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Current challenges and future opportunities of phage therapy

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One sentence summary:

The remarkable potential of phage therapy for the control of antibiotic resistant infections within the One Health approach, the challenges currently faced and the potential solutions in development.

ABSTRACT

Antibiotic resistance is a major public health challenge worldwide, whose implications for global health might be devastating if novel antibacterial strategies are not quickly developed. As natural predators of bacteria, (bacterio)phages may play an essential role in escaping such a dreadful future. The rising problem of antibiotic resistance has revived the interest in phage therapy and important developments have been achieved over the last years. But where do we stand today and what can we expect from phage therapy in the future? This is the question we set to answer in this review. Here, we scour the outcomes of human phage therapy clinical trials and case reports and address the major barriers that stand in the way of using phages in clinical settings. We particularly address the potential of phage resistance to hinder phage therapy and discuss future avenues to explore the full capacity of phage therapy.

Keywords: clinical trials, phage resistance, phage engineering, phage cocktails, regulatory framework, One-Health

INTRODUCTION

The discovery of antibiotics in 1928 and their introduction in clinical practice has revolutionized the field of medicine. Since then and for decades, antibiotics were used to treat a wide range of severe infections, saving millions of lives (Davies and Davies 2010). However, nobody predicted what was about to come a few decades later. As a consequence of antibiotic overuse and misuse, bacteria managed to develop multiple antibiotic resistance mechanisms, and the golden age of antibiotics has come to an end (Davies and Davies 2010; Malik and Bhattacharyya 2019). We are currently facing a post-antibiotic era, in which common infections or minor injuries can become fatal (WHO 2014). Recent reports state that more than 2.8 million antibiotic-resistant infections occur each year in the United States and that more than 35,000 people die as a result (Centers for Disease Control). In Europe, approximately 33,000 people die every year from antibiotic-resistant infections (Cassini *et al.* 2019). If no action is taken, the World Health Organization estimates that drug-resistant infections could kill about 10 million people per year by 2050. The search and development of new and effective antibacterial compounds is urgently required to avoid such a threatening future, and (bacterio)phages might play a major role in tackling this global crisis.

Phages are bacterial viruses and the most abundant entities on Earth (Clokie *et al.* 2011; Fernández *et al.* 2019). While the use of phages in human therapy begun soon after their discovery by Frederick Twort and Félix d'Hérelle over a century ago, their application in clinical practice in Western countries was quickly overshadowed by the introduction of antibiotics (Chanishvili 2012; Gordillo Altamirano and Barr 2019). In places such as Georgia and Poland, phage therapy remained active until today, mostly via two major phage therapy centres: the Eliava Institute of Bacteriophages, Microbiology and Virology (Tblilisi, Georgia) and the Ludwik Hirszfeld Institute of Immunology and Experimental

Therapy (Wroclaw, Poland) (Rohde, Wittmann and Kutter 2018). Many patients with antibiotic-resistant infections are traveling from multiple places in the world to these centres to receive individualized phage treatments as a last hope (Rohde, Wittmann and Kutter 2018). Despite all the success cases of patients treated with phages documented to date, the introduction of phage therapy in Western countries still faces major obstacles, especially regulatory issues (Fauconnier 2019). Now, efforts to make phage therapy widely available are ongoing and a number of clinical trials are being conducted in Europe and in the United States (Sybesma *et al.* 2018; Fauconnier 2019). In this review, we will first discuss the current state of phage therapy in the Western world and then address the major challenges faced by phage therapy and the future opportunities in this field.

THE CURRENT STATUS OF PHAGE THERAPY

The clinical use of phages to treat a wide range of infections begun in the early 1920s. However, inconsistent results reported about phage trials during the 1930s and a lack of controls and inappropriate characterization, production and purification of phage preparations raised important concerns about the safety and efficacy of this therapy (Gordillo Altamirano and Barr 2019). As such, phage therapy remained active only in a few countries of Eastern Europe, where studies have provided substantial evidence of the efficacy of phages to treat certain infections with no adverse effects reported (Sulakvelidze, Alavidze and Morris 2001; McCallin and Brüssow 2017). Still, the lack of confirmation in line with evidence-based medicine, i.e. clinical trials, fuels the reluctance of regulatory agencies and clinicians from Western countries on the use of phage therapy (Sybesma *et al.* 2018). To establish phage therapy as a feasible alternative to antibiotics, clear efficacy data from randomized controlled clinical trials is required (McCallin *et al.*

2019). To tackle this situation, an increasing number of clinical trials have been carried out over the last years but only a few are currently completed (Furfaro, Payne and Chang 2018; Rohde, Wittmann and Kutter 2018; Sybesma *et al.* 2018).

In 2009, Wright et al. reported a randomized, double-blind, placebo-controlled phase I/II clinical trial approved by both UK Medicines and Healthcare products Regulatory Agency (MHRA) and the Central Office for Research Ethics Committees (COREC) (Wright *et al.* 2009). This trial was carried out on 24 patients with chronic otitis to assess the efficacy and safety of a phage preparation composed of six phages for the treatment of otitis caused by antibiotic-resistant *Pseudomonas aeruginosa*. By the end of the trial (day 42), all the clinical indicators (e.g. inflammation, ulceration, discharge type and quantity, and odour) improved in patients treated with phages, but only three of the 12 patients receiving phage treatment were apparently cured. Importantly, no serious adverse effects were reported (Wright *et al.* 2009). Also in 2009, Rhoads and colleagues reported another randomized, double-blind controlled study that addressed the safety (and not efficacy) of a phage cocktail targeting *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* for the treatment of venous leg ulcers (VLU) (Rhoads *et al.* 2009). This first phage therapy trial in the United States involved 42 patients with VLU. Patients were topically treated with either phage cocktail or saline solution (control) for 12 weeks with a follow-up period of up to 24 weeks. No adverse effects were associated with phage treatment, but no significant differences were found on the rate and frequency of healing between phage-treated and control groups. This is not surprising as the phages were not tested for infectivity on the bacteria causing the VLU. According to the authors, the efficacy of the phage preparation should be evaluated in a phase II efficacy trial with a larger sample and with wounds infected with bacteria susceptible to the phage cocktail (Rhoads *et al.* 2009).

105 The largest clinical trial on phage therapy conducted in Europe and performed under both
106 Good Manufacturing Practices (GMP) and Good Clinical Practices (GCP) was the
107 PhagoBurn trial, launched in 2013. In this multicentre randomized controlled phase I/II
108 clinical trial , 27 patients suffering from burn wound infections were recruited from
109 hospitals located in France and Belgium to be randomly treated with phage therapy (a
110 cocktail of 12 lytic phages) or standard care (1% sulfadiazine silver emulsion cream) to
111 compare the efficacy and tolerability of both treatments in patients with wounds infected
112 by *P. aeruginosa* (Jault *et al.* 2019). Both treatments were topically administered for
113 seven days with a 14 days follow-up period. Overall, the phage cocktail was able to
114 decrease bacterial burden in burn wounds but the progress was slower than in the control
115 group (standard treatment). On the positive side, no adverse effects were found in the
116 phage-treated group. The limited efficacy of the phage cocktail was reported to be caused
117 by a significantly drop of the phage titre after GMP manufacturing, leading the
118 participants to receive a much lower concentration of phages than initially estimated.
119 More importantly, the susceptibility of wound bacteria to the phage cocktail was not
120 assessed prior to treatment. In those patients in which phage treatment failed, bacteria
121 were later found to be resistant to low phage doses (Jault *et al.* 2019).

122 Nestlé (Switzerland) also performed a phase I/II trial in collaboration with the Dhaka
123 Hospital of the International Centre for Diarrheal Disease Research, Bangladesh (Sarker
124 *et al.* 2016). This randomized double-blind, placebo-controlled trial was conducted
125 between 2009 and 2011 to assess the safety and efficacy of oral administration of a T4-
126 like phage cocktail or a placebo, in children hospitalized with acute bacterial diarrhoea.
127 Although the oral coliphages could reach the intestine, no phage replication was observed,
128 and the treatment had no beneficial effects. At the time, the authors attributed the failure
129 to improve diarrheal outcome to the low host range coverage of the phage cocktail (i.e.

some strains were not infected) and also the need of higher oral phage doses (Sarker *et al.* 2016). Indeed, oral application of phages without any protection (e.g. encapsulation of the phages or neutralization of the stomach acid) prior to administration reduces the phage numbers reaching the intestine to levels that might be insufficient for a visible therapeutic effect. Later, it was also found that *E. coli* was not the main cause of acute bacterial diarrhoea, and therefore even an efficient phage treatment of *E. coli* would not result in improved diarrheal outcome (Satter *et al.* 2017; Nelson *et al.* 2018). This Nestlé trial and the clinical trial developed by Rhoads et al. highlight the importance of identifying the etiologic agent(s) causing infection and of checking for phage susceptibility prior to treatment. Therefore, phage therapy clinical trials must be carefully designed to avoid potential problems that might impair the outcome of the treatment. Recently, Ooi et al. reported a clinical trial aiming to assess the safety, tolerability and preliminary efficacy of a phage cocktail composed of three lytic phages, applied intranasally in patients with recalcitrant chronic rhinosinusitis (CRS) caused by *S. aureus* (Ooi *et al.* 2019). In this open label, phase I clinical trial, only patients carrying a clinical isolate sensitive to the phage cocktail were considered. Overall, the twice-daily intranasal irrigation of phages was safe and well tolerated by the nine patients through the 14 days treatment, with no serious adverse events reported. While the preliminary efficacy observations seem promising (two of the nine patients had eradication of infection), the authors highlighted the need for a randomized clinical trial to determine the optimal dose regimen and demonstrate the efficacy of the phage cocktail (Ooi *et al.* 2019). The high safety of phage therapy has already been reported in multiple patients from the phage therapy unit in Poland (Międzybrodzki *et al.* 2012; Rogóż *et al.* 2019).

While most clinical trials have failed to provide unequivocal evidence of the efficacy of phage therapy, the number of case studies in which phage therapy was successfully used

155 to treat life-threatening infections is increasing (Table 1) (Sybesma *et al.* 2018; McCallin
156 *et al.* 2019). Some of these successful cases have reached the media (Dedrick *et al.* 2019;
157 Strathdee, Patterson and Barker 2019), fostering the interest of the global community in
158 this therapy. One of these newsworthy cases concerned a 68-year-old man who suffered
159 from necrotizing pancreatitis complicated by an *Acinetobacter baumannii* multidrug-
160 resistant infection (Schooley *et al.* 2017). Despite multiple rounds of antibiotic treatments,
161 the patient condition rapidly deteriorated over time. Therefore, the *A. baumannii* strain
162 isolated from the patient was used to screen for phages in two different laboratories,
163 which made possible to compose phage cocktails tailored for the patient. Phage
164 administration (via catheters into the abdominal cavity and also intravenously) rapidly
165 reverted the clinical condition of the patient by clearing the infection (Schooley *et al.*
166 2017). Phage therapy documentaries have also been broadcasted on television in many
167 countries (Djebara *et al.* 2019). As a consequence, the Queen Astrid military hospital in
168 Brussels, Belgium, has experienced a huge increase in external phage therapy requests
169 since 2017 (Djebara *et al.* 2019). The majority of these requests were initiated by the
170 patients themselves and came mostly from the Netherlands followed by Belgium and
171 France. Among the 260 phage therapy requests received by the hospital between 2013
172 and 2018, only 15 patients, who were infected with bacterial pathogens susceptible to the
173 available phages, received treatment but these data were not yet reported (Djebara *et al.*
174 2019).

175 The rising interest in phage therapy by patients and physicians and the consequent
176 increase of requests for phages from all over the world highlights a growing need for the
177 establishment of phage banks with well characterized phages that could facilitate access
178 by the international community. Some phage banks have already been established, such
179 as the Félix d'Hérelle Reference Center for Bacterial Viruses at the University of Laval

(Québec, Canada), the Leibniz Institute DSMZ German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the Bacteriophage Bank of Korea (Yongin, South Korea), the American Type Culture Collection (ATCC) Bacteriophage Collection (Virginia, USA), the National Collection of Types Cultures (NCTC) Bacteriophage Collection (Salisbury, UK) (McCallin *et al.* 2019; Sacher 2019), and the Fagenbank (Delft, Netherlands). It is important that phage researchers feed these global phage banks to have a larger coverage of (pathogenic) bacterial species.

CURRENT CHALLENGES IN PHAGE THERAPY

Quality and safety requirements

The success of phage therapy is highly dependent on the safety of phage preparations, which raises manufacturing and formulation challenges (Fig. 1A). For broad medical applications, phages would need to be produced in large scale under Good Manufacturing Practices (GMP) approved by regulatory agencies (Regulski, Champion-Arnaud and Gabard 2018). Although the production of phages for therapy must comply with the strict regulations that are usually applied for pharmaceutical products to ensure the high quality standards appropriate for their intended use, no clear guidelines were yet developed specifically for phage manufacturing (Mutti and Corsini 2019). To address this issue, a group of phage researchers have set some quality and safety requirements for sustainable phage therapy products (Pirnay *et al.* 2015). One of the requirements is to avoid phages encoding for lysogeny, virulence factors or antibiotic resistance. However, this might limit the use of phage therapy in some fastidious bacteria for which no strictly virulent phages have been found so far, such as *Clostridium difficile* (Hargreaves and Clokie

2014).The presence of impurities such as endotoxins in phage preparations should also be avoided or be below a threshold (Pirnay *et al.* 2015). Several purification methods have been developed and optimized to remove these toxic elements from phage preparations (Hietala *et al.* 2019), but none has reached optimal results so far.

It is important to note that as phages are biological entities, the development of robust manufacturing processes in compliance with GMP is also essential to avoid variability among phage preparations (García *et al.* 2019; Mutti and Corsini 2019). Another important aspect is the quality control of phage stock preparations. This should be regularly assessed by checking for their stability (shelf life), sterility and cytotoxicity, as well as by performing periodic pH measurements (Merabishvili *et al.* 2009; Pirnay *et al.* 2015). Although recent progress in phage manufacturing has revitalized phage therapy in Western countries, there is still a long way to go before a general approval is reached for the use of phage therapy (Regulski, Champion-Arnaud and Gabard 2018).

Stability of phage preparations

The stability of phage preparations is a key requirement for successful treatment and also for the regulation of phages as pharmaceuticals. A potential phage candidate for therapy should have a good shelf life, i.e., it should be stored in a formulation that ensures activity without significant drop in phage titre during processing and long-term storage (Fig. 1B), as such decrease might compromise the outcome of the treatment (Malik *et al.* 2017; Merabishvili, Pirnay and De Vos 2018; Jault *et al.* 2019). Several strategies have been developed and optimized to improve phage stability and the most common include spray-drying, freeze drying, extrusion dripping methods, emulsion and polymerisation

techniques (Malik *et al.* 2017). However, phage stability in different formulations (e.g. liquids, gels, powders) is highly variable, especially among different phage types (Leung *et al.* 2017; Gonzalez-Menendez *et al.* 2018; Merabishvili, Pirnay and De Vos 2018). An alternative strategy to improve the storage shelf life of phages is their encapsulation on different matrices such as liposomes, alginate, cellulose or other polymers (Malik *et al.* 2017; Cortés *et al.* 2018). Phage encapsulation strategies are important not only to achieve longer shelf life but also for therapeutic purposes. Because treatment efficacy highly depends on phage concentration at the site of infection, protecting phages from the harsh conditions found in the human body is vital to avoid phage inactivation during treatment due to e.g. low pH or clearance mechanisms associated with the immune system (Malik *et al.* 2017; Dąbrowska 2019). In fact, the immune system plays a crucial role in phage clearance or inactivation from animal and human bodies. Most studies on the immune response to phages have focused on the development of phage-specific antibodies (adaptive immunity). These have been shown in many cases to decrease the circulation of phages, but other studies have reported no antibody formation or no effect of the formed antibodies on the ability of phages to clear the infection (Dąbrowska 2019). *In vitro* and *in vivo* studies have demonstrated the ability of encapsulated phages to persist for longer periods at low pH, enhancing the efficacy of oral administration in animal models (Yongsheng *et al.* 2008; Ma *et al.* 2012; Colom *et al.* 2017; Otero *et al.* 2019; Vinner *et al.* 2019). More studies are required to understand protection given by encapsulated strategies against immune clearance of phages. The protection of phages is also important for certain combined therapies that can inactivate phages when applied together and impair the outcome of the treatment. As an example, burn wound care products and their active ingredients usually exhibit high acidity that can negatively affect the activity of phages in wounds (Merabishvili *et al.* 2017).

Another issue of phage stability is the occurrence of spontaneous mutations in phage stocks stored for long periods or accumulated during phage production and manufacturing, which can impair viral fitness (Drake 1966; Botka *et al.* 2019). Although difficult, it would be helpful to predict phage evolution during production to set up a manufacturing process that would minimize the mutation rates in phage genomes (García *et al.* 2019).

Fast phage screening methods

Due to the high specificity of phage activity, finding a phage that targets a particular strain often requires the screening of large phage collections (Fig. 1C). The most traditional method to detect phage activity against a strain is the double layer agar (DLA) method, in which different phages are spotted on top of a lawn of the bacteria of interest (Cornax *et al.* 1990; Kropinski *et al.* 2009). Depending on the growth rate of the particular strain to target, results may take up to 48h to show and therefore the DLA method is not convenient in a therapeutic context where fast diagnosis is crucial. High-throughput and fast-screening methods are desirable to rapidly identify phage(s) able to efficiently infect the target strain(s).

Multiple methods have been developed for the detection and quantification of phages, via direct or indirect measurements, but few seem to have application in a clinical setting. For example, real-time PCR (qPCR) methodologies (Del Rio *et al.* 2008; Ly-Chatain *et al.* 2011) have been developed for fast and sensitive detection of phages and for the identification of infection via detection of increasing phage concentrations. But qPCR methods require a set of primers and optimized conditions for (almost) every phage,

which is neither high-throughput nor feasible when testing large (and fast expanding) phage collections against a target strain.

Flow cytometry has also been used to reveal phage infection via detection of cells with low-density cell walls (Michelsen *et al.* 2007). Low-density cell walls have been observed as a consequence of phage infection in *Lactococcus lactis*. The method allows for fast and early detection of phage infection, but is low-throughput and most likely not universal for all bacterial species and/or phages. Some other works have detected phage propagation indirectly via measuring enzyme release from bacterial cells due to phage-induced cell lysis. Intracellular enzymes such as adenylate kinase and adenosine 5'-triphosphate (ATP) or β-galactosidase have been tested as measurements of infection by *E. coli* phages (Stanek and Falkinham 2001; Guzmán Luna *et al.* 2009). Enzyme release is detected by the generation of a bioluminescence or colour signal after cleavage of a specific substrate. These assays are highly sensitive, generating a detectable signal in a short time (≈3h) even when starting with a low phage amount. Such methods are compatible with high-throughput and, in theory, work with any phage but may need to be optimized (e.g. enzyme/substrate selected) for each bacterial species.

The aptitude of surface plasmon resonance (SPR) techniques to measure and quantify molecules bound to surfaces was explored to study the interaction between phages and bacterial host (García-Aljaro *et al.* 2008). For this method, bacteria are immobilized on gold sensor chips using avidin-biotin, and binding of phages to the bacteria and consequent bacterial lysis can be detected and measured with high sensitivity in just 2 h. As it is, however, the method is not compatible with high-throughput screening as only a strain-phage pair can be tested simultaneously. A microfluidics adaptation of the method, in which multiple channels are created to test multiple phages simultaneously, could provide an interesting solution.

Cell respiration can also be used as a reporter for cellular growth and, consequently, for phage infection. Using this principle, Henry and colleagues developed the OmniLog™ system, in which cell respiration is measured using redox chemistry via reduction of a tetrazolium dye that produces a colour change measured in microtiter plates (Henry *et al.* 2012). Successful phage infection is detected by a reduction in colour due to reduced bacterial growth and respiration. Such method is simple and high-throughput, but might be limited to aerobic bacteria.

A simple approach was also recently suggested based on the analysis of optical density kinetics in bacterial cultures for the detection and quantification of phages (Rajnovic, Muñoz-Berbel and Mas 2019). This method detects phages at low amounts with a response time of 3.5 h, and is susceptible of miniaturization and automation for high-throughput applications that can be implemented in routine analysis. A possible drawback is that it relies solely on a change in optical density of the bacterial culture, which is not always observable for lytic phages.

In the future, a simple and fast high-throughput method for phage screening should be established and implemented in clinical settings and in phage banks, if phage therapy is to be widely used as a treatment option.

Efficacy of phages against biofilms

In nature and in the human body, bacteria are most often found in the form of a biofilm. A biofilm can be defined as a population of bacteria attached to a surface and embedded within a self-produced matrix (Hobley *et al.* 2015). In biofilms, bacterial cells closely collaborate as a strategy for survival and persistence in harsh environments (Costerton *et*

al. 1995), e.g. providing increased tolerance to antibiotics (Costerton, Stewart and Greenberg 1999; Stewart and Costerton 2001). Phage-bacteria interactions have been mostly studied in planktonic cultures, but these interactions have been shown quite distinct for bacteria in a biofilm form. Studies have revealed the therapeutic potential of phages to control both mono-species (Curtin and Donlan 2006; Fu *et al.* 2010; Alves *et al.* 2015; Melo *et al.* 2016) and dual-species biofilms (Sillankorva, Neubauer and Azeredo 2010; Gutiérrez *et al.* 2015b; Lehman and Donlan 2015), but multiple works have also unveiled the impressive complexity and diversity of phage-biofilm interactions.

Within biofilms, bacteria are protected by a matrix composed mainly of polysaccharides, lipids, extracellular DNA, and proteins (Hobley *et al.* 2015; Seviour *et al.* 2019). The matrix is a major factor influencing the ability of a phage to successfully disturb a biofilm (Darch *et al.* 2017), via several suggested mechanisms. The matrix can adsorb phages (Bull *et al.* 2018) or simply form a physical barrier for phage diffusion (González *et al.* 2018; Dunsing *et al.* 2019), preventing phages from reaching and infecting the living cells within the biofilm (Fig. 1D). Phages have, however, developed strategies to counteract the limiting effects of the matrix on their activity (Pires *et al.* 2017a). Many phages encode polysaccharide-degrading enzymes known as depolymerases, which are used to degrade capsular polysaccharides of bacteria and thereby give the phage access to its receptor on the bacterial cell surface. Some depolymerases can also degrade exopolysaccharides of the biofilm matrix and improve access of the phages to the bacterial cells (Harper *et al.* 2014; Gutiérrez *et al.* 2015a). The activity of depolymerases tends to be very specific for a certain polysaccharide type, and the use of a phage cocktail encoding for different depolymerases may represent a good treatment solution, and even enhance the activity of other non-depolymerase producing phages (Schmerer, Molineux and Bull 2014).

346 The spatial organization of the biofilm is also a determinant factor for phage infection.
347 To form a biofilm, cells organize so that localized niches are created with distinct nutrient
348 availability and consequently with bacteria of distinct motility, metabolic state, and gene
349 expression, all of which affect the capacity of phages to infect biofilm cells. The diffusion
350 of the phage through the biofilm is limited by the close proximity of the cells, which may
351 cause multiple phages to infect the same host cell and decrease the number of progeny
352 phages the cell generates (Taylor, Penington and Weitz 2017). Still, it is also possible that
353 local infection of a biofilm leads to a significant disruption of the biofilm structure,
354 ultimately leading to its dispersal and easier removal.

355 The establishment of nutrient gradients often leads to the generation of dormant persister
356 cells in the deeper layers of the biofilm, where nutrient resources are scarce. Phages
357 infecting these metabolically inactive cells are expected to be unable to propagate as they,
358 in principle, cannot use the (inactive) replication machinery of the cell (Łoś *et al.* 2007;
359 Pearl *et al.* 2008). However, a *Staphylococcus* infecting phage was recently shown
360 capable of propagating in dormant staphylococcal cells, a feature expected to be present
361 in other phages yet to discover (Melo *et al.* 2018; Tkhilaishvili *et al.* 2018). Additionally,
362 phages can remain within the persister cells until they exit the state of dormancy, being
363 then able to propagate as normal (Pearl *et al.* 2008).

364 Gene expression in biofilms is frequently controlled by quorum sensing, which involves
365 the use of extracellular signal molecules that sense population density to coordinate gene
366 expression (Ng and Bassler 2009). Quorum sensing can be used by bacteria to respond to
367 phage infections, for example by regulating expression of CRISPR-Cas systems
368 (Patterson *et al.* 2016; Høyland-Kroghsbo *et al.* 2017) and of phage receptors (Høyland-
369 Kroghsbo, Maerkedahl and Svenningsen 2013; Tan, Svenningsen and Middelboe 2015),
370 and also by regulating the production of biofilm matrix (Parsek and Greenberg 2005).

Some phages have developed strategies to exploit the bacterial quorum sensing system to guide their lysis-lysogeny decision either by encoding receptors for the bacterial quorum sensing molecules (Silpe and Bassler 2019) or by expressing their own extracellular signalling molecules once inside the bacteria (Erez *et al.* 2017) . By sensing the bacterial population, phages can sense a favourable or unfavourable environment for lytic development.

Biofilms are also known to release outer membrane vesicles (OMVs) in high number. These OMVs may contain outer membrane proteins used as receptors by some phages, and therefore work as a decoy for phage infection, protecting biofilm cells from phages (Manning and Kuehn 2011; Reyes-Robles *et al.* 2018). Nevertheless, phages that use receptors other than outer membrane proteins (e.g. lipopolysaccharides) are not affected by such strategy.

Dispersion of bacteria from a biofilm for colonization of a new niche is an important step of the biofilm life cycle. Phages may be interesting solutions to control the spreading step of a biofilm infection, as some phages unable to eradicate a biofilm can still inhibit dispersal of migrating bacteria and the establishment of new colonies (Darch *et al.* 2017).

Most *in vitro* work in biofilms has been performed using single strains. Natural biofilms, however, are often multi-strain or multi-species, which significantly affects the biofilm spatial organization and the interaction with phages. The specific outcome of phage infection in a multi-species biofilm seems to strongly depend on the bacterial species composing the biofilm (e.g. whether they establish synergist or antagonist interactions). Some studies have reported the ability of phages to target the susceptible host in the biofilm independently of the presence of a non-susceptible strain (Harcombe and Bull 2005; Kay *et al.* 2011; Gutiérrez *et al.* 2015a). A few works, however, suggest the

presence of insensitive strains to provide spatial structure-associated protection to the sensitive bacteria against phage infection, thereby reducing the efficacy of phage treatment (Tait, Skillman and Sutherland 2002; Testa *et al.* 2019). Broad host range phages (Kim *et al.* 2012) as well as phages carrying depolymerases (Pei and Lamas-Samanamud 2014) may be particularly efficient against multispecies biofilms. In the latter case, the diversity and heterogeneous distribution of exopolysaccharides on a multi-species biofilm may limit depolymerase activity.

The complexity of phage-biofilm interactions is increased by evidence of promoted biofilm formation induced by exposure to certain phages (Lacqua *et al.* 2006; Tan, Dahl and Middelboe 2015; Henriksen *et al.* 2019). Two scenarios have been proposed for this phenomenon. In the first scenario, changes in biofilm are thought to occur as a consequence of the specific bacterial receptor used by the phage. Mutations in these receptors occur as a response to infection and may lead to changes in the biofilm cells that result in increased biofilm formation (Scanlan and Buckling 2012; Fernández *et al.* 2017; Henriksen *et al.* 2019). The second scenario suggests that some phages may benefit from increased biofilm formation, with entrapment of phages in the biofilm matrix providing protection against harsh environmental factors (Agún *et al.* 2018; Gabiatti *et al.* 2018). In this scenario, an increase of the biofilm is beneficial for both bacteria and phage.

Overall, the potential of phages to control biofilm infections is clear. However, the complexity and diversity of phage-biofilm interactions limit broad conclusions and call for more research before phage therapy becomes a real solution for biofilm-related infections.

Evolution of bacterial resistance to phages

One of the major concerns in phage therapy is the possible emergence of bacteriophage-insensitive mutants (BIMs) that could hamper the success of this therapy (Fig. 1E). Over the last years, several studies have addressed the problem of bacterial resistance to phages, demonstrating that the emergence of phage-resistant mutants is frequent and almost unavoidable (Oechslin 2018; McCallin and Oechslin 2019). The resistance mechanisms used by bacteria to counter-attack phage evasion include, among others: (i) prevention of phage adsorption by loss or modification of bacterial receptors; (ii) prevention of phage DNA entry by superinfection exclusion systems; (iii) degradation of phage DNA by restriction-modification (R-M) systems and other related systems (BREX, DISARM, etc) or by CRISPR-Cas systems; (iv) use of abortive infection systems that block phage replication, transcription or translation; or (v) cyclic oligonucleotide-based anti-phage signalling systems (Labrie, Samson and Moineau 2010; Bernheim and Sorek 2020).

A number of *in vitro* studies have reported the emergence of BIMs within a short period of time after phage treatment (Fu *et al.* 2010; Le *et al.* 2014; Oechslin *et al.* 2016; Pires *et al.* 2017b). In most of these studies, bacterial resistance to phages was caused by mutations on genes encoding phage receptors, which include lipopolysaccharides, outer membrane proteins, capsules, flagella, pili, among others. The emergence of phage-resistant variants has also been noticed *in vivo* in several animal models as well as in human pilot studies and case reports (Oechslin 2018; McCallin and Oechslin 2019). However, some studies have highlighted the fact that the evolution of resistance observed *in vitro* does not resemble what actually happens *in vivo*. For example, Oechslin et al. studied the efficacy of a phage cocktail in the treatment of *P. aeruginosa* endocarditis and observed that BIMs emerged *in vitro* but not *in vivo* (Oechslin *et al.* 2016). According to

the authors, this occurred probably because the bacterial mutations on phage receptors rendering them resistant might incur fitness costs, with the bacteria becoming less virulent and therefore easier to eliminate by the immune system. Other authors have also reported the attenuated virulence of BIMs in consequence of modifications in cell surface receptors for other bacterial species (Filippov *et al.* 2011; León and Bastías 2015; Sumrall *et al.* 2019).

Bacterial resistance to phages can be circumvented using different approaches (McCallin and Oechslin 2019). The most common is the combination of multiple phages, preferentially targeting different receptors and with complementary host ranges, in a single preparation, which is usually known as a phage cocktail. In addition to displaying a larger coverage against a particular bacterial species, such cocktails can also arrest the emergence of BIMs. These are the main reasons behind the preferred use of phage cocktails over single phage preparations in therapy. Phage cocktails might have a fixed composition covering a broad host range (*prêt-à-porter*) or a customized formulation designed for a particular patient (*sur-mesure*) (Pirnay *et al.* 2011). Another strategy commonly used to deal with the problem of resistance during phage treatment is the replacement of the phage against which the bacteria developed resistance by a phage that is active against the resistant variant. While this is not easy for antibiotics, when it comes to phages it can be quite simple given their abundance and diversity in nature as a result of their constant co-evolution with bacteria (Rohde, Wittmann and Kutter 2018). Lastly, the combination of phages with antibiotics or other antimicrobial agents can also be used to avoid the development of bacterial resistance and to improve the therapeutic efficacy (see below for more detail) (Torres-Barceló and Hochberg 2016; Tagliaferri, Jansen and Horz 2019).

Regulatory framework of phage therapy

Regulatory authorities have classified phages as biological substances and, as such, phages fall within the scope of the pharmaceutical legislation (Pelfrene *et al.* 2016; Reindel and Fiore 2017). The regulatory framework in the European Union and in the United States stipulates that a marketing authorization is required for medicinal products prepared industrially or manufactured by a method involving an industrial process (Fig. 1F). As such, marketing a phage product requires proof of both safety and efficacy, and also of quality by manufacture under GMP (Directive 2001/20/EC; Pelfrene, Sebris and Cavaleri 2019). GMP compliance requires extensive financial resources (Pelfrene *et al.* 2016; Jault *et al.* 2019) and is therefore a critical obstacle for hospitals or non-for-profit phage therapy centres. Current legislation calls also for predetermined qualitative and quantitative evaluation of every constituent of the medicinal product. For phages, recommended criteria (Parracho, Burrowes and Enright 2012; Pelfrene *et al.* 2016) include the absence of prophages and antibiotic resistance in the bacteria used to produce the phage(s), the lytic (non-temperate) and specific activity of individual phages on the target bacteria, the control for impurities (e.g. endotoxins, residual reagents) in phage preparations, and the test for potency and purity of the phages. This strict regulation is somehow suitable for phage cocktails of fixed composition (*prêt-à-porter*) manufactured at industrial scale, but is certainly inadequate for patient-specific, customized, phage cocktails (*sur-mesure)* whose composition is variable and not intended for large-scale distribution (Directive 2001/83/EC; Pelfrene, Sebris and Cavaleri 2019)(Pirnay *et al.* 2011).

Discussions between phage sponsors and regulatory agencies are ongoing to set more satisfactory regulations for personalized phage therapy. The European Union currently

allows for a few exceptions on the requirement to obtain a product license, which apply to the magistral formula (any medicinal product prepared in a pharmacy in accordance with a prescription for an individual patient (Nahler 2009a)) and the officinal formula (any medicinal product which is prepared in a pharmacy in accordance with the prescriptions of a pharmacopoeia and is intended to be supplied directly to the patients served by the same pharmacy (Nahler 2009b)), and for any advanced therapy medicinal product (ATMP, medicinal product which is either a gene therapy medicinal product, a somatic cell therapy medicinal product, or a tissue engineered product), if prepared on a "non-routine basis according to specific quality standards, and used within the same Member State in a hospital under the exclusive professional responsibility of a medical practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient" (Directive 2001/83/EC; Pelfrene, Sebris and Cavaleri 2019). An exemption is applied also for compassionate use, a treatment option that allows an unauthorized (in development) medicine to be made available to groups of patients who have a disease with no satisfactory authorized therapies and who cannot enter clinical trials. However, compassionate use is only allowed for medicines undergoing clinical trials or that have entered the marketing authorization application process (Compassionate use | European Medicines Agency; Pelfrene, Sebris and Cavaleri 2019).

Due to the current unsatisfactory regulatory framework, Member States of the European Union are finding national solutions for phage therapy regulation. The Belgian authorities are pioneering phage therapy regulations in Western countries by establishing a national regulation of magistral preparation of tailor-made phage medicines (Pirnay *et al.* 2018). The regulation requires issuing of a monograph that judges in written form the quality of the phage active pharmaceutical ingredient (API) to be used for the preparation of the

517 medicinal product. Every stock of the phage therapy medicinal product is then tested by
518 a Belgian approved laboratory to confirm the phage(s) comply with the phage API
519 monograph(s), issuing a certificate of analysis that approves its use. A pharmacist then
520 uses the certified phage stock for preparing a customized medicinal product based on the
521 prescription of a physician (Pirnay *et al.* 2018). This process has already allowed the
522 implementation of phage therapy in Belgium, but it is not yet ideal as all responsibility is
523 given to the prescriber and the pharmacist (Fauconnier 2017). Similar regulatory
524 principles were already in practice, for example, in Georgia and Russia. In Georgia,
525 ready-to-use phage medicines require a marketing authorization according to regular
526 regulation, while customized phage preparations may be prepared as magistral
527 preparation in an authorized pharmacy (Parfitt 2005). The Russian pharmacopeia includes
528 a monograph on phages for prophylactic and therapeutic use (Russian Pharmacopoeia
529 OFS.1.7.1.0002.15).

530 Other countries are also finding similar solutions. France has issued recommendations for
531 the use of phage medicinal products under the nominative Temporary Authorization for
532 Use (ATUn) (Phagothérapie). An ATUn can be issued by hospital pharmacies, for a
533 single patient who cannot participate in a clinical trial, at the request and under the
534 responsibility of the prescribing physician, allowing for the use of a medicinal product
535 without market approval if its efficacy and safety balance is presumed favourable for the
536 patient, in the absence of any approved treatment. In the United States, phages can be and
537 have been used following the Food and Drug Administration (FDA) emergency
538 investigational new drug (eIND) pathway (Schooley *et al.* 2017; LaVergne *et al.* 2018).

539 Further clinical evidence of the success of phage therapy in human trials conducted to
540 modern standards would help foster regulatory advance (Pelfrene *et al.* 2016), but current
541 regulatory issues affect also the conduct of clinical trials. A new provision in the

542 regulatory framework of the European Union may facilitate clinical trials with phage
543 medicinal products, by exempting GMP requirements in the preparation of investigational
544 medicine products (IMPs), "where this process is carried out in hospitals, health centers
545 or clinics legally authorized in the Member State concerned to carry out such process and
546 if the IMPs are intended to be used exclusively in hospitals, health centers or clinics taking
547 part in the same clinical trial in the same Member State" (Regulation (EU) No 536/2014).

548 In summary, current regulations will certainly undergo serious modifications before a
549 fully practicable regulation is implemented for phage therapy, as well as other customized
550 medicinal products meant to be tailored to an individual patient.

551

552 # THE FUTURE OF PHAGE THERAPY

553 ## Phages in One Health approach

554 It is estimated that at least six out of ten known infectious diseases in humans are
555 originated in animals (Zoonotic Diseases | One Health | CDC). Moreover, the selective
556 pressure on phytobacteria drives evolution in a vast number of defence mechanisms,
557 which can result in increased virulence towards humans, especially those with advanced
558 age, immunodeficiency, or cancer (Erken, Lutz and McDougald 2013; Falkinham, Pruden
559 and Edwards 2015). The One Health concept recognizes that the health of humans and
560 animals as well as our environment are all intertwined. To improve the lives of all living
561 species, the One Health program proposes the integration of human medicine, veterinary
562 medicine and environmental science (http://www.onehealthinitiative.com/). Agriculture
563 and food safety are also included in this holistic and multi-sectoral approach to tackle
564 antimicrobial resistance (Baum *et al.* 2017; Hernando-Amado *et al.* 2019). Although

565 microorganisms will inevitably develop resistance towards antibiotics as a consequence
566 of genetic mutations or horizontal gene transfer, the problem of resistance is worsened by
567 the misuse of antibiotics since their discovery. A clear example is the use of antibiotics
568 as growth promoters at livestock farms, which impelled the European Union to create
569 stricter regulations to control their widespread usage (Kittler *et al.* 2017). To mitigate the
570 spread of antimicrobial resistance, new alternative therapeutics under the One Health
571 view are needed. Since their discovery, phages are being applied for the control of
572 bacterial proliferation in several microbiomes, such as humans (as reviewed above),
573 animals (Oliveira, Sereno and Azeredo 2010), several environmental settings (e.g.
574 wastewater treatments) (Withey *et al.* 2005), and on food industry (Abuladze *et al.* 2008).
575 A good example of the global use of phages are the diverse application opportunities in
576 food industry, where they can be used at all stages of food processing, from slathering
577 and crops to food transportation (reviewed by (Goodridge and Bisha 2011)), even
578 improving the shelf life of food products (Alves *et al.* 2019). In fact, several phage-based
579 products to be applied in food-stuff have already received the GRAS (generally
580 recognized as safe) classification by the Food and Drug Administration (FDA) in the
581 United States (Sarhan and Azzazy 2015). Therefore, the use of phages is consistent with
582 the One Health approach as they can be applied in different settings (e.g. food, animals
583 or crops) thus preventing the overuse of antibiotics and the dissemination of antibiotic
584 resistance to humans (Kittler *et al.* 2017).
585

586 ## Emerging approaches

587 The use of phages for the control of bacterial infections might be improved via
588 combination with other agents, especially when targeting the complex biofilm

589 communities (Koo *et al.* 2017). These combined therapies have often the advantage of
590 limited development of resistance towards agents with distinct modes of action due to the
591 fitness cost associated with resistance against multiple factors (Torres-Barceló and
592 Hochberg 2016; Chaudhry *et al.* 2017).

593 Probably the most obvious combination is that of phages and antibiotics (Fig. 1G). When
594 used simultaneously, phages and antibiotics have shown synergistic effects and
595 effectiveness against planktonic cells (Bedi, Verma and Chhibber 2009; Nouraldin *et al.*
596 2016; Jansen *et al.* 2018; Yazdi, Bouzari and Ghaemi 2018) and (especially old) biofilms
597 (Bedi, Verma and Chhibber 2009; Rahman *et al.* 2011; Chaudhry *et al.* 2017; Akturk *et*
598 *al.* 2019), where the individual treatments had restricted success. In cases where repeated
599 treatment with phages increased biofilm production, the combined use of phage and
600 antibiotics resulted in biofilm eradication (Henriksen *et al.* 2019). Structural changes in
601 the biofilm caused by one or both agents may be behind the enhanced efficacy. For
602 example, removal of peripheral cells by the phage may lead to increased resource
603 availability for inner cells and improve their metabolic state, making the cells more
604 susceptible towards phages and certain antibiotics (Chaudhry *et al.* 2017). Antibiotics
605 may also themselves cause changes in the biofilm architecture and thereby enable
606 increased invasion of biofilms by phages (Díaz-Pascual *et al.* 2019).

607 Synergism between antibiotics and phages does not happen for all phage-antibiotic
608 combinations (Knezevic *et al.* 2013; Kamal and Dennis 2015; Jansen *et al.* 2018) and
609 high doses of antibiotics can also antagonize phage propagation (Dickey and Perrot 2019).
610 This is particularly evident when using antibiotics that target cell protein synthesis
611 (Akturk *et al.* 2019). But in some cases, even though no synergism in antimicrobial
612 activity is observed, the combined use of phages and antibiotics significantly reduces or

even prevents the development of antibiotic- and phage-resistant bacteria (Coulter *et al.* 2014; Dickey and Perrot 2019).

While several studies have looked into the effect of phage-antibiotic therapies, few have developed a rational approach to explore the bacterial response to these agents. An example of such strategy is the isolation of phages targeting specific outer membrane proteins that are used by bacteria as multidrug efflux pumps. Development of resistance to this phage would require the bacteria to change the efflux pump and therefore increase sensitivity against certain antibiotic classes (Chan *et al.* 2016). This approach was successfully employed to save a patient suffering from a chronic prosthetic vascular graft infection caused by *P. aeruginosa*, in which phage OMKO1 binding to efflux pump proteins was used in combination with ceftazidime; evolution of phage resistance led to increased antibiotic sensitivity and the infection was resolved (Chan *et al.* 2018). Approaches like this are not only efficient but also extend the lifetime of our current antibiotics.

Phages can also be co-administered with enzymes for improved activity. For example, depolymerases can be used together with phages that do not naturally express them to improve their activity against biofilms (Gutiérrez *et al.* 2015a). DNAse enzymes can also be used together with phages to degrade the DNA component of the biofilm matrix and improve phage activity (Hughes *et al.* 2006). Other successful cases combined phages with chlorine (Zhang and Hu 2013), triclosan, chlorhexidine, hydrogen peroxide (Agún *et al.* 2018), cobalt (II) sulphate (Chhibber, Nag and Bansal 2013), xylitol (Chhibber, Bansal and Kaur 2015), honey (Oliveira *et al.* 2017), and probiotics (Woo and Ahn 2014).

The modification of phage genomes is also being explored to improve phage therapy outcomes (Fig. 1H). This approach is being fuelled by recent advances in the synthetic

637 biology field, with many techniques now available to engineer phage genomes (Martel
638 and Moineau 2014; Ando *et al.* 2015; Pires *et al.* 2016; Kilcher *et al.* 2018). The host
639 range of a phage is one of the main targets to engineer. While the high host specificity of
640 phages is advantageous by preventing targeting of beneficial bacteria, it also implies that
641 it is almost impossible to target all strains within a given species using a single phage.
642 Tailored control of a phage's host range is therefore a major goal in phage therapy.
643 Working towards this goal, several studies have swapped receptor-binding protein genes
644 between phages of different families, successfully exchanging the host range of the phage.
645 This has been possible between phages infecting the same (Yoichi *et al.* 2005; Mahichi
646 *et al.* 2009) or different species (Ando *et al.* 2015). Others had fused a heterologous
647 receptor binding domain to the receptor binding protein of a phage, thereby increasing
648 the phage host range (Marzari *et al.* 1997; Heilpern and Waldor 2003).

649 Phages can also be engineered to deliver specific cargo to enhance the phage
650 antimicrobial activity. For example, enzymes such as dispersin B and lactonase have been
651 engineered into phage T7 to increase the phage activity against biofilms (Lu and Collins
652 2007; Pei and Lamas-Samanamud 2014). Dispersin B, a glycoside hydrolase, is expressed
653 at high levels during T7 infection and released upon cell lysis into the biofilm
654 environment, where it degrades the matrix; by doing so, dispersin B increases the phage
655 efficacy on removing both bacteria and matrix from the biofilm (Lu and Collins 2007).
656 Lactonase was also engineered into phage T7, but to interfere with the bacterial quorum
657 sensing, making use of its ability to inactivate the quorum sensing acylated homoserine
658 lactones (Pei and Lamas-Samanamud 2014). Inactivation of the quorum sensing
659 molecules interferes with biofilm formation and leads to improved biofilm control by the
660 engineered phage. Curiously, this strategy was shown to work in multi-species biofilms,
661 where quorum sensing molecules of one species also increase biofilm formation of the

second species, and inhibition of the molecules by the lactonase reduces biofilm formation in both species. This may therefore be an interesting alternative treatment against multi-species biofilms in the future.

While most engineering efforts have centred on lytic phages, temperate phages have also been the subject of a few engineering experiments for phage therapy purposes. The most obvious approach consists on genetically modifying phages to become exclusively lytic. This has been accomplished by deletion of the genomic module responsible for the establishment of lysogeny (Dorscht *et al.* 2009; Zhang *et al.* 2013; Kilcher *et al.* 2018). The creation of virulent mutants of otherwise temperate phages can easily extend the number and diversity of phages available for therapeutic purposes. A great example of the value of this approach is the recent use of a cocktail composed of one natural lytic phage and two engineered temperate phages to successfully treat a 15-year-old patient with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection (Dedrick *et al.* 2019). The temperate phages were engineered to become lytic via removal of the repressor of the lytic cycle, and the cocktail was administered intravenously and was well tolerated. Genetically engineered phages are not readily accepted for phage therapy due to the inherent ethical issues of genetically modified organisms (GMOs) but this case study clearly shows that engineering approaches are useful. The possibility of using temperate phages engineered into lytic forms in phage therapy increases the number of phages available for therapeutic use, by reducing/removing the risk of transduction of bacterial genetic information (e.g. virulence-related genes) mediated by temperate phages (Monteiro *et al.* 2019).

Temperate phages have also been engineered to deliver synthetic gene networks, exploiting their natural capacity to integrate into the host bacterium chromosome, where the phage expresses the molecule of interest. Phages have been modified as adjuvants to

antibiotics, by codifying dominant antibiotic sensitive genes (Edgar *et al.* 2012) or CRISPR-Cas systems (Bikard *et al.* 2014; Yosef *et al.* 2015) that revert antibiotic resistance in bacteria, or by codifying CRISPR-Cas systems designed to target bacterial cells (Park *et al.* 2017).

Overall, engineering approaches can potentially improve the antimicrobial properties of phages and create innovative strategies for fighting bacterial infections. The consequences of genetic manipulation of phage genomes must be carefully addressed, but phage engineering strategies should be effectively considered as a therapeutic option. Additionally, engineered phages have easier patentability than natural phages, and may therefore have more commercial interest.

Can phage resistance become a global problem?

Phage therapy frequently raises the question of whether the global use of phages could lead to a widespread problem similar to antibiotic resistance. A definitive answer does not exist.

First, phages will unlikely be used as a first line treatment against bacterial infections as it happens with antibiotics. In a future perspective, phage therapy is expected to be applied only in clinical cases of patients who experienced the failure of antibiotic treatments. Additionally, contrary to antibiotic therapy, phage preparations for therapeutic applications are expected to be developed in a personalized way by formulating phage cocktails that might delay the emergence of bacterial resistance to phages.

In the scenario of phages being extensively used in the future both as therapeutic and as environmental bio-control agents, it is possible that a strong selective pressure is imposed

towards the development of resistant bacteria. Still, it seems improbable that no phage will be available in nature to infect a bacteria that has become resistant to a previous phage. In fact, the long and continuous co-evolution of phages and bacteria (Dion, Oechslin and Moineau 2020) have resulted in bacteria evolving a range of mechanisms to avoid phage predation, and in phages developing effective counter-strategies to evade the antiviral systems (Samson *et al.* 2013). This arms race between phages and their bacterial hosts will not come to an end and, despite the emergence of resistant bacteria, phages will certainly find a way to ensure their propagation. The use of strategies as combined therapies and genome engineering may be an additional aid to prevent the spread of phage resistance. Still, further studies are required to guarantee that the global use of phages will not eventually compromise its efficacy.

FINAL REMARKS

In an era of global crisis for antibiotics, phage therapy has emerged as a potential alternative with already proven cases of clinical success. The generic use of phages for biocontrol meets the One Health Approach and is well aligned with the recently established European Green Deal (European Commission 2019) that recommends reducing significantly the use of antibiotics in food production. On the other hand, scientific advances have contributed to a better knowledge of phage-bacteria interaction enabling a safer and more efficient phage therapy. So, the conditions needed for the reintroduction of phage therapy as a therapeutic practice are met. Nevertheless, the widespread use of phage therapy creates additional challenges that go beyond the clinic standpoint and carries extra demands. These include (i) the need of increasing phage collections of reference phage banks; (ii) the development of efficient phage screening

methods for the fast identification of the therapeutic phage; (iii) the establishment of efficient phage therapy strategies that tackle infectious biofilms; (iv) the set-up of phage production protocols that assure quality and safety of phage preparations; and (v) the guarantee of stability of phage preparation during storage and transport.

As infectious diseases have no borders, a global action plan to make phage therapy worldwide available is needed. This obviously requires an active collaboration between countries for overcoming logistic and regulatory challenges, and between clinicians and scientists for filling current gap knowledges and fostering advances in the field.

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