Phage GRCS rescued both diabetic and non-diabetic mice by 90 and 100%, respectively. Phage was more efficient in reducing cell counts than oxacillin.	
Mice (BALB/c)	i.p.	*E. faecalis*	A single injection using a low MOI 1 h post-infection rescued all mice. Phage treatment affected the balance of the gut microbiota community in a dose-dependent manner.	Cheng et al. (2017)
Mice (BALB/c)	i.p.	*A. baumannii*	Both phage Abp1 and polymyxin B rescued all treated mice. The phage found in liver and kidney at high titres suggested *in vivo* replication.	Yin et al. (2017)
Mice (BALB/c)	i.p.	*V. parahaemolyticus*	Mice treated with phage 1 h after inoculation with bacteria were protected from systemic infection and death caused by i.p. administration of bacteria (MOI 10).	Jun et al. (2014)
Mice (ICR)	i.p. and i.m.	*P. aeruginosa*	Two different phages were used. Although i.m. administration showed better pharmacokinetics, i.p. injection was more efficient rescuing mice.	Heo et al. (2009)
Mice (C57BL/6)	i.p. vs. Oral (gastric tube)	*K. pneumoniae*	Evaluation of i.g vs. i.p. delivery during the course of liver infection. i.g. delivery offered higher protection at early phage administration (30 min), while i.p. injection demonstrated better results at late phage administration (6–24 h).	Hung et al. (2011)
Rat (Sprague Dawley, pups)	i.p. or s. c.	*E. coli*	Phage administered 7 or 24 h post-infection resulted in 100 and 50% of rats survival, respectively.	Pouillot et al. (2012)
Mice (BALB/c)	i.v.	*S. enterica* serovar Paratyphi B	Phage isolated from faeces of patients with gastroenteritis had life-saving effect in mice when administered right after inoculation with bacteria at MOI 1 or in delayed treatment (2 weeks after infecting mice with sublethal dose of bacteria). Phage eradicated bacteria from the animals.	Capparelli et al. (2010)
Mice (BALB/c)	i.v.	*E. coli*	A single phage dose was highly efficient when administered 1 h after bacterial challenging.	Schneider et al. (2018)
Other infections				
Rat (Wistar)	i.v.	*P. aeruginosa*	Endocarditis. Administration of the phage cocktail 18 h after bacterial challenge by either continuous or bolus injection decreased median vegetation bacterial titres by 3.0 and 2.3 log CFU/g, respectively, within 6 h of therapy.	Oechslin et al. (2017)
Rat (Wistar)	i.m.	*E. faecalis*	After periapical lesion induction, treatment with phage cocktail formulated with poloxamer P407 reduced 99% of the intracanal viable bacterial counts, with reduced periapical inflammation and improved healing following phage treatment	Shlezinger et al. (2019)

the wound model in mice (Basu et al. 2015; Yin et al. 2017) including burn models (Kumari et al. 2010, 2011; Chadha et al. 2016), tumour surgery wounds (Dabrowska et al. 2014) and footpad injury (Trigo et al. 2013). Nevertheless, the possibility of phages to combat infected diabetic foot ulcers was also assessed in two different animal models—rat and porcine. As these infections are often polymicrobial, a phage cocktail covering three different pathogens was administered topically, and although successful in both models, improvements were more significant in rats (Mendes et al. 2013).

The majority of the *in vivo* phage therapy studies for SSTIs were performed using phage cocktails rather than single phages. Nevertheless, Chadha et al. (2016) compared the effect of single phage versus phage cocktail therapy in a burn wound infection model in mice. The authors isolated five new *Klebsiella pneumoniae* phages and applied them topically, either individually or in combination. Although monotherapy was able to reduce bacterial load, the phage cocktail demonstrated better results.

In several studies, only a single phage administration was applied using different multiplicities of infection (MOI), that represent the ratio of phage particles to bacterial cells. In a murine burn wound model it was found that when phages were topically applied at MOI of 200, the level of phage-derived protection was higher than that offered by chemicals or antibiotics (Kumari et al. 2011). In another study, using a hindpaw infection in diabetic mice, Chhibber et al. (2013) demonstrated the effect of a single administration of phages (MOI of 100) in combination with linezolid. A significant effect was noted on resolving the course of infection by following the development of infection, namely the bacterial load, lesion score, histological examination and myeloperoxidase activity (Chhibber et al. 2013). Also Trigo et al. (2013) demonstrated that a single subcutaneous (s.c.) injection of a relevant phage decreased bacterial burden and negative

symptoms in mouse models of footpad infection with *Mycobacterium ulcerans*.

Chadha et al. (2017) demonstrated the potential of treating burn wound infections with a liposome-encapsulated cocktail of five *K. pneumoniae* newly isolated phages. The encapsulated cocktail was more efficient in reducing bacterial load than free phage cocktail being able to rescue all the animals tested. The authors suggested that this difference was possibly related with liposomes higher retention within the body (Chadha et al. 2017).

The route of administration of phages has been shown to have an important impact in the outcome of the wound treatment. A cocktail of three phages was administered by MacVay et al. (2007) in a single dose by three different routes, intramuscular (i.m.), intraperitoneal (i.p.) and s.c., after mice had been burned and infected with a fatal dose of *P. aeruginosa*. Although all the routes of administration increased mice survival, i.p. injection was the most successful one. The differences in the efficacy may be a consequence of different pharmacokinetics of phage after different application routes. As the authors noticed, i.p. administration allowed phages to disseminate in the system earlier and at a higher dose than the other routes of administration (McVay et al. 2007).

Despite several studies used topical or systemic administration of phages, only a few have compared their efficacy. Recently, the s.c. application versus topical application of phage Abp1 was tested in mice wounds infected with *Acinetobacter baumannii*. The authors observed that the topical application of phage resulted in smaller wounds than in s.c. injected mice (Yin et al. 2017), suggesting that topical application is probably a good choice in wound treatment.

Mendes et al. (2013) pointed out that a phage cocktail administered topically was more effective when added after a wound debridement treatment. Although all the phage treatments were able to significantly reduce bacterial cell counts and improve wound healing in animal models, the efficiency of the phage cocktails varied according to the target bacteria as it was not so effective against *A. baumannii* ($<$1 log reduction) as it was against *P. aeruginosa* (4 log reduction) and *S. aureus* (3 log reduction) (Mendes et al. 2013).

The aforementioned studies have shown the potential and efficacy of phages to treat bacterial infections of skin and soft tissues, namely using topical but also parenteral application of phages (Figure 1). However, it is important to highlight that the majority of these studies were tested on models of acute infection, mainly due to the difficulties in establishing chronic models of infection. There are other emerging pathogens causing SSTIs, namely *Cultibacterium acnes*, *Mycobacterium marinum*, *Mycobacterium szulgai*, *Treponema pertenue and Actinomadura madurae*, for which there are no data available and consequently, further studies on these microorganisms should be performed.

Eye, ear, and dental infections

Eye infection can occur when pathogens invade and colonize the cornea or the conjunctiva. Methicillin-resistant *S. aureus* (MRSA) is the most common eye pathogenic agent, causing for example conjunctivitis, preseptal cellulitis and/or lid abscess, keratitis, endogenous endophthalmitis, orbital cellulitis and blebitis (Blomquist 2006). Other bacterial pathogens such as *E. faecalis* and *P. aeruginosa* also cause these types of infections (Gorski et al. 2009). Although the risk of spreading is high and millions of people suffer from conjunctivitis on a daily basis, epidemiological data are limited.

Recently, the *P. aeruginosa* phage KPP12 was isolated and characterized, and its ability to treat keratitis in mice was assessed. KPP12 was administered as eye drops of 10^8 PFU/ml to *P. aeruginosa* infected eye. The results showed that a single phage dose controlled the infection, preserving the corneal structure (Fukuda et al. 2012). More recently, a phage cocktail composed of two phages was also applied as eye drops to combat a *P. aeruginosa* keratitis infection in mice. The results revealed that the phage treatment was successful when the cocktail was applied within 3 h post-infection using an MOI of 100 (Furusawa et al. 2016). Although there are few reports on phage therapy in eye infections, both studies have shown efficacy with no side effects reported, emphasizing that phage-containing eye drops can be a promising approach to treat eyes with antibiotic-resistant infections (Figure 1).

Otitis is a general term for inflammations and infections of the inner, middle or outer ear. Acute otitis media is a common problem and a leading cause of health care visits and antibiotic prescription in children, where it affects up to 75% of children up to the age of 5 years (Klein 1994). *P. aeruginosa* is one of the more prevalent chronic externa and media otitis causative agents. In a veterinary study, a cocktail of six different phages was developed and used as a treating agent in 13 dogs with chronic *P. aeruginosa* otitis (Hawkins et al. 2010). The cocktail was topically administered into the auditory canal in a concentration of 1×10^5 PFU/ml of each phage. The clinical score and bacterial counts

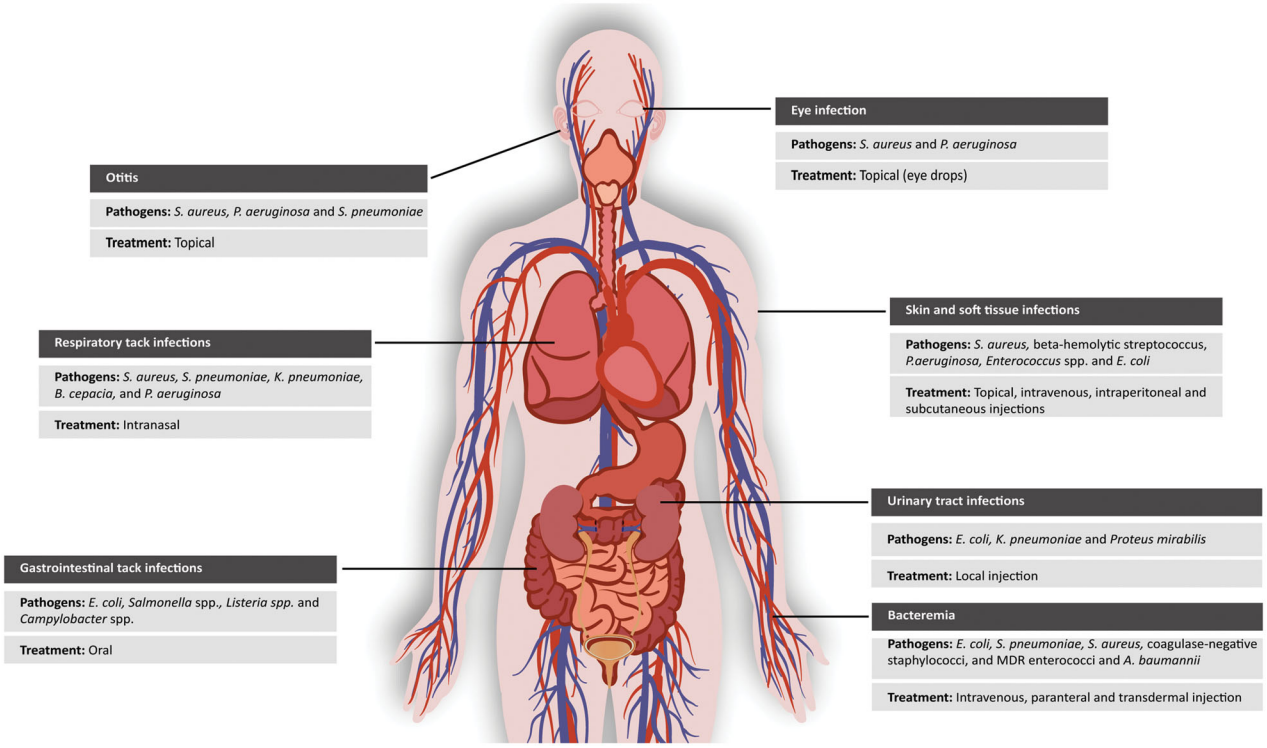

Figure 1. Different studied phage applications to treat the most common types of infection.

were decreased at 48 h after treatment, with no adverse effects detected.

Despite the lack of other studies of otitis in animal models, the topical application of phage to cure outer ear infections seems to be justified. In more inaccessible zones such as the media or the inner ear, phage inhalation could be a valuable therapeutic complement (Mirandola et al. 2013). Also, further tests in other relevant otitis-causing agents should be addressed, namely against *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis* (Broides et al. 2009).

Dental infections are infections that occur at tooth or tooth-supporting structures and that can be easily spread throughout the surrounding tissue. These infections usually occur after trauma, development of dental caries or dental procedures where bacteria gain access to the dental pulp and spread to the adjacent tissues. In the USA, it is estimated that more than 20% of the population have untreated dental caries, and around 75% has at least one dental restoration during their lifetime (Dye et al. 2012). The prevalence of caries and consequently of dental infections is dynamic during lifetime being more present in adulthood in comparison with childhood (Beltran-Aguilar et al. 2005; Pourhajibagher et al. 2017). In a recent study, a poloxamer P407 formulation containing enterococci phages EFDG1 and EFLK1 phages were applied in a rat root canal infection model. The formulation was injected into the root canals being further spread intracanal during gelation process and finally sealed. The treatment lasted for 3 weeks and the formulation reduced by 99% enterococci cell counts (Shlezinger et al. 2019). Even with few studies applied on this type of infections, further studies could target other relevant pathogens including streptococcal and non-streptococcal bacteria (e.g. *Bifidobacterium* spp., *Scardovia* spp. and *Actinomyces* spp.) and anaerobic pathogens more present on the subgingival area, namely *Bacteroidaceae* spp. and spirochaetes (Lamont et al. 2018). Moreover, the use of phage therapy has been suggested to be applied to other oral pathologies, namely periodontitis (Pinto et al. 2016).

Respiratory tract infections

Respiratory tract infections (RTIs) are generally distinguished as upper respiratory (if located in the nose, sinuses or throat) or lower respiratory (if located in bronchi or lungs) infections. Depending on the type of infection (e.g. sinusitis, pneumonia), the way of contraction (i.e. nosocomial versus community-acquired), and other factors (e.g. whether patients are immunocompromised), several microbes can colonize the mucosa. In the upper respiratory tract there is a predominance of viral infections, but they often pre-dispose patients to secondary and severe bacterial infections (Juven

et al. 2000). In fact, bacterial infections are observed in 60% of patients with upper RTIs, with *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *K. pneumoniae* and *Streptococcus pyogenes* being the most commonly involved pathogens (Stensballe et al. 2003; Wardlaw et al. 2006) (Figure 1). Bacterial infections are even more common in the lower respiratory tract and more life-threatening, especially for children under 5 years. About 20% of children hospitalizations are diagnosed with respiratory diseases, and of those, 90% are pneumonias (World Health Report 2008). This scenario leads to 2.2 million childhood deaths annually, according to the latest surveillance report from the WHO (Williams et al. 2002; World Health Organization 2011). Less frequent, but equally important, are lung infections of patients suffering from cystic fibrosis (CF). CF is a genetic disorder characterized by increased mucus clogs in the airways and lungs. *Burkholderia cepacia* and *P. aeruginosa* play an important role in CF pathogenesis as opportunistic pathogens often displaying high antibiotic resistance. *P. aeruginosa* poses a special risk to CF patients as it represents 80% of the total pulmonary infections (Hoiby 2011). Although a smaller fraction of infections is caused by *B. cepacia*, the prognosis for CF-infected patients is poor, with an increased risk of death (Govan et al. 2007).

Upper respiratory infections

Probably due to lower severity, the application of phages against upper respiratory infections is less represented, compared with infections located in the lower sites of the respiratory tract (Table 2). To our best knowledge, only four studies have aimed to decolonize nares, three from *S. aureus* and one from *P. aeruginosa*. Two types of animal models were used, mouse and sheep. In both, the nasal tissue was exposed to bacteria and subsequently to phages, after which the tissue was extracted for histological analysis and assessment of the bacterial load. The results showed that, although phages were able to decolonize nares from *S. aureus*, the effect was similar to that achieved with antibiotics or chelating agents. Chhibber et al. (2014) compared intranasal (i.n.) administration of phage (MOI of 1) to the treatment with mupirocin and a significant reduction (>2 log reduction) of MRSA from nares of mice 7 days after challenge was observed. Combination of both phage and mupirocin led to the complete eradication of MRSA even at day 5. Drilling et al. (2014) studied the efficacy of topical application of a phage cocktail flushed daily for 3 days to treat sinusitis in a sheep model. The significant reduction of biofilm formed on the extracted sinuses showed an effective short-term treatment of the sinus mucosa affected by *S. aureus* mucosal biofilms. EDTA is a metal chelator with therapeutic potential against biofilms, so the authors compared its effect with the phage cocktail. It was also shown that EDTA-treated nares demonstrated a similar effect (to that in phage-treated nares), and no synergy was obtained when EDTA was combined with phages.

These two studies highlight that applications of a single phage and phage cocktails are possible means to reduce the nasal bacterial load. However, the use of phage cocktails covering a wider host range is crucial for successful nasal decolonization in clinical settings. The administration of higher phage doses would possibly be beneficial for sinusitis treatment as well as the phage combination with other active agents, as demonstrated with mupirocin.

To investigate the safety of longer-term treatments, Drilling et al. (2017) conducted a second study where they flushed the frontal sinus of sheep contaminated with *S. aureus* with a phage cocktail for 20 days. No side effects, inflammatory responses or tissue damage were observed. Similar findings were obtained by Fong et al. (2019), who applied a *P. aeruginosa* phage cocktail twice daily to the frontal sinus of sheep for 3 weeks, with no adverse effects observed.

Taken together, the phage therapy in the field of rhinology to control sinonasal bacterial biofilms seems to be a safe and promising approach, independently of animal model, type of phage (single or cocktail) or dose (single or multi) when administered intranasally (Figure 1).

Lower respiratory infection

Lower respiratory infections can be life-threatening and therefore have been extensively studied by phage scientists, in mice and mink models (Table 2). Mouse models have been widely used for testing phages in pulmonary infections caused by *K. pneumoniae, B. cepacia*, *P. aeruginosa, A. baumannii* and *S. aureus*. Cao, Wang, et al. (2015) demonstrated that mice infected with a MDR *K. pneumoniae* (10^8 CFU/mouse) could be controlled by phage 1513 (10^7–10^9 PFU/mouse) applied i.n. 2-h post-infection. Also, the cytokine levels (tumour necrosis factor α and interleukin-6) in the lung tissue of phage-treated mice were remarkably lower than those of the control mice, demonstrating the anti-inflammatory effect of the treatment and thus documenting the potential of phage therapy as alternative treatment in pneumonia caused by MDR pathogens. The mink model of haemorrhagic pneumonia was also explored by Cao, Zhang, et al. (2015). The authors collected *P. aeruginosa* strains from minks with lung infection

and isolated a lytic phage PPA-ABTNL against that bacterial strain. Minks were challenged with 10^8 CFU of *P. aeruginosa* and treated i.n. with phage (MOI of 1–100) 2 h later. A MOI of 10 proved to be enough to treat haemorrhagic pneumonia with an 80% survival rate scored 12 days after phage administration.

The phage administration dose to treat RTIs can be detrimental. Debarbieux et al. (2010) implemented a *P. aeruginosa* acute lung infection in mouse models and, in curative treatments, applied phages i.n. Phages had a protective effect when applied 24 h before bacterial infection. Moreover, they saved mice when applied 2 h after inoculation with bacteria. This positive effect was however dose-dependent, as 100% of the animal survival was achieved only with a MOI of 10. Jeon et al. (2016) also demonstrated a dose-dependent effect in the treatment of *A. baumannii*-induced pneumonia in mice. The authors showed that the i.n. application of phage 30 min after bacterial inoculation could clear the pathogen from the lungs within 3 days and that the survival rates of mice varied from 100%, 50% to 16% when treated with phage at MOI of 10, 1 or 0.1, respectively.

Regarding the administration route, generally, a protective effect of phages has been observed after their administration by the i.n., i.p., or i.v. route or endotracheally. The most popular route to treat respiratory infections was i.n. administration (Table 2). Alemayehu et al. (2012) applied i.n. a phage cocktail that was effective in killing lux-tagged PAK mucoid CF strain in murine lungs. The light emitted by labelled pathogen cells from the control mice reached a maximum at 6 h after infection, and it was significantly decreased in mice treated with phage during the same period. In addition to therapy with a phage cocktail, treatments with single phages have shown to be equally successful. The group of Debarbieux also proved that a single i.n. application of phage to immunocompetent mice 2 h after infection could rescue 95% of treated mice infected by a MDR mucoid *P. aeruginosa* strain (named CHA) isolated from a CF patient of Grenoble hospital in France (Morello et al. 2011). Roach et al. (2017) reported an interesting study showing that the functional immune system was essential for the effectiveness of phage therapy. The authors applied phages i.n. against *P. aeruginosa* induced pneumonia using several mice strains and conditions: (i) wild type healthy immunocompetent, (ii) MyD88-deficient, lymphocyte-deficient and (iii) neutrophil-depleted mice. The results demonstrated that neutrophils were required to control phage-sensitive and emergent phage-resistant bacteria, emphasising the need to complement phage therapy in order to increase its efficacy in patients with immunodeficiencies.

All the examples mentioned above demonstrated the high applicability of the i.n. administration route, but other routes of phage administration have also been studied. Oduor et al. (2016) showed that *S. aureus*-induced pneumonia in mice can be treated by injecting phage i.v. Phage was administered at 10^8 PFU/ml and it reduced the bacterial load from 8 CFU/g in the non-treated group control to 0.5 CFU/g in the lung tissues. The authors also showed that clindamycin (8 mg/kg of body weight) or combined treatments with phage could also reduce the bacterial load, but was not as efficient as phage-alone treatment. Antagonistic effects may be explained by the bacteriostatic effect of clindamycin that can hinder protein synthesis in bacteria together with subsequent phage proliferation. Carmody et al. (2010) compared i.n. and i.p. administration routes in mice, revealing that i.p. administration of BcepIL02 phage was more effective to control acute *Burkholderia cenocepacia* lung infection. However, Semler et al. (2014) contradicted that study, by showing that aerosol dissemination of the phage KS12 was more effective than i.p. injection of phages to treat *B. cenocepacia* RTI. A similar MOI was used, indicating that the differences observed can possibly be attributed to the bacterial strain, infectious dose, phage species or other factors related to animal experiments such as the mice strains. One elegant approach to improve i.p. efficacy was demonstrated by Singla et al. (2015), who improved pharmacokinetics of phage by entrapping virions into liposomes. The authors achieved effective therapy with liposome-entrapped phages (3 days post-infection) or prophylactics (24 h prior infection) against *K. pneumoniae*-induced lobar pneumonia. In contrast, free phages (nonliposomal) were only effective when administered 1 day post-infection or 6 h prior infection. Reduction of the inflammatory markers further supported the superior effect of liposomes as delivery vehicles of phages.

A possible solution to improve phage delivery is dry-powder products, which could facilitate storage, transport and standardize phage administration. Chang et al. (2017) and Chang et al. (2018) validated this option by formulating a phage spray with lactone and leucine to promote higher stability during spray drying and protection from moisture, respectively. This phage preparation administered via the endotracheal route could reduce by 5 log the bacterial load in the lungs of mice infected by *P. aeruginosa* after 24 h. No adverse effects were observed in healthy mice.

Despite the general success of phage therapy in *in vivo* models, one important issue is related to the fact that in the studies discussed herein phages were only applied a few hours (usually 2 h) after infection, which can only be translated to starting treatment at an early stage of infection. To address this issue, Waters et al. (2017) reported the first study showing the effectiveness of phage treatment up to 7 days post-infection. The authors reported that phages administered i.n. at a MOI of 10 could completely clear the *P. aeruginosa* from infected lungs either when applied at 24, 36 or 48 h post-infection, encouraging the potential use of phage therapy against established long-term chronic lung infections.

Another important concern is the lack of clear guidelines to choose phage strains applicable in therapies. Therefore, Henry et al. (2013) proposed, for the first time, a reliable index to predict phage treatment efficacy. Using the bioluminescent *P. aeruginosa* PAK strain, a well-established mouse lung infection model and a real-time imaging system, the *in vivo* effectiveness of phage therapy was assessed. In this model, the authors demonstrated that phage infectivity *in vitro* cannot always be directly extrapolated to good *in vivo* efficacy. The authors also observed that phages isolated directly on the targeted host were the most efficient, supporting a personalized approach favouring optimal treatment.

Concerning the safety aspect of phage treatment, Dufour et al. (2019) used a murine *E. coli* caused acute pneumonia model and assessed the treatment with phages or antibiotics (ceftriaxone, cefoxitin and imipenem-cilastatin). The authors demonstrated that while both treatments could significantly reduce the lung infections, they had similar endpoints. Interestingly, they also reported that phages could control the infection more quickly than the antibiotics and phage treatment did not result in an increase of innate inflammatory response (due to the release of bacterial debris) when compared to the antibiotic treatments.

Taken together, phage effectiveness to treat RTIs seems to be strongly dependent on the infection type and route of phage administration. Generally, the i.n. administration seems to provide more promising results. While some common respiratory pathogens have been studied (e.g. *K. pneumoniae*, *B. cepacia, P. aeruginosa*, *A. baumannii*, *S. aureus*), surprisingly, no studies on the deadliest respiratory bacterial pathogens, such as *S. pneumoniae*, *H. influenzae* or *Mycoplasma pneumonia*, have been reported so far. Moreover, there are no current animal models that mimic the increased formation of mucus clogs in the airways and lungs, typical of CF disorder, and that can have a significant impact on phage efficacy.

Gastrointestinal tract infections

Gastrointestinal tract infections (GTIs), also known as gastroenteritis, are inflammations of the stomach and/or intestines that result from acute enteric infections and diarrheal diseases. Based on epidemiological studies, it is estimated that 5 billion cases of gastroenteritis occur annually, which result in 1.4 million deaths worldwide (Elliott 2007; Lozano et al. 2012). Furthermore, GTIs represent the leading cause of infant mortality. Outbreaks of gastroenteritis are caused by frequent bacterial pathogens such as *E. coli*, *Campylobacter jejuni, Salmonella* Enteritidis and *Listeria monocytogenes*, and also by less frequent but still significant *Clostridium difficile* and *Vibrio cholerae*. These infections often occur as a result of poor sanitation, lack of safe drinking water or contaminated food (Figure 1) and are typically known as food poisoning.

To treat human GTIs, phage effectiveness has been studied in animal models (mice, rabbit and hamster). Generally, phages are able to survive in the GI tract, and most importantly, they protect animals from GTIs. In mouse models, Denou et al. (2009) showed that a T4-like coliphage cocktail (10^9 PFU/ml) administered with drinking water to infected animals could reach the duodenum and the distal parts of the gastrointestinal tract without causing mice weight loss or abnormal behaviours, which is a prerequisite of the phage effectiveness to control GI tract pathogens. Nevertheless, Maura et al. (2012) clearly demonstrated that bacterial susceptibility to phage infection is not uniform throughout the gut. Feeding mice with three different *E. coli* O104:H4-infecting phages in the drinking water 24 h after bacterial challenge, the authors showed that the O104:H4 55989Str strain present in the ileum was sensitive to all phages, but the cells recovered from faeces were permissive to only one. In addition, the therapeutic treatment with a phage cocktail had only a transient effect as the bacterial levels in the ileum and faeces restored their initial concentrations after the third day, even though the phage titre was maintained relatively high (Nikkhahi et al. 2017). *E. coli* probably becomes resistant as a response to phage predation, similarly as observed with *Campylobacter* in the avian gut (Scott et al. 2007). However, other reports have shown the success of phages in controlling diarrheal diseases in mice, although in some cases antibiotic treatment (ciprofloxacin) was found to exert a similar or even better

antimicrobial effect (Jaiswal et al. 2014; Nikkhahi et al. 2017).

In a rabbit model of cholera infection, Jaiswal et al. (2013) demonstrated that phage cocktails could reduce *V. cholerae* burden when given 6 and 12 h after bacterial challenge. Similarly, Jaiswal et al. (2014) compared phage cocktail with antibiotics and with oral rehydration treatments to control *V. cholerae* infection, by applying each treatment orally over a 3-day period. Whereas oral rehydration had no effect, phages could reduce the bacterial burden (by 3 $\log_{10}$/g), but maximum protection was achieved by ciprofloxacin treatments (reduction of >5 $\log_{10}$/g). In a hamster model, Nale et al. (2016) showed that a phage cocktail could significantly reduce *C. difficile* colonisation at 36 h post-infection and it delayed the onset of disease symptoms by 33 h. This demonstrates the potential of phages to control bacterial infections in the gut.

The choice between a mono-phage therapy and phage cocktails seems to favour the latter for the possibility of covering a wider range of strains capable of causing clinical diseases. Although both strategies have successfully been used to control enteric pathogens, phage cocktails have been the most often reported. The time of administration, however, seem to be relevant. While reports show that phages are able to reduce pathogens several hours post-infection, prophylactic applications seems to be less efficient. This was shown in the experiment by Jaiswal et al. (2013) where phages reduced the shedding of *V. cholerae* when given 12 h post-infection, but had no effect when added prior to bacterial challenge. It is possible that at least some phages maintain their ability to infect bacteria relatively briefly when administered orally; thus concentrations of active phage drop to inefficient levels before they encounter sensitive bacteria.

Concerning the mode of administration, many models comprised phages applied continuously with drinking water or by serial dosing, but administration of single phage doses by oral or intracloacal gavage is also possible. Nikkhahi et al. (2017) evaluated the effect of a single phage dose (10^9 PFU/mouse) to treat salmonellosis induced by *Salmonella* Enteritidis, applying them with an oral gavage syringe 4 days after challenge. Phage treatment protected mice from salmonellosis and prevented weight loss in mice as a disease symptom. Ciprofloxacin used as a positive control had a similar protective effect but it did not protect against weight loss. Similar results demonstrating the effectiveness of single doses of phage were obtained by Vahedi et al. (2018), who controlled enteropathogenic *E. coli* in mice, and by Jun et al. (2014), who successfully treated mice infected with *Vibrio parahaemolyticus* (marine bacterium causing infections associated with consumption of raw oysters). Jaiswal et al. (2013) also showed that phages administered orally to rabbits by gastric tube 6 and 12 h post-infection controlled *V. cholerae* infections.

Taken together, phages seem to have a potential to control enteric pathogens as demonstrated in many animal models, bacterial hosts and types of administration (Table 2). Since in some cases only a transient improvement has been reported, the use of phage cocktails and multiple applications are recommended. Of note, reports generally suggest that some phages are naturally resistant to stomach conditions, at least to the extent that allowed for the positive effects of treatment (Bruttin and Brussow 2005; Litt and Jaroni 2017). However, extreme pH of the stomach varies between rodents (3.5–5.2) and humans (1.5) (Kararli 1995; Fallingborg 1999; Beasley et al. 2015). Therefore, administration of phage with anti-acids (Koo et al. 2001), engineered phages displaying lipids on their surfaces (Nobrega et al. 2016) or microencapsulated phage formulations (Ma et al. 2008; Islam et al. 2018) to increase phage tolerance to acidity can be considered. It is also important to highlight that phages offer the possibility of reducing gut pathogens with a low impact on microbiota composition when compared with antibiotics (Galtier et al. 2016).

In the future, more detailed phage-host interaction studies in the gut should be further explored, testing various dosages and schedules, and studies should be extended to other gastrointestinal pathogens. Specifically, *Helicobacter pylori* is a pathogen responsible for acute gastritis and peptic ulcer diseases. Despite being one of the most prevalent human pathogens, no lytic phages active on *H. pylori* have been isolated so far. Furthermore, some well-known (e.g. *Shigella sonnei* and *Serratia marcescens*) and newly identified bacterial species (e.g. *Campylobacter concisus*, *Edwardsiella tarda*, *Aeromonas hydrophila*) should also be considered as targets for phage control (Schlenker and Surawicz 2009).

Urinary tract infections

Urinary tract infections (UTIs) are amongst the most common bacterial infections, affecting annually around 150 million people worldwide (Stamm and Norrby 2001). It has been estimated that just in the US, 10 million patients are admitted to hospital facilities with UTI symptoms, causing health care costs of roughly US$3.5 billion annually (Foxman 2014). Such infections are associated with morbidity in females (all ages), infant

boys and older men (Flores-Mireles et al. 2015). UTIs are infections affecting any part of the urinary tract, such as the kidneys, ureters, bladder and urethra. These infections are usually caused by *Enterobacteriaceae*, namely *E. coli*, *K. pneumoniae* and *Proteus mirabilis* (Flores-Mireles et al. 2015).

There are a few studies regarding the efficacy of phage therapy against UTIs. *Escherichia coli* is the main causative agent of UTIs, and to control this type of infections, Nishikawa et al. (2008) used phage therapy on a mouse model. In that study, a lethal dose of an uropathogenic *E. coli* strain was injected into the bladder. The authors observed that when phages were injected into the peritoneal cavity, they were secreted into the urine. The authors also observed that phage efficacy in controlling the infections varied depending on the phage strain and on the dose. The best results were achieved with phage T4 at an MOI of 60, resulting in survival of 100% of mice (Nishikawa et al. 2008). Using a similar administration method, two phages were used to combat *Cronobacter turicensis* UTI. This cocktail reduced by 70% the bacterial burden on mice kidneys. Moreover, other infection parameters were reduced using phage therapy (Tothova et al. 2011).

The lack of *in vivo* studies in UTI-relevant models of infection suggests that a lot of studies are still to be performed, including those of dose effect, administration route, single phage versus phage cocktails, time of administration, other locations of infection and other pathogens. Nevertheless, the positive results described so far are promising for the possible applications of phage therapy against UTIs.

Bacteraemia

The presence of viable bacteria in the bloodstream is called bacteraemia. If the host immune system fails to eliminate bacteria from the blood, this results in sepsis (Bone 1991). During recent years, the incidence of either community- or hospital-acquired bacteraemia has increased considerably (Friedman et al. 2002). Bacteraemia aetiology varies according to geographic location, environment, age, sex and physiological state of the patient; mortality ranges from 4 to 41.5% (Christaki and Giamarellos-Bourboulis 2014). *Escherichia coli*, *S. pneumoniae*, MRSA, coagulase-negative staphylococci, and MDR enterococci are among the most frequently isolated pathogens causing systemic infections (Kollef et al. 2011) (Figure 1). The treatment of these infections is difficult, mostly due to the spread of MDR strains with a limited number of effective antibiotics available. To overcome this problem, several studies have been performed to assess the efficacy of phages in the treatment of bacteraemia in different *in vivo* models (Table 2).

The majority of studies have been based on models of systemic infection in mice. Nevertheless, in 2009, Heo et al. (2009) validated the use of a *Drosophila melanogaster* model to evaluate the efficacy of phage therapy. The systemic infection was caused by a lethal dose of *P. aeruginosa*. Two phages administered by feeding were able to significantly delay or prevent fly death. Due to this positive effect, both phages were injected into mice by either the i.m. or i.p. route 6 h after bacterial challenge. A set of doses was studied. Although i.m. administration showed better pharmacokinetics, i.p. injection was more efficient in rescuing mice from septicaemia caused by *P. aeruginosa*.

Tiwari et al. (2011) studied phage PA1Ø using different MOIs (1, 10, and 100), injecting the phage i.p. into both immunocompetent and neutropenic mice infected with *P. aeruginosa*. While the phage therapy extended the life time of neutropenic mice only for a short time, normal mice were all rescued by phage treatment. The authors also suggested that the combined effect of phages and neutrophils was crucial for an efficient bacterial killing in the reported model (Tiwari et al. 2011). However, in another study it was demonstrated that phage therapy effectively controlled *S. aureus* infections in neutropenic mice (Sunagar et al. 2010) and immunosuppression did not impair phage efficacy. A single injection of GRCS phage in streptozotocin-induced diabetic mice was able to save 90% of the infected mice, even when the treatment was delayed to 4 h after bacterial challenge.

Although the majority of the studies of phage therapy relied on the use of a single phage, it was demonstrated that a cocktail of three *K. pneumoniae* phages injected i.p. had a stronger life-saving effect compared to mono-phage therapy (Gu et al. 2012). This result was attributed to the reduced mutation frequency of *K. pneumoniae* during the cocktail treatment. Besides reducing the level of bacterial counts in the bloodstream and the consequent associated mortality, phages were also able to alleviate gut microbiota imbalance provoked by bacterial challenge (Cheng et al. 2017).

Due to the rapid mortality caused by systemic infections, phage treatment has typically been applied shortly after bacterial challenge. Almost all the models recently reported relied on phage application up to 4 h after bacterial inoculation (Pouillot et al. 2012). In a lethal *E. coli* murine model, when a single dose of phage particles was i.v. injected 10 and 60 min

following bacterial challenge, 100% and 95% mouse survival was observed, respectively. However, no mice were rescued when phage administration occurred 3 h post-infection (Schneider et al. 2018). In contrast, one report presented later use of phage therapy. In a rat model of *E. coli* sepsis, EC200 phage was administered 7 h or 24 h after bacterial challenge and resulted in survival of 100% and 50% of rats, respectively (Pouillot et al. 2012). It was recently suggested that the most proper routes for phage delivery to combat bacteraemia are i.v., parenteral and transdermal administration (Oliveira et al. 2015), and for the majority of the cited studies i.v. injections were successfully used to combat systemic infections (Figure 1).

How pre-clinical studies in animal models correspond to results of recent human tests and clinical trials

Much of the knowledge of phage therapy practices is derived from Eastern Europe, namely from Georgia and Poland. At the Eliava Institute of Bacteriophages, Microbiology and Virology in Georgia, the use of phage cocktails is a standard medical practice used both for preventing and controlling bacterial infections. However, several studies are not well documented or accessible in English (Kutter et al. 2010). In Poland, at the Hirszfeld Institute of Immunology and Experimental Therapy, historical and modern observations of the potential of experimental phage therapy in humans are documented (Miedzybrodzki et al. 2012). In the US and Western Europe, only some studies have reached the point of human testing under the standards of regulatory agencies. In the last 10 years, at least four phase I/II clinical trials involving topical and oral phage administration have been conducted. One of the first phase I clinical trials, reported in 2009, was developed at the Southwest Regional Wound Care Centre (USA) using a phage cocktail targeting *S. aureus*, *P. aeruginosa* and *E. coli* against venous leg ulcers, and aimed at assessing the safety of phage therapy (Rhoads et al. 2009). In this trial, no significant differences between test and control groups were reported in terms of frequency of adverse effects. Similar conclusions were taken from human volunteers from Bangladesh administered with a T4-like cocktail composed of nine phages (10^7 or 10^9 PFU doses) or placebo (Sarker et al. 2012). In the randomized, double-blind, placebo-controlled study reported by Wright et al. (2009), including 24 patients with chronic otitis caused by *P. aeruginosa,* the efficacy of the phage cocktail Biophage-PA was tested. The authors reported statistically significant differences with a decrease of the *P. aeruginosa* counts in phage-treated patients, associated with an improvement of the clinical indicators. Furthermore, no adverse effects were reported. Encouraging results regarding the safety of phage therapeutics are in line with those from animal models, where adverse effects of phage treatments are typically not observed.

More recently, the results of the phase I-II clinical trial Phagoburn, which ran from 2015 to 2017 with the objective to treat burn wounds infected with *P. aeruginosa,* were reported (Jault et al. 2018). In this study, 27 patients were recruited, from which 13 were treated with the phage cocktail with a predicted MOI of 10 and 14 with standard of care (1% sulfadiazine silver emulsion cream). The phage cocktail comprised 12 natural lytic anti-*P. aeruginosa* phages and was administered topically for 7 days. Although one participant of each group died from treatment-unrelated reasons, generally the phage cocktail was less efficient in reducing the bacterial burden than standard of care. The authors explained that these unsuccessful results were probably due to the small patient sample size, the low phage dose administered due to the loss of the titre of the initial phage preparation (during storage) and the low susceptibility of bacterial isolates to the low phage doses. According to the authors, further studies addressing these issues are needed (Jault et al. 2018). Of note, the importance of phage dose for topical treatment of infected wounds has been suggested by animal models, where the successful experiments reported MOIs ranging between 10 and 200.

A placebo-controlled, double-blind trial has been developed to investigate the efficacy of the phage cocktail Pyo bacteriophage against UTIs in patients planned for transurethral resection of the prostate. In this trial, three groups of 27 patients have been planned: (a) placebo solution; (b) Pyo bacteriophage cocktail or; (c) antibiotic treatment. This trial was registered in 2017, and the outcomes of this study are still awaited (Leitner et al. 2017). Finally, a phase II trial (identifier code: NCT02664740) in progress aims to compare the efficacy of standard treatment associated with a topical anti-staphylococcal bacteriophage cocktail versus standard treatment plus placebo for diabetic foot ulcers monoinfected by methicillin-resistant or susceptible *S. aureus* (MRSA or MSSA) as measured by the relative reduction in wound surface area (%) at 12 weeks.

No results from full-scale clinical trials involving intravenous phage administration have been reported so far. Nevertheless, a few individual cases of compassionate phage therapy have been documented. In 2017, there were two cases where phage therapy was applied

as last resort treatment to human patients. In a patient with a *P. aeruginosa*-caused septicaemia, a cocktail of two phages with activity against the patient's strain was administered. The cocktail was administered i.v. every 6 h for 10 days and the patient's wound was irrigated with the same cocktail every 8 h during the same period. All infection parameters improved almost immediately after the treatment (Jennes et al. 2017). In another case report, nine phages were used to treat a 68-year-old diabetic patient with necrotising pancreatitis caused by a complicated MDR *A. baumannii* infection. Two different phage cocktails were developed, one for i.v. administration and another for percutaneous administration into the abscess cavities. Eight days after treatment, phage-resistant phenotypes of bacteria emerged; thus a third cocktail for i.v. administration was developed. After a few days of phage treatment, the patient demonstrated improvement in all fronts (Schooley et al. 2017). A few years before, in 2015 Fadlallah et al. (2015) reported a successful treatment of a corneal infection caused by *S. aureus* at the Phage Therapy Centre in Tbilisi. The patient was treated with *S. aureus* phage SATA-8505 that was administered by eye drops, nasal spray and i.v. for 4 weeks. Six months after treatment, the patient's ocular signs stabilized and no *S. aureus* was detected on ocular and nasal cultures. This is in line with encouraging effects of phage therapy in animal models of eye infections. More recently, another successful clinical case was reported with the administration of engineered phages for the treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus* infection. A significant improvement of the overall clinical state was reported after 32 weeks of prolonged treatment with a three-phage cocktail in multiple doses administered i.v. and topically (Dedrick et al. 2019). On another recent study case, three lung transplant recipients with life-threatening infections caused by *P. aeruginosa* (two patients) and *Burkholderia dolosa* (one patient) received phage therapy in combination with antibiotics. Cocktails comprising different lytic phages were selected for each individual. Phages had different dosing routes and frequencies, depending on cocktails and patients. Although one of the patients died due to infection relapsing, phage therapy was associated with a clinical improvement of the patients not observed with antibiotic therapy (Aslam et al. 2019). Very recently another successful case was reported involving phage and antibiotic combined therapy. A patient with a left tibial infection caused by *A. baumannii* and *K. pneumoniae* was treated with phages (i.v. injection) targeting both strains along with meropenem and colistin. Few days after treatment it was possible to observe tissue healing and elimination of positive cultures. The treatment outcome prevented patient's leg from amputation (Nir-Paz et al. 2019). The first obvious conclusion drawn from these clinical studies and trials is that phage therapy seems to be safe as no adverse effects were identified in any of the reported studies. The same was concluded from the pre-clinical studies in animal models. In terms of its efficacy, the results are quite variable and very dependent on the type of infection. Chronic infections are generally more difficult to tackle than acute infections. These infections are characterized by the presence of biofilms, which are sessile bacteria in a low metabolic state embedded in a self-produced polymeric matrix that are difficult to access by potential antimicrobials, including phages (Pires et al. 2017). Chronic infections are also more difficult to mimic in animal models. Nevertheless, both pre-clinical and clinical trials demonstrated successful outcomes with topical application of phages usually associated with prior debridement (when it was possible) and using high phages titres. Phage concentration seems to be a key factor for the outcome of phage therapy, since *in vivo* trials demonstrated better therapeutic efficacy when high phage titres were applied, usually at an MOI of 10 and concentrations greater than 10^7 phage active particles per treatment (10^7–10^9 PFU). The administration of a cocktail of phages has usually proven to be better than a single phage application. Monophage therapy is probably more likely to lead to the emergence of phage resistance in bacteria. It is also less likely that a random clinical isolate of bacteria will be susceptible to one phage (than to several); thus monotherapy probably requires pre-testing for susceptibility of the infecting bacteria to the phage, and sometimes *in vitro* and *in vivo* susceptibilities do not coincide (Chibani-Chennoufi et al. 2004). Other treatment variables such as the optimal timing of administration, duration of treatment and route of administration are difficult to generalizse based on pre-clinical studies. For example, treatment of systemic infections in mouse models only proved to be effective when phages were administered a short time after bacterial challenge (Gu et al. 2012). Conversely, the few cases of compassionate treatment of systemic infections in humans have been successful independently of the timing of phage application. However, in most clinical cases, patients are also receiving antibiotic therapy and therefore it is difficult to distinguish between the effect of phages and antibiotics alone, or a potential synergistic effect. Although phage-based clinical products are commercially available in some Eastern European countries, in Western Europe or

USA no phage products for clinical applications have yet reached the commercial stage (Pelfrene et al. 2019). In the future, the compilation of clinical trials data, namely phase III results, will surely boost phage therapy in Western medicine.

Challenges and limitations

The increasing amount and significance of phage therapy studies over the last decades, and the promising outcomes that have been achieved so far, have been paving the way to bring phages into clinical practice. Phages can play a vital role in the treatment of antibiotic-resistant infections, one of the greatest threats to global health. Despite the great potential of phages, there are still several constraints that limit their broad application and acceptance in clinical practice. Some issues can be an advantage or a disadvantage, such as the limited phage host range, which allows for saving commensal microbiota in treated patients but also causes difficulties in rapid selection for an appropriate phage to combat an infection. Furthermore, the emergence of phage-insensitive bacterial mutants as well as the relatively rapid clearance of phages by the immune system may reduce phage efficacy *in vivo*. Also, the stability of various types of phages must be addressed in the coming years of phage research (Loc-Carrillo and Abedon 2011; Lu and Koeris 2011; Oliveira et al. 2015).

The problem of the narrow lytic spectra of phages is often circumvented by the individual selection of phages for each patient infected by specific bacteria, or by the use of phage cocktail formulations, consisting in the combination of multiple phages with complementary host ranges and features (e.g. targeting different bacterial receptors) in a single preparation. Besides expanding their spectrum of activity to target a wide range of bacteria, phage cocktails also prevent the emergence of phage-resistant variants (Chan et al. 2013). Another option consists of monitoring the bacterial population variation and applying consecutive treatments with different phages effective against the isolated infecting bacteria.

The choice of the administration dose and route has a relevant impact on the outcome of treatment since its efficacy is highly dependent on the phage concentration achieved at the site of infection (Malik et al. 2017). Strategies to enhance the stability of phages in unfavourable pHs or temperatures and to avoid phage clearance from the body in order to maintain active phages at infective doses are being explored. A possible approach includes the encapsulation of phages on different matrices (e.g. in liposomes, alginate, cellulose and others). *In vivo* studies using liposome-encapsulated phages have reported better persistence of phages at the infection site, increased stability and enhanced therapeutic efficacies (Colom et al. 2015; Chhibber et al. 2018). Such strategies are also important to prolong shelf life during storage of phages. Most phage preparations are used as liquid formulations and the stability of these formulations is generally limited, which is not a desirable feature for phages to become regulated pharmaceuticals, where stable dosages and well-defined pharmacokinetics and pharmacodynamics are crucial (Vandenheuvel et al. 2015; Malik et al. 2017).

Another challenge concerning phage therapy is safety in terms of phage preparations as they are applied to patients. One reason is the presence of endotoxins or other toxic bacterial products in crude phage lysates that are released upon bacterial cell lysis and that can trigger a harmful immune response. Effective purification methods to remove bacterial contaminants from phage preparations are in high demand for therapeutic phages (Van Belleghem et al. 2017). Another issue is the fact that the majority of proteins encoded in phage genomes do not have assigned functions, which leaves practitioners with the unsolved problem of predictions that may only go as far as the current list of identified undesired genetic elements. Moreover, many bacterial infections are related to the development of bacterial biofilms, which are very difficult to treat as a consequence of the protective effect of the exopolymeric matrix, which maintains the three-dimensional biofilm structure, and the different metabolic states of biofilm cells (Stewart and Franklin 2008). Since it is very challenging to mimic real clinical biofilms, most of the phage studies on biofilms have been performed using *in vitro* or *ex vivo* models, and consequently there is a lack of knowledge about how phages interact with these bacterial communities. Therefore, phage efficacy studies that investigate the interaction of phages with bacterial cells under different metabolic states are still required.

To enhance the antibacterial properties of phages and to circumvent some of the limitations concerning their application, several strategies have been exploited. A possible approach is the combination of phages with other antimicrobial agents, for example, antibiotics, natural products, antiseptics or enzymes. Besides the additive or synergistic effect that might occur between them to enhance the bacterial killing, this strategy could also arrest the proliferation of resistant variants (Torres-Barcelo and Hochberg 2016). Furthermore, the recent advances in the synthetic biology field have opened a new window of opportunity to

develop phage-engineering tools that can be applied to build synthetic phages with novel and improved functions. Several phage-engineering tools were developed and have been successfully applied to tailor phage genomes in order to expand their host range, improve their bactericidal efficacy and anti-biofilm properties, reduce phage toxicity and immunogenicity, revert bacterial resistance to antibiotics or enhance the bactericidal activity of antibiotics (Hagens et al. 2004; Lu and Collins 2007, 2009; Edgar et al. 2012; Pei and Lamas-Samanamud 2014; Ando et al. 2015).

Concluding remarks

It is important to highlight that the phage therapy studies conducted in animal models are imperfect representations of the infections that occur in humans. For instance, to demonstrate phage efficacy, lethal doses of bacteria are typically administered to animals, which results in rapid death, and the phage treatment is usually initiated immediately after bacterial challenge. Also, there are no mouse strains with increased mucus clogs in the airways and lungs that can have a significant impact on phage effectiveness to control pathogens associated with CF disorders. Also, the physiology of animals is obviously different to that of humans, including differences in anatomy, secretion (e.g. intestinal pH) and microbiomes (Dabrowska 2019). Nevertheless, while animal studies do not replicate human condition perfectly, they are generally efficient and safe. Moreover, the human clinical trials conducted so far have reported no adverse effects concerning the use of phages, which corresponds to observations in mice. Despite all the advances in phage therapy and many convincing examples of its efficacy, there are still many challenges to overcome before phage therapy being accepted in clinical practice. Clear guidelines need to be put in place for the approval of both natural and engineered phage-based products. This might encourage companies to invest in phage research, which is currently limited.

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