Andrzej Górski · Ryszard Międzybrodzki
Jan Borysowski *Editors*

Phage Therapy: A Practical Approach

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Editors
Andrzej Górski
Ludwik Hirszfeld Institute of Immunology
Polish Academy of Sciences
Wroclaw, Poland

Ryszard Międzybrodzki
Ludwik Hirszfeld Institute of Immunology
Polish Academy of Sciences
Wroclaw, Poland

Jan Borysowski
Department of Clinical Immunology
Medical University of Warsaw
Warsaw, Poland

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This book is dedicated to Ludwik Hirszfeld, the founder of our institute and a pioneer of phage therapy in Poland.

Preface

The excitement about the prospects of phage therapy (PT) has been growing worldwide, fueled by the recent reports of its successful application in severe cases of bacterial infections. Reviews on PT appear almost every month, most of them written by newcomers to the field or authors who have never seen or treated any patient. This book, however, is edited by authors of whom two have seen, consulted, and treated in the past years almost 700 patients with various bacterial infections resistant to antibiotic treatment in accord with the strict ethical and legislative rules of both the European Medicines Agency (EMA) and national regulations governing healthcare and medical research.

Monitoring the progress of PT clearly shows the widening gap between the very promising results achieved in experimental animal models of bacterial infections and those derived from human clinical studies. While numerous reports have confirmed the therapeutic value of PT in animal models of acute bacterial infections, this success has not been paralleled by comparable achievements in human treatment. So far, no successful clinical trial completed according to all current EMA/FDA regulations has provided a required proof of effectiveness of PT in human disorders. On the other hand, several cases of spectacular results of PT have recently been reported including successful treatment of disseminated infections with resistant *Acinetobacter baumannii*, *Mycobacterium,* and *Pseudomonas*. However, critical analysis of data presented in those reports does not exclude that those beneficial effects were indeed only phage dependent and unrelated to other drugs concomitantly administered to the patients (e.g., antibiotics). That such an adjunctive effect of those drugs administered with phage may be relevant for patients' outcome is clearly shown in another recent report.

While the formal proof of PT effectiveness in the human clinic is eagerly awaited, other potential PT applications have appeared on the horizon. Those developments stem from our hypothesis published in the 2000s on phage-associated anti-inflammatory and immunomodulatory activities which also enable phages translocating from the gastrointestinal tract to mediate those effects in situ in local organs and tissues. This assumption opens a pathway toward application of PT in autoimmune

disorders, allergy, and transplant rejection as well as post-transplant complications (graft-versus-host disease). Therefore, "phage repurposing" might be another attractive option for PT. Hopefully, both well-known antibacterial activity and still hypothetical phage applications will find therapeutic applications in the future.

Wroclaw, Poland Andrzej Górski
Wroclaw, Poland Ryszard Międzybrodzki
Warsaw, Poland Jan Borysowski

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Editors and Contributors

About the Editors

Jan Borysowski, MD, PhD, works at the Department of Clinical Immunology, Medical University of Warsaw. His interests include phage therapy and medical ethics, especially ethical aspects of compassionate use. He has published over 40 papers and seven book chapters. His papers have been published, among others, by *BMC Medicine, Frontiers in Immunology*, and *Frontiers in Microbiology*. Moreover, he was the lead editor of the book *Phage Therapy. Current Research and Applications* published in 2014 by Caister Academic Press.

A. Górski, MD, PhD, is head of the Bacteriophage Laboratory and Phage Therapy Center. His main interests are phage therapy and immunobiology of phage. In the 2000s, he presented a hypothesis on protective action of endogenous phages which involves not only their antibacterial but also anti-inflammatory and immunomodulatory action. Subsequently, he formulated a hypothesis on phage translocation from the gastrointestinal tract to other tissues where phages could mediate their effects. In the past few years, he has published articles envisaging the potential role of phages in the treatment of autoimmune disorders, allergy, and graft-versus-host disease ("phage repurposing") (A. Górski et al., Med Res Rev 2019, https://doi.org/10.1002/med.21593). He was a coeditor of a book on phage therapy (see above).

Ryszard Międzybrodzki, MD, PhD, has been engaged in preclinical studies on bacteriophage application in the treatment of bacterial infections as well as experimental phage therapy. Since 2013, he has also worked at the Department of Clinical Immunology, Medical University of Warsaw. He has published over 70 papers and five book chapters related to therapeutic phage application including new perspectives of clinical use of bacteriophages (anti-inflammatory and immunomodulating phage effects). He was a coeditor of the book *Phage Therapy. Current Research and Applications* published in 2014 by Caister Academic Press.

Contributors

Derek F. Amanatullah Department of Orthopaedic Surgery, Stanford University Hospital, Redwood City, CA, USA

Joana Azeredo CEB - Centre of Biological Engineering, LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal

Jeremy J. Barr School of Biological Sciences, Monash University, Melbourne, VIC, Australia

Sylwia Bloch Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Jan Borysowski Department of Clinical Immunology, Medical University of Warsaw, Warsaw, Poland

Marco Cavaleri Office of Anti-infectives and Vaccines, Human Medicines Evaluation Division, European Medicines Agency, Amsterdam, The Netherlands

Krystyna Dąbrowska Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Daniel De Vos Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium

Aleksandra Dydecka Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Vincent A. Fischetti Laboratory of Bacterial Pathogenesis, Rockefeller University, New York, NY, USA

Wojciech Fortuna Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

The Medical University of Wroclaw, Wrocław, Poland

Jérôme Gabard Saint Barthélemy d'Anjou, France

Andrzej Górski Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Patrick Jault Clinique de la Muette, Paris, France

Randall Kincaid National Institute of Allergy and Infectious Diseases, Bethesda, Maryland, USA

Petar Knezevic University of Novi Sad Faculty of Sciences, Novi Sad, Serbia

Katarzyna Kosznik-Kwaśnicka Department of Molecular Biology, Faculty of Biology, University of Gdańsk, Gdańsk, Poland

Sławomir Letkiewicz Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Jan Dlugosz University in Czestochowa, Czestochowa, Poland

Marzanna Łusiak-Szelachowska Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Danish J. Malik Chemical Engineering Department, Loughborough University, Loughborough, UK

Robert Manasherob Department of Orthopaedic Surgery, Stanford Hospital and Clinics, Redwood City, CA, USA

Shigenobu Matsuzaki Kochi Medical School, Kochi University, Nankoku, Kochi, Japan

Shawna McCallin Unit of Regenerative Medicine, Department of Musculoskeletal Medicine, Service of Plastic, Reconstructive, & Hand Surgery, University Hospital of Lausanne (CHUV), Lausanne, Switzerland

Luís D. R. Melo CEB - Centre of Biological Engineering, LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal

Maia Merabishvili Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium

Ryszard Międzybrodzki Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

The Medical University of Wroclaw, Wrocław, Poland

Rodrigo Monteiro CEB - Centre of Biological Engineering, LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal

Agnieszka Necel Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Bożena Nejman-Faleńczyk Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Frank Oechslin Département de biochimie, de microbiologie, et de bio-informatique, Faculté des sciences et de génie, Université Laval, Québec City, QC, Canada

Eric Pelfrene Office of Anti-infectives and Vaccines, Human Medicines Evaluation Division, European Medicines Agency, Amsterdam, The Netherlands

Diana Priscila Pires CEB - Centre of Biological Engineering, LIBRO - Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal

Jean-Paul Pirnay Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium

Roger D. Plaut Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, Maryland, USA

Johan Quintens Vésale Bioscience, Noville sur Mehaigne, Belgium

Paweł Rogóż Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Verica Aleksic Sabo University of Novi Sad Faculty of Sciences, Novi Sad, Serbia

Zigmars Sebris Regulatory Affairs Office, Human Medicines Evaluation Division, European Medicines Agency, Amsterdam, The Netherlands

Scott Stibitz Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, Maryland, USA

Nina V. Tikunova Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russian Federation

Gracja Topka Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Jumpei Uchiyama School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan

Jonas D. Van Belleghem Laboratory Bacteriology Research, Department of Clinical Chemistry, Microbiology and Immunology, University Ghent, Ghent, Belgium

Mario Vaneechoutte Laboratory Bacteriology Research, Department of Clinical Chemistry, Microbiology and Immunology, University Ghent, Ghent, Belgium

Gilbert Verbeken Laboratory for Molecular and Cellular Technology (LabMCT), Queen Astrid Military Hospital, Brussels, Belgium

QA/QC and regulatory affairs, Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium

Kilian Vogele Systems Biophysics and Bionanotechnology-E14, Physics-Department and ZNN, Technische Universität München, Garching, Germany

Beata Weber-Dąbrowska Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Alicja Węgrzyn Department of Molecular Biology, Faculty of Biology, University of Gdańsk, Gdańsk, Poland

Grzegorz Węgrzyn Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland

Maciej Żaczek Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Part I

Bacteriophages as Antibacterial Agents

Production of Phage Therapeutics and Formulations: Innovative Approaches

Maia Merabishvili, Jean-Paul Pirnay, Kilian Vogele, and Danish J. Malik

1 Introduction

There is an increasing global health threat posed by pathogenic bacteria developing resistance to frontline antibiotics. The development pipeline for conventional broad-spectrum antibiotics is not looking promising. This is due to a number of well-known challenges including inadequate market incentives for pharmaceutical companies to develop new antibiotics and to be able to recoup their R&D costs. However, development of rapid sequence-based diagnostics may allow the use of narrow-spectrum antibiotics such as bacteriophages for therapeutic purposes. There has been a renaissance in the field of bacteriophage research for the development of phage therapeutics which is currently drawing significant interest from a broad range of researchers and policymakers involved in medical and health sciences. The media coverage and prominence given to the global antimicrobial resistance (AMR) crisis are one of the main reasons why a century-old technology is back in the spotlight (O'Neill 2016; Rios et al. 2016; Lin et al. 2017).

Worldwide production of phage preparations by pharmaceutical companies started in the late 1920s (Summers 1999; Sulakvelidze and Kutter 2005). Felix d'Herelle (who is attributed to have codiscovered bacteriophages along with Frederick Twort) is considered to have been the initiator of the phage therapy

M. Merabishvili (✉) · J.-P. Pirnay
Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium
e-mail: maia.merabishvili@mil.be; jean-paul.pirnay@mil.be

K. Vogele
Systems Biophysics and Bionanotechnology-E14, Physics-Department and ZNN, Technische Universität München, Garching, Germany
e-mail: ga25sod@tum.de

D. J. Malik
Chemical Engineering Department, Loughborough University, Loughborough, UK
e-mail: d.j.malik@lboro.ac.uk

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concept. At the inception of phage therapy, poor outcomes were attributed to low-quality phage preparations, and this very much undermined public confidence during this nascent period of phage therapy development (Kuhl and Mazure 2011). In 1938 d'Herelle published detailed instructions, which can be considered as guidelines on how to ensure production of high-quality phage preparations (Kuhl and Mazure 2011). The advice given by d'Herelle is equally relevant today and should provide a cautionary lesson in overlooking issues related to formulation of stable phage preparations that could have a direct impact on therapeutic efficacy. In a recent high-profile randomized controlled trial (RCT) on the efficacy and tolerability of *Pseudomonas aeruginosa* bacteriophages to treat burn wounds (PhagoBurn trial), lack of stability of the formulated phage product after manufacture of the cGMP (Current Good Manufacturing Practice) batch was observed (Jault et al. 2018).

2 Overview of Innovative Production Systems for Phage Therapeutics

With growing interest towards phage therapy, global demand for large-scale production of phage therapeutics will also increase. Manufacture of commercially useful quantities of phages at low cost necessitates development of continuous production techniques and a move away from batch processing in order to improve productivity and reduce process footprint. Regulatory agencies (e.g. the US Food and Drug Administration, FDA) have strict requirements regarding controlling the product quality within specified limits for pharmaceuticals. A fundamental understanding of the underlying kinetics and the influence of processing conditions on product quality attributes is an essential prerequisite to ensure implementation of on-line or at-line process control strategies as part of a Quality by Design (QbD) framework now favoured by USFDA for the manufacture of pharmaceuticals.

There are two main technological pathways for phage propagation: either using liquid or semisolid growth media. A number of international patents and patent applications describe methods and devices for phage manufacturing suitable for commercial scale propagation, purification and formulation of phages (Ghanbari and Avenback 1997; Weber-Dabrowska et al. 2002; Boratynski et al. 2008; Sulakvelidze et al. 2001, 2004; Mosa et al. 2013; Shaw 2017).

Typically phages propagated in liquid cultures are produced in batch fermenters, e.g. shaken flasks, and more recently in disposable wave bags used for tissue cell culture; there are no real issues with regard to residence times and complex control strategies (Sauvageau et al. 2010; Bourdin et al. 2014). The downsides for industrial-scale batch fermentation include higher capital costs, large process footprints, labour-intensive operation, the proportion of downtime compared with production time which can be high, lack of process control and variability of product quality (Sauvageau et al. 2010). Continuous upstream production of phages using chemostat systems has heretofore received little attention in the published literature which instead has focused on using such systems for studying coevolution processes (Lindemann et al. 2002; Oh et al. 2005; Nabergoj et al. 2018). Decoupling the

bacterial host propagation from phage production removes the selection pressure for bacterial mutation and should allow stable long-term steady-state operation of the process (Husimi et al. 1982).

Semi-continuous production approaches using such a strategy include a two-stage self-cycling process for the production of phages (Sauvageau and Cooper 2010; Krysiak-Baltyn et al. 2018). Synchronous host populations at high cell concentrations are produced in the first reactor operated in batch mode. These are then passed on for infection with phages in a second batch reactor using a relatively simple control strategy based on the host population approaching near stationary phase prior to half of the fermentation volume being transferred to the second reactor for infection. Fresh nutrients are subsequently added to the first reactor to continue host cell propagation in the exponential growth phase while the second infection vessel amplifies the phages prior to the process being repeated in a cyclic fashion.

Continuous processes are better suited to increase volumetric productivity. In the study by Mancuso et al. (2018), the residence times of the two bioreactors were independently controlled by using different reactor volumes even though the same flow rate was maintained through them. Continuous production of high titres of *Escherichia coli* T3 phages (10^{11} pfu/mL) was achieved using two continuous stirred tank bioreactors connected in series and a third bioreactor as a final holding tank operated in semi-batch mode to finish the infection process. Host bacterial growth was decoupled from the phage production reactor downstream of it to suppress production of phage-resistant mutants thereby allowing stable operation over a period of several days. The novelty of the process was that manipulation of the host reactor dilution rates (range 0.1–0.6 h^{-1}) allowed control over the physiological state of the bacterial population with high dilution rates resulting in bacteria with considerably higher intracellular phage production capability yielding significantly higher phage productivity. The pilot-scale chemostat system allowed optimization of the upstream phage amplification conditions conducive for high intracellular phage production. The effect of host reactor dilution rates on phage burst size, lag time and adsorption rate was evaluated. The host bacterium physiology was found to influence phage burst size thereby affecting the overall process productivity. Mathematical modelling of the dynamics of the process allowed parameter sensitivity evaluation and provided valuable insight into factors affecting the phage production process which may be used at an industrial scale for improved process control.

Baldwin and Summer (2011) in the earlier patent describe a continuous flow phage proliferation process by providing additional phage portions from the outflow back into the reactor vessel along with extra doses of host bacteria, thereby ensuring accumulation of large commercial quantities of the desired bacteriophages.

Synergetic activity of phage and antibiotics towards bacterial pathogens is a well-known phenomenon that has been highlighted in a number of studies describing in vitro and in vivo models (Comeau et al. 2007; Ryan et al. 2012; Knezevic et al. 2013). An earlier patent claimed a tenfold increase in the yield of phages propagated in *E. coli* strains in the presence of sublethal doses of beta-lactam and quinolone antibiotics (Comeau et al. 2007; Krisch et al. 2007).

Another technological pathway comprising application of semisolid cultures is characterized by relatively small operating volumes, reduced downstream processing

and higher phage yields leading to overall reduced costs of production. One of the earlier patent applications (Bujanover 2003) described use of media with a low percentage of hydrocolloids (<0.3%) followed by multiple extractions with phage-specific buffer resulting in 55 L of final product with a phage titre of 10^{12} pfu/mL.

A recent patent application (Elliott 2016) presents a phage production method using a 'vertical cassette' device, which consists of variable numbers (up to 32) of 2-sided cassettes filled with semisolid media intended for phage propagation. Phages are extracted from all cassettes simultaneously within the same device suspended in an appropriate extraction buffer. The phage propagation process in this apparatus resulted in commercially useful quantities of phages; 60 L (for 23 cassettes) of product with high-phage titre around 10^{11} pfu/mL were reported to have been produced using such a system. Phage preparations with high titres may be purified and subsequently diluted to the desired therapeutic titres, typically in the range of 10^6–10^8 pfu/mL (Merabishvili 2014).

Purification procedures described for commercial manufacturing of phages typically comprise a wide variety of filtration and chromatography unit operations including microfiltration and tangential flow filtration (operated as ultrafiltration or diafiltration) (Voroshilova et al. 1992; Reshetnikov et al. 2002; Sulakvelidze et al. 2004; Lehnherr and Bartsch 2007; Muller 2009), ion-exchange and size-exclusion chromatography (Weber-Dabrowska et al. 2002; Morris et al. 2003; Muller 2009; Smrekar et al. 2011a). Existing bioprocess engineering approaches used for the manufacture of biotherapeutics, e.g. monoclonal antibodies and vaccine manufacture, may be adapted for phage production; however, there are important differences including the relatively large size of phages (~100 nm) with implications for development of process unit operations. Conventional chromatographic materials, for example, are currently designed to allow proteins access to the internal pore structure and large surface area which are not readily accessible to large phage particles reducing the separation capacity of the resins (Smrekar et al. 2008). New innovations are therefore needed such as use of monolith-based chromatography supports to overcome such challenges (Smrekar et al. 2011b).

2.1 Synthetic Natural Phages

Phage specificity is considered to be one of the major issues of sustainable phage therapy approaches because it involves the analysis of the infecting bacterium's phage susceptibility to select the adequate phage(s) from large collections of wide host-range therapeutic phages (Pirnay et al. 2011).

Synthetic biology could offer a solution to reduce the specificity of phages (and antimicrobials in general) to target a larger array of bacterial pathogens while minimizing the killing of potentially helpful and commensal bacteria (Hwang et al. 2018). For instance, a yeast-based platform for phage tail/tail fibre protein switching was developed to engineer hybrid T7 phages with extended host range (Ando et al. 2015; Yosef et al. 2017). Other phage therapy problems that can be addressed using genetic engineering strategies (e.g. CRISPR-Cas editing tools) are negative

patient-phage interactions (e.g. anti-phage immune response) (Brown et al. 2017), the potential emergence and spread of bacterial phage resistance mechanisms and the release of harmful bacterial contents such as endotoxins (Hwang et al. 2018).

However, Western regulatory frameworks are currently struggling to cater for conventional phage therapy approaches (Verbeken et al. 2014), based on natural phages, and are bound to regulate genetically modified phages even more tightly (Brown et al. 2017). Recently, Belgium implemented a pragmatic phage therapy framework that centres on the magistral preparation (compounding pharmacy in the USA) of tailor-made phage medicines (Pirnay et al. 2018). Even though this framework—currently and technically—only allows for the inclusion of 'naturally occurring phages' as active pharmaceutical ingredients (APIs) in magistral preparations, there are indications that this pathway might be expanded to synthetic phage APIs, providing that they are indistinguishable from naturally occurring phages.

Figure 1 shows a tentative approach for the ad hoc and on-site production of personalized medicines containing synthetic naturally occurring bacteriophages (SNOBs). The first three steps, from sampling to bacterial whole-genome sequencing, are slowly percolating into the practice of clinical microbiology (American Society for Microbiology 2016). Next-generation sequencing (NGS) of large numbers of bacterial pathogens and of their infecting phages will broaden our understanding of how bacteria and phages interact and co-evolve. The subsequent prediction/selection of adequate therapeutic phages using computational methods and tools (e.g. new deep learning-based approaches), based on information from large natural phage and bacterial genome libraries (step four), will require some extensive research to reach clinically acceptable levels of accuracy. Recently,

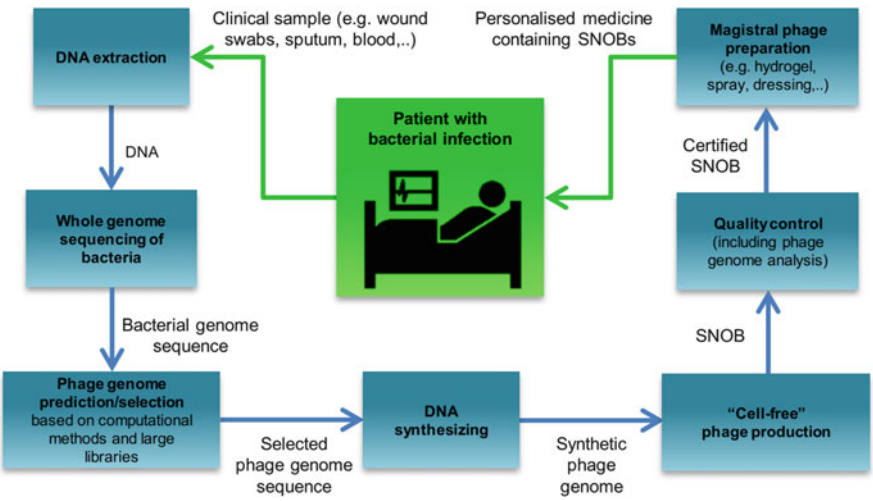

Fig. 1 Tentative approach for the ad hoc and on-site production of personalized medicines containing synthetic naturally occurring bacteriophages (SNOBs)

machine learning was used to predict bacteria-phage interactions based on proteomics (Carvalho Leite et al. 2017). The resulting predictive models reached accuracy, sensitivity and specificity values of over 90%. Synthesizing the genome(s) of selected naturally occurring phage(s) that lyse the infecting bacteria is technically feasible today but will also require some additional research to optimize and speed up the procedure. The current DNA synthesis technique is based on organic chemistry and directly produces oligonucleotides of about 200 bases long. The size of phage genomes ranges from 3.4 to almost 500 kb (Keen 2015). The de novo synthesis of even a small phage genome would thus require the synthesis of numerous 200 nucleotide pieces and stitching together polymerase chain reaction (PCR) products that carry overlapping nucleotides that template the order in which the fragments need to be assembled (Barbu et al. 2016; Lemire et al. 2018). Today, the final assembly of synthetic phage genomes can be performed by assembling these larger DNA fragments in either the yeast *Saccharomyces cerevisiae*, using yeast artificial chromosomes (YACs) (Ando et al. 2015), or by biochemical assembly (Gibson Assembly) (Gibson et al. 2009). These methods for synthesizing phage genomes are time-consuming and expensive and often require multiple attempts. Recently, scientists developed a new technique to synthesize DNA that is predicted to be easier, more accurate and faster (Palluk et al. 2018) and which would facilitate phage genome synthesis. This new technique is based on a DNA-synthesizing enzyme found in cells of the immune system that naturally has the ability to add nucleotides to an existing DNA molecule.

Next, these synthetic phage genomes could be rebooted to produce phage offspring through transformation of *E. coli* or *Listeria monocytogenes* L-forms or using cell-free transcription-translation (TXTL) systems (Barbu et al. 2016). Recently, *Listeria* L-form cells were used to enable cross-genus reactivation of *Bacillus* and *Staphylococcus* phages from their synthetic genomes (Kilcher et al. 2018), and the complete infectious phage T4 was rebooted from its 169-kb genome in a single test tube TXTL reaction (Rustad et al. 2018).

The resulting SNOB suspension needs to be purified (e.g. removal of endotoxins), and its identity and purity need to be ensured, using validated tests, according to the Good Manufacturing Practices (GMP) guidelines or the requirements laid down in the API monograph (Belgium) (Pirnay et al. 2018). As explained before, only 'naturally occurring phages' can be approved for use in magisterial preparations in Belgium. The question is whether infectious SNOBs produced from phage genome sequences derived from naturally occurring phages, according to the methodology described in this paragraph, will be considered as naturally occurring phages or as genetically modified organisms (GMOs), which need to be more tightly regulated. There are indications that, if these SNOBs comply with the requirements of the phage API monograph and if their genome sequences do not vary or only vary very slightly (comparable to natural mutations) from the original (starting) natural genome sequences, they might be considered to be naturally occurring phages, which can be used as active components of phage magisterial preparations (compounding pharmacies) for use in human medicine in Belgium.

The ad hoc and on-site production of SNOBs proposed here would—at least theoretically—have several advantages over conventional production systems using

natural phages and host bacteria. (1) There is no need to maintain physical therapeutic phage banks. A database containing an exhaustive number of therapeutic phage genome sequences would be sufficient. (2) This implies that if devices would be developed and marketed that could ad hoc and on-site select and produce SNOBs targeting the infecting bacteria, there would be no need for the time-consuming distribution of (tailored) phage preparations. (3) Since cell-free or L-form phage synthesis does not require phage attachment to a bacterial cell wall, there are indications that it would be possible to produce more diverse (cross-genus or even cross-species) phages in one cell-free phage production system. (4) Metagenomic datasets derived from human or environmental samples can contain (predicted) phage genomic sequences (Reyes et al. 2010; Amgarten et al. 2018), even when no phages could be isolated from these (or similar) samples using traditional phage isolation techniques (e.g. because the hosts used in the isolation techniques were not susceptible for the phages present in the samples). However, using these predicted phage genomic sequences, therapeutically interesting SNOBs could be produced. (5) Since cell-free phage production systems do not require intact bacteria, synthetic doppelgangers of natural phages, which were found to exclusively infect bacteria that can cause serious and potentially lethal disease and would normally require biosafety level-3 (bsl-3) biological containment precautions, such as *E. coli* O104:H4 strain from the 2011 outbreak in Germany (Merabishvili et al. 2012), could be synthesized in bsl-1 cleanroom conditions. (6) Cell-free phage production systems can be designed to produce smaller amounts of highly pure active agent reducing the risk of negative impact on patients (e.g. due to presence of endotoxins in the final preparations).

For the on-site production of bacteriophages, the storage of the primary material is essential, which in case of the cell-free system can be done in a lyophilized state (Salehi et al. 2016). In this condition it is easy storable, and by simply adding an aqueous phage DNA solution, the expression starts.

But, there might also be some pitfalls. For one, it is not clear whether SNOBs that are produced in bacterial cell (wall)-free systems maintain the same level of bacterial infectivity as their natural analogs. For instance, cell (wall)-free production systems that include one or more replication steps might select for SNOBs that are less (or not) capable of attaching to the bacterial cell wall and injecting their synthetic genomes hence failing to infect intact bacterial cells.

Overall, it seems that the proposed methodology for the production of SNOBs could potentially expedite the turnaround time for a phage antibacterial medicine and allow for sustainable (personalized) and timely phage therapy approaches.

3 Pharmaceutical Formulations of Phage Therapeutics

The demand for advanced and innovative pharmaceutical phage formulations will also increase as phage therapy becomes a more realistic healthcare option. The number of published studies exploring a wide variety of pharmaceutical phage

strategies has increased drastically in the last decade and that trend is most likely to be maintained in the future.

Developing formulations that incorporate bacteriophages for therapeutic applications requires an appreciation of the chemical and physical stresses phages may encounter both during processing and during storage once formulated. Phage inactivation and long-term reduction in phage titre upon storage are highly undesirable. The physical and chemical properties of the formulation need careful consideration when selecting a technique for encapsulation. Accurate loading of phages per particle requires particle monodispersity which is rarely achieved in practice; however, some techniques are considerably better than others (novel microfluidic approaches are discussed below). The particle morphology should be without deformities, and the particles shouldn't aggregate or uncontrollably fuse together with material in the surrounding environment. Overlooking these aspects may influence accurate dosing, e.g. during particle aerosolization for pulmonary applications using dry powder inhalers or rehydration of lyophilised or dried phage powders. There are many techniques and processes that may be used for stabilising, immobilising and encapsulating phages. Conventional approaches for preparation of stable dry phage formulations include freeze drying and spray-drying. However, novel approaches including microfluidic methods discussed here may permit high levels of innovation for production of micro- and nano-encapsulated phage delivery systems in solid dosage forms suitable for oral and pulmonary delivery or as nanoemulsions, e.g. in liposomes for topical delivery or incorporated within microneedle systems for needleless injection. Phages may be encapsulated in nanofibres as part of a smart wound dressing releasing phages in response to an infection or immobilised on surfaces for food biocontrol applications or diagnostic applications. We briefly discuss conventional approaches suitable for phage formulation and then highlight more novel methods that may allow a high level of innovation that both addresses unmet clinical needs but which would also enable creation of new intellectual property and value for the start-up companies venturing into the field of phage therapy.

3.1 Conventional Formulation Approaches

Conventional pharmaceutical formulations of phage therapeutics include liquid and lyophilized formulations (Merabishvili 2014). The simplicity of the manufacturing process and the wide variety of possible therapeutic administration routes result in broad acceptance of liquid formulations, and this is likely to be the case for the future. The quality and safety requirements for phage preparations are governed by their further application routes, e.g. these can substantially differ between intravenous and topical preparations. The expected shelf life of liquid formulations as a rule varies between 1 and 2 years at 4 °C. Stability of water-based phage suspensions is highly dependent on phage titres and added excipient compositions (unpublished data). Additionally, sensitivity of phages towards different environmental factors, including various salt concentrations, pH of solvents, is considered a highly specific feature (Łobocka et al. 2014). A number of pharmaceutically accepted excipients

and additives such as stabilization, tonicity, wetting, solubilizing and dispersion agents and preservatives may be used to improve the shelf life of liquid-formulated phage therapeutics.

Freeze drying (lyophilization) is routinely used in the pharmaceutical industry to dry proteins, vaccines, peptides or colloidal carriers such as liposomes, nanoparticles and nanoemulsions (Wang 2000; Abdelwahed et al. 2006). Freeze drying is typically done from solution and involves a freezing step followed by a drying step. During the freezing step, the phage containing solution is cooled, and ice crystals of pure water form resulting in freeze concentration of the remaining liquid. Preventing aggregation of bacteriophages and inactivation due to high osmotic pressure changes needs careful consideration here. At the end of the freezing stage, drying begins as a result of switching on a very low-temperature condenser which draws water from the sample over time. A primary drying stage results in direct sublimation of the ice crystals, and the remaining adsorbed water is removed in a secondary drying phase. In the freeze-dried glassy state, the rate constants of most chemical and physical degradation reactions are dramatically reduced thereby permitting long-term storage under refrigerated or ambient temperatures (25 °C). Freeze-dried material typically results in a cake that needs further processing, e.g. milling to achieve fine particles suitable for loading in dry powder inhalers (DPI) (Telko and Hickey 2005).

In the former Soviet Union, lyophilized phages were produced in the form of tablets and powders. Common excipients used in their manufacturing included calcium gluconate, glucose and skimmed milk (Ministry of Health of the USSR 1987).

Papers on phage and virus freeze drying have mainly focused on evaluation of formulations in order to stabilize phages for storage (Engel et al. 1974; Ackermann et al. 2004; Dini and de Urraza 2013; Merabishvili et al. 2013; Malenovská 2014). Amino acids (e.g. sodium glutamate (Engel et al. 1974)), peptides (e.g. peptone (Davies and Kelly 1969)) and proteins (casein and lactoferrin (Golshahi et al. 2010)) have been added to the formulations to improve phage viability upon freeze drying and following rehydration. Literature suggests that disaccharides, e.g. lactose (Golshahi et al. 2010), sucrose (Puapermpoonsiri et al. 2010; Dini and de Urraza 2013; Merabishvili et al. 2013; Malenovská 2014) and trehalose (Dini and de Urraza 2013; Merabishvili et al. 2013; Dai et al. 2014; Colom et al. 2015; Leung et al. 2016), improve phage survival during freezing as well as subsequent lyophilization. Rapid rates of freezing at relatively low temperatures (<-20 °C) have been found to result in better phage survival compared with slow freezing which has been attributed to less time for osmotic damage to occur (Davies and Kelly 1969).

Spray-drying processes atomize a liquid containing dissolved solids, converting it into a fine mist which is contacted with a hot dry gas (typically hot dry air) inside a drying chamber. Spray drying is a scalable industrial process technology and is widely used to produce fine powders for pharmaceutical applications including pulmonary delivery via dry powder inhalers (DPI) (Hoe et al. 2014). Dry powders are favoured for respiratory drug delivery because they show relatively long storage stability without requiring refrigeration (Chew and Chan 2002; Klingler et al. 2009) and DPI are simple to use and do not require regular cleaning and disinfection (Weers 2015). However, spray drying has never been used for manufacturing of phages on commercial scale.

Phages may not to be able to withstand high shear stress for too long; the nebulization process has been shown to result in loss of phage titre (Vandenheuvel et al. 2013; Leung et al. 2016; Carrigy et al. 2017). Phages are also sensitive to thermal stresses and have been shown to partially lose activity at temperatures higher than 60 °C (Davies and Kelly 1969; Jończyk et al. 2011). Low spray drying temperatures (~40 °C outlet air temperature) have been shown to result in higher-phage titre (Leung et al. 2017) with high loss of phage titre observed at higher temperatures (Walbeck 2008; Matinkhoo et al. 2011; Vandenheuvel et al. 2013; Leung et al. 2016). Literature on spray drying of phage suspensions typically includes excipients in the formulation to protect the phage from desiccation and thermal stress. Trehalose is the most frequently reported excipient used in the spray drying of phages, and it has been shown to result in spray-dried powders with high-phage titres and good storage stability (Matinkhoo et al. 2011; Vandenheuvel et al. 2014; Leung et al. 2016, 2017). Trehalose has low toxicity and protects biological materials, including proteins, probiotics and vaccines, against desiccation and thermal stress. Other excipients that have been used to improve the dispersibility of spray-dried phage containing powders include dextrans, lactose (common excipient used for DPI powders), glucose, sucrose, mannitol and leucine (Telko and Hickey 2005; Matinkhoo et al. 2011; Vandenheuvel et al. 2013; Leung et al. 2016). Addition of proteins, e.g. casein or leucine in combination with sugars, has shown good results too (Matinkhoo et al. 2011; Chang et al. 2017). Refrigerated storage of spray-dried powders was reported to yield higher titres of phages during long-term storage compared with storage under ambient conditions (Leung et al. 2017). Different phage strains formulated and spray-dried under identical conditions showed significant differences in the resulting phage titres suggesting the need for individually formulating each phage to be used in a phage cocktail (Vandenheuvel et al. 2013, 2014). A number of studies have reported spray-dried phage powders to have a suitable mass median aerosolized diameter (between 1 and 5 microns) for pulmonary delivery of phage to treat respiratory infections (Matinkhoo et al. 2011; Vandenheuvel et al. 2013, 2014; Leung et al. 2016; Chang et al. 2017).

3.2 Innovative Pharmaceutical Formulations

3.2.1 Methods Used for Bacteriophage Encapsulation in Micro- and Nanoparticulate Solid Dosage Forms

Bacteriophages may be encapsulated in protective micro- and nanoparticles to overcome adverse storage and physiological conditions en route to delivering the phage load to the site of infection. Controlled-release and sustained-release strategies for phage delivery applications may be achieved using a diverse array of strategies. These include systems based on diffusion-controlled release (e.g. solvent diffusion-based osmotic pumps), matrix dissolution and erosion-controlled systems and ion-exchange swelling-based systems. Phage-compatible formulation and encapsulation processes need to be carefully designed to prevent damage to viral capsid and DNA/RNA components and stabilization of the structure of viral capsid and tail

proteins to prevent loss of phage viability during manufacturing operations. It is important that the carrier encapsulating the phages is able to withstand adverse environmental conditions for the duration of exposure and is capable of delivering the bacteriophages at a suitable high dose to the relevant site where host bacteria are present (Ibekwe et al. 2008; McConnell et al. 2008).

A number of phage encapsulation studies using synthetic and natural polymers have utilized the emulsification route followed by solvent removal. The dispersed phase usually comprises of the core material and solvent carrying the active agent (bacteriophage) and the polymer and a second phase which allows for the break-up of the inner phase into droplets. The emulsion may be water-in-oil (W/O) more typical for phage encapsulation, but it could also be oil-in-water (O/W) (Puapermpoonsiri et al. 2009; Esteban et al. 2014). Once the droplets have formed, the solvent carrying the core material may be removed, which leaves behind solid particles containing the active agent in the core. Different mechanical emulsification techniques may be used to break-up the inner phase into droplets, such as high-pressure or rotor-stator homogenization and ultrasonication. When two immiscible liquids are put together, the shearing force breaks up the inner phase into small droplets. The droplet size uniformity is low as the shear applied does not remain uniform throughout the container resulting in droplet heterogeneity with some droplets being larger and some smaller. More advanced methods to produce uniform droplets based on microfluidic techniques have thus far not been widely used for phage microencapsulation (these methods are discussed later).

Alternatively, phage-containing polymer droplets have been produced via an extrusion technique (Ma et al. 2008). The droplet may form in the air or into another liquid. The dispersed phase containing the polymer and phage is extruded through a needle with a specific nozzle diameter determining the size of the resulting droplets (Tang et al. 2015). Atomization nozzles have also been used to generate smaller droplets (Colom et al. 2017). Extrusion techniques previously employed have extruded alginate-containing phage droplets into a bath of calcium chloride which causes ionotropic gelation (Ma et al. 2008, 2012; Dini et al. 2012). The process of gelation can occur in a number of ways depending on the properties of the polymer/hydrogel. Other gelling triggers apart from ionotropic gelation include heating, cooling and covalent cross-linking. Other techniques that have previously been used for phage encapsulation in synthetic and natural polymers include polymer precipitation, photopolymerization and thermal phase inversion (Bean et al. 2014; Esteban et al. 2014; Hathaway et al. 2015; Samtlebe et al. 2016).

Polymer phage encapsulation literature has focused largely on gastrointestinal infections. Ensuring delivery and subsequent release of a precise high dose of phages in the gastrointestinal tract remains an important challenge to ensure that phage-based modulation of the gut microbiome develops its full potential (Malik et al. 2017). The drivers for encapsulation are the need to protect phages from the harsh stomach environment rendering free phages inactive or at any rate resulting in reduction in phage titres. High doses of bacteriophages need to be delivered in a controlled manner at the target site in order to effectively reduce the concentration of target bacteria present there. This poses a considerable formulation and delivery

challenge. Animal studies focusing on gastrointestinal infections have shown poor efficacy outcomes due to the challenges associated with in vivo phage therapy. Oral administration of bacteriophages for human or animal use requires careful consideration of a number of factors including the acidic pH of the stomach, digestive enzymes (pepsin, proteases, lipases, amylase and trypsinogen), bile salts, pancreatic juices, residence time in different intestinal compartments (duodenum, jejunum, ileum) and phage permeability into the mucosal lining where the infection may reside.

Overcoming phage instability to gastric acid is a major concern in oral phage delivery for gut microbiome applications including food biocontrol; it has been addressed in some studies through administration of antacids, e.g. sodium bicarbonate prior to oral phage treatment or co-encapsulation of calcium carbonate (Jamalludeen et al. 2009). Formulations used for phage encapsulation require careful selection of constituents and encapsulation conditions to ensure phages remain viable and in order to prevent titre loss during processing and storage. Previous efforts targeting *Salmonella* in the food chain have attributed poor phage stability in the gastrointestinal tract for insufficient in vivo efficacy (Wall et al. 2010). *Salmonella* Felix O1 phage (belonging to the *Myoviridae* family) was shown to be highly sensitive to acidic pH, and it is not an exception. Similar results have been reported previously for number of other phages in gastrointestinal environment (Ma et al. 2008, 2012; Saez et al. 2011; Kim et al. 2015; Albino et al. 2014). This loss of phage activity highlights the need to protect phages from the harsh acidic environment of the stomach if controlled doses of phages are to be reliably delivered to treat salmonellosis in the infected gut. Felix O1 microencapsulated in alginate beads has previously been shown to survive and amplify in the gastrointestinal tract of pigs (Wall et al. 2010).

Carriers designed to protect the active agents, e.g. from the harsh gastrointestinal environment, may also be used to trigger their release. An important example of this is pH-dependent release of encapsulated phages (Stanford et al. 2010), exploiting the variation in pH throughout the GI tract; carriers may be designed to respond to specific pH which differs from the stomach (pH 1–3) to the small intestine (pH 5.5–6.5) and the colon (pH 6.5–7.2) (McConnell et al. 2008). Gastric emptying rates are another important factor and need to be considered in the design of phage encapsulation systems for oral use. Previous studies indicate that in humans gastric emptying of small microcapsules (less than 2 mm) is rapid (~0.5–1.5 h) and is not greatly affected by the digestive state of the individual (Davis et al. 1986). The harsh environment of the digestive system renders many sensitive therapeutic agents, e.g. proteins and phage, inactive when administered without due consideration to proper formulation (Philip and Philip 2010; Dini et al. 2012; Choonara et al. 2014; Amidon et al. 2015). Owing to the nature of the infection, fluctuations in physiological conditions may also need to be considered and may indeed be put to use to trigger release at the site of infection (McConnell et al. 2008). Consideration of particular infection-specific symptoms that might influence the delivery of the phages needs to be taken into account. For example, the onset of diarrhoea in humans causes the osmotic gradient between the epithelia and colon to decrease

resulting in increased fluid leakage and shorter transit times (Van Citters and Lin 2006). Changes in pH have been observed in the intestine during infection which can directly affect the microbial population as well as transit times in the GI tract (Hua et al. 2015).

3.2.2 Bacteriophage Encapsulation Using Membrane Microarrays

Advanced techniques may be applied for phage encapsulation to precisely control the size and architecture of phage containing micro- and nanoparticles. To-date methods used for phage encapsulation have not utilized state-of-the-art processing techniques that could be applied for the encapsulation of phages to achieve controlled dosage forms in uniform micro- and nanoparticles. Membrane emulsification (ME) using micropore arrays is one such process that allows formation of uniform drops by injecting a dispersed-phase liquid through a microporous membrane into the continuous phase (Fig. 2). Alternatively, a pre-emulsified mixture of the dispersed and continuous phase may be repeatedly injected through the membrane (Fig. 2). To encapsulate bacteriophages, the formed droplets, typically composed of a mixture of the wall-forming materials, solvent(s) and phage, could be solidified under mild agitation using various solidification reactions or processes, such as free radical polymerization, polycondensation, ionotropic/thermal gelation, cooling crystallization and molecular or particle self-assembly triggered by solvent evaporation (Vladisavljevic and Williams 2005). The advantages of ME over standard emulsification procedures using high-pressure valve homogenizers or rotor-stator devices are in higher drop size uniformity and lower energy inputs and applied shear, which can be useful to preserve phage integrity. It is well-known that bacteriophages are

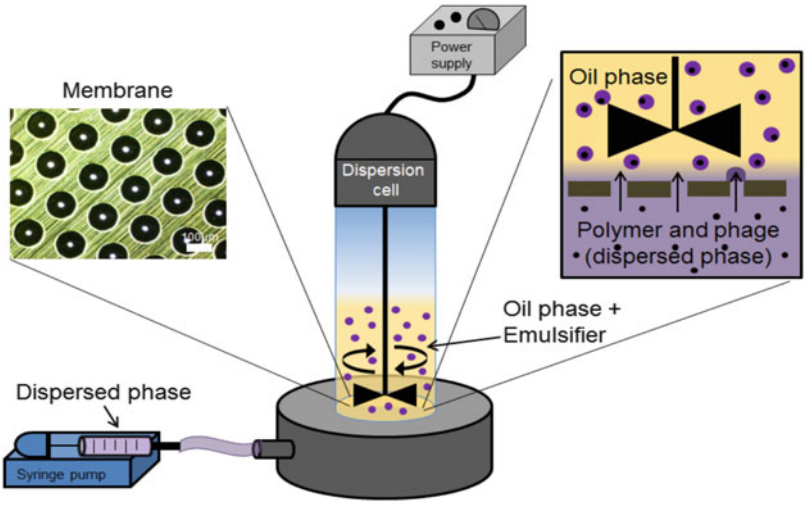

Fig. 2 Schematic representation of the membrane emulsification system used for microencapsulation of bacteriophages (Reproduced from Malik 2019)

sensitive to mechanical shearing (Vandenheuvel et al. 2013; Leung et al. 2016). In direct ME, the shear rate on the membrane surface is ~10^3–10^4 s^{-1}; however, uniform drops may be obtained without any shear by spontaneous droplet formation driven by Laplace pressure gradients (Kukizaki 2009). The shear rate in high-shear mixers and colloid mills is typically ~10^5 s^{-1} and can exceed 10^7 s^{-1} in microfluidizers. The most common membranes used in ME are Shirasu porous glass (SPG) and microsieve metallic membranes (Vladisavljević et al. 2012). Shear on the membrane surface required for drop detachment can be generated in various ways including (1) using a paddle stirrer placed above the membrane surface (Kosvintsev et al. 2005), (2) rotating membrane (Vladisavljević and Williams 2006) and (3) oscillating membrane (Holdich et al. 2010). In the oscillating ME system, the tubular membrane can oscillate tangentially clockwise or counter-clockwise (Silva et al. 2015) or radially upwards and downwards (Holdich et al. 2010), with frequencies ranging from 10 to 90 Hz.

3.2.3 Microfluidic Microencapsulation of Bacteriophages

Fabrication of bacteriophage-encapsulated microparticles using microfluidic technology has recently been shown to permit precise control over the particle size, phage dose per particle and tailoring of the release profile for targeted delivery and controlled release of phages (Vinner et al. 2017; Vinner and Malik 2018). The low controlled shear in the microfluidic droplet generation unit potentially allows 100% phage encapsulation and production of highly uniform droplets. The significant potential of microfluidic microencapsulation for phages is relatively unexplored with only three previously published papers to date (Boggione et al. 2017; Vinner et al. 2017; Vinner and Malik 2018). Little consideration has previously been given to the control of the particle size distribution, the phage loading per particle and the resulting heterogeneity of the release kinetics from each particle (Ma et al. 2008, 2012; Tang et al. 2013, 2015; Kim et al. 2015). A key advantage of the microfluidic microcapsule fabrication process is the precision with which uniform small microparticles (mean size 10–100 μm) containing encapsulated phages may be prepared under low shear conditions.

Droplet generation in a microfluidic device results in each droplet being produced at the same position; disruptive forces of the same magnitude act on each individual droplet, and these forces become negligible after pinch-off (Figs. 3 and 4). In conventional bulk emulsification systems, the droplets are continuously being subjected to shear which typically varies widely across the emulsification or homogenization unit (Jain et al. 2005; Surh et al. 2007; Sağlam et al. 2011). Continuous excessive exposure to shear results in the droplets breaking further into smaller droplets resulting in emulsions with a high degree of polydispersity (Kong et al. 2012). The microfluidic fabrication process provides a highly controlled uniform shear environment resulting in microparticles with low polydispersity (Vinner et al. 2017; Vinner and Malik 2018). Such systems may be scaled up by using multiple droplet generation units operated in parallel with little loss in droplet size control.

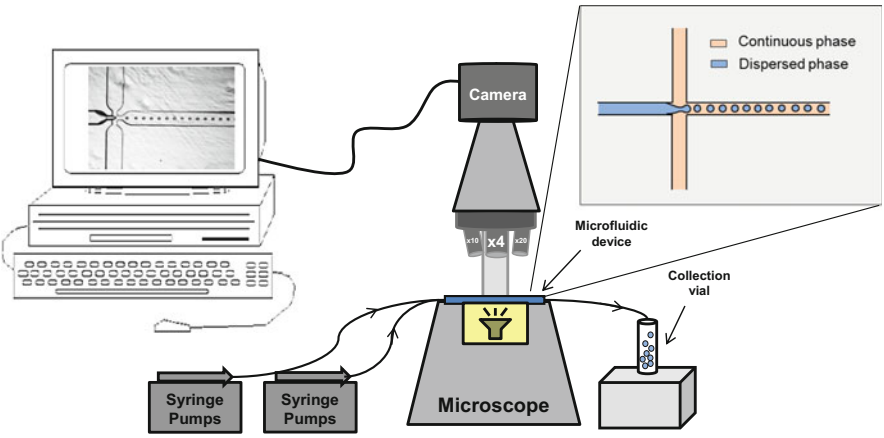

Fig. 3 Schematic of experimental setup to produce water-in-oil emulsion for phage microencapsulation (Reproduced from Vinner and Malik 2018)

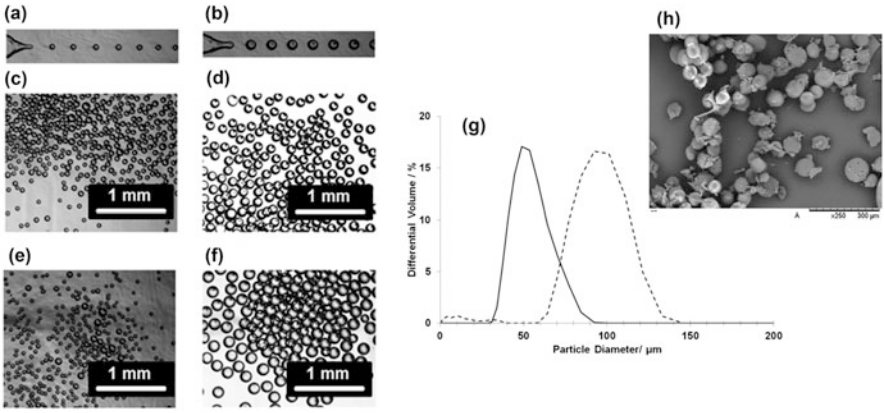

Fig. 4 Production of pH-responsive microparticles using a microfluidic droplet generation system. At-line optimization of hydrodynamic conditions allowed control over the droplet size, (**a**–**f**) in situ imaging showing generation of droplets of different sizes prepared using a microfluidic chip droplet generation unit, (**g**) particle size distributions of gelled pH-responsive microparticles, (**h**) scanning electron micrograph of freeze-dried pH-responsive microparticles (Reproduced from Vinner and Malik 2018)

3.2.4 Bacteriophage Encapsulation in Liposomes

Liposomes are considered as efficient vehicles for delivery of various types of drugs, biomolecules and genetic material. Liposomes were first described by Bangham in 1961 and published in 1964 (Bangham and Horne 1964), and a few years later, they were already the subject of intensive research focusing on pharmaceutical

applications. Liposomes are vesicles composed of phospholipid bilayers which enclose an inner aqueous compartment. A major asset of liposomes is conferred by their ability to merge easily with cell membranes and release their content inside the target cells. Phospholipids are also considered as GRAS (generally recognized as safe) ingredients. Liposomes are highly biocompatible, and they have been widely used in the last 50 years to encapsulate both hydrophilic and hydrophobic therapeutic agents in their aqueous core or within the lipid bilayers in multilamellar vesicles, thereby enhancing drug bioavailability and stability over time. Encapsulated liposomes are a promising drug delivery system and pharmaceutical carrier of choice for numerous applications (Torchilin 2005). There are relatively few current studies exploring encapsulation of phages into liposomes aiming to overcome the common hurdles associated with phage therapy delivery, in situ retention and targeting intracellular pathogens.

Typically, phages encapsulated in liposomes have been prepared using the conventional thin-film hydration method (Zhang 2017). Briefly, this entails dissolving lipids in an organic solvent (typically chloroform) followed by solvent evaporation under vacuum leaving a dry lipid film. Addition of an aqueous phage suspension to the dry lipid film results in the stacks of liquid crystalline lipid bilayers becoming fluid and swelling. Agitation of the solution results in detachment and closure of the lipid layers thereby forming relatively large and heterogeneous multilamellar liposomes. Other conventional methods of liposome production include reversed-phase evaporation, solvent-injection techniques and detergent dialysis (Huang et al. 2014). To control the size and dispersity of liposomes, various methods can be applied, in particular, high-pressure membrane extrusion, microfluidization, sonication and others (Colom et al. 2015; Bulbake et al. 2017).

Along with conventional methods of encapsulation of drugs and phages into liposomes, new technologies are being developed; these include supercritical fluid technology, supercritical anti-solvent (SAS) method, dual asymmetric centrifugation as well as microfluidic methods (Huang et al. 2014; Cinquerrui et al. 2018; Leung et al. 2018).

From a clinical viewpoint, the potential ability of liposome-encapsulated phages to enter live cells containing intracellular pathogens is of crucial importance, e.g. *Mycobacterium tuberculosis* or other intracellular bacteria, including *Staphylococcus aureus* or *E. coli*. The surface of liposomes may be functionalized to achieve various goals: (1) attachment of specific ligands for targeted delivery of liposome cargo; (2) attachment of hydrophilic polymers, e.g. polyethylene glycol (PEG) to increase systemic circulation time and slow release of bacteriophage cargo; (3) attachment of various labels to monitor the fate of the bacteriophage-loaded liposomes; (4) incorporation of positively charged lipid derivatives or positively charged polymers, e.g. to aid mucosal adhesion, thereby improving residence time in the gastrointestinal tract; (5) protection of bacteriophages from physical and chemical stresses; and (6) liposome-encapsulated bacteriophages may result in modulated immune response to the presence of the bacteriophages in the body.

Liposomes can differ in size, shape, charge, composition, solubility and lamellarity. All these parameters determine distribution of liposomes within the

body and their eventual pharmacokinetics outcomes (Aggarwal et al. 2009; Ernsting et al. 2013). Size and lamellarity vary as a result of the manufacturing process. Fluidity and superficial charge depend on the chemical nature of the lipids and their relative ratio.

Size range of liposomes is broad: vesicles can be as small as 20 nm, while size of giant uni- and multilamellar vesicles can reach few micrometres. Nano-sized liposomes, like other nanoparticles, are characterized with better ability to penetrate deep into the intestinal mucosa and human skin in comparison with micron-length scale particles (Takeuchi et al. 2005; Immordino et al. 2006; Thirawong et al. 2008). The residence time of nanoparticles has been shown to be longer compared to larger microparticles; larger particles are more prone to peristaltic action and rapid transit through the colon. Nanoparticles have been shown to interact with epithelial cells and show increased retention enabling selective delivery of therapeutic agents into the colitis tissue (Xiao et al. 2013). Conventional formulations have so far not exploited this ability causing deposition in regional areas. Smaller particle sizes are able to take advantage of endocytosis for internalization into the epithelial cells of the colon. They attach to specialised epithelial M cells which are responsible for antigen transport from the lumen to the immune system. These M cells are also responsible for uptake of nanoparticles by transcytosis (Pichai and Ferguson 2012). Microparticles have been shown to adhere to the inflamed mucosal wall, whereas nanoparticles are absorbed across the epithelial barrier (Schmidt et al. 2013).

Application route and targeted cells should carefully be considered while designing liposomes for bacteriophages. Zeta potential of particles is regarded as an important determinant (more than size) for successful transfection through various surfaces (Koping-Hoggard et al. 2001; Huang et al. 2005). If the aim of drug delivery is longer blood-circulating nanoparticles, e.g. antitumour medications, then choice should be made in favour of neutral particles (Li and Huang 2008). Modification of liposome surface, such as by PEGylation, also reduces their uptake by mononuclear phagocyte system (MPS) conferring advantage of longer circulation in blood and decreased recognition by the liver and spleen (Immordino et al. 2006; Aggarwal et al. 2009). In case of phages targeting intracellular pathogens, the goal of application of phage liposomes is increased uptake by MPS.

However, contradictory results have been reported regarding pharmacokinetics of liposomes with different zeta potential. According to Campos-Martorell et al. (2016), liposomes with only negative and neutral charges are capable to pass the blood-brain barrier, while others present results in favour of positively charged particles (Cavaletti et al. 2009; Chen et al. 2016). Intestinal mucosa exhibits a negatively charged surface, while in inflamed epithelial tissue, positively charged proteins prevail, and therefore adhesion potential of differently charged particles can create some obstacles in terms of the delivery route (Hua et al. 2015). Additionally, in the GI tract, liposomes can have electrostatic interactions with various other substances, such as negatively charged soluble colonic mucins, which can also influence their retention in different parts of the GI tract. Although there is some contradictory evidence regarding the uptake by healthy and inflamed intestinal tissue of liposomes exhibiting different surface charges, several papers suggest that anionic

liposomes exhibit better penetration properties through inflamed mucosa compared with cationic ones (Jubeh et al. 2004; Thirawong et al. 2008; Beloqui et al. 2013; Ahn and Park 2016). Unlike cationic particles which get immobilized on the mucus, anionic particles may be able to diffuse through the mucus network due to decreased electrostatic interactions (Hua et al. 2015). Other studies suggest that particles are entrapped within the mucin mesh no matter what charge they carry since the mucus displays negatively and positively charged proteins. Hence, if a non-adherent approach is needed, the nanoparticle surface could be decorated with specific moieties that confer the so-called 'stealth' properties (Cu and Saltzman 2009). Incorporating poly(ethylene glycol) (PEG) on the surface of nanoparticles has been shown to result in a hydrophilic surface chemistry that allows unobstructed diffusion of nanoparticles through the epithelium. An explanation for this is that surface modification prevents strong interaction of nanoparticles with the mucus which typically prevents diffusion to the colitis tissue (Cu and Saltzman 2009; Hua et al. 2015). Electrostatics cannot be considered as the only factor engaged in attachment and uptake process of liposome by host target cells; size and lipid content of liposomes also play role in this process (Jubeh et al. 2004; Calvagno et al. 2007; Cu and Satzman 2009).

One of the first attempts to encapsulate phages in liposomes was published in 2014 by Balcao et al. (2014). Myovirus phi2/2 (later designated as PVP-SE1) (Santos et al. 2011) active against *Salmonella* Enteritidis strains was encapsulated into 'lipid nanoballoons' of 85–200 nm in diameter and zeta potential values of ca. −12 mV. In the study lyophilized phages were encapsulated into the aqueous core of lipid vesicles by applying multiple water-in-oil-in-water (W/O/W) emulsion strategy using a homogenizer. Size stability of phage-entrapped liposomes over a 3-month period was reported; however, proof-of-encapsulation efficacy and antibacterial activity of phage liposomes were not provided.

Nieth et al. (2015) investigated the possibility of phage encapsulation and their uptake by eukaryotic cells. The initial aim was to develop a method for the efficient encapsulation of mycobacteriophage TM4 to be further used for therapy targeting intracellular *M. tuberculosis* cells. TM4 phage is a siphovirus with a head of 50–58 nm in diameter and tail of 170 nm in length; λ phage has similar dimensions as TM4. Nieth et al. (2015) applied two different methods on a model of fluorescently labelled lambda λeyfp phage. The researchers showed that liposome particles up to 5 μm in size prepared using the thin film hydration method did not encapsulate the phage efficiently. Two other methods for liposome formation were used, gel-assisted rehydration (Weinberger et al. 2013) and inverse emulsion (Pautot et al. 2003), to produce giant unilamellar vesicles (GUV); however, encapsulation yield of phages was still poor.

Gel-assisted method used PVA (polyvinyl alcohol) as a support layer for the lipids gave several advantages, in particular, higher speed of particle formation and some evidence of phage encapsulation (Weinberger et al. 2013). Inverse emulsion allowed step-by-step formation of particles from the inside to outside and allowed controlling compositions of each layer by creating asymmetric particles (Pautot et al. 2003). Both methods have proven to be effective for encapsulation of various

chemical compounds. In the case of λeyfp phages, both methods showed poor efficiency of encapsulation although the inverse emulsion method was better. Both methods produced liposome particles that were quite large and not homogenous in size, ranging from 5 to 50 μm and 10 to 15 μm, respectively. The US Pharmacopeia recommendation for intravenous applications is for the particle size to be below 500 nm, and particles larger than 5 μm are considered potentially dangerous as they exceed the diameter of blood vessels (United States Pharmacopeia 2007). Reducing the size of the vesicles by extrusion failed due to the low concentration of the phage-encapsulated GUVs, and further work is needed to achieve reliable production of phage-containing liposomes in commercial quantities.

Nieth et al. (2015) also showed higher uptake of phage-formulated liposomes by human monocytic cell line THP-1 in comparison with free phages and their co-localization within endocytic compartments, which may serve as a good indication for the high probability of phages to reach target mycobacterial phagosomes by phage-loaded liposomes. Intracellular pathogenic bacteria such as *M. tuberculosis* and *L. monocytogenes* (Schnupf and Portnoy 2007) have developed different mechanisms to escape phagosomes and get access to the cytosol of macrophages. The intracellular fate of these pathogens normally depends on the mobilized immune response affected by both bacteria and host (Majlessi and Brosch 2015; Queval et al. 2017). Therefore, phage-containing liposomes targeting intracellular bacteria could potentially fuse with the eukaryotic cell membrane and subsequently release the phages directly into the cytosol.

Researchers from India conducted several studies (Singla et al. 2015, 2016a, b; Chadha et al. 2017; Chhibber et al. 2018) with phages formulated in liposomes (limited evidence was provided on actual encapsulation of phage in these studies). In these studies liposomes were prepared using thin-film hydration method which has been shown previously to yield very low numbers of encapsulated phage (Nieth et al. 2015). Nevertheless, studies were performed on phages with different morphologies; in particular, podovirus and myoviruses active against two pathogens *Klebsiella pneumoniae* and *S. aureus* were tested (Singla et al. 2015, 2016b; Chhibber et al. 2018). Various liposomal formulations were tested, and encapsulation efficiency of up to 92% was reported in the case of podovirus and 87% in the case of myoviruses (Singla et al. 2016b; Chhibber et al. 2018). The size of liposomes was around 120 nm for the T7-like podovirus (Singla et al. 2016b) and 200–230 nm for myoviruses with the following size range of 57–90 nm of head diameter and 75–120 nm of tail length (Chadha et al. 2017; Chhibber et al. 2018). Stability studies were performed for liposome-formulated myoviruses active against *S. aureus*. The liposomes were reported to be stable over a 9-week period at 4 °C (Chhibber et al. 2018).

In the above studies, the major purpose of encapsulation was to increase efficacy of phage therapy due to higher retention rates in situ and secure better delivery of phages into infection sites, such as lung, skin and soft tissue wounds. Experiments were performed in murine infection models. Applications of liposome-containing phage preparations were done intraperitoneally or locally. Both methods were reported to be effective in the eradication of infections.

Singla et al. (2015) reported higher therapeutic and prophylactic activity of liposomes loaded with phages (LP) in comparison with free phage preparations

when applied intraperitoneally against lung infections caused by *K. pneumoniae*. LP were found to be capable of preventing infections when applied 48 h prior to bacterial challenge, while for free phages (FP), this period was only 6 h. In the case of already established infections, advantage of LP over FP was reported: 3 days vs. 1 day. Liposome-formulated phages were reported to have a stronger influence on the immune response of the host in comparison with free phages by enhancing anti-inflammatory response and inhibiting production of pro-inflammatory cytokines. The mechanisms behind this effect although not completely clear could be due to the increased activity of encapsulated therapeutic agents and elevated uptake of cationic liposomes by cells (Basnet et al. 2012).

Based on in vitro and in vivo (animal) experiments, Singla et al. (2016a) showed that liposome-formulated *K. pneumoniae* podovirus was able to penetrate biofilms in an ex vivo model and synergistic effect in combination with amikacin (~95% bacteria killing) was noted. The study also reported high phage retention rates in situ, and resistance towards anti-phage mouse antibodies was noted.

A recent study (Chadha et al. 2017) evaluated the therapeutic efficacy of liposomes with an encapsulated phage cocktail consisting of five myoviruses active against *K. pneumoniae* in a murine burn wound model. A cocktail of phages in a liposome formulation (LCP) showed stronger retention and a higher efficacy in comparison with liposome-free cocktail of phages (CP). Animals treated with LCP did not suffer mortality, while in the CP-treated group, mortality reached 100% after 4 days (phages were applied 24 h postinfection).

In a different study (Chhibber et al. 2018), the efficacy of two encapsulated myoviruses active against *S. aureus* was tested in a diabetic excision wound model in mice. Liposome-formulated phages were applied locally and showed higher healing ability compared with free cocktail of phages (FCP). Wounds treated with LCP were shown to heal in 10 days versus 15 days when the FCP was applied. A 2-log increase in phage titre at the infection site was reported for LCP-treated wounds. Results suggested longer retention of LCP and faster healing.

Phage encapsulation in liposomes is also of interest for the treatment of gastrointestinal infections. An example of this is the protection of *Salmonella* phages from gastric acids and to prolong their intestinal residence time (Colom et al. 2015). Two podoviruses (60–66 nm of head size) and one myovirus (68 and 114 nm of head and tail size respectively) were formulated using cationic liposomes prepared using a thin-film hydration method (Colom et al. 2015). Positively charged particles may have several advantages: high mucoadhesiveness, better encapsulation of negatively charged phage particles and improved dispersion in aqueous media. A high yield of liposome-associated phages (around 50%) was reported, and the nanoparticles were in the size range 309–326 nm with a net positive charge of 31.6 and 35.1 mV (pH 6.1). In this study liposome-formulated phages were freeze-dried, and the proportion of phages that survived this treatment exceeded those that were not formulated in liposomes (free phages) by between 44 and 86%. Liposome-formulated phages were shown to have higher stability upon exposure to simulated gastric fluid (in vitro data) and prolonged efficacy against *Salmonella* infection in broiler chickens (in vivo data).

Drawbacks of the reviewed methods (discussed above) include low encapsulation efficacy, large size of liposomes and high percentage of phage inactivation during processing. Leung et al. (2018) tried using a microfluidic micromixing approach for liposome production. Two phages active against *P. aeruginosa*, each of them representing different morphological families, *Podoviridae* and *Myoviridae*, with sizes of ~65 and ~220 nm, respectively, were evaluated for nanoencapsulation in liposomes. Size ranges of liposome nanoparticles were 135–218 nm and 261–448 nm, for podo- and myovirus, respectively. Vesicles carried slightly positive charge (2–4 mV) on their surface. Reported encapsulation yield of 50–59% seems very high. No more than one phage was encapsulated per liposome and empty liposomes were also noted (observed using Cryo-TEM). Efficiency of phage release from the liposomes and their antibacterial activity was not evaluated.

Cinquerrui et al. (2018) also evaluated the use of a microfluidic based technique for the encapsulation of bacteriophages in liposomes. The liposome size was shown to be controllable by changing the formulation (different amounts of cholesterol added to stiffen the membrane bilayer) and regulating the hydrodynamic conditions in the microfluidic device. Liposomes having mean sizes between 100 and 300 nm were prepared. Encapsulation of two model phages was undertaken, an *E. coli* T3 podovirus (size ~65 nm) and a myovirus *S. aureus* phage K (capsid head ~80 nm and phage tail length ~200 nm). The yield of encapsulated T3 phages was 10^9 pfu/mL and for phage K was much lower at 10^5 pfu/mL. The encapsulation yield for *E. coli* T3 phage was affected by aggregation of T3 phage at high titres. *S. aureus* phage K was found to interact with the liposome lipid bilayer resulting in large numbers of phage particles bound to the outside of the formed liposomes instead of being trapped inside them. Inactivation of the liposome-bound *S. aureus* K phage was undertaken while retaining the activity of the encapsulated phage in order to estimate the yield of microfluidic encapsulation of large tailed phages. This work suggests that previous published studies on phage encapsulation in liposomes may have overestimated the yield of encapsulated tailed phages. Additionally, very few studies have shown convincing data on the separation of free, encapsulated, and liposome-associated phage particles.

The above studies show that in spite of the high therapeutic potential of phage liposome preparations, there are quite a number of factors that make encapsulation of phages into liposomes a challenging field worthy of further investigation. These factors include the following: relatively large size of phage particles (50–200 nm), orders of magnitude bigger than any encapsulated chemical drug; aggregation of phages at high titres makes encapsulation in small liposomes difficult; interaction of tailed phages with lipid bilayers is problematic; and influence of external physico-chemical factors, e.g. use of solvents, in liposome production negatively affects antibacterial activity of phages (Toh and Chiu 2013; Cinquerrui et al. 2018; Leung et al. 2018). Furthermore, liposome formulations would need to be shown to be stable during storage and to be prepared under sterile conditions for therapeutic use.

3.2.5 Encapsulation of Bacteriophages in Electrospun Fibres

Electrospun fibres have biomedical importance because of their high specific surface area, porosity, extreme flexibility and softness. This type of fibres can serve as

delivery vehicles for different bioactive agents, such as anticancer drugs, antibiotics or others. Electrospun fibres with encapsulated antimicrobials, including bacteriophages, allow sustained release of active components into environment and can be applied as wound dressings in medicine and bioactive packaging material in food production (Zhong et al. 2010). Nanofibres are also used as scaffolds in tissue repair and regeneration process (Mohamed and Xing 2012). Manufacturing of phage-encapsulated polymer fibres (diameters ranging from tens of nanometers to microns) may readily be achieved using electrospinning, by drawing a charged polymer-in-solvent solution onto a grounded electrode while evaporating the solvent.

Studies have shown the feasibility of encapsulating phages in nanofibres using the electrospinning technique, and different natural and synthetic polymers have been evaluated for encapsulating phages in fibres including cellulose diacetate (Korehei and Kadla 2014), polyethylene oxide (PEO) (Korehei and Kadla 2013, 2014), polyvinyl alcohol (Salalha et al. 2006; Kuhn and Zussman 2007) and polyvinyl pyrrolidone (Lee and Belcher 2004; Dai et al. 2014). Good phage encapsulation results have been reported using water-soluble or chloroform-soluble polymers (Korehei and Kadla 2013; Dai et al. 2014). Rapid dehydration during electrospinning was shown to damage phages. Use of buffer and incorporation of sugars, e.g. trehalose, were shown to improve protection of phage (Dai et al. 2014). Protection of phages in core-shell fibres using coaxial electrospinning technique was shown to be particularly promising (Korehei and Kadla 2013, 2014). Increasing polymer molecular weight or blending different polymers (e.g. cellulose acetate/PEO) was shown to result in changes in the phage release kinetics from electrospun fibres (Korehei and Kadla 2014). Korehei and Kadla (2014) varied the molecular weight (MW) of PEO (100–600 kDa) and blended hydrophilic PEO with the hydrophobic polymer cellulose diacetate (CDA). *E. coli* T4 phage encapsulated in fibres produced using different PEO/CDA showed slow release of phage depending on the PEO MW and the ratio of PEO/CDA. Higher PEO MW (results in increased polymer entanglements) and greater proportion of hydrophobic CDA (slower rate of polymer swelling and erosion) resulted in slower release of phage.

The main reported mechanism of phage release from encapsulated fibres is typically swelling of fibres upon exposure to solvent followed by polymer erosion accompanied by phage diffusion and release. Encapsulating phage in hydrophilic water-soluble polymers results in rapid phage release due to polymer dissolution, whereas use of hydrophobic polymers or blends may allow tailoring of phage release to ensure slower release over a prolonged period.

The polymer and solvent combination used for fibre production was found to be an important factor in the formation of nanofibres and retention of phage viability therein. Lee and Belcher (2004) spun fibres (diameters 100–200 nm) containing *E. coli* M13 phage which retained infectivity against bacterial host when resuspended in buffer. The phage-containing fibres were spun from an aqueous polyvinyl pyrrolidone buffered solution containing phage; the fibres were captured as a non-woven fibrous mat. Concentration of viable phage in the fibres was not reported. Salalha et al. (2006) and Kuhn and Zussman (2007) encapsulated T4, T7

and λ *E. coli* phage in electrospun nanofibres (diameter 250–400 nm) prepared from aqueous poly(vinyl alcohol) (PVA) solutions. Nanofibre-encapsulated phage was shown to retain viability and remained stable for 3 months stored at −20 and −55 °C and nearly so at 4 °C. Salalha et al. (2006) suggested that this method was a facile way of preserving phage. Immediately after electrospinning, a 2-log loss in phage viability was reported (T4 ~ 1%, T7 ~ 2% and λ ~ 1%). The losses in phage viability during electrospinning may be attributed to drying stresses due to rapid evaporation of solvent and drastic changes in osmotic pressure experienced by phage. Dai et al. (2014) also compared phage stabilization using electrospinning with freeze drying. Phage encapsulated in electrospun fibres (made from SM buffer with trehalose) was found to have similar titre compared with freeze-dried powder samples. Storage of phage T7 in electrospun fibres for 8 weeks (stored at 20 °C) resulted in a 3-log reduction in phage titres for samples prepared from SM buffer with/without trehalose.

3.2.6 Immobilization of Bacteriophages on Surfaces

Bioactive surfaces with immobilized phages is a relatively new technology with high potential to be used for detection, identification, capture and deactivation of target bacterial pathogens in medicine and food industry.

Biosensors with different sensing platforms, such as optical detection, surface plasmon resonance (SPR), mass-based techniques (quartz crystal microbalance), magnetoelastic biosensors or microcantilevers (Singh et al. 2009; Brovko et al. 2012; Wang et al. 2016; Malik et al. 2017), can be applied for rapid detection of pathogens in food production. In these systems, phage-based biosorbents are considered to carry several advantages over sensors with other biological materials (e.g. antibodies, enzymes, organelles) due to their high specificity, low cost and easiness of production process (Hosseinidoust et al. 2014). Most of the phage-based platforms for biosensors are designed to detect bacterial pathogens involved in food contamination, such as *Salmonella* typhimurium, *E. coli* and enterotoxin-producing *S. aureus*. Density of immobilized phages and their orientation are the main challenges in the development of highly sensitive phage biosorbents. Although phage immobilization on surfaces may readily be achieved through passive adsorption (Bennett et al. 1997; Balasubramanian et al. 2007), the process is inefficient with loss of phage activity due to poor orientation of phage tails needed to recognize and infect target bacteria. Therefore, a number of strategies have been developed to attach phages with their heads to various biosensor surfaces, including physisorption and chemical adsorption or combination of both.

Electrostatically driven physisorption of phages implies immobilization of negatively charged phage heads on positively charged surfaces. Minikh et al. (2010) immobilized T4 phages on a commercially available highly electropositive non-woven filter media based on alumina nanofibres. Detection threshold of pathogen in the mixed samples with other bacterial species was reported to be around 10^3 cfu/mL of *E. coli*. Cademartiri et al. (2010) modified the inherently anionic surface of silica from highly anionic to highly cationic with different chemical groups and observed dependence of phage attachment ability on surface charge, as

well as on presence of various chemical moieties on the modified surface, e.g. highly anionic carboxylate silica surface efficiently binds phages and retains their infectivity at high level. No significant difference was observed in adsorption density of phages with different morphology towards highly cationic silica saturated with amine groups, but infectivity of phages was affected. Diverse affinity of phage tails with silica can be explained by dissimilarities in charge or recognition receptors and needs further investigation. Anany et al. (2011) and Lone et al. (2016) utilized the electrostatic interaction between the anionic capsid head of *E. coli* O157:H7 and *Listeria monocytogenes* phage cocktails and polyvinylamine-treated cellulose membranes. Modification of cellulose membranes resulted in greater numbers of infective phage oriented correctly. Phage immobilized cellulose can serve as bioactive packaging material in different areas, including food packaging, e.g. ready-to-eat meat, ready-cut fruits and poultry (Lone et al. 2016).

Recently, Richter et al. (2016) applied an electrical potential to align *E. coli* phage T4 and drive them to the electrode surface resulting in head-down, tail-up orientation resulting in increased sensitivity of phage for bacteria and a low-detection limit for *E. coli* of 10^3 cfu/mL.

Techniques for the attachment of phage to gold substrates have been investigated to increase their diagnostic utility as part of quartz crystal microbalance (QCM), surface plasmon resonance (SPR) transduction-based diagnostic platforms (Singh et al. 2009) or direct detection as part of virus electrodes (Yang et al. 2006).

Tawil et al. (2013) and Choi et al. (2018) compared physisorption with chemical binding of phages to gold surface and observed obvious advantage of covalent binding. In both studies gold surface was modified with similar and well-established method of applying combination of catalysts 11-mercaptoundecanoic acid 11-MUA and 1-(3-dimethylaminopropyl)-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide EDC/NHS, which can couple carboxylic acid to amine groups to form amide bonds between phage and substrate. Detection platforms used in the studies were different. Tawil et al. (2013) developed their method for SPR to detect MRSA and observed a 1000-fold increase in the activity of covalently bound phages vs. directly adsorbed phages. The technique designed by Choi et al. (2018) was intended for ferromagnetoelastic biosensors with *Bacillus cereus* phage biosorbents. After modification, a fourfold enhancement in phage density to the gold surface was achieved, although infectivity was shown to be time dependent and quite low.

Chemical biotinylation of the phage capsid head has also been used for oriented immobilization of a *S.* Enteritidis phage on streptavidin-labelled magnetic beads (Sun et al. 2001). In another study, Tolba et al. (2010) used phage display technology to introduce affinity tags on the capsid head to immobilize *E. coli* phage on streptavidin-coated magnetic and cellulose beads, but genetically modified phage showed a decreased burst size and an increased latency period.

However, according to the patent by Boss and Lieberman (2006), streptavidin presents tetrameric protein and serves as a potential food source for bacteria; therefore surfaces coated with streptavidin lack specificity and can give unacceptable number of false-positive results. Authors of the patent used commercially available silica-based magnetic microspheres and modified the surface with a long-chain alkyl

amine diaminodipropylamine (DADPA) and silanized amine (3-APTMS). Phages were attached to the surface using two mechanisms: via unsymmetrical cyanine dye fluorophore (SYBR) configured to penetrate a phage head and bind to nucleic acid with DADPA and amine-coupling chemistry created by modifying the silica surface with 3-APTMS.

Handa et al. (2008) employed chemical vapour deposition to make a smooth aminosilane monolayer on a glass substrate. Subsequently, monolayers of covalently bound *Salmonella*-specific phage were attached to the aminosilane monolayer using sulfo-NHS and EDC chemistry (Handa et al. 2008). The phage infectivity was maintained at high levels, as tail receptors were fully accessible and functional due to extreme elasticity of the monolayer.

Cooper et al. (2015) used carbodiimide-oriented covalent attachment of a tailed *P. aeruginosa* bacteriophage to magnetized multiwalled carbon nanotubes. Preliminary results showed antimicrobial efficacy of bacteriophage-nanocomposite conjugates against *P. aeruginosa*.

Tawil et al. (2015) reported on the synthesis of colloidal phage-gold nanoparticle complexes for the detection of a single *S. aureus* bacterium in a mixture of *S. aureus* and *E. coli* using dark-field microscopy.

A recent patent (Applegate et al. 2009) describes a method of immobilization of reporter phages on corona-treated polymers (polyethylene) with the help of UV-light exposure polymerization. Such phage-embedded polymers may prove to be effective biosensors for food applications. Immobilized reporter phages cause the infected target cells to emit light or fluorescence, allowing detection of bacteria in a sample employing different methods, including colorimetry, fluorimetry and luminometry.

Wang et al. (2016) explored immobilization of T4 phage on polyhydroxyalkanoate (PHA) polymer surfaces. The material is food compatible and biodegradable. The surface of PHA was modified using oxygen plasma, and phages were attached through covalent binding. Plasma-treated polymers showed 2-log higher infectivity than others prepared with chemical modification methods, including NHS/EDC treatment.

Jabrane et al. (2008) explored the possibility of designing a hydrogen peroxide biosensor with gravure-printed phage papers. Bio-ink consisted of phage suspension mixed with carboxy methyl cellulose solution used as viscosity modifier. T4 phage used as a model system was printed on lightweight papers coated with clay and calcium carbonate. Papers only with the highest phage concentration of 10^7 pfu/mL in bio-ink showed antibacterial activity on bacterial lawns.

Different printing techniques including gravure printing, blade coating and piezoelectric inkjet printing have been evaluated for *E. coli* and *Salmonella* phages to be used in a paper dipstick detection platform (Griffiths et al. 2015; Anany et al. 2018). Piezoelectric inkjet printing on commercially available cationic ColorLok papers proved to be most efficient. Phage amplification assay based on classic culturing and q-PCR methods showed the detection limit for such systems was 10–50 cfu/mL in the sample material. However, not all tested phages survived the printing process, and the shelf life of the phage papers was not long (<1 month). The method proved to have high potential for rapid detection of food pathogens. In the future further optimization of bio-ink composition and the printing process is required.

The potential of phages for biosensing applications including low-cost medical diagnostics may require their coupling with novel materials (thin-film constructs, magnetic, metallic, polymer nanoparticles, quantum dots) (Peltomaa et al. 2016). Microbial biofilms on indwelling medical devices, such as urinary and central venous catheters, stents, implants and invasive health-monitoring devices, are responsible for a majority of medical device-associated infections and are considered as a significant healthcare problem. One anti-biofilm formation strategy includes the surface attachment of antimicrobial agents, including phages to polymer surfaces used in such devices. Pearson et al. (2013) and Urban and Elasri (2014) covalently attached lytic *E. coli* and *S. aureus* phages through reaction of primary amine groups on the capsid head with R-COOH surface groups (generated using microwave plasma in the presence of maleic anhydride) on polyethylene and polytetrafluoroethylene surfaces. After immobilization, both tested phages retained high infectivity against their host bacteria.

Dixon et al. (2014) studied the influence of a broad range of potential clinical interferents, including host extracellular polymeric substances (EPS), albumin, fibrinogen and human serum on covalently immobilized phages on aminosilane-coated glass surface. This type of surface can serve as a model substrate to study adsorption of biomolecules. Two different phages active against *S.* Typhimurium and *E. coli* showed variable stability in the tested environments, indicating phage dependency. Phage activity was negatively affected in the presence of EPS and blood serum.

Another recent patent focused on the controlled covalent attachment of bacteriophage to a hydrogel coating material for regulating biofilm development with examples focusing on biofilm reduction on urinary (Foley) catheters (Donlan et al. 2016). Phages active against various urinary pathogens, including *Proteus mirabilis*, *E. coli*, *P. aeruginosa* and *K. pneumoniae*, were successfully attached to the activated hydrogel coat of silicone catheters, which showed high ability of preventing biofilm formation in vitro and in vivo models. A similar approach was used against *Staphylococcus epidermidis* (Curtin and Donlan 2006).

An earlier patent application gives examples of phage immobilization on nylon sutures for wound healing purposes (Scott and Mattey 2008). A number of sequential patents and patent applications from the same group of authors (Mattey and Wilkinson 2013; Chadwick and Mattey 2012; Mattey and Chadwick 2016; Mattey and Bell 2017) describes a wide range of phage immobilization methods on various polymer (e.g. nylon, polyethylene, cellulose) surfaces, including either preactivation by corona discharge and permanganate oxidation or application of coupling agents such as carbodiimide or glutaraldehyde for amino and carboxyl surface groups containing polymers and pretreatment with vinylsulphonyethylene of cellulose and other hydroxyl-containing polymers. Such phage-functionalized polymers can find applications in different types of wound dressings, bandages, implants, plasters and sutures. When phages are immobilized on the surface of beads or microspheres, they can also be used in creams and gels either with new formulations or in products already existing on the market. Phages immobilized on less than 5-μm size micro-/nanospheres were applied systemically in animal models and showed high efficacy in eliminating blood infections. In a recent patent application (Mattey and Bell 2017),

investigators show that immobilized phages carry clinically relevant advantages compared with free phages, such as decreased production of pro-inflammatory cytokine (IL-1 alpha), increased survival in serum, reduced antibody response and prolonged persistence in the body. Researchers investigated immobilization of *Salmonella* phages on 100-nm nylon particles to facilitate killing of intracellular bacteria in mice macrophage cells (Mattey and Chadwick 2016). Decreased or complete elimination of intracellular *S.* Typhimurium was reported.

Nogueira et al. (2017) reported on covalent immobilization of phages on polycaprolactone (PCL) nanofibres (non-woven textile) via acid-amine reactions. The PCL textiles have biomedical importance because of high elasticity and low biodegradability. Head-to-tail orientation of phages active against *P. aeruginosa* attached to PCL showed an effective 6-log reduction of bacterial cells immediately following contact.

Immobilization of bacteriophages into nanofibres made from honey, polyvinyl alcohol and chitosan (HPAC) along with other products such as propolis (Pr) and bee venom (BV) has been reported (Sarhan and Azzazy 2017). Efficacy of these nanofibres was evaluated in vitro and in an in vivo wound model and compared with conventional treatment. The combination HPAC-BV with phages showed high antibacterial activity and wound-healing ability.

Immobilization of bacteriophages in stimuli-responsive surfaces where release is triggered in response to an external stimulus has been reported (Bean et al. 2014). A bilayer hydrogel was prepared where the lower layer was agarose with embedded lytic phage K active against *S. aureus*, while the second upper layer was formed by photo-cross-linkable hyaluronic acid methacrylate (Bean et al. 2014). Majority of *S. aureus* strains produce the enzyme hyaluronidase which would degrade the hyaluronic acid upper layer releasing phage K into the environment killing the bacterial cells.

A number of patents describe covalent immobilization of bacteriophage cocktails into biodegradable amino acid-derived polymers (polyesteramides) together with antibiotics, pain relievers and anti-inflammatory agents (Katsarava et al. 2006, 2016). The dressing is reported to contain enzymes (e.g. trypsin, collagenase, fibrinolysin) which help to hydrolyze the polymer thereby releasing the bioactive components into the wound. A wide variety of wound dressings can be formulated using these polymers including perforated/unperforated films, gels, powders and ointments. Polyester urea can be used for the manufacture of implantable medical devices too. Commercially available product such as PhagoBioDerm has been around for a long time and has been shown to be effective in the treatment of infected poorly healing wounds, including burns (Katsarava and Alavidze 2001; Markoishvili et al. 2002; Jikia et al. 2005).

4 Summary and Conclusions

Scalable cost-effective manufacturing processes for the commercial production of phage products as well as development of stable pharmaceutical formulations are needed to meet future industrial demand. There is a pressing need to develop solid dosage forms to precisely deliver high concentrations of phages targeting MDR

bacterial infections. Phage susceptibility to environmental stresses is an important limitation that needs careful consideration for the future success of phage therapeutics. Encapsulation processes may be low cost and simple such as spray drying or highly advanced such as those based on microfluidic fabrication of highly uniform microcapsules. Freeze drying of microencapsulated phages may allow improvement in product storage shelf life. Immobilization and encapsulation technologies have the potential to enable precise control over phage loading and production of highly uniform micro- and nanoparticles, in fibre format or immobilized on various surfaces. The automised and onsite production of synthetic phages, might overcome phage specificity issues.

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Phage Pharmacokinetics: Relationship with Administration Route

Shigenobu Matsuzaki and Jumpei Uchiyama

1 Introduction

Bacteriophages (phages) are viruses that specifically infect bacteria and are currently believed to be the most abundant living entity on earth (ca. 10^{31} particles) (Suttle 2005). They play many important biological roles in both marine and freshwater aquatic environments, including control of bacterial numbers, horizontal transfer of bacterial genes, and recycling of carbon and nitrogen materials in the earth's ecosystem (Suttle 2005).

However, phages exist not only in the external environment but also in the inner environments of human and animal bodies (Abeles and Pride 2014; Navarro and Muniesa 2017). The natural phages resident in the human body are thought to play a role in maintaining human health through control of bacterial numbers and features in the human body by bacteriolysis and lysogenization, respectively (Cardin et al. 2017).

The increase of antimicrobial resistance (AMR) in pathogenic bacteria has accelerated in recent years (The World Health Organization [WHO] 2014), leading to a requirement for alternatives and/or supplements for antibiotics. One of the methods with the greatest potential is phage therapy, in which the phages are used as a bacteria-killing agent (Matsuzaki et al. 2014a; Ofir and Sorek 2017; Górski et al. 2018b). Phage therapy is described in the action plans of some countries such as USA and Japan (2015 and 2016, respectively) as an alternative method to counter AMR.

Georgia, Poland, and Russia have a long history of using phage therapy against human bacterial infections (El-Shibiny and El-Sahhar 2017). In contrast, in the West, the reevaluation of phage therapy started only around 1980, concomitant with the escalation of the problem of AMR (Smith and Huggins 1982). Although phages are

S. Matsuzaki (✉)
Kochi Medical School, Kochi University, Nankoku, Kochi, Japan
e-mail: matuzaki@kochi-u.ac.jp

J. Uchiyama
School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan

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not yet approved in the West as therapeutic drugs for treatment of human bacterial infections, several clinical trials have been performed (Cooper et al. 2016), and the effectiveness of phage therapy in individual patients or small groups of patients has been reported in case reports (see below).

In phage therapy, the phages have traditionally been administered by topical (wound infection, otitis, eye infection), intravenous (septicemia), transnasal (lung infection), transrectal (prostatitis), transurinary (bladder, kidney), or oral (intestinal and systemic infections) routes (Alisky et al. 1998; Weber-Dabrowska et al. 2000). When they are administered by topical, intravenous, transnasal, and transurinary routes, phages can directly contact the target pathogens and destroy them. In contrast, when administered by oral or transrectal routes, the administered phages need to move from the gut to the blood and lymph through the epithelial cell layers, unless they are targeting an intestinal infection.

In this paper, we will describe recent reports of phage therapy carried out using several phage administration routes against human diseases (Table 1) and discuss the direct and indirect evidence of movement from the gut to blood by phages administered orally or transrectally.

Table 1 Administration routes in the recent human phage therapy

Year	Country	Administration route	Disease	Target bacteria	References
2016	USA	Topically (dripped, covered with gauze)	Toe ulcers	*S. aureus*	Fish et al. (2016)
2017	Georgia	Topically (spray, cream) and orally	Skin disorder	*S. aureus*	Zhvania et al. (2017)
2017	USA	Percutaneous drainage and intravenously	Systemic infection	*A. baumannii*	Schooley et al. (2017)
2017	Belgium	Intravenously	Systemic infection	*P. aeruginosa*	Jennes et al. (2017)
2018	USA	Topically (around the ulcer once)	Digital osteomyelitis	*S. aureus*	Fish et al. (2018)
2018	Georgia	Orally and via inhalation	Cystic fibrosis	*Ach. xylosoxidans*	Hoyle et al. (2018)
2018	Belgium, France, Switzerland	Topically (an alginate template)	Burn infection	*P. aeruginosa*	Jault et al. (2018)
2018	USA	Intravenously	Craniectomy site infection	*A. baumannii*	LaVergne et al. (2018)
2018	Poland	Intrarectally	Prostatitis	*E. faecalis*	Górski et al. (2018a)

2 Topical and Inhalation (Transnasal) Administration of Therapeutic Phages

Recently, several instances of using phage therapy against infections in human patients have been reported not only from Georgia, Poland, and Russia but also from the USA, Belgium, France, and Switzerland. Topical administration seems to be the most acceptable route, because the phages do not enter the blood directly. As discussed below, in Georgia, topical or inhalation administration of phages is often accompanied by simultaneous oral administration.

Fish et al. (2016), in the USA, reported six representative results of treatment with phage Sb-1, which has been used for many years at the Eliava Institute in Georgia, against intransigent diabetic toe ulcers infected with *Staphylococcus aureus*. The phages were dripped into the wound cavity, and the wound was covered with gauze that was soaked with 0.1–0.5 mL of the phage suspension. This treatment was given once a week, and most patients recovered within an average of 7 weeks. Fish et al. (2018) subsequently reported successful Sb-1 phage treatment of a patient with digital staphylococcal osteomyelitis in diabetic foot ulcers infected with *S. aureus*. The patient was injected with 0.7 mL (a total of 4.9 mL) of phages into the soft tissue around the ulcer once a week for 7 weeks and recovered.

Zhvania et al. (2017) in Georgia reported phage therapy in a young patient who developed Netherton syndrome, a rare congenital disease including a severe skin disorder that is associated with multidrug-resistant *S. aureus* infection. The group applied phage Sb-1 or Pyo bacteriophage, a cocktail including several *S. aureus* phages, to the patient's limbs using a spray and a cream that included around 10^7 phages. The patient also received the phages orally after neutralization of gastric acid. Therapeutic effects were detected by the 7th day of phage application.

Hoyle et al. (2018) in Georgia reported phage therapy in a patient with cystic fibrosis who was infected with multidrug-resistant *Achromobacter xylosoxidans*. Phage suspension ~10^8 (plaque-forming units, pfu), consisting of two different phages, was administered orally and via inhalation using a nebulizer twice daily for 20 days. This course was repeated four times at 1, 3, 6, and 12 months. The patient's symptoms improved significantly.

Jault et al. (2018) reported the results of the PhagoBurn project, which aimed to treat *Pseudomonas aeruginosa* and *Escherichia coli* infection of burn wounds using phages; they reported the results only against *P. aeruginosa* infections. Hospitals in Belgium, France, and Switzerland were involved in the project, which was the first randomized and controlled double-blind phase 1/2 trial of phage therapy. They used a cocktail of 12 natural lytic *P. aeruginosa* phages (PP1131; 1×10^6 pfu/mL), which were embedded in an alginate template that was directly applied to the wound. This treatment was compared with 1% sulfadiazine silver emulsion cream as standard care. The topical treatments were given daily for 7 days. The phage treatments did reduce the number of bacteria, although the rate of reduction in the number of *P. aeruginosa* in the wound was slower than that with standard care; however, the rate of adverse effects seemed to be lower for phage treatment than standard care. However, a verdict about the effectiveness of this treatment awaits the result of a subsequent trial with a higher number of phages (Breederveld 2018).

With respect to topical administration, Merabishvili et al. (2017) reported that the disinfectants used with topical administration of phages need to be selected carefully, because some disinfectants inactivate the therapeutic phages.

3 Intravenous Administration of Therapeutic Phages

In intravenous administration, phages are injected directly into the blood, so no translocation is required. However, this route has had more difficulty in achieving acceptance than other routes, although its safety has been demonstrated (Speck and Smithyman 2016).

Schooley et al. (2017), in the USA, presented a case report describing intravenous and percutaneous administration of phages to treat systemic infection with multidrug-resistant *Acinetobacter baumannii* in diabetic patients with necrotizing pancreatitis. They administered ϕPC (a cocktail of phages AC4, C1P12, C2P21, and C2P24) through percutaneous drainage catheters. From the 3rd day, in addition to percutaneous administration of ϕPC, ϕIV (a cocktail of phages AB-Navy1, AB-Navy4, ABNavy71, and AB-Navy97) was administered intravenously. After this treatment, the patient recovered from a coma. Because of the subsequent appearance of phage-resistant strains of *A. baumannii*, ϕIVB (a cocktail of phages ϕPCABNavy71 and ABTP3ϕ1) was used. This combination treatment was continued every 5–8 h for a total of 59 days. The patient recovered. Ninety days after cessation of phage therapy, the lytic activity of ϕIVB in the patient's plasma was measured. Even if a cocktail of 5×10^9 pfu was administered, the titer decreased rapidly to 1.8×10^4 pfu/mL, 4.4×10^3 pfu/mL, 3.3×10^2 pfu/mL, and 20 pfu/mL by 5, 30, 60, and 120 min, respectively, indicating that antibodies against the phage cocktail might be present in the plasma.

LaVergne et al. (2018), in the USA, also reported intravenous phage therapy in a patient with *A. baumannii* infection of a craniectomy site. Although, unfortunately, the patient died, this report included important suggestions for future effective phage therapy against different foci of infection caused by *A. baumannii*.

Jennes et al. (2017) in Belgium presented another case indicating that phages administered intravenously were effective against systemic infections caused by *P. aeruginosa* in a patient with acute kidney injury. This patient received 50-μL BFC1 cocktail, which included two *P. aeruginosa* phages (14/1 and PNM) and one *S. aureus* phage ISP (Merabishvili et al. 2009), administered as a daily 6-h intravenous infusion for 10 days, and the wound was irrigated with 50 mL of the cocktail every 8 h for 10 days. After treatment, the patient's blood cultures became *P. aeruginosa* negative, and they recovered function.

4 Oral and Intrarectal Administrations of Therapeutic Phages

When therapeutic phages are administered by topical and intravenous routes, they can directly interact with the targeted pathogens to destroy them. In contrast, for oral phage administration against systemic infections and intrarectal administration of

phages against prostatitis, the phages do not directly encounter their target bacteria but must transit from the gut to the bloodstream. Although the mechanisms of phage translocation are not understood enough, oral administration has been shown to be effective against systemic infections such as sepsis and meningitis (Slopek et al. 1987; Alisky et al. 1998; Weber-Dabrowska et al. 2000; Górski et al. 2006). Generally, when phages are given orally, they are administered after neutralization of stomach acid, because the low pH in the stomach may inactivate the administered phages (Alisky et al. 1998; Weber-Dabrowska et al. 2000). In fact, this process was shown to prompt the movement of the phages from stomach to intestine in an animal model (Międzybrodzki et al. 2017).

Weber-Dabrowska et al. (1987) reported about the pharmacodynamics of phages administered orally in humans. First, 20-mL phage liquid was administered orally to human volunteers 30 min after neutralization of gastric acid. The initial virus titers in sera were 2.1×10^9 pfu/mL, 1.5×10^9 pfu/mL, and 7.5×10^7 pfu/mL for *S. aureus* phages ϕ131 and 767/Fory and *P. aeruginosa* phage Ps/68, respectively. Five days after administration, the titers in sera had reduced to 1.7×10^7 pfu/mL, 1.2×10^7 pfu/mL, and 2.1×10^5 pfu/mL, respectively, showing that although the titers decreased by about 99%, these phages were kept at comparatively high titers. Orally administered phages targeting *Staphylococcus*, *Klebsiella*, *Escherichia*, *Pseudomonas*, and *Proteus* were detected in the blood of about 84% of patients (47/56). Interestingly, in some patients (9/26), phages detected in the blood seemed to be excreted in urine.

Transit of phages from the gut to blood has also been observed in both healthy and neutropenic mouse models (Geier et al. 1973; Watanabe et al. 2007; Matsuzaki et al. 2014b; Międzybrodzki et al. 2017), although the rate seems to be relatively low. Furthermore, Nishikawa et al. (2008) indicated the possibility that in a mouse model, phages administered intraperitoneally moved to the blood and several organs and then into urine.

Interestingly, Międzybrodzki et al. (2017) showed that translocation of phages might be largely dependent on phage and animal species. They used two phages, *E. coli* phage T4 and *S. aureus* phage A5/80, and two animals, a rat and a mouse. First, they observed that orally administered phages T4 and A5/80 could move from the stomach to small intestine and that this transit was increased by neutralization of gastric acid, as described above. Second, they also observed translocation of phage A5/80 into blood in the mouse, but this was considerably lower for T4 phage. Third, translocation was not detected for either phage in the rat. These results are consistent with reports that orally administered T4-related phages are present in the feces but not in the blood of human volunteers (Bruttin and Brüssow 2005; Denou et al. 2009).

One possible reason why the translocation of phage T4 was observed only at very low frequency in mice or at lower than detection limit in humans may be the presence on the T4 head of the immunoglobulin-like nonessential Hoc protein (Barr 2017). The T4 head adheres to the mucus layer via Hoc, leaving the tail tip free. Thus, T4 can adsorb to *E. coli* in the intestine to complete the infection cycle. This linkage of T4 with the mucus layer is a possible reason for the difficulty of phage T4 in transiting to the blood from the gut (Barr 2017; Międzybrodzki et al.

2017); if no mucus layer is present, T4 will translocate from the gut to the blood through transcytosis (see below).

Intrarectal administration of phages against human enterococcal prostatitis has also been shown to be effective (Letkiewicz et al. 2009, 2010; Górski et al. 2018a). In this case, the phages are also thought to transit from the rectum to the blood in the same manner as the above case.

5 Phages Resident in the Human Body

Phages are commonly present everywhere bacteria are present. The number of phages in the aqueous environment (10^6–10^8 particles/mL) has been reported to be about ten times the number of bacteria (average 10^6 bacteria/mL) living in the same location. Electron microscopic observation of thin section samples of concentrated phages (Bergh et al. 1989; Proctor and Fuhman 1990; Suttle 2005) suggests that about 20–40% of marine bacteria are killed by phages each day, indicating that phages play an important role in determining the number of bacteria in aquatic environments.

There are also vast numbers of bacteria and phages present in the human body, including the oral cavity, skin surface, nasopharynx, genitourinary tract, and intestinal tract (Navarro and Muniesa 2017). Recently, the microorganisms resident in the human body have been analyzed by metagenomics (direct sequencing of all the DNA included in a sample using next-generation sequencing), general PCR, and random cloning/sequencing. The microorganisms analyzed by next-generation sequencing include bacteria, viruses, fungi, and protozoa. Most of the viruses resident in the human body were demonstrated to be phages. These phages may be present as free lytic phages, free phages induced from lysogenic/pseudolysogenic bacteria, or phages in a lysogenic/pseudolysogenic state, in which their DNA is integrated into the host bacterial genome or present as an episome. Next-generation analyses frequently do not distinguish these. Barr (2017) reported that resident phages were present throughout the body including the blood.

5.1 Gut

The first phage was discovered by d'Herelle in 1917 in the stools of a patient recovering from dysentery caused by *Shiga* bacillus (d'Herelle, translated in 2007). He thought that the bacteriolytic activity of the phage was the cause of the disappearance of the pathogen from the stools of recovering patients. Whether the phages were a part of the microflora of the patient's intestine or were ingested with food remains unknown. However, this was not only the first report of a phage but also of the possible roles of a phage in controlling numbers of pathogenic bacteria and leading to the spontaneous cure of bacterial infections in human intestine.

The contents of the adult human intestine are estimated to weigh about 2 kg, including 10^{14} bacteria (Lepage et al. 2013; Mills et al. 2013). Based on earlier

reports, Dalmasso et al. (2014) estimated that there are ca. 10^{15} phages in the human large intestine. Most of the phages present in the intestine seem to be in the form of integrated or episomal phage DNA, because epifluorescence microscopy showed only 10^8–10^9 virus-like particles (VLP) per 1 g feces. The total number of free phages in the intestine was estimated to be ca. 2×10^{11}–10^{12} particles/2 kg feces; therefore, the ratio of free phage/bacteria was calculated to be ca. 0.01–0.001, which was very low compared with the aqueous environment described above.

Most of the phages resident in the intestine belonged to the order *Caudovirales* (Dalmasso et al. 2014), the tailed phages. The order *Caudovirales* consists of three families: *Myoviridae* (phages having an icosahedral head and a tail with a contractile sheath), *Siphoviridae* (phages having an icosahedral head with a flexible long tail), and *Podoviridae* (phages having an icosahedral head with a short tail) (Ackermann 2006), although the taxonomy is currently being modified by the International Committee on Taxonomy of Viruses (ICTV). Interestingly, these results are consistent with those of Ackermann, who reported that 96% of phages isolated from all origins belong to *Caudovirales* (Ackermann 2006).

Phages belonging to *Microviridae* (small cubic phages without a tail), which have a single-stranded DNA genome, have also been commonly observed in human feces (Kim et al. 2011; Dalmasso et al. 2014). A representative of this family is a famous phage, ϕX174 (Ackermann 2006). Filamentous phages belonging to the family *Inoviridae* have also been reported in the feces of healthy adults (Dalmasso et al. 2014).

Several reviews have shown the relationship of gut phages with human health (Mills et al. 2013; Dalmasso et al. 2014; Scarpellini et al. 2015; Manrique et al. 2016, 2017; Mahony et al. 2018). Interestingly, the presence of phages has also been associated with the effectiveness of fecal transplantation for treatment of *Clostridium difficile* infection in the intestine (Lucas López et al. 2017; Łusiak-Szelachowska et al. 2017; Zuo et al. 2018; Anonye 2018).

5.2 Oral Cavity

Edlund et al. (2015) demonstrated that phages are the most abundant members of the oral virome. He estimated that there are 6×10^9 bacteria and 2×10^{11} viruses in the oral cavity. Epifluorescence microscopy showed 10^8 VLPs per mL of saliva (Pride et al. 2012) and 10^7 VLPs per mg of dental plaque (Naidu et al. 2014).

Genetic analyses by next-generation sequencing showed that most of these VLPs were phages. Interestingly, the dynamics between *Streptococcus* spp., a major component of the oral cavity microflora, and its phages showed a Lotka–Volterra competition model-like oscillation (Edlund et al. 2015). This predator–prey relationship is widely observed in natural organisms.

5.3 Skin, Genitourinary Organs, and Nasopharynx

Specific bacterial flora is present on the skin surface, in genitourinary organs, and in the nasopharynx, although they are less abundant than those in the gut and oral cavity. However, as in other regions of the body, the most representative members of the virome are phages (Hannigan et al. 2015; Liu et al. 2015; Wang et al. 2016; Miller-Ensminger et al. 2018; Van Zyl et al. 2018).

5.4 Blood

The blood and lymph were believed to be sterile in humans. However, recent examinations of the virome in healthy and unwell humans have indicated the possible presence of phages in their blood (Gaidelyte et al. 2007; Dinakaran et al. 2014; Fancello et al. 2014; Barr 2017).

Breitbart and Rohwer (2005) examined the virome in the human blood by careful isolation of virus DNA purified by CsCl density gradient ultracentrifugation. Subsequent shotgun cloning and DNA sequencing demonstrated that sequences similar to those of *Clostridium perfringens* phage φ3626 (family *Siphoviridae*, temperate phage) (Zimmer et al. 2002), *Streptococcus pneumoniae* phage EJ-1 (family *Myoviridae*, temperate phage) (Díaz et al. 1992), *Methanobacterium* phage psiM2 (family *Siphoviridae*, temperate phage) (Pfister et al. 1998), and *Chlamydophila pneumoniae* phage φCPAR39 (family *Microviridae*, no integrase gene) (Sait et al. 2011) were present in the blood. Phages φ3626 and psiM2 infect members of the gut flora, and phages EJ-1 and φCPAR39 infect the bacteria present in the respiratory tract. These phages are presumed to be present in the blood as a result of movement from the mucous layers of the intestine or respiratory system as free phage and/or in a host-associated state.

Bogdanovic et al. (2016) compared the viruses present in the blood of children with acute lymphoblastic leukemia with those in healthy controls using next-generation sequencing of the blood DNA. They detected several phages that infected the genus *Propionibacterium*, members of which inhabited the skin. In the patients, a phage similar to PHL113M01 (*Siphoviridae*; Pa6virus, no integrase gene) (Farrar et al. 2007) was detected. In addition, phages similar to PHL111M01 (*Siphoviridae*, no integrase gene), PHL010M04 (*Siphoviridae*, no integrase gene), PHL071N05 (*Siphoviridae*, no integrase gene), PA6 (*Siphoviridae*, no integrase gene), and P1.1 (*Siphoviridae*, no integrase gene) were also detected. Because these phages lacked an integrase gene, they were thought to have moved from the skin or mucus as free phages.

Moustafa et al. (2017) suggested that although phages infecting many bacterial species were detected in the blood of more than 8000 healthy humans, most may have been contaminants acquired during DNA sequencing. However, they did not exclude the possibility of phage translocation.

In contrast, Li et al. (2012) reported that although no phages were found in the blood of healthy controls, many phages were detected in the blood of HIV/AIDS

patients, suggesting that an immunodeficient status prompted the movement of phages from specific loci in the human body to blood.

The above observations suggest strongly the possibility that the phages present on human surfaces body enter the bloodstream through epithelium cells.

6 Translocation Routes of Resident Phages

Several possible routes by which phages present on human body surfaces can migrate into blood have been proposed (Barr 2017): first, that the resident phages are able to migrate directly into blood through injured epithelial cell layers of each organ, and second, that phages are able to migrate into blood with their host bacteria. In this case, the phage DNA is integrated into bacterial DNA or is present as an episome. Lytic phages in the latent phase of their life cycle may also move to the blood by this route. The phages will be released from the host bacteria after their migration into blood, like the Trojan horse of Greek mythology (Barr 2017).

Recently, Nguyen et al. (2017) demonstrated a third route: migration into blood by transcytosis through epithelial cells. Using a quantitative in vitro system, they examined whether the applied phages could pass through artificially formed cell layers. The cell layers were constructed using cell lines derived from several organs: MDCK (dog kidney), T84 cells (human colon), CaCo2 cells (human colon), A549 cells (human lung), Huh7 cells (human liver), and hBMec cells (human brain). Phages T3 (*Podoviridae*), T4 (*Myoviridae*), T5 (*Siphoviridae*), T7 (*Podoviridae*), P22 (*Podoviridae*), SPO1 (*Myoviridae*), and SPP1 (*Siphoviridae*) were investigated. Phages T3, T4, T5, T7, and P22 infect *E. coli* and *Salmonella*, which are Gram-negative bacteria, while phages SPO1 and SPP1 infect Gram-positive *Bacillus subtilis*.

Phage T4 could pass through all these types of cell layers derived from the gut, brain, liver, kidney, and lung. The migration rate (migrated pfu/applied pfu) was around 0.1%. Morphological analyses showed that the mechanism was transcytosis with trafficking through the Golgi apparatus in the cell and that the preferred migration direction was apical to basal. Phage T4 was shown to pass through the epithelial layer in about 10 min. Phages T3, T5, T7, SPO1, SPP1, and P22 were also shown to pass through an MDCK cell layer.

This report suggested that phages, belonging to *Myoviridae*, *Siphoviridae*, and *Podoviridae*, are able to migrate into the blood through the epithelial cell layers of the gut, lung, kidney, liver, and brain, independent of the phage morphology. Furthermore, based on these results, the authors estimated that 3.1×10^{10} phage particles migrate from the gut into the body each day. Considering the above estimate of a total of ca. 2×10^{11}–10^{12} free VLP phages in the intestine, this implies that 1/7–1/70 of the free phages in the intestine migrate into the blood each day, if the mucous layer does not prevent phage migration.

Because the migration of phages through epithelial cells derived from the lung was also demonstrated, a portion of the phages present in the respiratory tract may migrate into the blood. This may also be a reason for phages being frequently detected in human blood.

These reports indicate that if orally or transrectally administrated phages reach the intestine, they can migrate from the gut to the blood via one of the above three routes, especially transcytosis. This means that they will encounter their target bacteria at the foci of disease in the deep part of the body, destroy them, and release many progeny phages. In fact, the phage translocation of phage from gut to blood has been reported (Górski et al. 2006).

7 Immunological Response Against Phages and Fate of the Phages

Phages moving into the blood can provoke several immunological responses (Krut and Bekeredjian-Ding 2018). First, they may induce production of antibodies against proteins required for virion construction, while antibodies alone against phage proteins that are associated with phage adsorption to bacterial receptors can inactivate the infectivity of phages (Żaczek et al. 2016). Generally, phage treatment may be completed before significant production of antiphage antibodies. However, if the treatment continues over several weeks, antiphage antibodies will be produced that can inactivate phages (Schooley et al. 2017).

Second, phages in the blood are eliminated by innate immune responses such as phagocytosis by polymorphonuclear cells, macrophages, and dendritic cells (Jończyk-Matysiak et al. 2017). Hodyra-Stefaniak et al. (2015) suggested a possible phage-inactivation mechanism in which a phagocyte stimulated by infection with the host bacteria inhibited the therapeutic phages. They mathematically simulated phage pharmacokinetics considering the interactions between four factors: phages, bacteria, innate immunity, and adaptive immunity. These interactions must be considered for successful phage therapy.

8 Immunomodulating Effect of Phages Moving into the Blood

Advantageous effects of phages in the blood have also been suggested (Górski et al. 2017, 2012). There are many reports showing immunomodulating effects of phages in the blood, including inhibition of inflammation caused by bacterial infections (Weber-Dabrowska et al. 2000). Recently, Van Belleghem et al. (2017) demonstrated that the immunomodulating effect of phage administration was regulated at the level of transcription of immunity-related genes in monocytes.

9 Conclusion

In Georgia, Poland, and Russia, therapeutic phages have, for many years, been successfully administered through topical, intravenous, oral, and transrectal routes. Recent clinical trials of applications of phage therapy around the world have also

shown it to be effective independent of the route of administration. Oral administration is especially valuable because of its simplicity and general versatility. The mechanisms of phage translocation into the blood and the immunomodulating effect of phage are being elucidated molecular-biologically and cytologically. Unfortunately, however, there is very little information on the phage dynamics in the human body. Further analyses of the pharmacodynamics of phages in current human trials will contribute to improving the success rate of phage therapy.

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Bacterial Resistance to Phage and Its Impact on Clinical Therapy

Shawna McCallin and Frank Oechslin

1 Introduction

The faltering of antibiotics is projected to cause an unprecedented public health crisis by 2050, with estimates that more than 10 million deaths will be due to resistant infections each year (O'Neil 2016). Since their discovery in 1928, antibiotics have revolutionized medicine, and the consequences of returning to a pre-antibiotic era are understandably a cause for alarm. The first antibiotic, penicillin, was discovered from a natural encounter between staphylococci and the mold *Penicillium*, with the latter releasing the antibiotic substance that killed the former (Ligon 2004). Initial efforts at purifying penicillin delayed its introduction into human medicine, and by the time of the publication of the first clinical applications in 1943, resistance to the antibiotic had already been documented (Rammelkamp and Maxon 1942; Abraham and Chain 1988). A. Fleming himself warned that misuse of antibiotics would encourage the development of resistant strains during his Nobel Prize speech in 1945. It would have been difficult to fathom at that time the degree to which antibiotic resistance has escalated to in our current-day situation.

The surest option to curtail the gravity of the antibiotic resistance crisis is to develop alternative treatment strategies, with one of the most obvious being phage therapy. The obviousness of phage therapy stems from the fact that it was discovered prior to antibiotics, has been used in clinical medicine in some countries for nearly a century, and has shown promise in in vivo animal studies and compassionate use cases (albeit with poor performance in structured clinical trials to date). While such considerations do not deter the development of other antimicrobial strategies, the

S. McCallin
Unit of Regenerative Medicine, Department of Musculoskeletal Medicine, Service of Plastic, Reconstructive, & Hand Surgery, University Hospital of Lausanne (CHUV), Lausanne, Switzerland

F. Oechslin (✉)
Département de biochimie, de microbiologie, et de bio-informatique, Faculté des sciences et de génie, Université Laval, Québec City, QC, Canada

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empirical evidence and historical experience with phage that already exists may contribute to a more rapid clinical implementation in order to respond to current medical needs. Much effort is ongoing to evaluate the therapeutic potential of phage therapy for the treatment of antibiotic-resistant infections, which in part requires evaluating the risk of bacterial resistance to phage and its impact on clinical outcomes.

Phages are natural environmental predators of bacteria, and, as it follows Darwinian theory of evolution, such pressure will result in the selection of resistant bacteria able to survive those conditions. Bacteria are exposed to both chemical (antibiotic) and viral (phage) environmental pressures in nature, and antibiotic resistance has been documented in isolated microbial ecosystems millions of years old (Pawlowski et al. 2016). Unlike static antibiotics, phages have the propensity to coevolve with their bacterial hosts. This coevolutionary mechanism between phage and bacteria is a perpetual arms race, with altering peaks in populations of phage or bacteria. The natural occurrence of this arms race is well documented for seasonal patterns of *Vibrio cholerae* and its phages in Bangladesh, where an environmental bacterial outgrowth is followed shortly after by a flourish of phage until a phage-resistant variant (PRV) appears (Faruque et al. 2005).

Alternating patterns of coevolution have also been repeatedly documented in vitro. Luria and Delbruck noted that phage lysis of bacterial populations was selected for PRVs to allow for bacterial regrowth (Luria and Delbruck 1943). The underlying genetic changes that give rise to coevolutionary events often involve recognition elements (receptor and tail fibers) and have also been documented for *Escherichia coli* and phage T3 (Perry et al. 2015), PP01 (Mizoguchi et al. 2003), or lambda phages (Spanakis and Horne 1987; Meyer et al. 2012). Depending on the nature of mutations in PRVs, phage may or may not have the ability to coevolve, and many studies have supported the occurrence of such evolutionary dead ends where coevolution does not occur (Mizoguchi et al. 2003; Oechslin et al. 2016).

Despite the wealth of studies detailing the development of phage resistance in vitro, it is not possible to extrapolate their conclusions to therapeutic application. Even within these studies, factors such as nutrient availability or growth conditions (shaking or static incubation, carbon sources) have been shown to influence the apparition of resistance (Schade et al. 1967; Bradley 1972; Barrangou et al. 2007; Filippov et al. 2011; Jackson et al. 2017; Koonin et al. 2017). This chapter aims at first presenting the concepts necessary for detecting and understanding phage resistance mechanisms and then at analyzing its documentation in therapeutic literature.

2 Detecting Resistance

The development of resistance during treatment entails that bacteria were sensitive to the phage upon initial exposure, and this initial sensitivity, as detailed later, is an essential condition for successful clinical applications. Several methods can be used to determine phage sensitivity or resistance, which indicate the extent of bacterial lysis and/or phage replication. The most widely used methods are the spot or drop

test, efficiency of plating (EOP), viable counts of phage and/or bacteria, and turbidity reduction as a visual indicator of bacterial lysis by phage, all methods which have been used since the time of phage discovery in the 1920s (Table 1). Alternatively, colorimetric indications of bacterial growth can also be used to determine phage sensitivity and are being revived in recent studies (Tengerdy et al. 1967; Estrella

Table 1 Standard methods used to determine phage sensitivity of bacterial strains

Method	Description	Materials	Advantages	Disadvantages
Drop test (spot test)	Drops of phage are placed onto a bacterial overlay and observed for lysis	Soft agar; agar; Petri dishes	Inexpensive; highly used	Qualitative, read by eye
EOPs	Phage titration on strains compared to a standard; expressed as percent	Soft agar; agar; Petri dishes	Inexpensive; more quantitative than drop tests	Requires more Petri dishes; time-consuming; human error in counting
Viable counts	Bacteria and phage are enumerated using standard techniques	Soft agar; agar; Petri dishes	Inexpensive; quantitative	Must stop infection dynamics for proper counting; time-consuming
Turbidity reduction	Bacterial growth in liquid culture; can be read by eye or plate reader	Spectrophotometer; tubes or 96-wells plates	High throughput	Expensive infrastructure for plate reader; influenced by growth conditions
Metabolic activity	Bacterial growth in liquid culture; can be read by eye or plate reader	Plate reader; tubes or 96-well plates	Easy to interpret; high throughput	Expensive infrastructure for plate reader; influenced by growth conditions
In silico prediction[a]	Genomic sequences of patient isolate and phages are compared for matches	Genomic sequencer; extracted DNA; sequenced phages; computing capacity	Intelligent design; high throughput	Not yet developed; computational burden; repetitive in the case of resistance
Potassium release[a]	Electric signals generated by potassium levels are detected to indicate phage infectivity and lysis	Microchips; software; plate reader	Fast	Not yet developed; not able to detect resistance

[a]These methods are currently under development and have not yet been used for phage therapy applications

et al. 2016). Specifically, the method entails measuring the reduction of tetrazolium salts as an indicator of the metabolic activity of bacteria. Some methods, such as turbidity reduction and colorimetric tests, have been automatized by the use of plate readers, which increase the throughput of screening and render the inference of phage sensitivity more quantitative than visual observation.

These same tests are used both to select phages to be combined into a product or be used for treatment and then to monitor the sensitivity of a bacterial isolate over time during the course of treatment. Ultimately, it is desirable that the method requires little infrastructure, is easily interpretable, and produces consistent results. Each of the different methods mentioned provides certain advantages and constraints in terms of time, material, and expense, which are detailed for each method in Table 1. It is notable that current phage sensitivity methods are conducted in rich growth media, which may, or may not, reflect the real propensity to develop resistance during treatment. Further methods are available to characterize more detailed growth parameters of bacteria-phage interactions, such as one-step growth or burst size estimations, but they are not performed on a routine basis even for phage characterization and certainly not for monitoring resistance in therapy (Kropinski 2018).

The development of a reliable system for phage sensitivity testing is an area of ongoing innovation, where researchers are trying to develop faster and more easily interpreted methods. One area of future diagnostics is the use of in silico predictions of bacteria-phage interactions with genomic data, where the genome of patient isolates is sequenced and screened against an existing phage genomic database to identify matches (Leite et al. 2018). The detection of intracellular components from host bacteria released due to phage lysis, such as potassium, is another method that could be measured, as by changes in electrical current. Methods that are currently used usually follow the course of phage infection for 16–24 h or more in order to determine phage sensitivity or resistance: many strains that show initial sensitivity to the phage may develop resistance after hours of incubation. While this lag time could delay treatment for urgent infections, it is difficult to develop a method that would balance the need for speed with the physiological processes of bacteria and phage resistance.

3 Mechanisms of Bacterial Phage Resistance: Mutations, Evolution, and Costs

Current knowledge on the mechanisms of phage resistance comes primarily from laboratory studies. Bacteria can resist phages mainly through the following mechanisms: (1) spontaneous mutations or phase variation of surface receptors, therefore preventing adsorption of phages to their bacterial host, (2) specific cleavage of incoming phage DNA by bacterial restriction-modification systems (RMS), or (3) by bacterial adaptive immunity such as the CRISPR-Cas system (clustered regularly interspaced short palindromic repeats loci, coupled to CRISPR-associated genes) (Labrie et al. 2010). The potential impact that these mechanisms may have on

therapy depends on the time to development of resistance and its specificity to certain phages, the toll resistance takes on bacterial fitness, and the ability of the phage to counteract resistance.

3.1 Mutations

Spontaneous mutations of surface molecules can prevent phages from adsorbing and subsequently injecting their DNA into their host bacterium. It is also the most frequent mechanism driving both resistance to phage and phage-bacteria coevolution (Koskella and Brockhurst 2014). Indeed, the first step of viral infection is adsorption of the viral particle via a lock-key mechanism between the receptors present on the phage tail fibers that interact with receptors present on the bacterial surface. Completion of the phage life cycle results in the killing and lysis of the host bacterium, resulting in a selection pressure that evolves both phage to increased infectivity and bacteria to phage resistance (Buckling and Rainey 2002). Spontaneous mutation of bacterial surface components that act as phage receptors, such as lipopolysaccharides (LPS), outer membrane proteins, cell-wall teichoic acids (WTA), capsules, and other bacterial appendices, such as flagella, many of which may also be virulence factors (e.g., LPS), can result in phage resistance (Bertozzi et al. 2016). The ultimate effects of these mutations are dependent upon the function of the structure to the host and the extent of the modification.

Phage resistance acquired through mutation of surface LPS has been observed in multiple bacterial species. In *E. coli*, PRVs were observed to have altered LPS composition after phage PP01 infection with reduced production of high- and low-mass LPS (Filippov et al. 2011). Resistance conferred by LPS modification was also observed for *Pseudomonas aeruginosa* (Le et al. 2014; Oechslin et al. 2016). The loss of the O-antigen that is required for phage adsorption was observed to be due to large chromosomal deletions encompassing the *galU* gene, which is involved in LPS synthesis. In addition, Filippov et al. used site-directed mutagenesis to demonstrate that *Yersinia pestis* can resist phage through alteration of different parts of the LPS (Filippov et al. 2011). LPS surface alteration was also observed to be phase variable in *V. cholerae* and used by the bacteria to escape O1 antigen-specific phages in nature (Seed et al. 2012). In this case, resistance was the result of single-nucleotide deletions in two genes critical for O1 antigenic variation.

Outer membrane proteins have also been observed to be involved in bacterial resistance toward phage, for example, in the case of the *E. coli* OmpC protein. It was observed that during the interaction between *E. coli* O157:H7 and bacteriophage PP01, OmpC silencing, in addition to LPS alteration, enabled the bacteria to escape phage infection (Mizoguchi et al. 2003). Similar observations were reported for *V. cholerae*-resistant variants that had decreased expression of the membrane protein OmpU (Seed et al. 2014).

During gram-positive bacterial infection, phages often use teichoic acids in order to adsorb on the bacterial surface to initiate infection. This is, for example, the case for phages that use the glucosylated teichoic acids of *Bacillus subtilis* or the *N*-

acetylglucosamine (GlcNAc) side chains of *Staphylococcus aureus* (Tipper et al. 1965; Young 1967; Yasbin et al. 1976). Spontaneous mutants of *Bacillus anthracis* to phage AP50c repeatedly had altered forms of the cell-anchoring protein, CsaB, which, while not the receptor protein itself, is thought to be responsible for linking receptor proteins to the cell surface (Bishop-Lilly et al. 2012). Phages of *S. aureus* largely target structures within the WTA on the host surface, and both the inactivation of the TagO protein that is responsible for WTA biosynthesis and altered glycosylation patterns have resulted in phage resistance in vitro (Xia et al. 2011; Chang et al. 2015; Uchiyama et al. 2017). A role of cell-wall components for phage adsorption has also been determined for *Enterococcus faecalis* and phage NPV1, where phage resistance is conferred by mutations in the *epa* gene cluster that is responsible for rhamnose cell-wall polysaccharides (Ho et al. 2018). Much of the information on resistance to these phages comes from mutational studies to identify host receptors rather than arising through infection dynamics, which is likely due to the importance of WTA structures to host viability, although in vitro resistance has been detected (Bishop-Lilly et al. 2012; Estrella et al. 2016; Jo et al. 2016).

Capsular polysaccharides can be involved in both receptor function and adsorption prevention. The capsular layer can act as an important mechanism of defense against phages, such as for the K1-capsule in *E. coli* that physically blocks recognition of LPS by phage T7 (Scholl et al. 2005). However, the capsular layer can conversely also act as a phage receptor, like in the case of phage K1–K5 that can recognize K1 and K5 antigens (Scholl et al. 2001). In addition, capsules are important virulence factors that help pathogenic bacteria to evade or counteract host defense during the infection (Jann and Jann 1992).

Other structures, like bacterial flagella or pili, can act as phage receptors. The flagellum can act as a primary receptor that helps the phage to adsorb to its secondary receptor located on the surface of the bacteria. Adsorption to the flagellum is generally reversible and helps the phage to move alongside its base where a second adsorption event takes place (Schade et al. 1967; Guerrero-Ferreira et al. 2011). In a similar way, the pilus can also be used as primary receptors, and its contraction is believed to bring the phages closer to the bacterial envelope (Bradley 1972).

In addition to spontaneous mutations of bacterial surface proteins, other bacterial resistance mechanisms are known to target virtually all infection steps of the phage life cycle. Innate (non-specific) resistance mechanisms, such as abortive infection or restriction-modification systems, are examples of such downstream mechanisms (Labrie et al. 2010). However, none of these systems have the ability to react to or evolve resistance to phage during the course of an infection and thus are less important in the context of resistance acquisition during phage therapy. Their implication could be confined to impact the host ranges of the phage rather the time course of resistance development, therefore making phage sensitivity testing an important prerequisite for phage therapy.

Adaptive systems such as the CRISPR-Cas system could, however, have a larger impact in the development of resistance during therapy. This system was reported in 45% of bacterial genomes and can cleave incoming phage DNA to provide adaptive resistance during infection (Barrangou et al. 2007; Koonin et al. 2017). Foreign

nucleic sequences, known as spacers, will first be integrated into the CRISPR locus and then will further serve as a guide for Cas-nuclease cleavage of subsequent foreign DNA that matches the spacer sequence. Spacers that are incorporated in the host genome define the specificity of the immune response of the host and its progeny (Jackson et al. 2017). It should be emphasized that a large number of anti-infective mechanisms are still to be discovered, as recently reported by Doron et al. (2018).

Deletion of bacterial surface receptors usually results in total resistance toward the phage or at least partial resistance in the case of primary receptor mutation and/or production of extracellular matrix or capsules that results in interference with phage adsorption (Labrie et al. 2010). For these reasons, the loss of the phage receptor can lead to an evolutionary dead end if the phage does not have the possibility to develop a counter resistance during the process of infection (reviewed in Dennehy (2012)). However, experiments using continuous chemostat culture have demonstrated the possible coexistence of phages with their respective PRVs, like for the case of *E. coli* O157:H7 and phage PP01 (Mizoguchi et al. 2003). In this specific case, phage resistance was observed to be associated with two resistant variant populations having an alteration in their LPS structure or decreased expression of their OmpC surface protein in addition to mucoid-type colonies. In parallel, phages were observed to evolve different host ranges for bacterial PRVs, which suggest multiple coevolutionary cycles, permitting parallel phage and bacterial expansion and mutual counterselections.

Antagonistic coevolution was also observed during many bacterial generations using *Pseudomonas fluorescens* and phage SBW25φ2. In this case, the bacterial host became resistant to a wider range of phage genotypes as phages infected a wider range of host genotypes, producing reciprocal increases in host resistance and phage infectivity (Buckling and Rainey 2002; Hall et al. 2011a, b). In addition, such an arms race was also observed to become weaker with subsequent generations due to the fitness costs associated with generalist adaptive mutations (Hall et al. 2011a, b). Indeed, a side effect of phage resistance is the fitness cost that may be associated with the specific mutation conferring resistance. For example, in *E. coli* and lambda phage, infection that will select for bacteria with reduced LamB porin expression also alters maltose uptake and can be detrimental in a maltose poor media (Spanakis and Horne 1987).

Resistance derived from de novo mutations that result in modification of the target phage surface receptors and prevents its adsorption also usually results in phenotypic effects. For example, PRVs resulting in loss or modification of their LPS structure can lead to so-called rough phenotypes (Kim et al. 2014); resistance through defective pili results in bacteria with altered twitching motility phenotype (Oechslin et al. 2016); production of alginate can result in PRVs having a mucoid aspect (Scanlan and Buckling 2012); production of capsular polysaccharides promotes aggregation at the bottom of the culture tubes (Capparelli et al. 2010); and phage resistance in *P. aeruginosa* is often associated with melanized phenotypes (Le et al. 2014; Oechslin et al. 2016).

However, the phage receptors that are present on the bacterial surface often act as virulence factors. For this reason, strains with receptor modification will be resistant to phage, but may also exhibit reduced virulence as discussed in the following chapter (León and Bastías 2015; Oechslin 2018).

4 Resistance Development During Therapeutic Application

4.1 Animal Studies

The need for alternatives to antibiotics extends beyond human medicine to animals and livestock, which have been recently targeted for their overuse of antibiotics and thus contribution to the resistance crisis (Martin et al. 2015). Most of the work done in phage therapy for animals deals primarily with gastrointestinal infections, as well as the control of pathogens rather than therapeutic treatment. Field studies or preclinical experiments in animals have the morbid advantage of being able to sacrifice study animals and deeply explore bacteria-phage interactions within the body at specific anatomical sites, which are obviously not fathomable for clinical applications. These studies therefore offer more investigation into the development of phage resistance than what can be learned through therapeutic application. The field and in vivo studies discussed here do not fully cover the vast literature on animal models conducted in controlled environments, but the several studies included here have yielded pertinent information on the development of phage resistance in vivo (also reviewed in Oechslin (2018)).

4.1.1 Livestock Gut Decolonization

Many well-documented studies on phage therapy and the emergence of resistance started with the control of gut-colonizing pathogenic bacteria in livestock animals including cattle, pigs, and poultry. This was the case with a series of studies done by Smith and Huggins on oral phage administration to prevent *E. coli*-induced diarrhea in colostrum-fed calves (Smith and Huggins 1983). Phage therapy could prevent diarrhea when given 8 h after bacterial inoculation, although it was able to resolve intestinal symptoms in only half of the animals when administered at the onset of the diarrheal symptoms. Interestingly, resistant bacteria were recovered from the small intestine in the case of calves failing to show a clinical response to phage application; yet the resistant strains did not cause diarrhea when reinoculated to healthy colostrum-fed calves. The decreased virulence was explained by the loss of the K-antigen, which is a known virulence factor for enteropathogenic strains and can act at the same time as a phage receptor (Taylor and Roberts 2005). Of note, similar results for the treatment and prevention of *E. coli* diarrhea in calves were described in a second study by Smith et al. (1987). However, K-positive resistant variants were also isolated in addition to K-negative variants and were observed to be as virulent as the parent strain.

Phage therapy has also been used to control *Salmonella* spp. and *Campylobacter* spp. gut colonization and infection in poultry (Sklar and Joerger 2001). It was able to

only reduce, but not eliminate, bacterial colonization in the case of *Salmonella enterica*. Treatment failure was not only attributed to phage resistance observed posttreatment, but also possibly linked to other factors, including the intracellular lifestyle of the bacteria. Similar observations were made by Atterbury et al., who observed a benefit by increasing the phage titer of the preparations without, however, achieving bacterial eradication (Atterbury et al. 2007). Interestingly, a positive correlation was observed between phage concentration and the emergence of phage resistance, with higher resistance rates following application of higher phage titers. In addition, PRVs were still able to colonize the gut. Similar observations were also reported by Carvalho et al., including a quick reversion to the original sensitive phenotype after resistance appeared, which could possibly explain why resistant variants can still colonize the gut (Carvalho et al. 2010). Similar conclusions were also reported for the treatment of *Campylobacter jejuni*, for which the observed decrease in bacterial load was dependent on the amount and time of phage administration (Loc Carrillo et al. 2005). As for *S. enterica*, resistance reversion was observed due to phase variation by inversion of a large genomic sequence that restored gut colonization capability (Scott et al. 2007). Phase variation was also observed to be associated with capsular polysaccharide production during *C. jejuni* and phage F336 application, although resistance was not associated with decreased gut colonization capabilities (Sørensen et al. 2012).

Taken together, these studies raise important questions about the selection of phage resistance in the intestine and its possible implication for phage therapy. Indeed, the complexity of the intestinal environment and its physiochemical conditions, including viscosity, the concentration dependency of phage resistance development, and phenotypic reversion, must be considered for future phage therapy applications.

4.1.2 Experimental Therapy in the Intestine

The efficacy of phage therapy and the emergence of resistance have also been investigated in different mouse models of intestinal colonization, which have produced interesting findings in terms of phage-bacterium dynamics in the gut. In a first study employing a 21-day oral administration of a cocktail composed of three different bacteriophages to mice colonized with enteroaggregative *E. coli*, the bacterial titer was not observed to decrease even though phage amplification was observed over the course of the experiment (Maura et al. 2012). Interestingly, bacteria recovered on day 21 were still susceptible to the phages present in the cocktail. Another study using phage T4 oral administration during a long-term period of 240 days reported that phage-resistant bacteria emerged after only 92 days and constituted 100% of the isolated colonies. In addition, PRVs were observed to persist over the 240 days of the experiment even when phage therapy was stopped after 92 days. In a very interesting study done by Duerkop et al. using germ-free mice colonized with *E. faecalis* V583, phage therapy was observed to decrease the fecal bacterial load after 24 h by threefold, and the level of colonization remained stable after 48 h (Duerkop et al. 2016). Phage resistance was observed to increase during the time of phage therapy: while 15% of the colonies were

susceptible after 24 h, 100% were resistant after 48 h. After sequencing the PRVs, resistance was revealed to be associated with multiple mutations in the integral membrane protein PIPef that promotes phage infection. In an attempt to prevent intestinal colonization or cholera-like diarrhea in infant mice and rabbit models, Yen et al. used a phage cocktail composed of three different phages (Yen et al. 2017). Oral administration up to 24 h before cholera infection reduced intestinal colonization and prevented cholera-like diarrhea even though PRVs could be observed. Resistance was associated with mutations in the O-antigen gene and outer membrane protein OmpU, although none of the isolates were resistant to all three phages.

4.1.3 Acute Infections

Besides models dealing with the gastrointestinal tract, several studies have also evaluated phage therapy in several models of acute infection. In an early study on phage therapy done by Smith and Huggins using a mouse model of meningitis, mortality was significantly lower when administering phage treatment 16 h after infection rather than antibiotics (Smith and Huggins 1982). Importantly, although no colonies isolated from mice brain were observed to be antibiotic resistant, PRVs were observed in 5 out of the 36 mice tested. Phage variants were K1-antigen negative, which suggests decreased infectivity, as described before (Smith and Huggins 1983).

In a study done by Pouillot et al., a model of murine neonatal sepsis was used to evaluate phage subcutaneous injection after rat pups were intraperitoneally infected with the virulent *E. coli* O25b:H4-ST131 strain (Pouillot et al. 2012). Interestingly, phage resistance was observed when the treatment was delayed 24 h post-infection, although their virulence was reduced in a sepsis model. In a model mouse liver abscess, Hung et al. did not observe the emergence of phage resistance after single-dose administration that could efficiently protect mice in a dose-dependent manner (Hung et al. 2011). Of note, PRVs could be selected in vitro during time-kill curve experiments, but their virulence was significantly attenuated in vivo.

Similar observations were done by Oechslin et al., where the efficacy of an antipseudomonal phage cocktail was evaluated in a model of rat endocarditis (Oechslin et al. 2016). Indeed, bacterial regrowth due to phage resistance could be observed after 24 h in vitro due to the selection of PRVs having acquired mutations either in the *galU* gene coding for LPS synthesis or in the PilT ATPase involved in pilus retraction. Interestingly, both resistant variants were less able to infect sterile rat valves, indicating that phage resistance comes at a high fitness cost. PRVs were not observed in vivo either before or after phage therapy treatment, which decreased the bacterial load by 2.3–3 log colony-forming units (CFU) depending on the mode of phage administration. Finally, the emergence of PRVs in vitro with reduced virulence that were not observed in vivo during phage treatment using two different phages (PPpW-3 and PPpW-4) was also confirmed with ayu fish orally infected by *Pseudomonas plecoglossicida* (Park et al. 2000). PRVs selected in vitro were less virulent when injected intramuscularly in the fish. Moreover, bacteria could be eliminated in fish receiving phage therapy, and the isolates recovered from control fishes were still susceptible to the two phages used in the experimental treatment.

4.2 Clinical Therapy

The first administration of bacteriophage for the treatment of a bacterial infection in humans dates back to 1917 (d'Hérelle 1917, 1931). The use of phage became more widespread at this time, prior to the introduction of antibiotics, after which point it was further developed by the Soviets, where it is practiced to this current day in countries of the former Soviet Union (Parfitt 2005; Summers 2012; Kutateladze 2015). The documentation of early phage therapy investigations is intermittent, with difficulties in original source and language availability. However, the ability of bacteria to develop resistance to phage and the importance of phage sensitivity to treatment outcomes have been documented since the 1930s (Eaton and Bayne-Jones 1934). Despite the wealth of historical literature, this chapter focuses on recent experiences with clinical phage therapy since 2000.

Phage therapy has experienced a revival of sorts due the increasing resistance to antibiotics, with a surge in activity over the past several years. However, no phage products have yet received a marketing authorization in Western countries to permit their use in clinical medicine, and only three formal clinical trials have been completed, although several phase ll studies have been announced for planned start dates in 2019. This limits the current use of phage therapy beyond countries where it has been historically approved, therefore causing a scarcity in available data on phage or the development of phage resistance in human medicine. However, phage therapy is increasingly being used as compassionate means to experimentally treat patients with antibiotic-resistant infections, particularly in Poland, Belgium, France, Australia, and the United States (Leszczynski et al. 2006; Letkiewicz et al. 2010; Khawaldeh et al. 2011; Jennes et al. 2017; Schooley et al. 2017; Lyon et al. 2018). These reports do little to contribute to a greater understanding of efficacy, but occasionally provide more details on each treatment than clinical trials, such as the need for phage modification due the apparition of PRVs or reverting antibiotic sensitivity.

4.2.1 Resistance Detected in Modern Phase ll Clinical Trials

Three modern clinical trials have been completed for phage products since 2009, covering burn wound and chronic otitis infections of *P. aeruginosa* and *E. coli* diarrhea (Table 2; Wright et al. 2009; Sarker et al. 2016; Jault et al. 2019). In formal trials, the product composition, application and dosage regimens, and analyses, such as phage sensitivity testing, are predetermined as part of the clinical trial protocol prior to patient enrollment. Two of the three studies did not include phage sensitivity testing as an enrollment criterion, therefore making it difficult to ascertain if phage resistance of patient isolates, when detected, was present prior to or developed as a result of phage administration. However, microbiological analysis of bacterial isolates after phage administration revealed clinical insensitivity to phage in some cases, which both supports the importance of sensitivity testing a priori and hints at some limitations of employing fixed-composition phage products designed to maximize host range.

Table 2 Summary of published clinical reports of phage therapy with relevant investigations into the development of phage resistance

Study	Pathogen	Phage product	Sensitivity testing		PRV analysis	References
			Pre-	Post-		
RCT, phase ll	*P. aeruginosa*	Fixed 6 phages	Yes	No	No	Wright et al. (2009)
RCT, phase ll	*E. coli*	Fixed 10 phages	No	Yes	No	Sarker et al. (2016)
RCT, phase ll	*P. aeruginosa*	Fixed 12 phages	No	Yes	No	Jault et al. (2019)
Summary reports	*S. aureus*, *E. coli*, *E. faecalis*, *P. aeruginosa*	Personalized	Yes	Yes, partial	na	Międzybrodzki et al. (2012), Górski et al. (2016)
Pilot study	*E. coli*, *Enterococcus*, *Streptococcus*, *P. aeruginosa*, *Staphylococcus*, *Proteus*	Commercial Pyobacteriophage	Yes	No	No	Ujmajuridze et al. (2018)
Case report	*P. aeruginosa*	Personalized 6 phages	Yes	Yes	na	Khawaldeh et al. (2011)
Case report	*P. aeruginosa*	Personalized 2 phages	Yes	Possibly	No	Duplessis et al. (2018)
Case report	*S. aureus*	Commercial staphylococcal monophage, Fersis, Pyobacteriophage	Yes	Yes	No	Zhvania et al. (2017)
Case report	*A. baumannii*	Personalized 4, 4, and 2 phages	Yes	Yes	No	Schooley et al. (2017)

PhagoBurn

The PhagoBurn trial was the first, multicentric European clinical trial for phage therapy (Jault et al. 2019). The trial investigated the efficacy of topical application of a 12-phage cocktail to reduce *P. aeruginosa* in burn wounds compared to a standard-of-care (SOC) antimicrobial cream of silver sulfadiazine. The study suffered production setbacks, could not reach enrolment populations, and did not report evidence of efficacy, among others. Authors did note that the lack of susceptibility testing prior to phage treatment decreased the number of patients who achieved the primary endpoint of the trial (Jault et al. 2019). The reason for not including phage sensitivity as an enrollment criterion is likely due to the fact that the cocktail was expected to have a broad epidemiological coverage of *P. aeruginosa* strains. Authors also noted that pre-sensitivity testing would have complicated the clinical protocol. However, in terms of phage resistance, 50% of 73 bacterial colonies from 10 patients in the phage treatment group were fully or intermediately resistant to the test product. Interestingly, four of the ten patients harbored colonies with different phage susceptibility profiles. As these colonies were isolated at day 0, it is likely that the patient isolates were phage resistant prior to phage administration.

Acute Pediatric *E. coli* Diarrhea

The other trial that did not include phage pre-sensitivity testing investigated the utility of phage for the treatment of pediatric *E. coli* diarrhea in Bangladesh (Sarker et al. 2016). This trial administered either a commercial phage cocktail targeted against *E. coli* and *Proteus* spp., an in-house T4-phage cocktail, or placebo to children with microbiologically diagnosed, acute *E. coli* diarrhea. The test product was applied orally, three times daily for 4 days without gastric neutralization. The trial was terminated early, as no indication of efficacy of phage application was observed on diarrhea parameters, such as disease severity or resolution, at an interim review. When *E. coli* colonies were isolated from patient stool after phage administration, only 50% were sensitive to phage, although harboring sensitive isolates did not correlate with higher stool titers. Both this study and PhagoBurn did not report data to support a therapeutic effect of phage therapy (albeit with plausible explanations), and both cited issues with phage-resistant bacteria, thus highlighting the importance of phage sensitivity testing for trial inclusion. The fact that isolates varied in phage sensitivity within some patients also indicates that multiple colonies should be included for this testing.

Chronic *P. aeruginosa* Otitis

The only formally structured completed trial that did include phage sensitivity testing as an inclusion criteria dates back from 2009, where a phage product, Biophage-PA, consisting of *P. aeruginosa* phages, was tested in a small phase I/II for its efficacy to treat chronic otitis (Wright et al. 2009). A total of six phages were tested individually against the patient isolate to ensure sensitivity, and then 1×10^5 plaque-forming units (PFU) per phage was administered together as a phage cocktail in a single dose to the ear. Patient samples were analyzed microbiologically 7, 21, and 42 days after phage application. A significant difference was observed between

the 12 patients receiving the phage product and the 12 receiving placebo in terms of clinical improvement scores and average bacterial counts.

The use of mean counts across patients may mask individual clinical responses. Indeed, close inspection of the individual patient bacterial counts over time in phage recipients revealed that two patients displayed an increase in bacterial load from day 0 to day 7 and the number of bacteria detected increased for six patients between days 7 and 21, despite initial sensitivity to phage. Unfortunately, no phage sensitivity testing was performed on the bacterial isolates at these different time points to test for the apparition of PRVs. Authors also noted an average 200× amplification of the test product phage components, although this was not reported per patient. Endogenous phage was detected in five of both placebo and phage recipients.

Collectively, there is little data to analyze about the development of phage resistance from recent clinical trials of phage therapy. This is largely due to the lack of pre-sensitivity testing to phages, as well as a lack of detailed microbiological analysis throughout the course of treatment. As mentioned previously, phage sensitivity is an essential requirement for phage therapy to even have a chance at providing a therapeutic effect and, therefore, should be required for all future clinical investigations. Even if phage sensitivity is included as an enrolment criterion, continual testing is required throughout treatment to investigate for the development of PRVs. If phage therapy is ever to be fully understood, thorough microbiological analysis of PRV strains should be done to shed light on how frequently resistance develops, what mechanisms are responsible for it, and how these changes might influence the pathogenicity or virulence of infecting bacterial strains.

4.2.2 Phage Resistance in Pilot Studies and Case Reports

Much more numerous than clinical trials are case reports, pilot studies, or summary reports of phage use (Table 2). Phage sensitivity testing preceded clinical application in these instances, usually for the formulation of personalized preparations as few preformulated products are currently available. However, reporting on compassionate use and smaller studies is often inconsistent or incomplete, making a comparative analysis difficult. Several reported cases that help to illustrate examples of phage resistance in human therapy are detailed below in chronological order of publication.

P. aeruginosa Urinary Tract Infection (UTI)

A six-phage personalized bacteriophage cocktail was used for the treatment of one patient in Australia in 2011 with a refractory UTI caused by *P. aeruginosa* (Khawaldeh et al. 2011). The phages were selected and prepared by the Eliava Institute at a titer of 1×10^6 PFU/mL. The preparation was administered directly into the bladder in doses of 20 mL every 12 h for a total of 10 days. Urine samples were collected frequently and elaborated for the detection of viable bacterial and phage counts, as well for bacterial DNA. The sensitivity of the patient isolate to the phage cocktail was confirmed three times, at days 1, 3, and 7, and concomitant antibiotic therapy (colistin and meropenem) was applied from the 6th day after the onset of phage therapy. No resistance to phage was detected, and bacterial titers continued to decrease over the 1st week of treatment until day 8 when no viable titers of

P. aeruginosa were cultivable. Phage titers increased after administration until they were no longer detectable, which occurred shortly after the sterilization of *P. aeruginosa* from the urine. Additionally, the authors investigated the clonality of the infection with DNA fingerprinting to show that all isolates were identical in their banding pattern, as well as in their sensitivity to the phage cocktail and antibiotics over time. Lastly, the presence of a secondary pathogen, *E. faecalis*, was monitored by PCR: its concentration did not vary with phage administration, but finally decreased after *P. aeruginosa* was eradicated and meropenem exposure was prolonged.

This is one of the few studies, despite being one of the earliest reported, which duly documented many aspects critical for understanding phage therapy and the development of resistance, with both bacterial and phage titers and clear testing for continued sensitivity over the course of treatment. Therefore, although it does not provide resistance data due to its absence, it represents a well-documented instance of the compassionate use of phage therapy that helps to clearly indicate that resistance did not occur. The types of information reported within this case report would be useful for all future cases of clinical applications.

S. aureus Skin Infection

A 16-year-old patient from France with Netherton syndrome, a complex skin condition, was treated with phage therapy at the Eliava Phage Therapy Center in 2016 (Zhvania et al. 2017). The skin condition caused the patient to suffer from chronic skin infections from antibiotic-resistant *S. aureus*, as well as allergies to most standard dermatologic products and antibiotics. Both the *Staphylococcus* bacteriophage and Pyobacteriophage commercial products from Eliava were well tolarated and applied locally via soaked bandages and impregnated creams or orally (10 mL each daily) after stomach acid alkinization. The treatment regimen was long and divided into two phases: first two, 20-day treatments separated by an interim 2-week break and second with alternating 2-week periods of phage administration with rest over 3 months. The authors tested the sensitivity profile of the infecting strain and indicated that while no resistance was detected after 1 month, a change in sensitivity at 3 months led them to exchange the Pyobacteriophage product for another commercial preparation, Fersis. The difference in activity of these two products was surprising, considering that a recent metagenomic comparison of these therapeutic preparations revealed that they contain highly similar phages against *S. aureus* (McCallin et al. 2018). Closely related members of these *S. aureus* phages, the *Spounavirinae*, are also the sole component of a clinical-grade product being developed by AmpliPhi Biosciences, AB-SA01 (Lehman et al. 2019). This preparation, which contains three phages sharing between 94 and 97% genetic identity, was also selected for their difference in host range and therefore indicates that these small genetic differences can lead to different clinical efficacies, as shown by this case report (Zhvania et al. 2017; Lehman et al. 2019). Ultimately, the overall bacterial load in different sites of the body was decreased by phage treatment, and the patient's severity of symptoms was greatly reduced, leading to an

improvement in quality of life. The authors note that chronic cases may benefit from periodic treatments with phage therapy to manage the underlying condition.

P. aeruginosa Bacteremia

Duplessis et al. reported the intravenous use of a two-phage cocktail against multidrug-resistant (MDR) *Pseudomonas* bacteremia for the treatment of a hospitalized 2-year-old patient (Duplessis et al. 2018). Phage sensitivity was performed prior to treatment, and the phages were selected from 25 active phages due to their lytic activity and targeting of different bacterial receptors. In a first course, phages were administered every 6 h at a dose of 3.5×10^5 PFU for 36 h for a total of 6 doses, several days after which blood cultures became and remained sterile for *P. aeruginosa* for 2 days. The presence of *Pseudomonas* reappeared after phage administration ceased, and phage therapy was recommenced, which again caused blood cultures to revert to negative. This continued for 5 days until confounding health problems led to a worsening condition with bacteria again being detected, and care was withdrawn, after which the patient died. It is not clear from the reported information if the bacterial outgrowth observed at the stage of clinical worsening appeared before or after the cessation of phage therapy, although authors noted that bacterial isolates from this time point were resistant to additional interventions (not specified).

A risk of performing compassionate treatment is an increased risk of treatment failure due to confounding medical conditions, which included DiGeorge syndrome, severe heart failure conditions, and the development of the flu for this particular patient. Despite the strain being resistant to antibiotics, concomitant therapy was continued throughout the course of treatment (meropenem, tobramycin, and polymyxin B) in order to maximize the possibility that the two antimicrobial strategies would have an additive effect for treatment. Authors reported on the time to positivity (TTP) as the measure for bacteria detected, which is commonly used for the diagnosis of bacteremia (Ning et al. 2016; Tang et al. 2017). Interestingly, the blood culture taken at the time when phage therapy was discontinued for the second time had a TTP nearly double of cultures isolated on 14 other days, which may indicate residual phage activity or the apparition of slow-growing PVRs, although it is not possible to verify such conclusions without microbiological analysis of the bacterial isolates.

Case Reports From IPATH

Several case studies have been communicated from the University of California, San Diego School of Medicine, which has led them to open an experimental therapy center, the Center for Innovative Phage Applications and Therapeutics (IPATH), as a result of their experiences with these compassionate cases (Schooley et al. 2017; Aslam et al. 2018; Furr et al. 2018; Wooten et al. 2018). In three instances, the reports of treatment performed there have mentioned the apparition of PRVs, although the full documentation of two cases is only currently available in short format. During the treatment of a lung infection in a cystic fibrosis patient, it was reported that a change in microbiological susceptibility to phage was noted for some

isolates of *P. aeruginosa*; however, no more details were available from this short-record format (Furr et al. 2018). Another record of compassionate use was for the treatment of a lung transplant recipient with a MDR *P. aeruginosa* infection (Aslam et al. 2018). Treatment was ultimately successful, but the cocktail composition was changed several times due to the apparition of PRVs.

A published case report from the same authors on the use of phage therapy for the treatment of a MDR *A. baumannii* abdominal infection provided quite possibly the most detailed analysis of aspects related to phage resistance to date (Schooley et al. 2017). Four phages were initially selected for treatment based on a large screening of phage collections for activity against the patient isolate and their previously determined host range spectrum. Resistance was detected at already 8 days after the initiation of treatment, and the phage composition was changed accordingly. In total, the patient was administered three different phage cocktails at different times and administration routes, which consisted of four, four, and two phages, respectively (one phage was retained from the second composition to the third). The first cocktail was applied intracavitary, while the subsequent preparations were applied intravenously. The changes to the phages used were due to the detection of phage resistance in vitro by monitoring bacterial growth of bacteria isolated at different time points in liquid culture; the therapeutic administration of the first cocktail shown to be inactive in vitro was continued. It was not reported if multiple isolates were tested or if pathogen clonality was investigated, therefore making it difficult to ascertain if resistance to the phage cocktails occurred in the parent strain background or if it merely selected for different isolates in a mixed infection.

The complexity of this case highlights the highly empirical nature of compassionate phage therapy. Publications of case reports have provided more information on phage resistance than formal clinical trials, yet multiple phage modifications, concomitant antibiotics, and underlying medical conditions make it difficult to compare cases or provide advice beyond the necessity to test for PRVs and modify phage compositions accordingly. Additionally, none of the abovementioned studies that did detect phage resistance during clinical treatment have gone as far as to follow-up with molecular characterization of the isolated PRV strains. Such analyses would provide information on lingering questions, such as if resistance develops via certain mechanisms or affects certain targets that would influence bacterial fitness. PRVs detected in vitro have been shown to have reduced virulence in vivo in several animal studies (Oechslin 2018). For this reason, the detection of PRVs by drop tests or liquid assays in rich media might not reflect their true clinical viability. While the purpose of compassionate use is to maximize therapeutic benefit for the patient and interventions should be empirically designed to do so, the opportunity to analyze clinical isolates and their PRVs should be exploited to also maximize future therapeutic benefits.

Pilot Study for UTI Treatment

A recently pilot study using phage for the treatment of UTIs caused by different bacterial pathogens was conducted in the prospect of designing a future formal clinical trial in Tbilisi, Georgia (Ujmajuridze et al. 2018). It is the only study to date which incorporates the adaption of phages to a set of clinical strains in order to

increase pathogen coverage prior to treatment. A commercial phage preparation, Pyobacteriophage (Eliava BioPreparations Ltd., Tbilisi, Georgia), underwent adaptation to clinical strains of *S. aureus*, *E. coli*, *P. aeruginosa*, *Streptococcus*, and *P. mirabilis* isolated from 130 patients undergoing transurethral resection of the prostate (TURP). The adaptation process increased the overall coverage of these isolates from 41 to 75%. The adapted preparation was then used to treat nine patients with sensitive bacterial isolates, and the primary outcome, pre- and post-bacterial viable counts, was recorded for eight. It is not clear if these patients' strains were included during the adaptation process or if they were nine new patients after the adaptation was complete.

The adapted Pyobacteriophage contained phages against the different pathogens in concentrations ranging from 10^7 to 10^9 PFU/mL, and treatment consisted of 20 mL applied directly to the bladder via a suprapubic catheter for 7 days, two times per day for 30–60 min. The results of phage application on pathogen load varied between the eight patients for which data was recorded: cultures became sterile for at least three patients, a decrease in original pathogen concentration was observed in four cases (although one patient developed an infection with a secondary pathogen), one patient's isolate showed no effect from PT, and no data was recorded posttreatment for one patient. Unfortunately, no data was reported on the phage sensitivity of the bacteria enumerated posttreatment nor for phage titers to indicate phage amplification, therefore making it again difficult to determine the development of phage resistance or, if it did, to understand how and what effect it may have had on treatment. The different results obtained between patients raise questions for phage therapy and the development of resistance. Considering that phage sensitivity was an inclusion criterion for the treatment population, the patient whose *E. coli* pathogen load remained the same throughout treatment is interesting in terms of resistance. Another case where the primary pathogen disappeared, but *E. coli* appeared is surprising because *E. coli* was a target of the adapted Pyophage prep.

Phage adaption was used in this pilot study to increase the activity of the preparation against a set of strains from a specific location, at a restricted time, and in a certain pathology. The principle of updating commercial phage preparations against relevant strains is common practice in countries with a history of phage therapy. This same concept could, in theory, be applied for adjusting a phage preparation for a single patient, time permitting, as a mechanism to counteract the development of phage resistance. However, multiple phages per pathogen are included in the commercial Pyobacteriophage preparation (Villarroel et al. 2017; McCallin et al. 2018). As this pilot study entails the adaptation of a cocktail, and not necessarily individual phages, the increased host range of the adapted preparation could be due to the selection of certain phages or population variants.

Summaries from the Polish Phage Therapy Unit (PTU)

One of the few institutes with a long-standing experience in phage therapy is the PTU in Poland, which has been treating patients compassionately with phages since the 1970s. They have published summaries of their experiences, with reports covering >1300 patients (Weber-Dabrowska et al. 2000, 2001, 2003; Międzybrodzki et al. 2012; Górski et al. 2016). The authors underline the initial sensitivity of the bacteria

to the applied phage as a requirement, with sensitivity to at least one phage from their collection being a prerequisite for receiving phage therapy at their establishment. In a study of the effectiveness of monophage therapy (the use of one phage per pathogen), a response to treatment was identified for 40% of 153 patients, although the rate of success was significantly associated with pathogen target and route of administration (Międzybrodzki et al. 2012). In a subset of 92 patients, authors investigated the development of resistance to phage during treatment in terms of phage typing profile, resistance to the applied monophage, and resistance to all phages against that pathogen in their collection. A change in phage profile was observed in 70, 100, 100, and 91% of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* isolates, respectively, therefore indicating changes in the pathogen clonality as a result of phage application. Resistance to applied phage was noted in 17, 43, 86, and 36% of strains of *S. aureus*, *E. faecalis*, *E. coli*, and *P. aeruginosa* isolates, respectively, although these values were lower in terms of resistance to all phages to 8, 21, 29, and 27% for the same pathogens, respectively. The development of resistance varied by pathogen, with the high level of resistance observed for *E. coli*, due to frequent changes to phages used for treatment. The difficulty in targeting *E. coli* with phages is reflected in the composition of their phage collection that targets 15 bacteria species, with 22% of all phages targeting *E. coli*. Despite the development of resistance observed for some patients, this has not deterred the continued use of phages for the treatment of antibiotic-resistant infections at this institution.

5 Ways to Overcome Resistance

As observed with the abovementioned cases in both humans and animals, the development of resistance to phage is a possibility, to varying extents, within the context of phage treatment. There are several strategies available to avoid or counteract resistance in order to reduce a negative impact on therapeutic outcomes. In terms of phage-only strategies, cocktail formation, phage substitution, and phage training are all strategies that have been employed to counteract phage resistance. By combining certain phages together or with other antimicrobial strategies, bacteria are less likely to be able to develop resistance, and thus there are ways to design treatments to maximize therapeutic effects. Should resistance develop, it is possible to substitute new phages with activity against the bacterial isolate or to adapt phages in vitro to increase their activity. Bacterial resistance to phage may have additional benefits for treatment that could render the development of resistance an intended effect of future phage therapy efforts.

5.1 Cocktail Formulation

The use of multiple phages together as a phage cocktail is commonly employed for phage products. Many commercial phage preparations from Eastern European countries are indeed cocktails and contain phages against different bacterial hosts,

as well as multiple phages against a single host species (McCallin et al. 2013, 2018; Villarroel et al. 2017). The number and diversity of phages to make a sufficient cocktail vary between bacterial hosts and indications, with certain species requiring high diversity, such as *E. coli*, while other pathogens, such as *S. aureus*, can be targeted with one or relatively few, genetically similar phages (McCallin et al. 2018; Lehman et al. 2019). On the other hand, 14 phage types with homology to *E. coli* phages were detected in a recent metagenomic sequencing of a commercial phage product, therefore indicating a high number of phages to target this pathogen (McCallin et al. 2018).

Cocktail composition can be formulated with the intention of having a broad spectrum of activity, such as in the PhagoBurn study, against a particular species or type of infection (Jault et al. 2019). However, selecting phages with different host ranges might not be sufficient to meet clinical needs. The aforementioned PhagoBurn study used a cocktail of 12 phages to cover a large panel of *P. aeruginosa* isolates, and yet many patients during the trial harbored insensitive strains to the phage cocktail, to the point that it was identified as a factor for patient withdraw (Jault et al. 2019).

Cocktail composition can also be guided by selecting phages that would decrease the likelihood of resistance developing. The preclinical development of a four-phage cocktail to target *S. aureus* took into consideration the ability of each component phages to mitigate the development of resistance to other components (Lehman et al. 2019). For this product, phages that could complement resistance were selected, and the overall mean apparent frequency of resistance was reduced in vitro, although not significantly.

Cocktail composition should be updated periodically in order to retain activity against epidemiological strains. A finding from the Polish experience with PT is that phage susceptibility of epidemiological strains does indeed vary over time (Międzybrodzki et al. 2012). This concept represents a major contradiction to current approval pathways for medicinal products, where phages are intended to have fixed, stable compositions. It is indeed a possibility that the therapeutic potential of phage therapy, and the associated risk of developing phage resistance, could be constrained by man-made regulations.

5.2 Phage Substitution

A common strategy to counter phage resistance during phage therapy is simply to replace the phage(s) to which the patient isolate has developed resistance against with an active one. Long-established phage therapy treatment centers, such as Eliava or the PTU, have large phage collections from which phages can be selected to formulate personalized therapies and adapt them accordingly. This type of modification requires the periodic sensitivity testing of the causative pathogen against the applied phage(s) and additional available phages that can be rapidly applied when needed. Phage substitution during active treatment therefore represents a personalized or tailored approach to phage therapy.

Changing the phages used in treatment has been observed in a number of clinical case reports in response to detected resistance (Schooley et al. 2017; Zhvania et al. 2017). The treatment of the MDR *A. baumannii* abdominal infection required three changes to the phages used throughout treatment, with resistance being detected after 8 days (Schooley et al. 2017). The added value that phage substitution could have in phage clinical trials, however, remains unexplored, as previous trials have used fixed-composition products. In any case, the permission to do trials with a personalized approach is currently unclear within the current regulatory framework that permits little modification of clinical protocols, especially not to the active product.

One may argue that if the original cocktail formulation is designed correctly, phage substitution would not be necessary. Indeed, the rationale behind the preclinical selection of phages to be included in the BA-SA01 from AmpliPhi Biosciences was that the four separate components would kill PRVs should they develop (Lehman et al. 2019). However, there is still not enough evidence to make generalizations of resistance developing during clinical treatment at this time. The lack of this information highlights a knowledge gap in phage research, where publications on the discovery and basic characterization of phages are numerous, and yet the intricate interactions between phage and host remain largely unknown for most species of bacteria.

5.3 Phage Training

Another approach that was proposed to overcome or minimize bacterial resistance is the use of "phage training" (Pirnay et al. 2012). Training or adaptation of a phage to its bacterial host can be achieved in vitro by serial rounds of coinfection using a continuous bacteriophage culture with the same original non-evolving host at each passage. Phage adaptation is also referred to as Appelmans' protocol since it is generally recognized that phage training protocols are based on Appelmans' experiments for the titration of bacteriophages developed in the 1920s (Appelmans 1921). Different studies reported that evolving the lytic phage ?2 toward its *Pseudomonas fluorescens* SBW25 host led to an increased phage growth rate, but not increased infectivity range (Poullain et al. 2008; Hall et al. 2011a, b). It is however observed that coevolution passages, where both phage and host are transferred, can result in the evolution of broader infectivity range (Poullain et al. 2008). Interestingly, Morello et al. reported that phage optimization toward a clinical strain after five consecutive passages in liquid culture improved both in vivo treatment efficacy and host infectivity on a panel of 20 *P. aeruginosa* cystic fibrosis strains (Morello et al. 2011).

Phage adaptation can also increase pathogen clearance in addition to tempered bacterial resistance evolution (Friman et al. 2016). The phage infection capacity against *P. aeruginosa* PAO1 of four different phages could be increased after six serial passages so that virtually all the original PAO1 population was susceptible to phage infection (Betts et al. 2013). This was the case even if the bacteria had evolved

in the presence of the phage for one transfer, indicating that phage training is a useful tool to overcome the initial step of bacterial resistance. Finally, it is important to notice that Betts et al. reported that when phages and bacteria were subcultured for a total of ten serial transfers, variable outcomes regarding infectivity and resistance were observed for these coevolutionary passages (Betts et al. 2014). Indeed, some phage infecting *P. aeruginosa* PAO1 became less infective against bacteria from previous time points, therefore suggesting that phage training can be a phage-specific process that has to be considered for further therapeutic applications.

This was exemplified by the study of Ujmajuridze et al. in which a commercial preparation called Pyobacteriophage was first adapted and then tested in nine patients having UTIs (Ujmajuridze et al. 2018). After testing the sensitivity of the cocktail regarding 118 patient strains, resistant or intermediate resistant strains were used in 4 adaptation cycles, which could increase the total sensitivity of the phage cocktail from 41 to 75%. The implementation of phage training in the therapy could be advantageous since it could also increase phage coverage for bacterial clones present within a population, like in the case of patients with cystic fibrosis that are infected by highly phenotypically diverse *P. aeruginosa* (Rohde et al. 2018). Phage training could also allow to decrease the number of phages used during therapy due to increased coverage of the circulating strain and prevention of phage resistance emergence, thus simplifying the production process (Rohde et al. 2018). However, genomic sequencing of adapted phages should be done in order to show that the adaptation process is truly selecting for phage variants and where such mutations are located.

5.4 Combined Activity with Antibiotics

The development and use of phage therapy are not intended to be a direct replacement of antibiotics and likely never will be. Currently, most indications being developed and all compassionate use cases only target antibiotic-resistant bacteria, although phages are equally active against antibiotic-sensitive strains. More likely is that phages and antibiotics will be employed for future treatments in tandem strategies, where they are either combined together or in subsequent administrations, in order to provide combinatory therapeutic effects of employing different antimicrobial strategies. The possible benefits of using the two strategies have been documented both in vitro, in animal models, and in some clinical case reports.

5.4.1 Phage-Antibiotic Synergy

Combining phage with antibiotics can result in a synergistic antimicrobial activity to improve therapeutic efficacy and prevent the emergence of phage resistance. It has been reported that some types of phages produce bigger lytic plaques when amplified with sublethal concentrations of antibiotics (Comeau et al. 2007). Synergism is defined as a combination of phage and antibiotic that produces at least a 2-log greater reduction in bacterial load than either strategy alone. This phage-antibiotic synergism, termed PAS (for review on the topic, see Torres-Barcelo and Hochberg

(2016)), was observed to be an efficient alternative for the treatment of *P. aeruginosa* infections, where different in vitro studies have demonstrated that combining both phage and antibiotics results in lower bacterial density than during single treatment alone due to limiting mutations that lead to resistance (Knezevic et al. 2013; Torres-Barceló et al. 2014). This positive synergism is proposed to result from a reduction in the bacterial population size due to phage predation, which could limit the ability of bacteria to resist antibiotic pressure (Torres-Barceló et al. 2014). Morphological alterations, including antibiotic-induced filamentation, are also suggested to facilitate phage access to its target or increase phage assembly and maturation, as also observed for *E. coli* (Comeau et al. 2007; Knezevic et al. 2013).

PAS has also been observed to be useful for the eradication of *Pseudomonas* biofilms (Nouraldin et al. 2016) and was recently confirmed for the first time in vivo in a rat model of *Pseudomonas*-induced endocarditis (Oechslin et al. 2016). In this study by Oechslin et al., the combination of phages and ciprofloxacin was highly synergistic in vivo with a >6-log reduction of CFU in treated animals compared to a 2.5-log reduction of CFU using phage alone. In addition, the combination of phages and ciprofloxacin was also observed to efficiently prevent the emergence of phage resistance in vitro. Synergistic effects for ciprofloxacin with phages have also been documented in *S. aureus* both for CFU reduction and the suppression of resistance development (Jo et al. 2016).

5.4.2 Resistance Reversion

The synergism expected with antibiotics and phage combinations may not be limited to a direct increased killing of the two combined antimicrobial strategies, but also to alternating patterns of resistance and resistance reversion to either phage or antibiotics. Chan et al. observed that the selection of phage resistance can restore antibiotic susceptibility of MDR *Pseudomonas* (Chan et al. 2016). By selecting a specific phage that uses the outer membrane porin M of the multidrug efflux systems, MexAB and MexXY, as a receptor, it can result in PRVs with altered efflux pump function and thus increased sensitivity to many different drug types. This phage has since been used successfully in the compassionate treatment of an aortic valve graft infection in combination with ceftazidime (Chan et al. 2018). Phage-induced mutations in the *epa* operon responsible for cell-wall components in *E. faecalis* simultaneously create an increased sensitivity to daptomycin, a lipopeptide antibiotic (Teng et al. 2009; Dale et al. 2015; Ho et al. 2018).

In terms of therapeutic applications in humans, phage has often been administered with antibiotics in order to maximize the chances of therapeutic benefit for the patient. The choice of antibiotic and dosage combinations is largely experimental, with decisions being logically based off results of phage sensitivity tests and antibiograms. After the administration of the first phage cocktail in the case report for *A. baumannii* infections, authors noted a change in antibiotic susceptibility of the patient isolate that had become sensitive to minocycline and resistant, to some extent, to the applied phages. The antibiotic was then added to the treatment regimen, and phage was continued (Schooley et al. 2017). Minocycline binds to bacterial ribosomal units to inhibit protein synthesis, and therefore phage resistance

mechanisms may have triggered a reversion of antibiotic resistance mechanisms, which can occur through efflux pumps, drug modifications, or ribosomal protection proteins (Garrido-Mesa et al. 2013; Nguyen et al. 2014). However, no information concerning the biological mechanisms responsible for the switch in resistance of the clinical isolates is available from this case report or any other. Such fundamental investigations into clinical isolates and the development of PRVs coupled with antibiotic susceptibility would provide a greater understanding of how to maximize PAS in future treatment strategies.

The use of antibiotics in compassionate cases on one hand stymies a clear causality between phage and infection resolution; on the other hand, it hints at the value of phage-antibiotic combinations in providing therapeutic benefit. Indeed, clinical outcomes have been more positive for case reports, which can choose phages and antibiotics ad libitum, than results observed with clinical trials. It has recently been shown that even the order in which antibiotics and phage are applied may have consequences for its combined therapeutic potential (Kumaran et al. 2018).

It was also observed that the use of subinhibitory concentrations of streptomycin can also increase the phage resistance mutation rate in *P. fluorescens* and, conversely, phage exposure could also increase the rate of mutation to streptomycin resistance. However, it is important to notice that no positive correlation between drug and phage resistance was observed in a large collection of laboratory or clinical *E. coli* isolates (Allen et al. 2017). These results hopefully suggest that the use of antibiotics in medicine or agriculture is unlikely to induce changes in phage resistance or phage-antibiotic cross-resistance in the environment.

6 Conclusions and Perspectives

Resistance to penicillin was detected prior to its incorporation into clinical medicine, a fact which has not negated the innumerable bacterial infections it has resolved over the past 80 years. The capacity of bacteria to resist phage during treatment has been documented, but is not yet generalizable to clearly determine to what extent resistance could affect clinical outcomes. Additionally, the high specificity of interactions between phage and bacterial host has raised previously unrecognized subtitles of infectious pathologies that could be previously ignored with broad-spectrum antibiotics. Resistance to phage has been shown to vary widely for different pathogens and may be influenced by product design, application method, and dosage.

The most informative data that can indicate appropriate use of phages comes from actual clinical applications, of which there are relatively few at this time. Even so, unstandardized reporting of phage therapy in humans or animals has limited our ability to understand both the likelihood of developing resistance to phage during treatment and the impact this resistance has on clinical outcomes. Without both pre- and post-phage sensitivity testing, it is not possible to ascertain if resistance develops throughout the course of treatment, and such analyses should be included in future studies. For studies that do detect resistance, it would be beneficial to characterize the molecular mechanisms that give rise to its occurrence.

Phages have the advantage of being strongly supported by fundamental research, including resistance mechanisms, and therapeutic development is now strongly supported by technological ability and innovation, compared to the time of phage discovery. Strategies such as cocktail formulation, phage substitution, phage training, or combination with antibiotics can be used to maximize therapeutic benefits of phage treatment. While this review covers only relationships between natural phage and their hosts, genetically engineered phages or phage lysins may hold even more potential to reduce risks of resistance. The importance of phage resistance on clinical outcomes will reflect the developmental pathway of phage therapy in terms of regulatory frameworks and logistics that stretch beyond biological mechanisms.

The underlying reason for the current search for novel antimicrobials is rooted in the ability of bacteria to develop resistance to past ones, therefore making the exploration of resistance to future strategies a logical investigation. However, not all resistance is created equal. While experience has shown that there is a veritable possibility of resistance to phages developing as a result of therapeutic application, the likelihood of resistance occurring can be counteracted—or even harnessed—to mitigate negative effects on treatment outcomes. Resistance should therefore not be a deterrent to phage therapy, but needs to be better understood and taken into consideration for designing future phage strategies.

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Part II

Bacteriophages and the Immune System

Phage Interaction with the Mammalian Immune System

Jonas D. Van Belleghem, Krystyna Dąbrowska, Mario Vaneechoutte, and Jeremy J. Barr

1 Background: Human Immune Response

Vertebrates are constantly threatened by the invasion of microorganisms and have evolved systems of immune defense to eliminate infective pathogens in the body. The mammalian immune system can be divided in two branches: the innate and acquired or adaptive immunity (Table 1) (Akira et al. 2006). The innate immune response is the first line of host defenses against pathogens and is mediated by phagocytes including macrophages and dendritic cells (DCs). Acquired or adaptive immunity is involved in elimination of pathogens in the late phase of infection as well as the generation of immunological memory. The main cells involved in the acquired or adaptive immune response are T and B cells (Medzhitov and Janeway 2000).

The innate immune system is believed to have predated the adaptive immune response on several grounds. First, innate host defenses are found in all multicellular organisms, whereas adaptive immunity is found only in vertebrates. Second, innate immune recognition distinguishes self from nonself perfectly. Third, the innate immune system uses receptors that are ancient in their lineage, whereas adaptive

J. D. Van Belleghem (✉)
Laboratory Bacteriology Research, Department of Clinical Chemistry, Microbiology and Immunology, University Ghent, Ghent, Belgium

Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, CA, USA

K. Dąbrowska
Bacteriophage Laboratory, Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

M. Vaneechoutte
Laboratory Bacteriology Research, Department of Clinical Chemistry, Microbiology and Immunology, University Ghent, Ghent, Belgium

J. J. Barr
School of Biological Sciences, Monash University, Melbourne, VIC, Australia

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Table 1 Innate and adaptive immunity. Adapted from Janeway and Medzhitov (2002)

Property	Innate immune system	Adaptive immune system
Receptors	Fixed in genome Rearrangement is not necessary	Encoded in gene segments Rearrangement is necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns	Details of molecular structure
Self-nonself discrimination	Perfect: selected over evolutionary time	Imperfect: selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of effectors
Response	Co-stimulatory molecules Cytokines Chemokines	Clonal expansion or anergy Cytokines

immunity appears to use the same effector mechanisms guided by clonally specific antibodies and T-cell receptors (TCR) encoded in rearranging genes of the Ig gene superfamily (Medzhitov and Janeway 1997). The virtues of having an innate immune system of pathogen recognition lies not only in the delaying tactics of inflammation upon infection, but also in the activation of the adaptive immune system only when the body is under attack by a specific pathogen (Janeway and Medzhitov 2002).

1.1 Innate Immunity

Innate immunity covers many areas of the host defense against pathogenic microbes and viruses, including the recognition of pathogen-associated molecular patterns (PAMPs) (Janeway 1989). In vertebrates, which are the only phylum that can mount an adaptive immune response, there are also mechanisms to inhibit the activation of innate immunity (Janeway and Medzhitov 2002). Innate immunity is an evolutionary ancient part of the host defense mechanisms, the same molecular modules are found in plants and animals, meaning it arose before the split into these two kingdoms (Hoffmann et al. 1999). Innate immunity lies behind most inflammatory responses; these are triggered in first instance by macrophages, polymorphonuclear leukocytes, and mast cells through their innate immune receptors (Janeway and Medzhitov 2002).

The innate immune system is the primary, or early, barrier to infectious agents and acts immediately upon recognition of a pathogen. It mounts an effective defense against infectious agents through the initiation of adaptive immunity, which is long-lasting and has immunological memory (Kumar et al. 2011). Invasion of a host by a pathogenic infectious agent triggers a battery of immune responses through interactions between a diverse array of pathogen-born virulence factors and the immune surveillance mechanisms of the host. Host-pathogen interactions are generally initiated via host recognition of conserved molecular structures known as pathogen-associated

molecular patterns (PAMPs) that are essential for the life cycle of the pathogen (Janeway and Medzhitov 2002; Kumar et al. 2011). However, these PAMPs are either absent or compartmentalized inside the host cell and are sensed by the host's germline-encoded pattern recognition receptors (PRRs), which are expressed on innate immune cells such as dendritic cells, macrophages, and neutrophils (Takeuchi and Akira 2010; Kawai and Akira 2010; Medzhitov 2007; Blasius and Beutler 2010).

Signaling receptors recognize PAMPs and activate signaling-transduction pathways that induce the expression of a variety of immune-response genes, including inflammatory cytokines (Takeuchi and Akira 2010; Kawai and Akira 2010; Ozinsky et al. 2000; Kumar et al. 2009). Toll-like receptors (TLRs) are the most widely studied PRRs and are considered to be the primary sensors of pathogens (Kumar et al. 2011). Based on their primary sequence, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPs: the subfamilies of TLR1, TLR2, and TLR6 recognize lipids, whereas the highly related TLR7, TLR8, and TLR9 recognize nucleic acids (Table 2).

1.2 Adaptive Immunity

Adaptive immunity is a relative newcomer on the evolutionary landscape. Because the mechanism of generating receptors in the adaptive immune system involves great variability and rearrangement of receptor gene segments, the adaptive immune system can provide specific recognition of foreign antigens, immunological memory of infection, and pathogen-specific adaptor proteins. However, the adaptive immune response is also responsible for allergy, autoimmunity, and the rejection of tissue grafts (Janeway and Medzhitov 2002). The adaptive immunity adds specific recognition of proteins, carbohydrates, lipids, nucleic acids, and pathogens to the underlying innate immune system, using the same activated, but not antigen-specific, effector cells generated by the innate immune recognition. The two systems, i.e., the innate and adaptive immune system, are linked in the use of the same effector cells (e.g., dendritic cells or macrophages) (Janeway 1989).

Activation of the adaptive immune system occurs only upon pathogen recognition by dendritic cells, where they play a pivotal role at the interface of innate and adaptive immunity (Pulendran et al. 2001). Pathogen recognition is mediated by innate receptors such as RLRs and NLRs (Kumar et al. 2009). Immature dendritic cells reside in the peripheral tissues, where they actively sample their environment by endocytosis and micropinocytosis (Orsini et al. 2003).

Unlike the innate mechanisms of host defense, the adaptive immune system manifests exquisite specificity for its target antigens. Adaptive responses are based primarily on the antigen-specific receptors expressed on the surface of T and B lymphocytes (Chaplin 2010; Bonilla and Oettgen 2010; Schroeder and Cavacini 2010). The adaptive immunity is mediated by immunoglobulins and T-cell receptors (TCRs) (Tonegawa 1983). A major challenge faced by the immune system is to identify host cells that have been infected by microbes that subsequently use the cell to multiply within the host. A major role of the T-cell arm of the immune response is

Table 2 TLR recognition of bacterial or viral components. Adapted from Akira et al. (2006)

Microbial components	Species	TLR usage	References
Bacteria			
LPS	Gram-negative bacteria	TLR4	Poltorak et al. (1998); Shimazu et al. (1999)
Diacyl lipopeptides	Mycoplasma	TLR6/ TLR2	Thoma-Uszynski (2001)
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/ TLR2	Thoma-Uszynski (2001)
Lipoteichoic acid (LTA)	Group B Streptococcus	TLR2	Alexopoulou et al. (2002); Ozinsky et al. (2000); Takeuchi et al. (2000, 2001)
Peptidoglycan (PG)	Gram-positive bacteria	TLR2	Alexopoulou et al. (2002); Ozinsky et al. (2000); Takeuchi et al. (2000, 2001)
Porins	Neisseria	TLR2	
Lipoarabinomanan	Mycobacteria	TLR2	Gilleron et al. (2003)
Flagelin	Flagellated bacteria	TLR5	Hayashi et al. (2001)
CpG-DNA	Bacteria and mycobacteria	TLR9	Hemmi et al. (2002)
ND	Uropathogenic bacteria	TLR11	Zhang et al. (2004)
Viruses			
DNA	Viruses	TLR9	Hochrein et al. (2004); Krug et al. (2004a, b); Lund et al. (2003); Tabeta et al. (2004)
dsRNA	Viruses	TLR3	Alexopoulou et al (2001)
ssRNA	RNA viruses	TLR7 and TLR8	Diebold (2004); Heil (2004); Hemmi et al. (2002)
Envelope proteins	RSV, MMTV	TLR	Kurt-Jones et al. (2000)
Hemagglutinin protein	Measles virus	TLR2	Bieback et al. (2002); Compton et al. (2003)
ND	HCMV, HSV1	TLR2	Kurt-Jones et al. (2004)

to identify and destroy infected cells. T cells can also recognize peptide fragments of antigens that have been taken up by antigen presenting cells (APCs) through the process of phagocytosis or pinocytosis. The immune system permits T cells to recognize infected host cells by the recognition of both self-component and a microbial structure. This is mediated by the major histocompatibility (MHC) molecules. MHC molecules (also called human leukocyte antigen (HLA) antigens) are cell surface glycoproteins that bind peptide fragments of proteins that either have been synthesized within the cell (class I MHC molecules) or have been ingested by the cell proteolytically processed (class II MHC molecules) (Chaplin 2010; Menéndez-Benito and Neefjes 2007; Davis and Bjorkman 1988; Watts 2004).

1.3 Immune Cell Communication: The Language of Cytokines and Chemokines

Cells of the immune system require communication networks that can, as required, act locally or at a distance, specifically or globally, and transiently or in a sustained manner. This immune cell communication is conducted mainly by cytokines and chemokines. The term *cytokine* defines a large group of nonenzymatic protein hormones whose actions are both diverse and overlapping and which affect diverse and overlapping target cell populations (Kelso 1998; Opal et al. 2000). Chemokines on the other hand are essential for the trafficking of immune effector cells to sites of infection. Moreover, their function is necessary to translate an innate immune response into an acquired response. Innate immune stimuli, through activation of TLRs, set in motion a genetic program that induces the expression of a subset of chemokines from resident tissue macrophages and dendritic cells and modulates the expression of chemokine receptors on dendritic cells (Luster 2002; Nomiyama et al. 2010).

1.3.1 Cytokines

Cytokines are local mediators produced by cells of the lymphoid and macrophage lineage as well as by epithelial and mesenchymal cells. Cytokines are involved in a variety of biological processes, including cell activation, growth, and differentiation, and they are central to the development of inflammation and immunity (Sartor 1994; Elson 1996). Cells of the innate immune system, such as macrophages and monocytes, are able to mount a rapid response to a danger signal, e.g., an infectious agent, by secreting several pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, IL-12, and tumor necrosis factor (TNF)-α. The cytokine milieu subsequently directs the development of adaptive immunity mediated by T and B lymphocytes (Papadakis and Targan 2000). Some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other cytokines suppress the activity of pro-inflammatory cytokines and are called anti-inflammatory cytokines (Dinarello 2000).

The concept that some cytokines function primarily to induce inflammation while others suppress inflammation is fundamental to cytokine biology and also to clinical medicine (Dinarello 2000; Opal et al. 2000). A dynamic and ever-shifting balance exists between pro-inflammatory cytokines and anti-inflammatory components of the human immune system. The regulation of inflammation by these cytokines and cytokine inhibitors is complicated by the fact that the immune system has redundant pathways with multiple elements having similar physiologic effects (Kasai et al. 1997; Munoz et al. 1991). The net effect of any cytokine is dependent on the timing of cytokine release, the local milieu in which it acts, the presence of competing or synergistic elements, cytokine receptor density, and tissue responsiveness to each cytokine (Dinarello 1998; Cannon 2000). Different immunogens induce the synthesis of different cytokines which in turn activate different immune effector mechanisms. Although every nucleated cell type can produce cytokines, most lineages express only a subset of cytokine genes (Kelso 1998; Cannon 2000).

Here we will discuss some (i.e., IL-1, IL-6, and IL-10) but not all interleukins. The IL-1 cytokine family comprises four main members IL-1α, IL-1β, IL-1 receptor antagonist (IL-1ra/IL-1RN), and IL-18 (Girn et al. 2007). The IL-1 family is primarily considered to be pro-inflammatory, as it can upregulate host defenses and act as an immunoadjuvant (Dinarello 1997b). IL-1β plays a significant role in inflammation; it has been implicated in enhancing expression of cell adhesion molecules on the endothelial surface and has consequently been deemed to be pro-atherogenic (Dinarello 1999). The only member of this family with paradoxical properties is IL-1RN, a naturally occurring cytokine antagonist, which plays an anti-inflammatory role in regulating IL-1 function (Dinarello and Thompson 1991; Perrier et al. 2006). IL1-RN blocks the action of IL-1α and IL-1β functional ligands by competitive inhibition at the IL-1 receptor level. IL-1RN is produced by monocytes and macrophages and is released into the systemic circulation in >100-fold excess than either IL-1α or IL-1β after lipopolysaccharide (LPS) stimulation (Dinarello 1998). The anti-inflammatory cytokines IL-4, IL-6, IL-10, and IL-13 inhibit the synthesis of IL-1β and stimulate the synthesis of IL-1RN (Dinarello 1997a).

IL-6 has long been regarded as a pro-inflammatory cytokine induced by LPS along with TNF-α and IL-1. It is often used as a marker for systemic activation of pro-inflammatory cytokines (Barton and Medzhitov 2002). Like many other cytokines, IL-6 has both pro- and anti-inflammatory properties. Although IL-6 is a potent inducer of the acute-phase protein response, it has anti-inflammatory properties as well (Barton et al. 1996). IL-6 attenuates the synthesis of the pro-inflammatory cytokines while having little effect on the synthesis of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β). IL-6 induces the synthesis of glucocorticoids and promotes the synthesis of IL-1RN and soluble TNF receptor release in human volunteers (Ruzek et al. 1997; Tilg et al. 1994). At the same time, IL-6 inhibits the production of pro-inflammatory cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ) and MIP-2 (Barton 1997).

IL-10 is the most important anti-inflammatory cytokine found in the human immune response (Opal et al. 2000). It is a potent inhibitor of T_H1 cytokines, including both IL-2 and IFN-γ, but also of IL-1, IL-6, and TNF-α (Hagenbaugh et al. 1997; Opal et al. 1998; Howard and O'Garra 1992; Lalani et al. 1997). IL-10 is a pleiotropic cytokine produced by a variety of cells, including T and B lymphocytes, thymocytes, macrophages, mast cells, keratinocytes, and intestinal epithelial cells. IL-10 is also a potent deactivator of monocyte/macrophage pro-inflammatory cytokine synthesis (Clarke et al. 1998; Brandtzaeg et al. 1996). It also inhibits cell surface expression of MHC class II molecules and the LPS recognition and signaling molecule CD14 (Opal et al. 1998).

The cytokine induced immune responses can be further regulated by suppressors of cytokine signaling (SOCS) and cytokine-inducible SH2 protein (CIS) family of intracellular proteins (Yasukawa et al. 2000; Larsen and Röpke 2002; Greenhalgh et al. 2002). In total, there are eight SOCS proteins (i.e., SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, SOCS7, and CIS) (Illson et al. 1998). The most well-characterized

SOCS family members, SOCS1, SOCS2, SOCS3, and CIS (Table 3), seem to act in a classical negative-feedback loop to inhibit cytokine signal transduction (Larsen and Röpke 2002). SOCS1 has an important regulatory function in macrophages and dendritic cells. The inhibitory activity of SOCS2 is not as strong as that of CIS (Metcalf et al. 2000). Both SOCS1 and SOCS3 can inhibit JAK tyrosine kinase activity.

1.3.2 Chemokines

Chemokines are small heparin-binding proteins that form a family of chemotactic cytokines that regulate migration and tissue localization of various kinds of cells in the body (Moser et al. 2004; Zlotnik and Yoshie 2000; Charo and Ransohoff 2006). In particular, they participate in inflammatory leukocyte recruitment, in lymphocyte recirculation and homing, and even in cancer metastasis (Gerard and Rollins 2001; Ben-Baruch 2008). Chemokines are known to have well-conserved four cysteines and are grouped into five subfamilies, CXC, CC, XC, CX_3C, and CX, based on the arrangement of the two N-terminal cysteine residues (Table 4) (Nomiyama et al. 2010). A single chemokine can bind to several chemokine receptors, whereas a single chemokine receptor can have multiple chemokine ligands (Zlotnik and Yoshie 2012). The recognition of chemokine-encoded messages is mediated by specific cell surface G-protein-coupled receptors (GPCRs) with seven transmembrane domains (Murphy 2002).

Infectious microorganisms can directly stimulate chemokine production by tissue dendritic cells (DCs) and macrophages as well as by many parenchymal and stromal cells. Conserved microbial PAMPs induce chemokines through PRR, such as TLRs or NOD1 and NOD2 (Girardin et al. 2003; Janeway and Medzhitov 2002). Classically the major inflammatory and immunomodulatory cytokines such as IL-1, TNF-α, IFNγ, IL-4, IL-5, IL-6, IL-13, and IL-17, induced in injury or infection, stimulate through their respective receptors the production of many different chemokines (Luster 1998; Rollins 1997; Baggiolini et al. 1997).

2 The Human-Phage Story: More than We Thought

2.1 Phage-Mammalian Host Interactions

The human body is colonized by commensal microorganisms; most of these microorganisms reside at body surfaces that are in direct contact with the environment, including the intestine, skin, and respiratory tract. Research efforts focused primarily on the bacterial component of the human microbiota and its associated genes have yielded a wealth of insight about the composition of human-associated bacterial communities (Duerkop and Hooper 2013). It has clarified how these resident bacteria interact with the immune system and how bacteria-immune system interactions are altered in disease (Hooper et al. 2012; Lozupone et al. 2012). Recently, it has become apparent that the microbiota of healthy humans also include viruses (White et al. 2012). Metagenomic studies have revealed that the human microbiome includes many viral genes (the virome) (Minot et al. 2011; Reyes et al.

Table 3 Cytokine induction and activation of SOCS proteins. Adapted from Alexander (2002) and Alexander and Hilton (2004)

SOCS protein	Induced by	Inhibits	References
CIS	IL-2, IL-3, IL-6, IL-9, IL-10, GM-CSF, GH, PRL, TSLP, EGF, CNTF, leptin, EPO, TPO	IL-2, IL-3, GH, IGF1, leptin, EPO	Adams et al. (1998); Aman et al. (1999); Bjørbæk et al. (1999); Emilsson et al. (1999); Isaksen et al. (1999); Lejeune et al. (2001); Okabe et al. (1999); Pezet et al. (1999); Ram and Waxman (1999); Sadowski et al. (2001); Shen et al. (2000); Starr et al. (1997); Yoshimura et al. (1995); Zong et al. (2000)
SOCS1	IL-2, IL-4, IL-6, IL-9, IL-10, G-CSF, GH, PRL, TSH, SCF, insulin, LIF, CT1, CNTF, EPO, IFN-α/β, IFN-γ, TNF	IL-2, IL-3, IL-4, IL-6, IL-7, M-CSF, GH, IGF1, PRL, TSLP, SCF, Flk ligand, insulin, LIF, OSM, CT1, EPO, TPO	Adams et al. (1998); Bjørbæk et al. (1999); Bourette et al. (2001); De Sepulveda et al. (1999); Endo et al. (1997); Hamanaka et al. (2001); Isaksen et al. (1999); Kawazoe et al. (2001); Lejeune et al. (2001); Losman et al. (1999); Morita et al. (2000); Naka et al. (1997); Park et al. (2000); Pezet et al. (1999); Sadowski et al. (2001); Shen et al. (2000); Song and Shuai (1998); Sporri et al. (2001); Starr et al. (1997); Trop (2001); Wang et al. (2000a, b); Zong et al. (2000)
SOCS2	IL-2, IL-6, GH, PRL, insulin, CNTF	GH, IGF1, LIF	Zong et al. (2000); Ram and Waxman (1999); Minamoto et al. (1997); Starr et al. (1997); Adams et al. (1998); Pezet et al. (1999); Sadowski et al. (2001); Bjørbæk et al. (1999)
SOCS3	IL-1, IL-2, IL-3, IL-6, IL-9, IL-10, IL-11, Il-22, GH, PRL, TSH, EGF, insulin, PDGF, BFGF, LIF, OSM, CT1, CNTF, leptin, EPO, TPO, TNF	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-11, GH, IGF1, PRL, insulin, LIF, OSM, CT1, CNTF, leptin, EPO, INF-α/β, INF-γ	Adams et al. (1998); Auernhammer et al. (1998); Auernhammer and Melmed (1999); Bjørbaek et al. (1998, 1999); Boisclair et al.

(continued)

Table 3 (continued)

SOCS protein	Induced by	Inhibits	References
			(2000); Cacalano et al. (2001); Cohney et al. (1999); Emanuelli et al. (2000); Hamanaka et al. (2001); Hong et al. (2001); Karlsen et al. (2001); Kotenko et al. (2001); Lejeune et al. (2001); Losman et al. (1999); Magrangeas et al. (2001a, b); Minamoto et al. (1997); Nicholson et al. (1999); Park et al. (2000); Pezet et al. (1999); Sadowski et al. (2001); Sasaki et al. (2000); Shen et al. (2000); Song and Shuai (1998); Starr et al. (1997); Terstegent et al. (2000); Wang et al. (2000a, b); Zong et al. (2000)

BFGF basic fibroblast growth factor, *CIS* cytokine-induced SRC-homology-2 protein, *CNTF* ciliary neurotrophic factor, *CT1* cardiotrophin-1, *EGF* epidermal growth factor, *EPO* erythropoietin, *G-CSF* granulocyte colony-stimulating factor, *GH* growth hormone, *GM-CSF* granulocyte-macrophage colony-stimulating factor, *IFN* interferon, *IGF1* insulin-like growth factor-1, *IL* interleukin, *LIF* leukaemia inhibitory factor, *M-CSF* macrophage colony-stimulating factor, *OSM* oncostatin M, *PDGF* platelet-derived growth factor, *PRL* prolactin, *SCF* stem-cell factor, *SOCS* suppressor of cytokine signaling, *TNF* tumor necrosis factor, *TPO* thrombopoietin, *TSH* thyrotropin, *TSLP* thymic stromal lymphopoietin

2010; Handley et al. 2012). Additionally, there are viruses associated with the intestine and the skin that replicate either in eukaryotic cells or in bacteria (Zhang et al. 2006; Minot et al. 2011; Reyes et al. 2010).

Bacteria that inhabit the intestine and skin are generally regarded as stable residents that confer metabolic and/or immune benefits to their hosts (Turnbaugh et al. 2009). The question can be raised whether a stable association between human healthy tissues and viruses can exist. It is interesting to consider whether phage predation of intestinal bacteria could alter community composition in ways that impact function of the immune system and influence the spread of pathogenic viruses (Duerkop and Hooper 2013; Ivanov et al. 2008; Mazmanian et al. 2005). Limiting pathogen colonization through niche occupation and resource use is part of how the microbiota impact host immunity. These indirect protective effects could extend to the viral members of the microbiota, of which there are an estimated 10^9 viruses per gram of feces. Some of these viruses target mammalian cells, but phages make up the majority of this viral community (Cadwell 2015).

Table 4 CC, CXC, CX_3C, and XC families of chemokines and chemokine receptors

Receptor	Chemokine ligands	Cell types	References
CCR1	CCL3, CCL5, CCL7, CCL14	T cells, monocytes, eosinophils, basophils	Mahad et al. (2004); Proudfoot et al. (2003); Trebst et al. (2001)
CCR2	CCL2, CCL8, CCL7, CCL13, CCL16	Monocytes, dendritic cells, (immature) memory T cells	Charo (2004); Charo and Peters (2003); Gu et al. (2000); Proudfoot et al. (2003)
CCR3	CCL11, CCL13, CCL7, CCL5, CCL8, CCL13	Eosinophils, basophils, mast cells, T_H2 cells, platelets	Daly and Rollins (2003)
CCR4	CCL17, CCL22	T cells (T_H2), dendritic cells (mature), basophils, macrophages, platelets	Calzascia et al. (2005); Flier et al. (2001)
CCR5	CCL3, CCL4, CCL5, CCL11, CCL14, CCL16	T cells, monocytes	Mahad et al. (2004); Proudfoot et al. (2003); Trebst et al. (2001)
CCR6	CCL20	T cells (T regulatory and memory), B cells, dendritic cells	Schutyser et al. (2003)
CCR7	CCL19, CCL21	T cells, dendritic cells (mature)	Cyster (2003, 1999); Mantovani (1999); Sozzani et al. (2000)
CCR8	CCL1	T cells (T_H2), monocytes, dendritic cells	Qu et al. (2004)
CCR9	CCL25	T cells, IgA^+ plasma cells	Calzascia et al. (2005)
CCR10	CCL27, CCL28	T cells	Homey et al. (2002); Wang et al. (2000a, b)
CXCR1	CXCL8 (IL-8), CXCL6	Neutrophils, monocytes	Gerszten et al. (1999); Liehn et al. (2013); Tachibana et al. (1998)
CXCR2	CXCL8, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6	Neutrophils, monocytes, microvascular endothelial cells	Gerszten et al. (1999); Liehn et al. (2013); Tachibana et al. (1998)
CXCR3-A	CXCL9, CXCL10, CXCL11	Type 1 helper cells, mast cells, mesangial cells	Flier et al. (2001); Sørensen et al. (1999)
CXCR3-B	CXCL4, CXCL9, CXCL10, CXCL11	Microvascular endothelial cells, neoplastic cells	Flier et al. (2001); Sørensen et al. (1999)
CXCR4	CXCL12	Widely expressed	Ma et al. (1998); Zou et al. (1998)
CXCR5	CXCL13	B cells, follicular helper T cells	Cyster et al. (1999); Müller et al. (2003)
CXCR6	CXCL16	$CD8^+$ T cells, natural killer cells, memory $CD4^+$ T cells	Matloubian et al. (2000); Shimaoka et al. (2000)
CX_3CR1	CX3CL1	Macrophages, endothelial cells, smooth-muscle cells	Bazan et al. (1997); Pan et al. (1997)
XCR1	XCL1, XCL2	T cells, natural killer cells	Kelner et al. (1994)

Although humans are routinely exposed to phages on a daily basis, concerns persist over their immunogenicity and overall safety, presenting an additional stumbling block for the adoption of phage therapy (Cooper et al. 2016). It becomes clearer that phages can do more than exercise antibacterial properties, they can become a part of the mucus layers (Barr et al. 2013, 2015) and even migrate through cell layers (Nguyen et al. 2017) or form an additional virulence factor elevating the bacterial fitness (Penner et al. 2016; Secor et al. 2015). When these phages enter the blood, they can interact with immune cells and induce innate and adaptive immune responses (Van Belleghem et al. 2017; Majewska et al. 2015; Miernikiewicz et al. 2013; Hodyra-Stefaniak et al. 2015).

2.2 Phages in the Mucus: Non-host-Derived Immunity

A critical immunological barrier protecting all animals against invading bacterial pathogens but also supporting large communities of commensal microorganisms are the mucosal surfaces (e.g., human gut and respiratory tract) (Linden et al. 2008; Johansson et al. 2008). The mucus is predominantly composed of mucin glycoproteins that are secreted by the underlying epithelium. The amino acid backbone of these proteins incorporates tandem repeats of exposed hydrophobic regions alternating with blocks bearing extensive O-linked glycosylation (Cone 2009). By offering both structure and nutrients, mucus layers commonly support higher bacterial concentrations than the surrounding environments (Martens et al. 2008; Poulsen et al. 1994). When invaded by pathogens, the epithelium may respond by increasing the production of antimicrobial agents, hypersecretion of mucin, or alteration of mucin glycosylation patterns to subvert microbial attachment (Gill et al. 2013; Jentoft 1990; Schulz et al. 2007). Besides bacteria, phages are also present in these mucus layers. Moreover, phage concentrations in mucus are elevated relative to the surrounding environment (Barr et al. 2013).

Phages in the human gut encode a population of hypervariable proteins (Minot et al. 2012). Approximately half of these encoded proteins possessed the C-type lectin fold previously found in the major tropism-determinant protein at the tip of the *Bordetella* phage BPP-1 tail fibers; six others contained Ig-like domains (Medhekar and Miller 2007). These Ig-like proteins, similar to antibodies and T-cell receptors, can accommodate large sequence variation (Halaby and Mornon 1998). Ig-like domains also are displayed in the structural proteins of many phages (Fraser et al. 2006, 2007). That most of these displayed Ig-like domains are dispensable for phage growth in the laboratory led to the hypothesis that they aid adsorption to their bacterial prey under environmental conditions (McMahon et al. 2005; Fraser et al. 2007). The increased concentration of phage on mucosal surfaces is mediated by weak binding interactions between the variable Ig-like domains on the T4 phage capsid and mucin-displayed glycans (Fig. 1). These Ig-like domains are present in approximately one quarter of the sequenced genomes of tailed DNA phages, i.e., the *Caudovirales*, and are only found in the virion structural proteins and are typically displayed on the virion surface (Fraser et al. 2006). Most of these structurally displayed Ig-like domains are dispensable for phage growth in the laboratory,

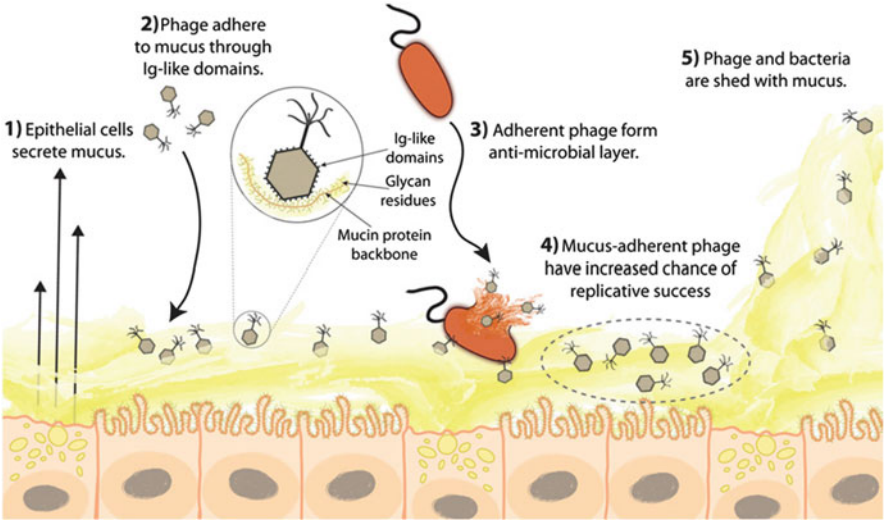

Fig. 1 The bacteriophage adherence to mucus (BAM model). (1) Mucus is produced and secreted by the underlying epithelium. (2) Phage bind variable glycan residues displayed on mucin glycoproteins via variable capsid proteins (e.g., Ig-like domains). (3) Phage adherence creates an antimicrobial layer that reduces bacterial attachment to and colonization of the mucus, which in turn lessens epithelial cell death. (4) Mucus-adherent phages are more likely to encounter bacterial hosts and thus are under positive selection for capsid proteins that enable them to remain in the mucus layer. (5) Continual sloughing of the outer mucus provides a dynamic mucosal environment. (Figure adapted from Barr et al. (2013))

which led to the hypothesis that they aid the phage in the adsorption to their bacterial host under environmental conditions (McMahon et al. 2005; Fraser et al. 2007). This concept was further extended showing that phages use the variable Ig-like protein to adhere to the ever-changing patterns of mucin glycosylation.

Furthermore, the presence of an Ig-like protein displayed on the capsid of T4 phage (highly antigenic outer capsid protein, Hoc) significantly slowed the diffusion of the phage on mucin solutions. Although phage particles, being inanimate and small, act as colloidal particles, they use subdiffusive motions instead of a Brownian motion. This was shown in experiments using phage T4, where the subdiffusive motions of phage T4 in mucus increase the frequency of host encounters. Thus, phage Ig-like domains that bind effectively to the mucus layer would be under a positive selection. These findings lead to the development of the bacteriophage adherence to mucus (BAM) model (Fig. 1), which provides a non-host-derived antibacterial defense (Barr et al. 2013, 2015).

2.3 Phage Transcytosis

The cellular epithelium forms another physical barrier, besides the mucosal surface, that separates the heavily colonized mucosa from the normally sterile regions of the

body. Due to their ubiquity within the epithelial mucus layer, phages are in constant and continual contact with the epithelial layers. The passage of commensal bacteria colonizing the intestine through the mucosa to local lymph nodes and internal organs is termed bacterial translocation and is a critical step in the pathology of various disorders (Guarner and Malagelada 2003; Wiest and Garcia-Tsao 2005). While bacterial translocation is a well-described phenomenon, little is known about the translocation of bacterial viruses.

Low internalization of bacteriophages by enterocytes and other endothelial cells was demonstrated for M13 phages (empty vectors used as a control in phage display) in vivo by Costantini et al. (2009) and in vitro by Ivanenkov et al. (1999). Clathrin-dependent endocytosis was proposed as the pathway, since chloroquine blocked then in vitro uptake (Ivanenkov et al. 1999). Since this type of endocytosis is strictly receptor-mediated, i.e., external objects must be bound to a membrane receptor to be dragged into pits, there is a reason to think that such phage uptake can be a consequence of specific phage-to-epithelium interactions.

Effective (Keller and Engley 1958; Wolochow et al. 1966; Reynaud et al. 1992; Jaiswal et al. 2014; Jun et al. 2014) or ineffective (Duerr et al. 2004; Bruttin and Brüssow 2005; Denou et al. 2009; Oliveira et al. 2009; Letarova et al. 2012; McCallin et al. 2013) systemic dissemination after oral administration has been demonstrated in vivo using nonengineered phages. This suggests that the translocation of natural phage from the gut to circulation is possible. It also shows it is dependent on specific conditions, probably comprising both physiological status of a host (Górski et al. 2006; Majewska et al. 2015) and the characteristics of the phage. Physical parameters of phage particles like their size and shape, can in some extant, influence the phage's ability to penetrate mammalian bodies. The most important factor seems to be the dose, which correlates strongly with the probability that an orally applied phage can be found in circulation or in tissues. This is in line with the fact that phages may differ in their ability to propagate on gut bacteria and this ability may further limit their systemic dissemination after application per os (Oliveira et al. 2009; Weiss et al. 2009).

It is important to consider whether phages can cross the mucosal barrier at sufficient numbers to bypass and interact with the cellular epithelium. It has been demonstrated, in vitro using cell lines, that phages can enter and cross epithelial cell layers by a nonspecific transcytosis mechanism, in an apical-to-basal direction (Nguyen et al. 2017). This transcytosis occurs across different types of epithelial cell layers (e.g., gut, lung, liver, kidney, and brain cells) and for diverse phage types and morphologies (e.g., *Myoviridae*, *Siphoviridae*, and *Podoviridae*). Roughly, 10% of epithelial cells endocytosed phage particles, which appeared to be localized within membrane-bound vesicles, as shown through microscopy analyses. The few cells that did endocytose phage particles appeared to contain large numbers of such vesicles. These endocytosed phage particles traffic via the Golgi apparatus before being functionally exocytosed at the basal cell layer. The transcytosis of phages across epithelial cell layers provides a mechanistic explanation for the systemic occurrence of phages within the human body in the absence of disease (Nguyen et al. 2017).

2.4 Intracellular Interaction of Phages with Mammalian Cells

The direct contact of phages with eukaryotic cells is accomplished through the penetration of phages in higher organisms. It is thus important to know whether these phages can interact or infect eukaryotic cells. Genuine infection seems unlikely, as elements of the phage tail structure only binds to specific receptors on the surfaces of their target bacteria. Intracellular replication is unlikely due to the major differences between eukaryotes and prokaryotes in regard to key intracellular machinery that are essential for translation and replication (Sharp 2001), nevertheless phage gene transcription and translation might be a possibility. Di Giovine et al. (2001) demonstrated that re-engineering of filamentous phage M13 enables it to infect mammalian cells. Although subsequent binding and internalization of the engineered phage was observed, no multiplication of the phage was detected (Di Giovine et al. 2001). Infection aside, as this is outside the scope of this chapter, it is feasible that phages can directly interact with the eukaryotic cell, either extra- or intracellularly. Phages have been attributed as being anti-tumorigenic. Genetic modification of phage M13 (designated WDC-2) led to the production of a tumorigenic phage that was able to bind 93% of the tested tumor cells (Eriksson et al. 2009). Moreover, the administration of the tumor-specific phage initiated the infiltration of neutrophilic granulocytes with subsequent regression of established B16 tumors in mice (Eriksson et al. 2007, 2009). The mechanisms of this phage-induced tumor regression are TLR dependent as no signs of tumor destruction or neutrophil infiltration were observed in tumors of MyD88$^{-/-}$ mice, where TLR signaling was abolished.

Cellular fractionation of epithelial cells, incubated with phage, has been performed by Nguyen et al. (2017) and showed complete perfusion of the eukaryotic cell, with phage particles seen within all endomembrane compartments. Phage particles are likely degraded, shuffled, and transported throughout the cell, providing ample opportunities to interact with eukaryotic cellular components. The question rises whether these interactions occur with the whole phage particle or with specific components of the phage such as the genetic material of the phage (e.g., dsDNA or ssDNA). The specific mechanisms here remain largely uninvestigated but could conceivably include recognition or binding with phage structural proteins or recognition, binding, transcription, or translation of phage nucleic acids (Lengeling et al. 2013). *E. coli* phage PK1A2 can actively bind and penetrate eukaryotic neuroblastoma cells in vitro, through an interaction and binding of cell surface polysialic acid. This cell surface polysialic acid shares structural similarity with the bacterial phage receptor (Lehti et al. 2017). These phage particles were able to be present in these cells for up to 24 h without affecting cell viability. Uptake of these phage particles may also lead to the activation of intracellular immunity, potentially priming the eukaryotic cell into an antimicrobial state or enhancing barrier function (Tam and Jacques 2014). Further research is needed within this area to elucidate intracellular phage-eukaryote interactions.

2.5 Phage-Mammalian Immune Response

Phages clearly interact with nontarget tissues to some extent. For example, at least some phages are taken up from the gastrointestinal tract into the blood and there is reason to think that such uptake can be a consequence of specific phage-to-epithelium interactions, as also appears to be the case given phage interaction with the reticuloendothelial system (Merril 2008; Górski et al. 2006; Duerr et al. 2004). Surprisingly, the early phage workers did not seem to be concerned about the immunological responses to phage therapy (Summers 2001).

2.5.1 Direct Phage-Mammalian Interaction

The fact that phages can directly interact with mammalian cells was first shown in 1940 by Bloch. He observed an accumulation of phages in cancer tissue and inhibition of tumor growth (Bloch 1940). Later on, it was demonstrated that phages can bind cancer cells in vitro and in vivo and attach to the plasma membrane of lymphocytes (Northrop 1958; Kantoch and Mordarski 1958; Wenger et al. 1978; Dąbrowska et al. 2004).

A hypothesis concerning the molecular basis of such interaction was coined by Gorski et al. (2003). He suggested that this interaction occurs through the presence of a Lys-Gly-Asp (KGD) tripeptide motif present in the phage T4 capsid protein gp24. This peptide motif acts as a ligand for the β3 integrins on cells. A genetic modification of phage M13 (designated WDC-2, containing a TRTKLPRLHLQS peptide motif) was reported to lead to the production of a tumor-specific phage that was able to bind 93% of tested tumor cells (Eriksson et al. 2009). Moreover, administration of this tumor-specific phage initiated the infiltration of neutrophilic granulocytes with subsequent regression of established B16 tumors in mice (Eriksson et al. 2007, 2009). The authors observed that the mechanisms of this phage-induced tumor regression were TLR-dependent as no signs of tumor destruction or neutrophil infiltration were observed in tumors of $MyD88^{-/-}$ mice, that lack TLR signaling.

2.5.2 The Cellular Immune Response Against Phages

Phage Can Induce Phagocytosis of Bacteria

It has been postulated that purified phages have anti-inflammatory effects via the suppression of reactive oxygen species (ROS) production and inhibition of NF-κB activity (Górski et al. 2012) and even affecting the cytokine production (Van Belleghem et al. 2017). Neutrophils and monocytes play an important role in host defenses against microbial pathogens, and ROS constitutes to their antimicrobial arsenal. Phagocyte-derived ROS may overwhelm the body's endogenous antioxidant defense mechanism when produced in excess, leading to oxidative stress and causes tissue damage. This forms a major contributing factor to the high mortality rates associated with sepsis and endotoxic shock (El-Benna et al. 2005; Riedemann et al. 2003; Pawlak et al. 1998; Sikora 2002). Hyperresponsiveness and immune cell apoptosis can be induced by ROS, while antioxidants can alleviate this effect (Betten et al. 2004; Malmberg 2004). Not much is not known about the effects

of bacteriophages on the ROS production, whereas the effect of bacteria and eukaryotic viruses on ROS activity have been described. A preliminary study performed suggested that phage T4 influences the phagocyte system (Przerwa et al. 2006), showing that phages could inhibit a ROS production in response to pathogenic bacteria (i.e., *E. coli*). This phenomenon appears to depend on specific phage-bacteria interactions, as phage F-8 (infecting *P. aeruginosa*) did not affect the ROS production induced by *E. coli* on the phagocytic cells. It could be argued that this reduction might be caused by a reduction of bacteria infected and lysed by the phage. This might also explain why phage T4 had an effect and not phage F-8 on the reduction of ROS induced by *E. coli.*

A more comprehensive follow-up study was conducted by stimulating polymorphonuclear leukocytes (PMN) with one of three different R-type *E. coli* strains (i.e., *E. coli* B and *E. coli* J5, both susceptible for T4, and *E. coli* R4, resistant to T4) and LPS derived from these three strains (Miedzybrodzki et al. 2008). The R-type strains were used as their LPS was able to activate the peripheral blood PMN ROS production (Kapp et al. 1987). Through this setup, a reduction in ROS production could be observed in the presence of phage T4 when PMNs were stimulated with either the live bacteria or their LPS. Moreover, this reduction was seen not only when T4 was able to infect the *E. coli* strains but also for the T4 resistant *E. coli* strain. Although the T4 resistant *E. coli* strain induced a less strong ROS production compared to the T4 susceptible strains. These results indicate that phage can directly interact with mammalian cells and could even have anti-inflammatory properties (Miedzybrodzki et al. 2008). Furthermore, the reduction of ROS by phage could be due to the T4 phage tail adhesion gp12, which specifically binds bacterial LPS. This could subsequently lead to a decrease in the availability of LPS and reduce its potential to induce an inflammatory response (Miernikiewicz et al. 2016).

When phages were administered together with the host bacteria, some studies showed that phages were able to stimulate bacterial phagocytosis, and this is attributed to certain opsonization of bacterial cells by phages. In addition, phages can remain active and infective when adsorbed onto the bacteria on intake by granulocytes (Kaur et al. 2014). Therefore, some authors have suggested that during phagocytosis, phages continue lysing the phagocytosed bacteria, helping the activity of phagocytic cells (Górski et al. 2012; Jończyk-Matysiak et al. 2015). Phages might also inhibit the adhesion of platelets and, to some extent, T cells to fibrinogen, a protein which plays an important role in transplant rejection, angiogenesis, and metastasis (Kurzepa et al. 2009).

Phage Innate Immune Response

The innate immune system, particularly the components of the mononuclear phagocyte system (MPS), could form a mechanism for removing phages that are circulating in the human body (Navarro and Muniesa 2017; Górski et al. 2012). Among the mechanisms responsible for the recognition of microbial and viral structures are the TLR (Kawai and Akira 2011). Viral nucleic acids act as PAMPs and are recognized by multiple TLRs. It could thus be postulated that phage DNA might be recognized by TLR9, which is responsible for the recognition of DNA

(Janeway and Medzhitov 2002), after phagocytosis of the phage. The intracellular phages are subsequently uncoated in the cytoplasm and the nucleic acid released. The MPS was credited for the rapid removal of administered wild-type phage λ from the circulatory system in humans (Merril et al. 1973). Moreover Merril et al. (1996) were able to identify certain phage λ mutants that was capable of circumventing the MPS immune response, these mutants prevailed for longer periods in the blood stream than the wild-type phage (Merril et al. 1996).

Immunological studies on the cellular immune response induced against phages have been conducted in recent year, in vitro as well as in vivo. However, it should be noted that many experiments concerning immune responses induced by phages have been carried out using phage lysates. This means that bacterial fragments, proteins, or LPS could still be present in these preparations, making it often difficult to determine whether the observed response can be attributed to the phage.

Mice treated intraperitoneally for 5.5 h with four T4 capsid proteins (i.e., gp23∗, gp24∗, Hoc, and Soc) showed that no cytokines were induced (Miernikiewicz et al. 2013). This lack of cytokine production might be explained by the early time point by which the mice were tested for the presence of cytokines or through the rapid removal of the phages from circulation. Another immunological study evaluated the cytokine production in mice induced by phage T7, after the mice were fed for 10 days with phage T7. A single dose was fed every 24 h, although an exact concentration was not provided by the authors (Park et al. 2014). Although this study had its limitations, the authors were able to demonstrate that phage T7 induced a very minor increase of inflammatory cytokine production in mice, but no histological changes were observed in the tissues of the gastrointestinal organs. As no caution was taken to the presence of endotoxins, the immune responses that were observed could be, partially, due to endotoxin contamination of the used phage stock.

2.5.3 Cytokine Response Against the Phage

Phages have the potential to induce cytokine responses, as indicated by several studies, often these studies make use of phage preparations that where not fully purified from bacterial endotoxins or proteins. The effect of phages on the production of tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) in human serum has also been studied, as well as the ability of blood cells to produce these cytokines in culture. The authors of this study used blood derived from 51 patients with long-term suppurative infections of various tissues and organs caused by drug-resistant strains of bacteria (Weber-Dąbrowska et al. 2000). These patients were treated with phages and blood samples were collected and tested for the presence of TNF-α and IL-6. The authors were able to observe a reduction in the production of these cytokines after long-term treatment (i.e., 21 days). Unfortunately, the authors were not able to show whether the observed immune response was due to the presence of the phage or due to the reduction of the bacterial count through their lysis by the phage. On the other hand, analysis of the cytokine production of mice treated intraperitoneally for 5.5 h with either a highly purified phage T4 or four phage T4 capsid proteins (i.e., gp23∗, gp24∗, Hoc, and Soc) showed that no inflammatory mediating cytokines were detected (Miernikiewicz et al. 2013).

The maturation of dendritic cells can be induced by *Cronobacter sakazakii* phage ES2 through the induction of the expression of IL12p40 via NF-κB signaling (An et al. 2014). This maturation presumably happens after the phagocytosis of the phage by the dendritic cells. The maturation of these dendritic cells play an important role in generating a cell-mediated immune response and subsequently in the production of phage specific antibodies. It has even been shown that phages have conserved anti-inflammatory properties. Using highly purified phages targeting two different pathogens, *P. aeruginosa* and *S. aureus,* it was shown that these phages induced very comparable immune responses (Van Belleghem et al. 2017). Especially the upregulation of the anti-inflammatory markers suppressor of cytokine signaling 3 (SOSC3), IL-1 receptor antagonist (IL1RN), and IL-6 was very similar between the different phages. These anti-inflammatory phage properties are also in line with some previous observations suggesting an immunosuppressive effect of phages in murine in vivo models of xenografts (Gorski et al. 2016). It should however be emphasized, that potential anti-inflammatory or immunosuppressive action of bacteriophages cannot be considered as comparable to physiological effects exerted by well-known anti-inflammatory or immunosuppressive drugs. Phage activity seems to be much weaker and it rather impacts the ecological balance in microbiota inside bodies than the physiological status of the organisms.

2.5.4 Anti-phage Antibody Production

It is very easy to generate phage antiserums by immunization of humans or animals with phage lysates (Górski et al. 2012; Bacon et al. 1986; Puig et al. 2001) and forms one of the major consequences of using phage as therapeutics (Kamme 1973; Smith et al. 1987; Górski et al. 2012). Soon after the discovery of phages, antibodies against the phage could be observed in humans and animals (Jerne 1952, 1956). Natural occurring bacteriophages are able to induce a humoral immunity, i.e., phage-neutralizing antibodies that were not stimulated by phage treatment were detected in the sera of different species (e.g., human) (Dabrowska et al. 2005). In fact, immunization with bacteriophage ϕX174 has been used extensively to diagnose and monitor primary and secondary immunodeficiencies since the 1970s without reported adverse events, even in patients in whom prolonged circulation of the phage in the bloodstream was observed (Ochs et al. 1971; Rubinstein et al. 2000; Shearer et al. 2001; Fogelman et al. 2000). Besides, the humoral response does not follow a simple schema of induction. It depends on the route of administration and on individual features of the phage. Moreover, it depends on the dose and application schedule and possibly on other, not yet specified, features (Górski et al. 2006, 2012; Dąbrowska et al. 2014; Łusiak-Szelachowska et al. 2014). The humoral response induced against phages can be devastating (Huff et al. 2010), but it has also been reported that the anti-phage activity of serum does not exclude a favorable result of phage therapy in humans (Łusiak-Szelachowska et al. 2014).

Initial safety studies of phage T4 performed on humans revealed no antibody induction in phage-related volunteers at all (Bruttin and Brüssow 2005). Evaluation of serum derived from 50 healthy volunteers who had never been subjected to phage therapy or were involved in phage work showed that 82% significantly decreased

phage T4 activity (Dąbrowska et al. 2014). In these positive sera, natural IgG antibodies specific to the phage proteins gp23∗, gp24∗, Hoc, and Soc were identified. It is not the highly antigenic outer capsid (Hoc) protein that induced the most of the humoral responses, but the antibodies specific to the major capsid protein gp23∗ (Dąbrowska et al. 2014).

The production of IgG, IgA, and IgM in human patients undergoing phage therapy was conducted where 20 patients were treated with the MS-1 phage cocktail (containing three lytic *S. aureus* phages, 676/Z, A5/80, and P4/6409) either orally or locally (Żaczek et al. 2016). Few patients produced elevated levels of IgG or IgM. Nevertheless, the presence of anti-phage antibodies did not translate into an unsatisfactory clinical result of the phage therapy. The small time-scale by which the patients were treated could explain the low antibody production against the phage cocktail. On the other hand, the elevated antibody production in a few patients could be due to a previous encounter of one of the phages used in the cocktail and the presence of an immunological memory.

Studies concerning the anti-phage antibody production in humans are rare, nevertheless an extensive study on the antibody production in mice are more common. The antibody production against a single phage (i.e., *E. coli* phage T4) in mice has been studied over a time period of 240 days (Majewska et al. 2015). The long-term oral treatment of mice with phage T4 demonstrated a humoral response, in contrast to previous human trials where no such responses were detected (Bruttin and Brüssow 2005). This response emerged by the secretion of IgA in the gut lumen but also as an IgG production in the blood (Majewska et al. 2015). The intensity of this response and the time necessary for its induction depend on the exposure to phage antigens, which is related to the phage dose. The specific IgA production seemed to be the limiting factor of phage activity in the. This was shown by the presence of phages in the feces when the secretory levels of IgA were low. When the IgA level, around day 80, increased, there were no active phages present in the feces.

The induction of serum IgG in these mice suggested that phages can translocate from the gut lumen to the circulation. This is further strengthened by the ability of phage to transcytose epithelial layers (Nguyen et al. 2017). It was even possible to isolate phages from murine blood after application of high phage doses (4×10^9 pfu/ml of drinking water).

Additionally, it is interesting to evaluate the immune responses induced to individual phage proteins, besides the whole phage particle. Majewska et al. demonstrated that phage T4 Hoc protein and gp12 strongly stimulated the IgG and IgA antibody production in the blood and gut, respectively, while gp23∗, gp24∗, and Soc induced low responses.

2.6 Relevance of Phage Host Immune Responses

It is becoming more evident that phages can interact with a mammalian or human host in diverse ways. The adherence of phages to mucosal surfaces provides an additional antimicrobial defense (Barr et al. 2013, 2015). The inclusion of symbiotic

phages within the mucosal surface provides the eukaryotic host with additional potential benefits, whereby the phages offer a selective antimicrobial defense. This operates at a much finer spectrum than some other broad-spectrum host secretions, such as the antibacterial lectin RegIII-ϒ (Vaishnava et al. 2011). The phages obtain a higher probability to contact epithelial cells and transcytose through them when they bind to the mucosal layers. The potential of phages to be internalized by eukaryotic cells raises the question whether they can induce intracellular immune responses. Might it additionally be possible that after internalization these phages can interact or even infect mitochondria? Although the presence of phages in mammalian cells has been observed (Nguyen et al. 2017; Di Giovine et al. 2001), replication of these viruses in theses cell types has not yet been observed.

Important implications for the use of phage in therapeutic settings are the observations that they can induce certain (anti-inflammatory) cytokines (Van Belleghem et al. 2017). The effect of phage anti-inflammatory properties on the outcome of a bacterial infection has been highlighted in in silico models. These phage immune responses could have a much broader effect; they could not only lead to a rapid clearing of a bacterial infection but could also lead to a higher persistence of the bacterial infection. Additionally, phages could even be used as nanocarriers for targeted drug delivery or display selected antigens (Majewska et al. 2015; Eriksson et al. 2007, 2009).

Phages can have a direct impact on sepsis, where the lytic activity of the phage can reduce the bacterial burden. The immunomodulating properties of the phage could lead to a, partial, dampening of the inflammatory response induced by the bacteria. The immune response could be further altered by using phage or phage-derived proteins that interact with bacterial components, e.g., endotoxins (Miernikiewicz et al. 2016). These phage anti-inflammatory properties could be exploited in the future to develop phage protein-based anti-inflammatory agents, leading to a possible new type of anti-inflammatory drugs with a new mode of action. These phage or phage-derived proteins could potentially possess less side effects compared to the classic nonsteroid anti-inflammatory drugs (NSAIDs).

3 Conclusion

It is becoming evident that phages can directly interact with mammalian cells and interact with the immune system. Current data indicates that high phage concentrations induce immune responses whereas low phage concentrations have less to none observable immune responses. If the anti-inflammatory property of phages is widespread between the different types of phages, this can have a profound effect on understanding different bacterial pathologies (e.g., *P. aeruginosa* infection of cystic fibrosis patients) but also further add to our current understanding of phage therapy. The study of phage-mammalian cell interaction may alter our view of the function of phages in the microbiota, showing the potential of phage anti-inflammatory properties to more rapidly remove a bacterial infection or lead to a

more severe infection. It is thus becoming clear that the study of phage-mammalian interactions leads to many new, exciting study opportunities.

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Humoral Immune Response to Phage-Based Therapeutics

Maciej Żaczek, Marzanna Łusiak-Szelachowska, Beata Weber-Dąbrowska, Ryszard Międzybrodzki, Wojciech Fortuna, Paweł Rogóż, Sławomir Letkiewicz, and Andrzej Górski

M. Żaczek (✉) · M. Łusiak-Szelachowska
Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland
e-mail: maciej.zaczek@hirszfeld.pl

B. Weber-Dąbrowska · A. Górski
Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

R. Międzybrodzki
Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland

W. Fortuna
Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Department of Neurosurgery, Wrocław Medical University, Wrocław, Poland

P. Rogóż
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

S. Letkiewicz
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (HIIET PAS), Wrocław, Poland

Medical Sciences Institute, Katowice School of Economics, Katowice, Poland

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1 Introduction

The nature of phage particles, which consist of nucleic acid and an immunogenic protein coat, clearly indicates that there must be a correlation between induction of neutralizing antibodies in the host and its exposure to phages (Van Belleghem et al. 2019; Krut and Bekeredjian-Ding 2018). These immunogenic properties of phages encourage authors to suggest possible interactions and further negative effects of human antiphage antibodies on treatment results (Sulakvelidze et al. 2001; Górski et al. 2012; Ly-Chatain 2014). Hedstrom and Kamme reported anti-*Staphylococcus aureus* phage antibodies in normal sera, whose titers increased in staphylococcal infections; this might have been caused by an immune response to phages released from lysogenic microorganisms (Hedstrom and Kamme 1973; Kamme 1973).

One of the earliest reports describing interactions among phage therapeutics and the humoral immune response in patients comes from the 1980s. Nonetheless, due to a limited number of centers conducting sanctioned, government-approved phage treatment with patients undergoing that type of experimental therapy, the results available in peer-reviewed journals have remained highly scarce for over two decades. In recent years, as research on phages has become extensive, a growing number of sources have published new data, with the vast majority coming from the Phage Therapy Unit (PTU) of the Medical Centre of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland (Górski et al. 2012; Łusiak-Szelachowska et al. 2014, 2016, 2017; Żaczek et al. 2016). No doubt, these days there is a growing publishing activity concerning phage interactions with the mammalian adaptive immune response toward therapeutic phage application.

Initial Polish reports (Kucharewicz-Krukowska and Ślopek 1987) presented data on the higher level of antiphage antibodies detected on the tenth day of therapy in 54.4% of patients treated orally. However, only 5.4% of them indicated high antiphage activity of sera (AAS). In the same study, almost 23% of examined patients had confirmed the presence of antiphage antibodies in their sera before treatment. Those antibodies, so-called "natural antibodies" (Górski et al. 2012), maybe a result of the high prevalence of bacterial viruses, which are the most abundant organisms in the biosphere and represent a ubiquitous feature of prokaryotic existence (Clokie et al. 2011). In fact, phages are well known for their presence even in such unprecedented niches as water supply systems (Weber-Dąbrowska et al. 2014), and therefore there is a high possibility of exposure to naturally occurring phages. Similar findings were described by Dąbrowska et al. (2014). Over 80% of healthy individuals, who had never been subjected to phage therapy (PT), were found to have antiphage (anti-T4) antibodies in their sera. Bochkareva et al. (2017) identified an increased level of IgG antibodies against *Acinetobacter baumannii* and *Pseudomonas aeruginosa* phages in healthy individuals whose work is associated with phages.

In a Georgian study, the level of phage neutralizing antibodies was measured after the fifth round of treatment with Pyophage cocktail containing phages targeting *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Proteus*, *P. aeruginosa*, and *S. aureus* (Villarroel et al. 2017) in a 7-year-old girl suffering from cystic fibrosis. The phage therapy was applied through inhalation using a nebulizer. In spite of multiple

rounds of treatment, anti-staphylococcal phage antibodies were detected at a low level with no antibodies directed toward *P. aeruginosa* phages (Kutter et al. 2014). It could be a result of impaired immunological function related to aggressive antibiotic therapy and other standard therapies such as anti-mucus medications and enzymes described by the authors in regard to this case.

Bruttin and Brussow (2005) suggested that triggering the immune response requires an application of phage preparations in high doses. At a dose of 10^5 pfu/ml applied orally no phage particle was detected in the human bloodstream, and thus no increased levels of anti-T4-specific antibodies were observed in all examined cases. Pescovitz et al. (2011) applied φX174 phage intravenously (4 times in a dose of 2×10^9 pfu/kg body weight) to immunize healthy subjects. Phage particles circulated 3–4 days after application until IgM antiphage antibodies completely cleared them before day 7. In comparison, healthy volunteers immunized intravenously with φX174 phage (0.02 mL/kg body weight with a standard preparation containing 10^{11} pfu/ml) cleared phage particles by 1 week after primary immunization. All had normal primary and secondary antibody responses, including immunologic memory amplification and switch from IgM to IgG antibody production. Of note, volunteers were exposed to the 8-month Antarctic winter-over model of spaceflight that was intended to alter human antibody responses. In spite of unfavorable conditions, no alterations were observed in any of the examined volunteers (Shearer et al. 2001).

This short introduction leaves no doubt in regard to the constant interaction between both naturally occurring and therapeutic phage particles and the adaptive immune system of higher organisms. The main goal of this chapter is to clarify to what extent such specific communication may impact the clinical outcome of the phage treatment, as it is of great importance in modern medicine.

2 Animal Studies

The relatively small number of patients subjected to phage treatment seriously limits the possibilities of testing the immunogenicity of phage therapeutics in human subjects. Animal models are not only more widely available but can also be planned more accurately. Conducting experimental treatment in humans faces several challenges inseparably connected with the number of patients' visits, duration of the treatment or even its termination by a physician or patients themselves in the case where PT did not meet their expectations (Żaczek et al. 2016).

Jerne described the presence of a specific antibody against bacteriophage T4 in normal mouse serum in the 1950s (Jerne 1956). Phage-mediated induction of the humoral response in animals was described in the 1960s in guinea pigs and rabbits immunized with *E. coli* phage φX174. In mice, the induction of anti-φX174 antibodies was dose dependent. Mice bred conventionally showed a higher level of immune response to those kept as germ-free animals (Krut and Bekeredjian-Ding 2018). Despite this fact, Geier et al. (1973) observed relatively rapid phage neutralization in germ-free models as well. Such mice fed with a high oral dose of 10^{12} pfu/

mouse of phage lambda showed only between 10 and 1000 pfu/ml of blood directly after exposure. Within 48 hours, phage particles were cleared from the blood and the peritoneum. Geier assumed that phage clearance in germ-free mice was caused mainly by the reticuloendothelial system (RES). However, a slight, twofold increase in antibody production in germ-free mice was observed after exposure to φX174 phage injected intraperitoneally by Stashak et al. (1970). Seemingly, the constant stimulation of the immune system by phages circulating in nature might be the factor enhancing the antiphage humoral response, but such adaptive immunity can quickly develop in organisms with no previous exposure to viral agents whatsoever. Noteworthy, the significance of anti-phage antibodies induced by virus administration is still unclear (Górski et al. 2007).

E. coli φX174 phage was utilized by Andrews et al. (1997) to immunize baboons. Phage was administered intravenously at a dose of 10^{10} PFU/kg of body weight in both healthy animals and those transplanted with allogeneic $CD34^{+}$ lymphocyte-depleted marrow cells. All showed normal responses to immunization with phage, indicating similarly increased levels of antibodies after primary and secondary immunization. Moreover, the typical pattern was noted in which IgM isotype switched to IgG isotype after the secondary immunization.

Antibodies specific to T4 phage have been detected in numerous murine models. A long-term study on T4 phage applied orally in very high doses (10^{9} pfu/ml, which gives 10^{10} pfu/mouse daily) showed a significant increase of IgG in sera and IgA in feces after 36 days and over 2 months, respectively. Mice treated with a phage dose of 10^{8} pfu/ml revealed a nearly nine times lower level of immunization on the 100th day of the experiment (Majewska et al. 2015).

A dose of 10^{9} pfu/mouse was necessary to detect an increase in IgG levels in mice against *P. aeruginosa* phages after single intraperitoneal injection, with the maximum reached after 30–40 days of administration (Wang et al. 2006). However, the author emphasizes that levels of the antibody against the phage were not significantly elevated at the time when the treated animals were protected by the phage. IgG levels declined gradually after 40 days. Biswas et al. (2002) showed that after application of 10^{10} pfu/mouse, anti-phage IgM and IgG only became detectable after three repetitive phage injections when treating acute bacteremia caused by *E. faecium*. Mice inoculated intraperitoneally with *P. aeruginosa* phage (10^{10} pfu/mouse) showed a boosted primary response (IgM) 5–10 days after phage application and a subsequent increased level of IgG, which was maintained at the maximum level 60 days after the beginning of the study. The authors conclude that such observations constitute a pattern typical for many other antigens (Hodyra-Stefaniak et al. 2015). Contrary to such expectations, Majewska et al. (2015) did not note an increase of IgM antibodies in mice before the IgG boost. A possible explanation lies in the route of phage administration in both studies (oral vs. intraperitoneal). It has been widely discussed that the way of phage application plays a crucial role in a humoral immune response, and hence adaptive immunity cannot be reduced to one simple schema both in animals and in humans.

Jain et al. (2017) utilized phage lysates to immunize guinea pigs. Adult animals were injected subcutaneously with 400 μl of *Brucella abortus* S19 phage lysate (10^{7}

pfu/ml). The titer of IgG antibodies reached a maximum level on the 45th day of treatment for all vaccinated groups and was significantly higher when compared to unvaccinated control groups. In the next step, immunized guinea pigs were infected intraperitoneally with the virulent *B. abortus* 544 strain. After 6 weeks, no growth of *B. abortus* 544 from the spleen of infected animals was observed. In a further investigation performed by the same author, the guinea pig sera samples, collected on the 90th day after immunization, were evaluated for protective efficacy in mice. It turned out that the passive transfer of antibodies to mice protected the animals against a bacterial pathogen. It indicates that antibodies themselves were capable of inhibiting the initial establishment of infection, which constitutes the crucial phase in pathogenesis. The author drew the conclusion that phage lysate, consisting of bacterial cell debris, induces the desirable protective response directed against the pathogen and is the key to developing a cross-protective immunizing preparation.

Although studies performed on animals provide us with essential data about the antiphage humoral immune response, those studies cannot be directly extrapolated to human models (Żaczek et al. 2016). Contrary to the tested animals, approximately 50% of patients undergoing PT have different types of immunodeficiencies (Łusiak-Szelachowska et al. 2017). Furthermore, it has been demonstrated that 90% of drugs successfully tested on animals failed in human trials (Shanks et al. 2009).

3 Phage Therapy Unit Experience

As mentioned previously, only a few centers in the world currently conduct government-approved phage treatment in humans. The PTU of the Medical Centre of the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy in Wrocław, Poland so far has admitted over 500 patients. It serves as a model for other centers in the world facing the issue of phage treatment as a "last resort method" and the ethical need to help suffering patients in whom antibiotic therapy and other standard therapeutic methods failed or cannot be applied. However, constant access to all patients during PT is sometimes limited. Qualified patients are not hospitalized at the PTU, with the number of visits varying from 1 to over 10, depending on the course of therapy (Międzybrodzki et al. 2012). Therefore, blood samples required for further immunological studies can be collected only during those visits. Such irregularity can greatly complicate the interpretation of the clinical findings. In spite of those concerns, our reports provide the most comprehensive set of standardized data on the humoral immune response to phage therapeutics in humans subjected to experimental PT.

3.1 Antiphage Activity of Human Sera

Antiphage activity in patients' sera (AAS) has been measured at the PTU since 2010 as a standard procedure. Additionally, sera of some patients have been tested using enzyme-linked immunosorbent assay (ELISA) to demonstrate whether PT can

contribute to the increase in antiphage antibody levels (Łusiak-Szelachowska et al. 2014; Żaczek et al. 2016).

Phage neutralization by human sera was calculated by phage inactivation (*K* rate), where *K* less than 5 was considered as weak phage neutralization, *K* between 5 and 18 as a medium level, and above 18 as a high level of phage neutralization.

Among 160 patients examined in the years 2010–2016, all indicated very weak AAS before PT ($K \leq 0.23$). Similar results were obtained in a group of healthy donors tested against various staphylococcal phages, both single phage preparations and phage cocktails (all 60 volunteers had $K \leq 1.73$). The differences occur when we analyze antiphage activity during PT, with particular emphasis on the route of administration, mono- or polyvalent phage therapeutics used in the course of therapy, the type of infection, duration of the treatment, and the phage dose (Łusiak-Szelachowska et al. 2014, 2016, 2017; Żaczek et al. 2016). Medium and high inactivation of phages by patients' sera decreased after therapy (Łusiak-Szelachowska et al. 2014).

3.2 Level of Antiphage Antibodies and Their Neutralizing Properties

A large part of the research on the humoral immune response at the PTU has been performed with a focus on neutralizing activity of human sera in response to PT (measured by the neutralization test). To obtain a fuller scope of an adaptive immune response in patients, some of them were also tested using the ELISA technique to quantitatively measure the level of antiphage antibodies (IgG, IgA, IgM isotypes) described as antibody units (AU) induced after phage application. One must be aware that phage–antibody interactions do not necessarily mean phage inactivation (Górski et al. 2012). Our results in this matter constitute probably the first report where the production of antibodies was compared to their neutralizing activity and the overall clinical outcome of PT (Łusiak-Szelachowska et al. 2014; Żaczek et al. 2016).

Twenty adult patients with various infections (mostly with bone and sinus infections) received the staphylococcal phage cocktail MS-1. Due to the individual character of PT in each patient and following difficulties with standardization of the procedure, we focused on sera samples obtained before therapy and on samples giving the maximum levels of absorbance in the ELISA test during the whole course of treatment.

A clear correlation between the increased level of antiphage antibodies revealed in ELISA and a higher rate of phage inactivation measured by neutralization test was observed for IgG and IgM antibodies. Of note, in almost every examined case, the boost of IgG and IgM was inseparable. Unfortunately, various timing of blood sampling made it impossible to observe a pattern typical for adaptive immunity (IgM antibodies involved in primary response and following increase of IgG levels). Nevertheless, in four patients exceptionally high levels of IgM (over 1000 AU) were detected during a course of local or local and oral treatment. Levels of IgG were

characterized by the greatest diversity during PT (from 30 to over 600 AU) despite the fact that all patients were treated with the same phage cocktail. A possible explanation lies in the different immune status of each patient with regard to different medical history. Undetectable levels of IgA were observed in sera in the majority of patients both before and during phage treatment, with the highest values elicited in two individuals suffering from bone infections, treated locally and locally and orally (34 and 12 AU, respectively). This phenomenon occurred even in patients with sinus infections, where the mucosal immune system was stimulated by local administration of phages. Apparently, IgA antibodies in sera were not involved in the humoral immune response during treatment, as even long-term exposure (several months) to phage antigens did not induce secretion of IgA (Żaczek et al. 2016). These results stand in opposition to studies performed on animals. Clark and March (2004) observed significant systemic (IgG and IgA antibodies) and local mucosal (IgA antibodies) immune responses against phage coat protein following oral delivery in rodents. IgA induction in mice was also observed by Majewska et al. (2015) after 79 days of exposure to T4 phage applied orally.

3.3 Factors Influencing the Humoral Immune Response

Our observations suggest that the large variation observed in levels of AAS is caused by several factors occurring both before and during PT (Fig. 1). Besides the evident impact those factors have on phage neutralization in patients, they might also have an indirect influence on the final results of PT.

3.3.1 Type of Infection

All patients with $K > 18$ treated locally with the *S. aureus* MS-1 phage cocktail had bone infections, indicating that the type of infection is at least indirectly related to the high AAS (Łusiak-Szelachowska et al. 2014). One must be aware that the type of infection is largely associated with other factors that influence the strength of the humoral immune response, primarily with route of administration. For instance, patients with bone infections had phage preparations administered mostly locally (compresses, fistula irrigation, sinus irrigation) due to the difficult penetration of phages within bone tissue. Oral and intrarectal administration (mainly applied in soft tissue infections and genitourinary tract infections) induced a weak humoral immune

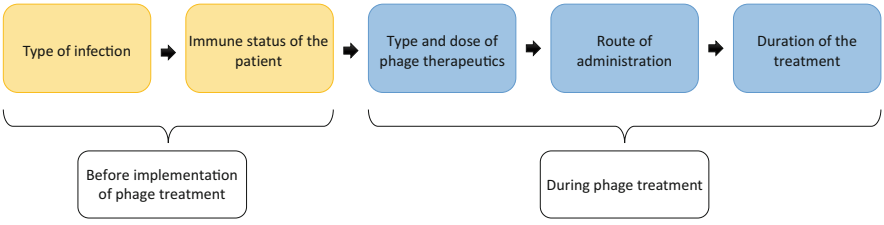

Fig. 1 Factors influencing AAS in patients undergoing PT

response irrespectively of the type of infection. In the group of 8 patients with urogenital tract infections, a low level of phage neutralization ($K \leq 2.39$) was observed 3 days after vaginal and intravesical phage application.

3.3.2 Route of Administration

The way phages are administered has been proved to be one of the most important factors in terms of the strength of AAS. Patients with oral or intrarectal administration as the only way of phage application showed a low level of AAS during PT with an outcome that is not related to the type of infection and phage preparation (monotherapy, phage cocktail, lysates, or purified phage preparations). Combined route of administration (oral and local) revealed comparable results. Among 21 patients only 2 of them indicated high antiphage activity of their sera ($K > 18$); the remaining 19 showed weak neutralization ($K \leq 4.53$) (Łusiak-Szelachowska et al. 2014). Patients administered orally and locally with staphylococcal phages had low AAS in the group treated with phage cocktail, whereas 12.5% showed high AAS in a course of monotherapy (Łusiak-Szelachowska et al. 2016). These results have not been confirmed in patients treated with staphylococcal phages entirely locally. In such cases, high AAS was observed in nearly 43% of patients using MS-1 phage cocktail that consists of three *Staphylococcus* phages (676/Z, A5/80, and P4/6409) and only in 17% of patients undergoing local monotherapy. In the years 2010–2013, sera of 56 patients administered phages locally were examined. In 13 cases, sera had high antiphage activity from 15 to 60 days of PT, while in 5 cases a medium level of AAS was observed. Even though local application clearly showed a higher induction of phage neutralization in some cases, the majority of patients (38 cases) still had a low level of AAS ($K \leq 3.7$). These results indicate the existence of important dissimilarities among tested sera samples.

A recently described case of a patient treated by intravenous phage administration also suggests the lack of clear association between production of antibody to phages and therapy outcome. Although antibody responses were not evaluated in this case, it is very likely that the patient who received prolonged intravenous PT developed antibodies, yet the therapy outcome was excellent (Schooley et al. 2017).

3.3.3 Immune Status of the Patient

It has been noted that patients qualified for PT have impaired immunity (Górski et al. 2012) possibly caused by chronic infections and the long-term previous standard therapies. Those patients might indicate lower levels of antiphage antibodies and AAS, which was first demonstrated almost 50 years ago (Ochs et al. 1971). In such cases, phages are believed to have longer viability after application (Borysowski and Górski 2008). Their translocation in patients might be also easier to achieve, as the gut barrier in disease is more permeable to microorganisms (Górski et al. 2006). Longer viability was described in a mouse study (Roach et al. 2017). Immunodeficiency did significantly ($p < 0.001$) prolong persistence of phage in the mouse lungs after a single inhaled monophage dose (10^9 pfu/mouse) was applied. Impaired antiphage antibody responses were observed in patients with asymptomatic HIV-1 infections (Rubinstein et al. 2000). The authors assumed that the blunted immune

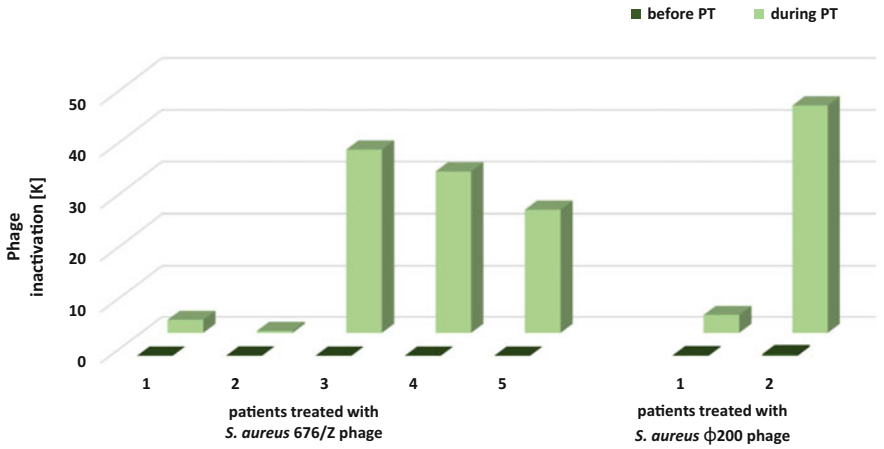

Fig. 2 Phage inactivation in patients with bone infection during local PT. *Description*. AAS was estimated as the rate of phage inactivation (K) during a 30-min reaction at 37 °C of phage with diluted serum as described earlier (Łusiak-Szelachowska et al. 2017). The phage neutralization by human sera was calculated by phage inactivation (K rate), where K less than 5 was considered as weak phage neutralization, K between 5 and 18 as a medium level, and above 18 as a high level of phage neutralization. The K rate was estimated using the equation $K = 2.3 \times (D/T) \times \log$ (P0/Pt), where K is the rate of phage inactivation, D is the reciprocal of the serum dilution, T stands for the time in minutes during which the reaction occurred (30 min in this case), P0 is the phage titer at the start of the reaction and Pt is the phage titer at time T

response to φX174 phage was the effect of concomitant short courses of zidovudine (ZDV), an antiretroviral medication used to prevent and treat HIV/AIDS. Rituximab, an antibody against the B-cell CD20 antigen, was implicated in the inhibition of the antibody response against phage φX174 too (Huh et al. 2019).

In our study, 15 patients treated at the PTU with monovalent *S. aureus* phages had a different level of AAS even in the group characterized by the same type of infection and route of phage application (Figs. 2, 3, and 4). On average, 47% of patients showed weak phage neutralization ($K < 5$), whereas 53% of them showed a high level of AAS ($K > 18$). A likely explanation of this phenomenon lies in the different immunological status of each patient.

Interesting data come from Russian studies. Antiphage antibody production was evaluated in infants and children up to 15 years of age after oral phage treatment. Interestingly, patients less than 1 month of age did not produce antibodies until 30–60 days after treatment. After that time antiphage antibodies were detected in only 20% of cases. Children aged 1 month to 1 year showed antibody presence in 4–60% of cases after 30–60 days oftreatment and for children aged 1–15 years the rate was up to 100%. It may indicate limited immune defenses in the fetus and the neonate and related to this possibly higher efficacy of PT (Furfaro et al. 2018). It leads to the conclusion that the patient's age may constitute another factor influencing the humoral immune response to applied phages. However, in this context age reflects the immune status of the patient (Fig. 1), which can also be affected by individual

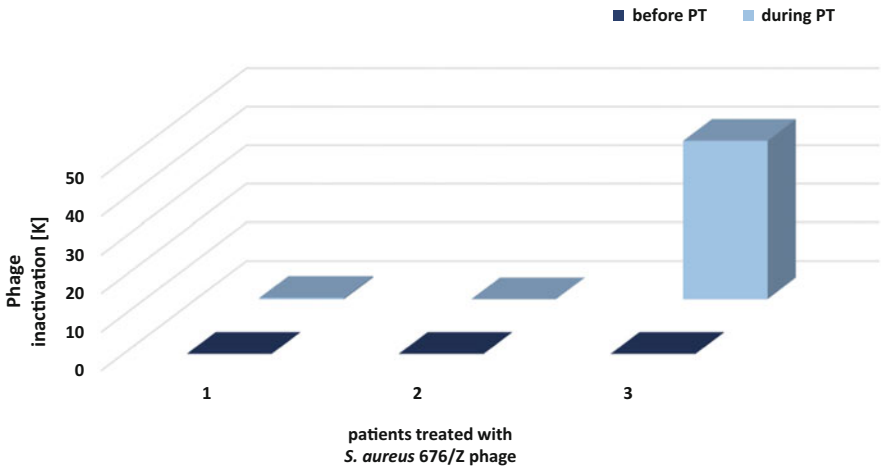

Fig. 3 Phage inactivation in patients with soft tissue infection during local and oral PT

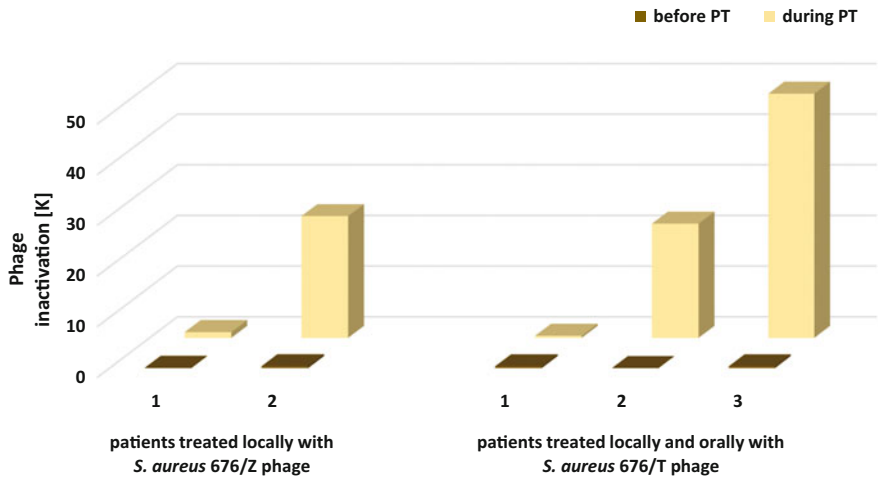

Fig. 4 Phage inactivation in patients with upper respiratory tract infection during local or local and oral PT

medical history as mentioned earlier in this section. Phage therapy based on the phage preparations from the PTU has been applied to children in Poland. Phage lysates have been administered to patients ranging from neonates to 20-year-olds with positive effects in the majority of cases. No humoral immunity studies in those patients have been performed though (Fortuna et al. 2008). On the other hand, Roach et al. (2017) suggested the necessity of synergy between the host immune system and bacteriophage for successful PT. The role of the recovery of the immune system in combating the disease was also mentioned by Górski et al. (2017).

3.3.4 Type and Dose of Phage Therapeutics

The mammalian adaptive immune response is characterized by extreme specificity in terms of antigen recognition. Some authors point to the differences among structural proteins of phage particles as a factor playing a role in activating the antibody response during PT (Krut and Bekeredjian-Ding 2018) and a major factor determining phage fate in vivo (Hodyra-Stefaniak et al. 2015; Majewska et al. 2015). Neutralizing IgG antibodies directed against T4-like phages showed affinity to several phage head proteins with high induction of anti-Hoc and anti-gp23 antibodies against T4 phage in mice. Production of gp24-specific antibodies was weak, while the weakest response was induced by Soc protein. Specific anti-gp23 and anti-Hoc antibodies substantially decreased T4 phage activity in vitro and to some extent in vivo (Dąbrowska et al. 2014). Among patients treated with MS-1 phage cocktail, consisting of three *S. aureus* phages, the most immunogenic was A5/80 phage, which elicited noticeably the highest level of IgG antibodies in two patients with a similar course of treatment (Żaczek et al. 2016). These results are in line with a previous report (Łusiak-Szelachowska et al. 2014) where the level of antiphage antibodies detected by ELISA was much higher for the *S. aureus* A3/R phage than for the *S. aureus* 676/Z phage. Differences in absorbance (450 nm) applied to all 19 examined patients' sera both before and during PT. The same author described the cross-reactivity of sera collected from 7 patients treated with specific staphylococcal phages. The high AAS to phages used in PT in 4 patients was not observed against other *S. aureus* and *E. coli* phages. In the 3 remaining patients, the high AAS was specific to all staphylococcal phages, but not to *E. coli* phage. The obtained discrepancies might be a result of both differences in the structure of proteins attached to the capsid surface and diverse immunological status among patients. Undoubtedly, protein expression is a characteristic feature of the individual phage, making antibody induction highly variable (Krut and Bekeredjian-Ding 2018).

Interesting knowledge for further development of PT was obtained in patients administered with purified phage preparations deprived of all contaminants that are present in more broadly used phage lysates. Such purified phage therapeutics are expected to be more suitable for patients and thus are considered in future applications. Phage lysates may contain macromolecules derived from the host bacteria and culture medium. Their presence raises an important concern regarding the safety of the therapy (Szermer-Olearnik and Boratyński 2015). Furthermore, some components of bacterial cells present in phage lysates, especially lipopolysaccharides (LPS), are known for their stimulating activity toward antiphage antibodies (Górski et al. 2012). Our preliminary results revealed the opposite outcome. Patients taking a purified phage cocktail showed the highest level of both AAS and AU measured in the ELISA test during treatment. These results clearly indicate that titers of purified phage preparations (about four orders of magnitude higher compared to therapeutic phage lysates) were responsible for the increased immune response. Apparently, the phage dose, not the degree of purification of phage solution, plays the most important role in immunogenicity of phage therapeutics during PT (Żaczek et al. 2016). Analyzing values of C-reactive protein

(CRP), a classic inflammatory marker used for evaluating patient's clinical status, reveals further interesting facts. Surprisingly, patients receiving purified phage preparations showed increased values of CRP during treatment compared to the values before the implementation of PT. These results are in contrast with the data obtained for patients treated with phage lysates, in whom CRP remained constant during treatment or was even lower when compared to the level prior to PT (Żaczek et al. 2016).

The value of purified phage preparations requires confirmation, as they appear to be more immunogenic and preliminary observations do not provide clear evidence for their therapeutic superiority over non-purified preparations. So far, no clear therapeutic advantages of using purified preparations have been noted (Łusiak-Szelachowska et al. 2017). Phage preparations intended for treating infections in humans should be safe, sterile, and endotoxin-free (Weber-Dąbrowska et al. 2016), and these requirements do not exclude the use of lysates that can be effectively deprived of endotoxins (Żaczek et al. 2016).

3.3.5 Duration of Treatment

Our recent studies reveal a positive correlation between the duration of PT and the level of phage inactivation. Spearman's rank correlation coefficient for *S. aureus* phages was 0.85, which means a strong connection between these two factors. The earliest day of PT with high AAS detected (measured as the median of the entire therapeutic period) was 41 (Łusiak-Szelachowska, unpublished data). Convergent observations were noted by Bochkareva et al. (2017). Repeated courses of PT in patients with healthcare-associated infections (HAIs) did not lead to substantial eradication of pathogens, even after positive initial results. The lack of further improvement was associated with a faster rise of corresponding antibodies.

3.4 Clinical Outcome of PT

The most significant lesson from our studies, contrary to what has been believed so far, is the lack of clear dependence between the level of antiphage antibodies and their neutralizing properties with clinical outcome of PT.

Interestingly, among 15 patients with high AAS examined at the PTU, nearly half of them (7 cases) ended treatment with favorable results, which suggests that the neutralizing effect of phage therapeutics is not associated with the clinical outcome of PT (Łusiak-Szelachowska et al. 2014). In further investigation, Żaczek et al. (2016) drew analogous conclusions. Some patients with the highest level of antiphage antibodies and the highest antiphage activity of their sera (bone infections treated locally or locally and orally) ended phage treatment with good clinical results or even with a full recovery. On the other hand, PT in patients with weak adaptive immunity during the entire course of treatment did not succeed. The unfavorable treatment results in patients with low AAS might be a result of deficits in their immune system generated by past or present infections and previous long-term antibiotic therapies. The effectiveness of phage treatment was evaluated according to the scale established

Table 1 Humoral immune response and clinical outcome of phage treatment in 16 selected examined patients

Patient	IgG [AU] during PT[a]	IgM [AU] during PT[a]	IgA [AU] during PT[a]	Phage inactivation [K] during PT[a]	Clinical outcome of PT
1	598.84	671.4	0	High	F
2	124.04	6.56	0.17	Low	F
4	662.56	136.68	0	Medium	E
5	48.87	52.82	0.34	Medium	F
7	345.1	1193.08	34.59	High	B
8	405.44	536.94	12.26	High	A
9	45.01	3.43	0	Low	B
10	121.74	1.79	0	Medium	A
11	30.94	13.15	0	Low	D
12	72.78	0	0	Low	B
13	30.59	0	0	Low	F
16	300.28	216.57	1.9	Medium	D
17	136.56	1267.52	0.16	Low	C
18	28.01	1.74	0	Low	F
19	32.1	14.53	0	Medium	E
20	85.13	0.06	1.59	Low	F

Adapted from Żaczek et al. (2016) with some changes

Description. The scale of the results of PT presented by Międzybrodzki et al. (2012) is as follows: A—pathogen eradication and/or recovery; B—good clinical result; C—clinical improvement, D—questionable clinical improvement; E—transient clinical improvement; F—no response to treatment; G—clinical deterioration

[a]Maximum values achieved during treatment

at the PTU where categories A–C are considered as positive responses and categories D–G represent inadequate responses to PT (Table 1).

Our findings have been partially confirmed by Russian scientists (Bochkareva et al. 2017). All 42 patients suffering from several healthcare-associated infections and hospitalized at a neurological intensive care unit showed an induced IgG antibody response to polyvalent phage cocktail. The applied phage therapy included two phage strains for each type of bacterium, *A. baumannii*, *Klebsiella pneumoniae*, *P. aeruginosa*, *S. aureus*, and was administered orally. IgG induction varied from 1/16 to 1/4096 dilution of the sera. Despite such variation in IgG boost, effective PT was proved in about half of the cases after the first 3-day course of treatment. Inconsistently with our findings, the induction of antiphage IgG took place after oral application, which, again, could be the reason for the unsettled immune status of hospitalized patients.

The immune analysis was based on the detection of the level of specific antiphage antibodies in human sera reacting with phage antigens by exploiting an indirect ELISA technique (Żaczek et al. 2016). The AU were calculated from absorbance values at 450 nm using the parameters estimated from each standard curve (Miura

et al. 2008). The average value of each duplicate (per sample) was calculated. The dilution giving an optical density at 450 nm of 1 was assigned as 1000 AU.

Krut and Bekeredjian-Ding (2018) found PT was more suitable for acute infections because the antibacterial effects of phages become effective before antibody formation. The author concludes that chronic infections, in which repeated treatments with the same phages boost the humoral immune response, can interfere with therapeutic efficacy. We do not have enough patient data to support that assumption. However, our experience suggests that prolonged treatment does not necessarily result in a failure in its outcome. One patient described by Żaczek et al. (2016) finished PT with a full recovery (category A according to the scale developed at the PTU) after a several-month course of continuous treatment with MS-1 phage cocktail. The patient suffered from a fractured ankle with ulceration. Therapy was accompanied by an increase in the level of IgG antiphage antibodies in the patient's sera after local administration. At the same time, neutralizing activity of antibodies (K rate) rose from low to medium. The key to success was to switch from the phage cocktail to the new monovalent staphylococcal 676/F phage lysate that was applied orally. Changing the phage preparation and the route of administration during therapy induced neither IgG nor IgM secondary boost, with phage inactivation decreased to a low level ($K < 5$). Likely, the new phage with similar lytic activity to the previous one did not stimulate a significant humoral immune response and turned out to be an effective tool in therapy. It indicates that the great variability across phage antibody-binding proteins may help in the eradication of bacterial pathogens in chronic infections. Administration of additional phages that do not have the same serological cross-reactivity in prolonged treatment was also described by Abedon et al. (2011).

4 Humoral Immunity Toward Phage-Borne Endolysins

Immunomodulatory features do not apply exclusively to phage particles entering the human body but have also aroused interest in the field of phage-borne endolysins after both mucosal and systemic administration (Fischetti 2005). Endolysins are double-stranded DNA bacteriophage-encoded peptidoglycan hydrolases produced in phage-infected bacterial cells toward the end of the lytic cycle (Borysowski et al. 2006). These enzymes can cause so-called "lysis from without" on susceptible Gram-positive bacteria in the absence of phage (Harhala et al. 2018). Lysins have been considered an important therapeutic alternative to phages and can be effective antibacterial agents by inducing rapid lysis of a bacterial cell wall (Borysowski et al. 2006, 2011). Similar to the majority of phages, lysins are characterized by a relatively narrow host range. This targeted killing feature of phage-derived lysins is another advantage over broad-spectrum antibiotics (Fischetti 2018). Moreover, they can be prepared with high purity and are non-toxic. Lastly, no bacterial resistance develops to these proteins, probably because they possess multiple domains for cell wall binding and hydrolysis (Kropinski 2006).

Correspondingly with PT, the formation of neutralizing antibodies follows the endolysin treatment as well. However, investigations have found no clear evidence to support the neutralizing effect of antibodies on phage lysins. Antibodies against streptococcal and anthrax phage endolysins obtained from hyperimmunized rabbits did not neutralize their antibacterial activity, likely because of the very high affinity of the enzymes to their substrates in the cell wall (Borysowski et al. 2006). Studies performed by Zhang et al. (2016) in mice revealed that a single intravenous injection of lysin (50 μg/mouse) derived from the *S. aureus* phage GH15 was sufficient against lethal infection with methicillin-resistant *S. aureus* (MRSA). Higher production of specific antibodies, peaking at 3 weeks after application, was noted but without any deleterious effect on the therapeutic abilities of lysin.

Preclinical and clinical trials with the anti-staphylococcal lysin SAL-1 have been performed in animal models and, lately, in humans. Although triggered antibody levels were noted after repeated intravenous injections of phage lysin, so far no clinically significant alterations have been observed either in mice or humans (Vázquez et al. 2018). GLP-compliant safety evaluation of a new phage endolysin-based candidate drug, SAL200 (containing phage endolysin SAL-1) was undertaken as well (Jun et al. 2014). Anti-SAL-1 antibody production was induced by the repeated intravenous administration of SAL200 for more than 1 week. Adverse clinical signs, including subdued behavior, prone position, irregular respiration, and vomiting, were observed in the dog experiments. As expected, those adverse effects were correlated with activation of the complement system and led to significantly decreased levels of C3 complement in the blood. This phenomenon occurred in all examined groups, regardless of the dose of injected lysin (2.5, 10, and 25 mg/kg). The author emphasizes that the adverse effects were slight to mild and were only transiently apparent after injection. The C3 complement levels in the blood samples obtained during the recovery period were similar to pre-dose levels and those of the control group.

An even more optimistic conclusion arises after analyzing efforts to combat pneumococcal bacteremia in a mouse model. Loeffler et al. (2003) found that the efficacy of the Cpl-1 lysin to protect mice was not significantly diminished after previous intravenous exposure and antibody production in response to the lysin injected at a dose of 2000 μg/mouse. Importantly, no effect on lytic activity was observed even when Cpl-1 was mixed with Cpl-1-specific hyperimmune rabbit serum. The author proposes that lysins could be used in situations where multiple administrations were necessary to treat certain disease situations, for instance, chronic infections. In case of losing potency due to an adverse immune reaction, one could imagine a switch to another enzyme in the course of lysin treatment. Harhala et al. (2018) observed a typical pattern for IgG induction for both pneumococcal-specific lysins, Cpl-1 and Pal, with a slow increase and a peak 30 days after intraperitoneal injection in mice in one dose at 0.3 mg per mouse (15 mg/kg) for each protein. Next, the titers leveled off. No increase in IgE levels was observed during the entire course of study (50 days). The author revealed that neither Cpl-1 nor Pal had any effect on activation of the complement system in blood samples collected from healthy human donors. These results demonstrate that Cpl-1

and Pal do not activate the first line of the non-cellular immune response in humans. Additionally, Cpl-1 lysin has shown efficacy against *S. pneumoniae*-induced endocarditis and meningitis in rats, and by aerosolized delivery in a mouse model of fatal pneumococcal pneumonia. The combinational use of Pal and Cpl-1 displayed in vitro and in vivo synergistic efficacy (Harhala et al. 2018).

In summary, in vitro and in vivo studies on different endolysins and pathogens confirmed that antibodies slow down the antimicrobial efficacy of lysins but do not abolish their activity completely (Maciejewska et al. 2018). Borysowski et al. (2006) conclude that, in addition, the immunogenicity of endolysins could be considerably reduced by conjugation to polyethylene glycol (PEG), as reported for lysostaphin. PEGylation of a protein is known to reduce antibody binding even more than tenfold. Possibly, endolysin could, because of the very rapid lytic activity, kill bacteria before the generation of antibodies.

5 Discussion

PT constitutes a unique experimental therapy program which accepts patients according to their actual health needs (Łusiak-Szelachowska et al. 2017). While we are fully aware that no major breakthrough is possible without designing and conducting clinical trials, the long-term research conducted at PTU cannot be underestimated. Our studies confirm earlier reports indicating that PT induces various levels of the humoral immune response without correlation with the clinical course. These assumptions are in line with the latest report published by Dedrick et al. (2019). In a 15-year-old patient with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection sera showed no evidence of phage neutralization after successful three-phage cocktail treatment applied intravenously. Only weak antibody responses to phage proteins were seen, which was probably caused by immunodeficiency syndrome typical for that disorder (Ratner and Mueller 2012). The adaptive response depends on several different factors that constantly interact with each other. As emphasized by other authors, the immunogenicity of phages themselves may vary, with some phages being only very weak immunogens, requiring the use of adjuvant and repeated administration in order to elicit detectable antibody responses (Sulakvelidze 2005). Nguyen et al. (2017) assume that naturally occurring gut phages are likely continuously dosed to the circulation at relatively low levels through transcytosis. Possibly, they could provide long-term immunogenic tolerance but their immunomodulatory effect is still largely unknown. Such tolerance might be an explanation for the lack of inflammatory response to phages. This feature has enabled the use of one coliphage, φX174, for the assessment of humoral immunity in diagnostics and monitoring of both primary and secondary immunodeficiency diseases (Ochs et al. 1971).

Dąbrowska et al. (2014) proposed three parallel mechanisms involved in inactivating phage particles through antihead immunization—aggregation, which makes phage proteins less accessible; synergy between antiphage antibodies and immune complement complexes, which could lead to destabilization of phage

capsids; the complement system could also constitute a steric hindrance for proper function of phage proteins engaged in infection. The author suggests that the immune complement system contributes to the annihilation of phages.

It cannot be excluded that in fact, high antibody responses have a positive prognostic value. Such high antiphage activity of human sera may reflect recovery of the immune system that can cope more effectively with infection (Górski et al. 2017). Jain et al. (2017) performed animal studies that seem to confirm that hypothesis. Guinea pigs inducing the highest humoral and cell-mediated immunity due to phage vaccination through the subcutaneous route were also able to cope with the *Brucella abortus* S19 pathogenic strain most efficiently. Hence, there is no single and simple schema that would represent all aspects of the strength of the humoral response to phage therapeutics.

In terms of safety, the possible toxicity of phage–antibody complexes should also be considered. The renal glomerulus is particularly predisposed to the deposition of antigen–antibody complexes, which may cause both acute and chronic glomerular injury with serum sickness, and acute and chronic glomerulonephritis. Notably, virus–antibody complexes may mediate vasculitis of different tissues including skin, kidney, the central and peripheral nervous system, and liver (Górski et al. 2015).

Evidently, carefully designed clinical trials are needed to shed more light on phage-dependent immune responses and their significance for the success or failure of therapy involving phage-based therapeutics. We strongly believe that all issues in regard to the humoral immune response in PT can be overcome through pharmacokinetic, pharmacodynamic, and tolerance studies that will provide a rational, scientifically based framework to obtain the best possible knowledge regarding phage applications in the future.

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Part III

Use of Bacteriophages to Combat Bacterial Infections

How to Achieve a Good Phage Therapy Clinical Trial?

Jérôme Gabard and Patrick Jault

1 Introduction

1.1 Phage Therapy Clinical Trials Challenges: From Empirical to Evidence-Based and Toward Personalized Medicines

Randomized and blind clinical studies are scarce in phage therapy (Sulakvelidze et al. 2001; Kutateladze and Adamia 2010; Moelling et al. 2018). Since the discovery of phages (Twort 1915) and the beginning of phage therapy (D'Herelle 2007; Summers 2016) less than a hand full of trials have been conducted. Indeed, most of the experience acquired for fighting bacterial infections with phages is based on empirical findings and real-life treatments (Kutter et al. 2010; Międzybrodzki et al. 2012; Lehman et al. 2019).

Clinical centers with the greatest expertise in phage therapy, such as The Georges Eliava Institute (Tbilisi, Georgia) and the Institute of immunotherapy from the Polish Academy of Sciences (Wroclaw, Poland) mostly published summary papers to list their numbers of treated cases over a period and to relate treatment outcomes according to bacterial species and infectious domains.

One may wonder why properly conducted phage therapy clinical trials are so scarce? There are several reasons to that.

1. Phage therapy was wiped out of European pharmacopoeia at the end of 1970s, when neither antimicrobial resistance was significant, nor phages considered as potential countermeasure.

J. Gabard (✉)
Saint Barthélemy d'Anjou, France

P. Jault
Clinique de la Muette, Paris, France

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2. At the same period in Western countries, gold standard trials became the reference as controlled, double blind, randomized, and multicentric studies with large cohorts (ICMJE | Recommendations | Clinical Trials).
3. Therefore, when reintroduced in early 2000, regulators and clinicians/statisticians considered that up-to-date phage therapy clinical trials must be controlled. One arm (product A) is compared to another (placebo or product B): Efficacy of B should be better, equal, or lower than A. Meanwhile, clinicians that have been practicing phage therapy for decades in countries where such routine treatments are available did not recognize the pertinence of these studies.
4. In Western countries, the low number of phage therapy trials means that clinical evaluation starts almost from scratch where tolerance is more important than efficacy. Tolerance of A must be equal or better than B to keep the benefit–risk balance positive or null.
5. Apart for phase-I studies in the USA, all clinical batches must be produced according to Good Manufacturing Practices (GMP) and under quality constraints fitting with the targeted infectious area (Mattey and Spencer 2008; Merabishvili et al. 2009; Pirnay et al. 2011, 2015), whereas so little experience was and still is available in the Contract Manufacturing Organization (CMO) community for making GMP phages.
6. Minimum Inhibition Concentration (MIC) is a world-wide recognized standard for determining antibiotic efficacy and MIC tends to be transposed to any other antibacterial compound being evaluated including to phages, although not always applicable (Abedon 2011, 2016, 2018).
7. The gold era of antibiotic development has been leading to the *motus vivendi* that antibacterial treatments must have a wide spectrum targeting, if not both genus, at least Gram-positive or Gram-negative bacterial species, whereas new treatments including phages often aim at single bacterial species or even specific strains within a species.
8. Current practices, inherited from antibiotics, consider frequency of administrations linked to time (i.e., β-lactamines) or concentration (i.e., aminosides) according to MIC, regardless of the self-multiplying properties of bacteriophages. All pharmacodynamic and pharmacokinetic data about phages need to be acquired (Abedon 2009; Abedon and Thomas-Abedon 2010).
9. Bacterial eradication is still seen as the gold standard for checking antibacterial performances, whereas rebalancing a host bacterial microbiota to favor competition and preserve diversity may be sufficient (Górski et al. 2017; Mirzaei and Maurice 2017; Duerkop 2018).
10. Historically, the design of antibiotic clinical trials is tailored for evaluating a single compound, when phage therapy may require studying different phages in multiple combinations, which can be adapted to each patient bacterial strain infection. This type of personalized medicine is not fitted for large cohort of patients nor standard trial design.
11. In the scarce efficacy trials and in compassionate cases, phages are used after multiple antibiotic failures and often on multi- (or toto) resistant bacterial

strains. Using them at an early stage or as a first line of anti-infectious therapy could improve their evaluation success rate.

Nowadays phage therapy faces two major challenges: (1) to convince by publishing successful clinical trials/cases and (2) to engage institutions and private investors for developing clinical trials; it is a vicious circle. Furthermore, some of "acquired habits" transposed from previous antibiotic clinical development needs to be rethought for proper and successful phage therapy clinical trials.

1.2 Individual Cases

The literature describing individual clinical cases is more abundant because of historical phage therapy in Eastern countries and the newly rehabilitated concept that phages can be used for salvage therapy in western ones. Actually, this practice didn't fully disappear after the end of the first phage era in Western countries at the end of the 1980s, but the few clinicians who used phages tent to "treat under cover." Drs Alain Dublanchet and Olivier Patey (Patey et al. 2018) in France were among them and decided to publish afterward 15 different cases treated from early 2000 to nowadays, with a focus on bone infections.

Apart from the thousands of undescribed cases of patients treated in Georgia and Russia since decades, a dozen of individual case studies or small cohort treatments have been reported. In Poland, Letkiewicz et al. used anti-*Enterococcus faecalis* bacteriophages to treat three patients with prostatitis (Letkiewicz et al. 2009), resulting in bacterial eradication, abatement of clinical symptoms, and lack of disease recurrence. In Australia, Khawaldeh et al. used phages successfully to treat an antibiotic refractory *P. aeruginosa* urinary tract infection on bilateral ureteric stents with bladder ulceration (Khawaldeh et al. 2011). No bacteriophage-resistant bacteria arose, and the kinetics of bacteriophage and bacteria in urine led to a self-limiting infection. In the early years of 2010s, the surgeon Randy Fish applied anti-*S. aureus* bacteriophages to treat diabetic foot ulcers on a small cohort of patients. Methicillin-resistant *S. aureus* (MRSA) infections were infecting grade II to IV diabetic foot ulcers (Fish et al. 2016). In addition to phages, the treatments included curettage and eventually partial toe amputation. Wound healing was excellent, and patients recovered from their infected open wounds within weeks to few months. Lately, at the Queen Astrid Elizabeth Hospital in Belgium (Jennes et al. 2017), the treatment of a life-threatening *P. aeruginosa*-induced sepsis was successful. The colistin-only-sensitive bacterial strain was eradicated in a patient with acute kidney injury demonstrating that phages can be administered safely to humans for treating systemic infections. In California, Schooley et al. used different cocktails of phages over several months to fight a very severe disseminated *Acinetobacter baumannii* antibiotic-resistant infection (Schooley et al. 2017), leading to full patient recovery. At last, recently in France, various hospitals including the Hospices Civils de Lyon used different phages (produced by Pherecydes Pharma Co.) to treat a dozen patients. Two recent articles detailed the first treatments performed in 2017. The first one was infected by a colistin-only partially sensitive *P. aeruginosa* bacterial

strain. His right sacro-iliac joint infection was eliminated after a single application of four phage strains, although the treatment was repeated three more times. The second one was cured from an iterative chronic prosthetic infection induced by an antibiotic sensitive *S. aureus* and a multidrug-resistant (MDR) *P. aeruginosa* strains, using a single administration of three phages against each bacterial species (Ferry et al. 2018).

Recently (in January 2019), an impressive successful treatment was also achieved at the university hospital Pitié Salpêtrière (Paris, France). An extra-dural empyema induced by a MRSA and refractory to standard anti-biotherapy was successfully treated with a combination of three anti-*S. aureus* phages (Pherecydes-Pharma) and dalbavancine antibiotic.

2 Standard Past and Current Clinical Trials with Fixed Products

The long-lasting experience of phage therapy with preparations, which rank from barely purified to GMP grade, demonstrated the lack of toxicity of phage therapy and the high level of tolerance of patients to the treatment. In the case of PhagoBurn, the three national regulatory agencies who granted trial approval (ANSM: Agence nationale de sécurité du médicament et des produits de santé (The French Medicine Agency), FAMPH: Federal Agency for Medicines and Health Products (The Belgium Medicine Agency), and Swissmedic) agreed for a clinical testing directly entering into phase II, with a primary end point focusing on a persistent bacterial burden reduction. Their approval was based upon documented animal studies where the high concentration of phages applied didn't result into any toxicity symptoms.

In 2018, Furfaro et al. drew a summary of human phage therapy trials carried out to assess tolerance and/or efficacy. The scheme shows targeted sites and bacterial species (www.clinicaltrials.gov or https://globalclinicaltrialdata.com/) (Fig. 1).

As regard clinical trials, in 1963 (in Tbilisi, Georgia) more than 30,000 children were enrolled in an oral prophylaxis trial against bacterial dysentery. About half of them living on one side of the street received Shigella phages, whereas children on the other side received placebo. Phage administration was associated with a threefold to eightfold decrease in dysentery incidence compared to placebo group, including a decrease of any other forms of diarrhea. A protective effect of the anti-*Shigella* phage preparation was suggested, especially in children younger than 3 years. The study was reported in a brief Russian publication (Babalova et al. 1968).

In 1990, Abul-Hassan reported phage therapy to control *Pseudomonas aeruginosa* infection in 30 cases of antibiotic resistant infections in patients with moderate to severe burns (Morozova et al. 2018). Infections were eliminated in 40% of the cases but, because of practical issues, the author recommended to restrict treatments to antibiotic resistant strains.

In Great Britain, a cocktail of six bacteriophages (produced by BioControl Co.) was administered and compared to placebo to 24 patients (2–58 years) suffering from chronic osteitis induced by MDR *P. aeruginosa* infections (Wright et al. 2009). This first controlled clinical trial of a therapeutic bacteriophage preparation showed efficacy and safety in chronic otitis because of chemo-resistant *P. aeruginosa*.

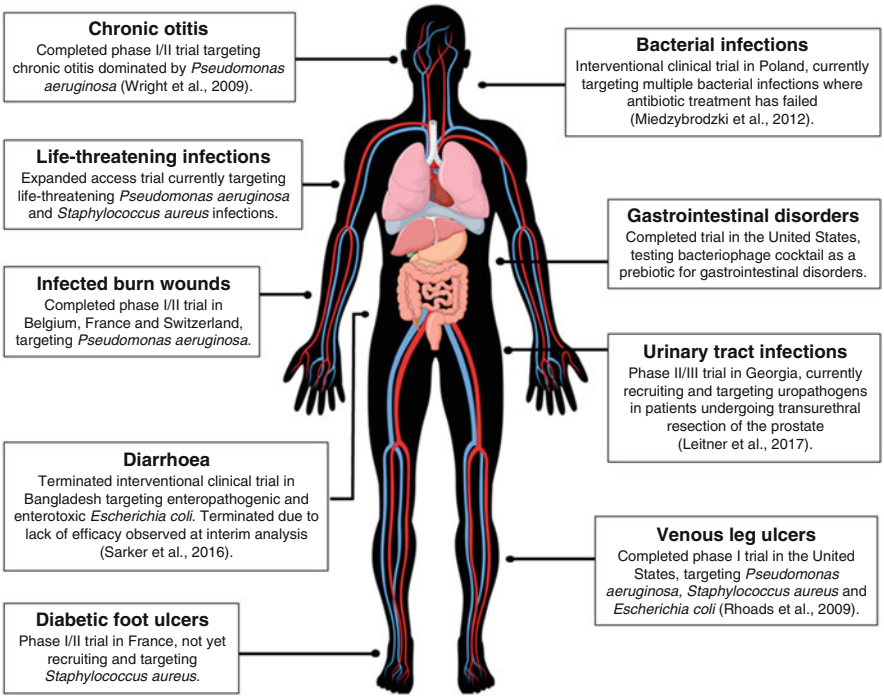

Fig. 1 Reported and documented phage therapy clinical trials reported at the end of 2018 (Furfaro et al. 2018)

In the early years of 2010s, NESTLE® supported an open study in Bangladesh to evaluate the potential of Russia-produced phages (Microgen Co.) to control *E. coli* induced intestinal tract infection (Sarker et al. 2016). Oral coliphages showed a safe gut transit in children but failed to achieve intestinal amplification and to improve diarrhea outcome, possibly due to insufficient phage coverage and too low *E. coli* pathogen titers requiring higher oral phage doses (Brussow 2005; Bruttin and Brussow 2005).

At last, the PhagoBurn project was designed to evaluate phage therapy against *P. aeruginosa* or *E. coli* infections in deep burn wounds in comparison to silver sulfadiazine standard of care. Because of cocktail stability issues, phage therapy was only successful in 50% of treated patients (Jault et al. 2019).

3 Toward Personalized Medicine Clinical Trials

Phage therapy remains unknown yet in most pharmacopoeias but Georgia, Ukraine, and Russia. In Poland, phage therapy benefits from the Helsinki Convention exception but is only authorized in a single hospital facility in Wroclaw, whereas in Georgia or Russia, phage therapy is a current medicinal practice. These examples

at the opposite of each other show that different national regulatory routes are applied today to authorize either hospital facility or general medicinal phage therapy (Verbeken et al. 2007, 2016a, b) practices.

But except for the multicenter (French, Belgium, and Swiss) PhagoBurn trial, no procedure was ever filed for approving a phage therapy clinical study across different countries. In 2013, PhagoBurn followed a standard approach recommended by various regulatory agencies: that is to enter clinical evaluation with a fixed phage product, displaying a wide spectrum of activity against the targeted bacterial species (*P. aeruginosa* or *E. coli*). Despite a favorable past empirical experience, the personalized treatments regulatory route was voluntary, set aside because of antibiotic clinical testing history and their "one-size-fits all" market positioning. The key advantage of such an approach was that the product was, in theory, standardized, identical for all patients, and much easier to evaluate in a clinical trial, compared to several combinations of different phages in various numbers. Major drawbacks were that (1) only a small number of phages from the cocktail may have been active against a given patient strain, whereas the remaining inactive phages could reduce the action of the active ones, (2) each active ingredient in the complex mix was impossible to monitor overtime, and (3) the combination of 12 different phages was unstable and degraded quite significantly overtime.

As the clinical trial was running, complementary ancillary data showed that phage therapy success was linked to an initial in vitro response of a patient strain to the phages of the cocktail (38–40) (Jault et al. 2019). The need to perform a preliminary diagnostic—the phagogram—before treating, became obvious. Concerns toward the "PhagoBurn-like" standard one-size-fits-all strategy were raised, as salvage therapy requests reemerged in France, Belgium, Switzerland, and USA. Thanks to the cooperation between clinicians, phages providers, and national regulatory agencies, compassionate personalized treatments were authorized using small sets of efficient phages tested against each patient's infectious strain. This major step forward in a few Western countries led the way to open a regulatory route to tailored phage therapy (Pirnay et al. 2011; Huys et al. 2013; Debarbieux et al. 2016). Then, one may certainly consider that the descendance of PhagoBurn is the possibility today to authorize a more practical phage therapy approach: the precision medicine treatments.

Belgium, with the support of the Federal Agency for Medicines and Health Products (FAMHP), started to implement a pragmatic phage therapy framework that focuses on the magistral preparation (compounding pharmacy in the USA) of tailor-made phage medicines. The first monography of "phage active pharmaceutical ingredients (API)" (Version 1.0) was released in 2018 (Pirnay et al. 2018).

In December 2016, ANSM also started to support and monitor the compassionate use of last-resort treatments for patients at risk of dying or losing functionality after antibiotic failure. Compared to PhagoBurn cocktails, these phages are not GMP: They are produced in a research laboratory but supported by a battery of quality controlled (QC) tests. Such case-by-case treatments are delivered once the clinician, the pharmacist, the company (Pherecydes Pharma), and the patient or its next of kin sign a consent form. Four anti–*P. aeruginosa* phages and three anti–*S. aureus* APIs

have been applied in various combinations targeting each bacterial species alone or together in a single patient. Although potentially active, solely suppressor, or inefficient, antibiotics have always been applied with bacteriophages to reduce patient risk. At the time of writing this paper, 10 antibiotic multidrug or toto-resistant patients have been treated with a favorable outcome for 8 of them.

In USA and Australia, the company Ampliphi Biosciences followed a similar path of last resort treatments approved by US Food and Drug Administration (FDA) or Australia's Therapeutic Goods Administration (TGA). With various research organizations providing phages (as the Navy), the company participated in the rescue of an emblematic patient whose recovery, as well as the treatment of five other patients, led the way to launch the North America's first Center for Innovative Phage Applications and Therapeutics (IPATH) in California (San Diego) in 2018.

4 Physician Versus Manufacturer Responsibilities

4.1 Clinical Trials

The one-size-fits-all clinical approach makes it mandatory to have an immovable drug, ready from the shelf and produced according to GMP. The drug product (DP) is a combination of a given number of phages at a fixed ratio. Each individual phage component is a drug substance (DS). All the steps of production including diluting, as well as, mixing DS before sterile filtration and primary container filling are carried out by the manufacturer. The DP is delivered in an unmovable specified way. All the risks associated with product production are in the hands of the manufacturer. The hospital pharmacist is only responsible of the reception and proper storage of the product in the hospital pharmacy. Clinicians are responsible for diluting the phage cocktail if required and of the administration.

Moving toward a personalized treatment opens the door toward mixing individual phages at patients' bedside. In that case, the DP is an individual phage conditioned into an individual batch of primary containers. The DS is the same bacteriophage before dilution, filtration, and filling. The drug product is delivered in variable combinations and variable dilutions according to each targeted therapeutic area that is, highly concentrated when targeting a systemic infection, versus significantly diluted when aiming at a large surface burn wound infected area. Hence, the pharmacist is responsible of mixing the individual phages to assemble the preparation and to dilute the mix to a proper volume in line with the type of administration. The risk of manufacturing is shared by both the producer and the pharmacist. The clinicians are responsible for the administration (Pirnay et al. 2018).

Reconciling GMP manufacturing of a magistral preparation with an easy to standardize one-size-fits-all product is unavoidable. However, final assembling of the DP is in the second case under the responsibility of the hospital pharmacist whereas it lies in the hand of the manufacturer in the first one.

4.2 Salvage Therapy

Nowadays, GMP phages are not always accessible. Because of the current focus on the most frequent bacterial infections in humans, such as *P. aeruginosa*, *S. aureus*, *E. coli*, or *A. baumanii*, GMP bacteriophages are not available against other bacterial species. In case of multiple antibiotic treatment failures, when the patient's life is at risk or if losing a limb functionality (through amputation for instance) is the prognosis, regulatory agencies may face clinician demands for phage therapy treatments but without GMP phages. Several Western regulatory agencies have been exposed to these situations during the past 10 years. ANSM, AFMPH, Swissmedic, and FDA to name a few did respond cautiously but favorably to some of these requests. The quality control department of the agencies looked carefully at the quality of these nonGMP phages but no medicinal regulatory context is really available for authorizing such cases. Consequently, the requesting clinicians, the hospital pharmacist, the patient, and the manufacturer end up being responsible of the risk taken. An informed consent must be signed between all these parties. Agencies supervise only the procedure.

5 Phage Therapy Efficacy Trials and Primary Endpoints

A regard efficacy and according to scientific literature available till early 2019, only three prospective, controlled, and randomized trials have been completed. All of them were conducted with a fixed mix of phages, a "cocktail."

The first two trials were conducted at a single center and placebo-controlled.

A double-blind study targeting chronic (2–58 years) MDR *P. aeruginosa* infections in otitis against placebo was achieved in 2008 (Wright et al. 2009), in a single specialist university hospital. The product was a mix of six different bacteriophages at a total titer of 10^5 PFU/ml. The cocktail was applied locally directly into the ear and concentration remained high at the infection site. This first controlled clinical trial of a preparation showed efficacy and safety in chronic otitis induced by antibiotic-resistant *P. aeruginosa* (Wright et al. 2009). The primary indicator was patient cure, a clinical outcome, whereas *P. aeruginosa* count was chosen as a secondary outcome after consulting with three regulating authorities (UK Medicines and Healthcare products Regulatory Authority, the European Medicines Agency (EMA) and the Food and Drug Administration (FDA)). Bacterial count was significantly lower in the phage treated group. No treatment related adverse event was reported.

The second one targeting *E. coli* including parallel groups compared the efficacy of a T4-like cocktail to Microgen ColiProteus cocktail or placebo in 6–24-month-old male. The commercial Russian phage cocktail consisted of at least 17 different phage types including T7 phage (McCallin et al. 2013) with approximated titers ranging from 10^3 to 10^6 PFU/ml. Coliphage and *Escherichia coli* titers and entero-pathogens were determined in stool and stool output and frequency were measured. Stool microbiota was studied by 16S rRNA gene sequencing; the genomes of four fecal

Streptococcus isolates were sequenced. Oral coliphages showed a safe gut transit in children but this second trial failed to demonstrate efficacy. No intestinal amplification was detected as well as no improvement of quantitative diarrhea outcome. The final phage concentrations after oral administration were likely much lower than expected because of product dilution into the intestinal track. Phage coverage was also considered as insufficient.

PhagoBurn was a controlled, randomized, multicentric, and double blind phage therapy trial according to Good Clinical Practices (GCP) and using Good Manufacturing (GMP) cocktails. Twenty-seven 32- to 70-years-old male and female third-degree hospitalized adults were enrolled. Although planned to evaluate in parallel groups two phage cocktails (PP0121: anti-*E. coli* cocktail with 13 different phages and PP1131: anti-*P. aeruginosa* cocktail with 12 other phages) against silver sulfadiazine standard of care, the patient recruitment pace left investigators with only one PP1131 to study. Its genuine concentration was 10^9 PFU/ml total including each phage at a single concentration of 8.33×10^7 PFU/ml. But the cocktail had to be diluted a thousand-fold at the point of care to reach an acceptable level of residual endotoxins into the DP because of a risk of potential systemic diffusion through deep burn wounds. Unfortunately, the genuine cocktail phage titer which was expected to be 10^6 PFU/ml but ended up being 10^2 FU/ml because of product instability (Jault et al. 2019). The primary endpoint was microbiological that is, a sustained reduction (by two quadrants) of the targeted bacteria in the wounds according to a semi-quantitative counting method (quadrant plating): phage therapy worked in 50% of the cases versus 83% with the standard of care. Afterward, an ancillary evaluation of the sensitivity of patient infections demonstrated on D_0 that about 50% of *P. aeruginosa* patient strains were sensitive to the low dose of PP1131 applied. The positive response of the *P. aeruginosa* strain to the phages of PP1131 on the initial treatment day matched with a successful microbiological primary outcome and patient cure. Without diagnostic at posteriori, the study would have been considered a failure.

In these studies, primary endpoints ranged from a qualitative clinical outcomes (patient cure), rated openly by the clinician in charge of the treatment, to quantitative evaluations such as the counting of the targeted bacteria performed by a blind microbiologist independent of clinicians (it is noteworthy that a comparative blind ancillary study counting bacterial on selective media didn't lead to discrepancies between the semiquantitative and quantitative methods in PhagoBurn). Clinical outcomes are often criticized because of their lack of objectivity, whereas quantitative ratings can lead to technical choices that affect success rate. For instance, in comparative studies, it is critical to cure the patient 1 day earlier than the standard of care, if product tolerance is much higher with phages than with the reference treatment? When times come to determine the phage drug's improvement of medical benefit compared to a current standard of care, a badly chosen primary endpoint may lead to a poor reimbursement level of phage therapy by payer stakeholders in several countries.

6 Switch from One-Size-Fits-All to Personalized Clinical Evaluations

How to design a clinical trial setup to evaluate various combinations of phages that may target a single or even several bacterial species?

In immuno-oncology the face of clinical trials has been changing drastically during this decade to integrate personalized medicine evaluation via biomarker-oriented drug development, specific alternative endpoint selection, challenges design, and risk-based monitoring (Mirnezami et al. 2012; Golan et al. 2017; Garralda et al. 2019). Different "master" protocols have been designed to assess multiple treatments on multiple diseases. A master protocol trial is often classified into Basket, Umbrella, or Platform trials based on characteristics of the study population (e.g., disease, histologic type, and molecular marker) and on both the type and number of study therapies. Such trials are designed to evaluate (Renfro and Sargent 2017; Woodcock and LaVange 2017; Hirakawa et al. 2018):

- A single targeted therapy on multiple diseases or disease subtypes: Basket
- Multiple targeted therapies for at least one disease: Umbrella
- Several targeted therapies for one disease during all the trial, and additions or exclusions of new therapies during the trial: Platform

Although, such definitions are not standardized yet and sometime overlap, they may offer a type of design which fits with personalized phage therapy evaluation constraints (See Fig. 2 below).

A Basket design addresses the specificity of phages (i.e., against a given bacterial species) and the need to test them against multiple infectious sites in the same trial to increase patient recruitment and reduce duration. In this scenario, a defined set of phages is evaluated against grouped infectious areas with similarities: burn wounds + venous ulcers + grade I and II DFU,. . . or urinary tract + prostates + pyelonephritis would form a Basket, and substudies would be conducted by types or areas within it. Often Basket trials are conducted as phase II single-arm, proof-of-concept (POC). Generally, the number of patients in subgroups is between 20 and 50.

An Umbrella design offers the possibility to test various phage combinations for personalized treatments in the same trial. Substudies could be conducted to evaluate targeted phage mixes that correspond to different bacterial response groups within patients that is, all the patient bacteria that respond to the same combination of phages after a phagogram. In that case, the whole set of phages is the "umbrella," under which substudies for each phage combinations are operated.

A Platform design allows to include new phages in the trial if patient infections end up being resistant to too many of the originally phage set, as the study progresses, and to withdraw inefficient phages from that set. In a platform trial, interim analyses would assess the efficacy or futility of each phage combination. Results would support excluding certain useless phages or adding new ones. Because efficient phages are chosen after a preliminary diagnostic and before inclusion, the platform design would help supporting the diagnostic result by

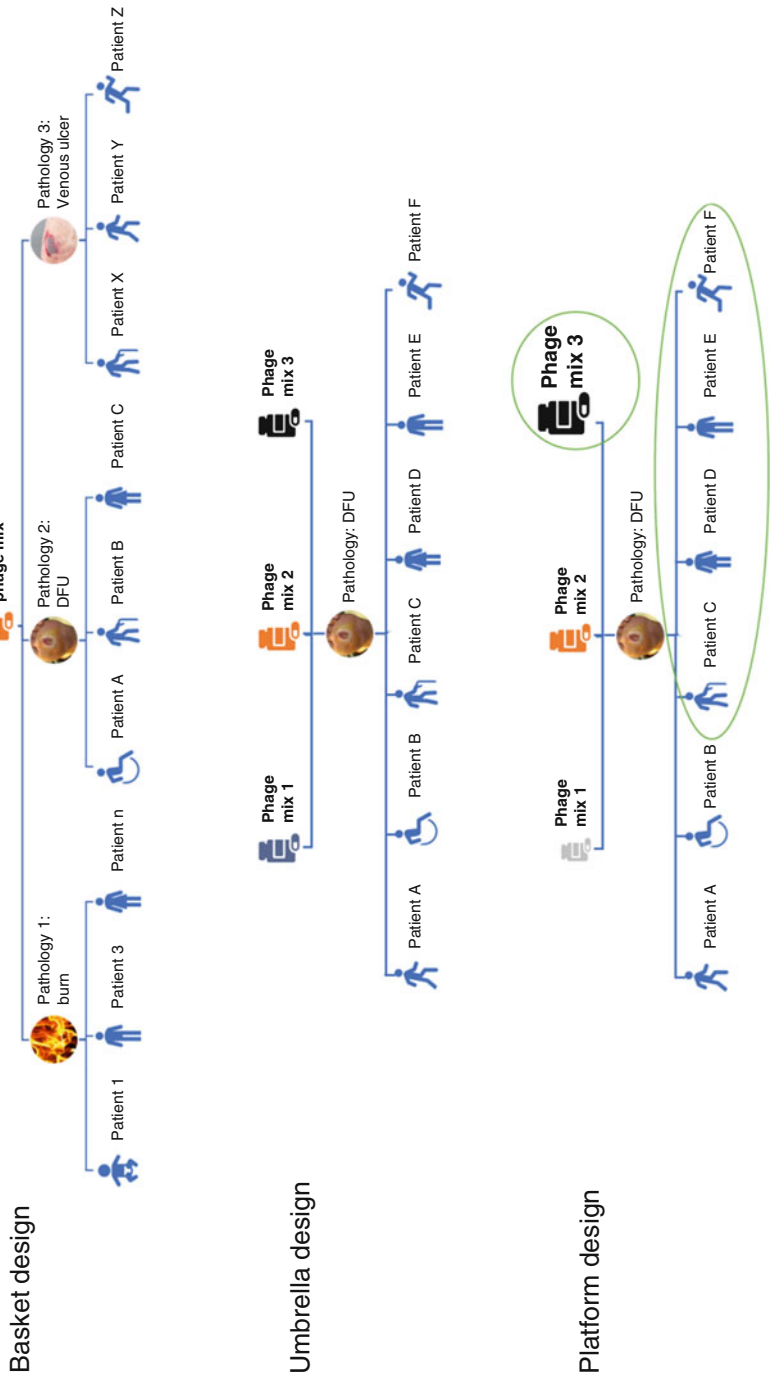

Fig. 2 Representation of Basket, Umbrella, and Platform phage therapy clinical trials

showing whether the choice of a phage combination was appropriate to achieve the clinical endpoint. In case a given combination ends up to often by a therapy failure that combination could be withdrawn from the set of evaluated treatments. Note that basket and umbrella designs mays be considered platform trials as well, if they allow adding or withdrawing new phages during the study.

Of course, these types of studies may end up with unequal numbers of patients treated in each subgroup. For instance, if a specific combination of phages is less efficient, its number of patients with likely become low at the trial end, whereas an efficient combination may lead to an overrepresented group. Consequently, statistical analysis methods may need to be adapted so that in subgroups were treatments appear to be inefficient, the number of treated patients is pulled to increase the data set and the power of statistical analysis. The same should apply to the subset of phages that succeeded. In case of a blind study, the monitoring of each phage combination may require unmasking clinical data during the study. However, with much more sophisticated databases and electronic Case Report Form (eCRF), it is possible to have multiple versions (i.e. blinded and unblinded) and to limit the access to the unblinded case to only certain people (like regulatory agencies or an independent monitoring safety board).

7 New Efficacy Trials to Come

As for the future, the FDA has accepted an Investigational New Drug application at the University of California San Diego School of Medicine to conduct the first US clinical trial of an intravenously administered bacteriophage-based therapy in collaboration with AmpliPhi Biosciences Company.

In France, after PhagoBurn, two Hospital Clinical Research Programs (HCRP) have been granted to test three anti-*S. aureus* phages (1) in diabetic foot ulcers (DFU) with as principal investigator Professor Albert Sotto from the University Hospital Center (UHC) of NÎmes and (2) in bone and joint infections (BJI) with as first principal investigator Professor Michel Dupon and Dr. Antoine Dauchy, from the UHC of Bordeaux. Originally planned in 2018, these trials have been postponed starting at the end of 2019, notably, because of switching from a fixed cocktail to adaptative treatments. In that case, the treatment can be all the combinations including one to three phages that is, three single phages, three pairs of phages, or one set of three phages applied together. The choice of the treatment is made after testing the sensitivity of the patient infectious strain to each individual phage in order to choose the most adapted treatment.

This approach leads to the challenge of evaluating different treatments on various infectious domains in limited patient populations (where antibiotics have been failing), where, at the end, the number of inclusions by therapeutic areas risk to be very low! In fact, the low number of potential patients pleads in favor of opening the trial designs to several therapeutic areas with a single phage cocktail and/or several combinations of phages.

8 What About Tolerance Testing in Phage Therapy Trial?

Since 2013, experiences with tens of different phages targeting three different bacterial species (*E. coli, P. aeruginosa, S. aureus*, *A. baumanii*, etc.) do not show any differences of tolerance from phage to phage in animal models or human treatments. No lytic phage has ever been responsible for toxicity issues in numerous studies. This is widely confirmed by various publications (Patey et al. 2018). For humans, evaluations have been performed with treatments ranging from large cocktails of more than 10 phages (like in the PhagoBurn study) to personalized treatments ranging from two to four different phages in case of salvage therapies. To name a few, the targeted infectious areas from these studies and other recent documented cases encompass, otitis, burn wounds, BJI, prosthetic decontamination, DFU, endocarditis, brain dura matter, lungs, sepsis, urinary tract infection (UTI), prostatitis, and so on. Modes of administration are as diverse as oral, topical, intramuscular, intravesical, intradermal, intravenous, nebulization, rectal, and so on. In none of these cases has phage toxicity being reported, although the quality of the products applied varied from coarsely purified preparations to GMP grade endotoxin undetectable products.

Tolerance to lytic phages appears to be excellent with a large therapeutic index. This data shows that phage therapy tolerance is not an issue and could be directly rated in an efficacy trial as a secondary endpoint, via a single set of data, even if various phage subgroups have been evaluated in the same trial. However, key points must be considered when such a clinical trial is to be set up. Because product tolerance is different from patient safety, the worst-case scenario must be planned, in the most suited facility to handle unwanted effects, to guarantees patient's safety including back-up treatment in case of failure with severe complications.

Of course, safety measures need to be adapted to the studied pathology. As such, when considering a clinical trial in grade I–II *S. aureus infected* diabetic foot ulcers, patients may be recruited in ambulatory hospital facilities without an emergency ward (Fish et al. 2016). On the other end, in severely burned patients infected by *P. aeruginosa* clinical trial like PhagoBurn, hospitalization with intensive care unit and safety conservatory measures is a must, in case of lack of efficacy or poor tolerance.

Potential expected safety hazards are especially linked to endotoxin residual content for Gram-negative and to hemolysin for Gram-negative infections, such as *Staphylococcus* spp. The administration of a well purified cocktail with a low level of residual bacterial toxins isn't followed by fever. Other screened usual clinical or biological parameters stay under thresholds of usual values. Despite clinical and biological good tolerances, it is cautious to consider that other side effects may be figured out with the increasing number of treated patients. Some of them are probably unknown, because under our current routine radars. One of them should be considered for future evaluations: the interactions of phages with immune system. Many publications suggest a modulation effect of phages in inflammatory response (Krut and Bekeredjian-Ding 2018) including a favorable clinical response, but without any biological routine records.

Naturally, pharmacovigilance system is mandatory and necessary to declare all adverse events and analyze accountability to tested product.

9 Get Away from MIC

Minimum inhibition concentration (MIC) has been and remains the gold standard to evaluate antibiotics. For inert chemical molecules or peptides, MIC fits with the need to identify the minimum efficient dose that a bacterial infection requires to be controlled in vivo. But the pharmacokinetics of antibiotics is not constant. The duration and frequency of the treatment depend on the molecule type: β-lactamines for instance are time-dependent and require frequent and close intakes or continuous administration, whereas aminoglycosides, which are concentration-dependent, require a rapid injection of a large bolus to achieve a target concentration.

However today, not all anti-infectious products do fit with this MIC. The Beam Alliance in Europe is working at defining new endpoints for anti-infective therapies according to patient perspective. They are defined in Fig. 3 below, as follows:

1. To avoid infection and prevent from getting sick and/or to enable surgical/medical interventions
2. To get diagnosed and take an informed and timely choice of treatment
3. To remove infection and resolve the disease symptoms as efficiently as possible
4. To avoid damage to the body and decrease the level of infection virulence
5. To avoid treatment failure and prevent long-lasting, recurrent, and life-threatening disease states
6. To avoid transmission and contagion and prevent the spread to relatives or the close environment.

Bacteriophages can be involved in step 1—to decolonize/avoid colonization (1b); step 2—to diagnose the bacterial infection and support the choice of a valid personalized treatment; step 3—to inhibit/kill pathogen (3a), to increase kinetics of pharmacologic effect (3b) and break existing resistance (3d) for instance by disrupting biofilm; step 4—to avoid microbiota dysbiosis (4a) by rebalancing the bacterial flora;

In addition, recombinant phages including CrispR/Cas9 constructs (i.e., from Eligo Biosciences Co.) may also act in step 5 to avoid resistant gene transfer (5b) in case, for instance, of *Shiga toxin*-producing *Escherichia coli* (STEC) intestinal tract infection.

Several of these steps require other endpoints than MIC, especially for phages. A minimum efficacy titer (MEC) must be determined before treatment to ensure that an adequate multiplicity of infection (MOI) is applied as regard phage bacterial strain sensitivity: Indeed, highly sensitive strains may be controlled by an MOI of 0.001 that is, one phage for 1000 bacteria when their progeny is produced in hundreds of copy per cycle in a short timeframe (10–20 min), whereas less sensitive strains may only respond to an MOI of 100 that is, 100 phages for one bacteria, if the propagation

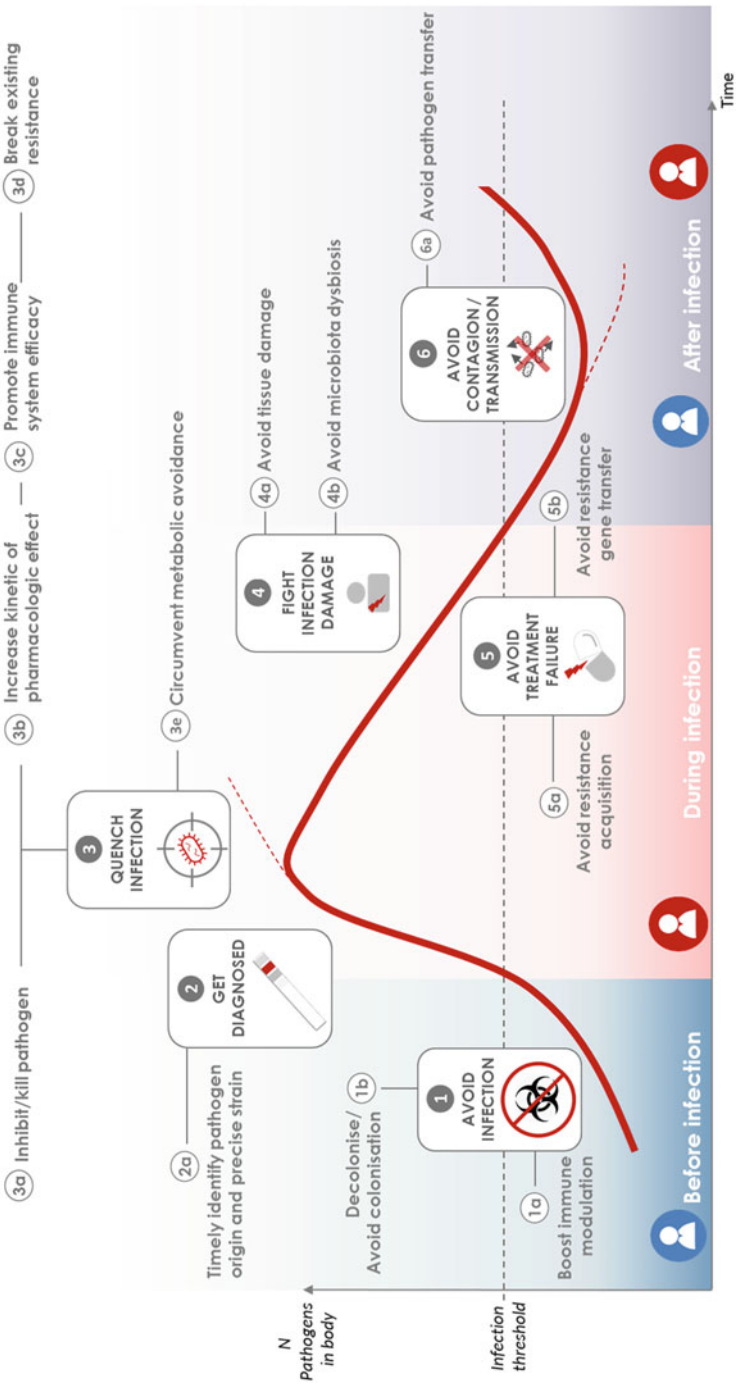

Fig. 3 The six ways innovative products address patient needs to combat antimicrobial resistance (AMR)

cycle of a phage is slow (<45 min) and its copy number low (10 phages par cycle). Hence, MEC is somewhat close to the MIC applied to antibiotics but different. MIC is the concentration of antibiotic required to maintain the active substance concentration above the dose required to kill the targeted bacteria. This concentration decreases overtime in function of molecule pharmacokinetics and requires multiple administrations according to antibiotic stability in the human body. In the case of phages, metabolization fate is counterbalanced by the speed of propagation of the phage in its bacterial host, that is, the length of phage lytic cycle and the number of phages produced at each cycle, sometimes called the burst size. Hence, this difference between a static and a living treatment makes phage dosing a much more complex issue than treatment with antibiotics (Abedon 2016). Because of the high diversity of bacterial infections, MOI and length of lytic cycles, choosing a phage titer treatment above 10^8 PFU/ml may be preferable if the approach is to be one-size-fits-all.

10 Phagogram

In case of personalized treatments, a preliminary diagnostic is required to evaluate the susceptibility of a patient bacterial infection to phages. This diagnostic called "phagogram," by analogy with antibiogram, is designed first to evaluate the activity of a given phage against the bacterial strain (yes or no answer) and second to determine its level of efficacy. Currently, phagograms proceed by two methods (Ferry et al. 2018).

Efficiency of each phage is tested using the efficiency of plating (EOP) according to the visualization of bacterial lysis when the strain is spotted on solid medium (spot test). In case of bacterial lysis with plaque-forming units (PFU), the EOP score is defined by the patient-strain/reference-strain phage titer. Bacteriophage is most efficient as the EOP score is closer to 1.

The killing assay helps in determining the ratio of phage/bacteria (multiplicity of infection: MOI) that is, the level of susceptibility of a phage against the patient strain. The patient's strains are cultured at a starting concentration of 1×10^6 CFU/ml w/wo bacteriophage. Each bacteriophage is added individually at three concentrations leading to different low, medium, and high MOI. In the following Fig. 4, two bacteriophages (Pherecydes Pharma) PP1493 and especially PP1815 are showing an excellent activity against a *S. aureus* patient strain treated in 2017 at the Hospices Civils de Lyon, with a chronic hip prosthesis infection (Ferry et al. 2018).

11 Randomization with Antibiotics

The experience of PhagoBurn on multi-resistant infections, as well as compassionate usage on multi-/toto-resistant strains show that the current positioning of phage therapy makes it mandatory to plan for preliminary and/or concomitant antibiotic treatments. This constraint makes it more difficult to randomize patient recruitments.

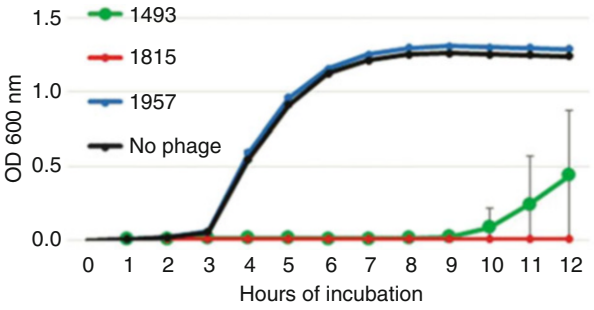

Fig. 4 Phagogram killing assay responses of three bacteriophages on *S. aureus* patient strains suffering from a relapsing hip infection. Phage PP1815 (red line) and PP1493 (green) stop bacteria (black) multiplication, whereas phage PP1957 (blue) does not

In the case of PhagoBurn, after a first random distribution in each arm (phage vs. silver sulfadiazine reference treatment), a secondary randomization based upon antibiotic treatment before inclusion was applied. These two-level stratifications end up splitting patient population into two different subgroups within a single arm, with a significant impact on the number of patients to recruit for achieving statistical hypothesis.

Because of the impossibility to randomize patients once treatment is started, the use of antibiotics during phage evaluation needs to be left open to the choice of the clinician. Indeed, if the infected area and treatment are local, one cannot exclude clinical conditions where a secondary infection emerges in another area, for instance the lungs whereas a burn wound is originally targeted. Even if this supplementary infection is carried out by the bacterial species targeted by the phage treatment, the lack of diffusion of the local studied treatment may impose to treat the secondary one by another local (when feasible) or systemic anti-infectious treatment. And even if the secondary infection is induced by another bacterial species than the studied one, possible interferences between secondary and phage treatment still remain: indeed, because antibiotics often lack specificity, the secondary treatment may very well affect the outcome of the phage treatment making it difficult to distinguish which treatment is responsible for what.

Therefore, the response of the patient strain should be carefully in vitro rated afterward to monitor its sensitivity toward phages or to the secondary antibiotic treatment. Swabbing and sampling patient microbiota during the whole study course is then highly recommended especially on D_0 (before phage treatment), on D_n (when a secondary antibiotic starts to be applied), and on D_f (once the treatment reached completion).

12 Monitoring Bacterial Strain Response to Phage

In the draft revision (n°3) of the European "Guideline on the evaluation of medicinal products indicated for treatment of bacterial infections, expected to be released at the end of 2019, it is expected that the frequency of selection of resistance may be estimated initially by exposing strains of species relevant to the indication(s) sought to drug concentrations below, at or above the MIC. It is recommended that the risk of

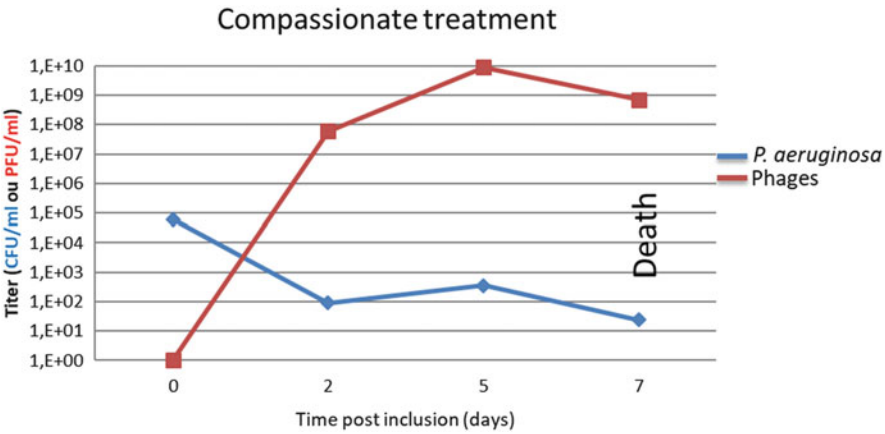

Fig. 5 Monitoring of bacterial response to phage during the treatment of a severely burnt patient following fire immolation. PP1131 phage cocktail multiplication (plaque forming unit: PFU/ml) counting is in red line. Patient *P. aeruginosa* strain growth is in blue line (colony forming unit: CFU/ml)

selecting for resistance is also evaluated in an *in vitro* pharmacodynamic model using drug concentration profiles that mimic those achieved or predicted in infected patients."

Phages are no exception to the rule. A well conducted clinical study should sample the targeted infected area to monitor the response of the patient bacterial strain to the phages before, during, and after the treatment. Attention should be paid to the possibility of carrying out such an evaluation specific of each phage entering in the composition of the treatment. In case of a complex cocktail using many phages, the evaluation is difficult because bacterial titration strains may end up lacking selectivity toward each phage: In that case, monitoring data bear the risk of lacking phage specificity.

As an example, the following figure displays the multiplication of active phages against a bacterial strain during a salvage therapy treatment which took place in November 2015 aside of PhagoBurn clinical trial. Severe burns covered 90% of the patient total body surface and 80% of them displayed a MDR *P. aeruginosa* infection. A last chance treatment with anti-P. aeruginosa phages (PP1131) was attempted, although survival probabilities were less than 2%. Figure 5 displays the monitoring of the phage multiplication in comparison to bacterial growth during the length of the treatment (7 days). Although the initial concentration was low in the range of $10^{1\ \text{to}\ 2}$ an intense phages multiplication took place within 2 days after the beginning of the treatment to reach a plateau at 10^{10}, as the bacterial concentration on the burnt wound decreased from 10^5 to 10^1 on the date of death.

Other approaches may be used to monitor the effect of bacteriophages on a pathology, especially when the clinical trial targets an intestinal infection. In the NESTLE® studies conducted on *E. coli*-induced diarrhea (McCallin et al. 2013; Sarker et al. 2016), in addition to standard phage titration and bacterial microbiology

species determination and counting, high throughput next-generation sequencing supported by bioinformatics were used. These fairly new approaches help having a general view on how a phage treatment may impact the balance of an intestinal microbiota. But they are still in infancy to study the phagome (virome), although a recent paper of van Zyl et al. (2018) demonstrates their value to identify new and shared phages among healthy volunteers skin. In addition, some of them relate to phage DNA material, whether phages are active or inactive or if their DNA is free, intact, or degraded, into the microbiota. They may result in an overestimation of the number of active phage particles and need to be correlated to standard titration techniques.

13 Conclusion

Although used continuously since more than 80 years in Russia and Georgia and for 100 years in Western countries, with close to 30-years interruption or underground treatments, modern phage therapy clinical trials are missing. Among the three modern efficacy trials performed during the last 10 years, only one targeting *P. aeruginosa* otitis was fully successful. PhagoBurn demonstrated that phage therapy is efficient at low doses on *P. aeruginosa*-infected deep buns, if the D_0 patient strains is sensitive to phages, making it obvious that a preliminary companion diagnosis, the phagogram, is essential. As for preserving our antibiotic arsenal (Jim O'Neil 2016) and increasing the chances of patient treatment success, such a diagnostic should become the norm for any kind of curative aimed anti-infectious product.

At the opposite, many individual and a series of cases in Russia, Georgia, Poland, France, Belgium, Switzerland, and the USA have been published. Lately several successful case reports have been detailed in some of these countries. Their compromised clinical situation made it mandatory to maintain inefficient, suppressive, or new (currently being evaluated) antibiotic treatments in addition to the bacteriophages. Phage refractory clinicians explain that such dual treatments are successful because of the supplemental antibiotic(s) but not of the phages.

Such a nonsense opposition is hopefully fading away. Nowadays our antibacterial arsenal is depriving from a validated, ecological, environmentally friendly, and natural therapeutic approach. If 1% of the money invested to fight cancer (close to $US6 billion for the American National Cancer Institute in 2019 https://www.cancer.gov/about-nci/budget and almost $US5 billion for the National Institute of Health (NIH) cancer research in 2018: https://www.aaas.org/news/deep-cuts-nih-other-life-sciences-fy-2018-budget-plan) would be injected into performing modern phage therapy clinical trials, "Saint Thomas would not be able to touch the Christ scars, emerging from the grave, because healing would by now be complete."

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Treatment and Prevention of Bacterial Infections Using Bacteriophages: Perspectives on the Renewed Interest in the United States

Randall Kincaid

1 Background

Diseases arising from bacterial infection have wreaked havoc in human populations throughout history. The most devastating diseases have been those that are highly communicable, such as bubonic plague, cholera and typhoid fever, leading to extensive mortality and societal disruption. However, many types of bacterial infection have been tolerated by humans during their evolution, albeit with significant consequences to the quality of life. For some individuals, particularly the very young and those with impaired immunity, infection could often be deadly. Fortunately, advances in medicine to minimize infection and to reduce the likelihood of exposure to dangerous bacteria have improved human health immensely, with truly transformative changes over the last century in the treatment (antibiotics) and prevention (vaccines) of such infections.

However, these remarkable achievements of medical science may be only temporary victories in the larger battle against bacterial pathogens. We now fully appreciate the remarkable plasticity of bacteria and their ability to circumvent well-designed cures through the development of resistance. As drug-resistant pathogens gain a foothold, and as the densities and movements of human populations increase, we must consider new approaches to manage bacterial disease in order to avoid a "post-antibiotic" era. The impact on modern medicine of broadly drug-resistant bacteria would be devastating, and we could revert to a time when many infections were untreatable. In response to such an ominous scenario, the United States government took steps in 2014 to address this concern, creating a

R. Kincaid (✉)
National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, USA

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strategic plan called Combatting Antibiotic Resistant Bacteria (CARB)[1] to coordinate and support efforts in this area, in collaboration with its international partners. This Presidential initiative includes comprehensive efforts to evaluate new therapeutic approaches and more effective diagnostic tools to tackle this emerging medical problem.

The National Institute of Allergy and Infectious Diseases (NIAID) has targeted antibacterial resistance as one its highest disease priorities. In its evaluation of the current status and future challenges posed by antibacterial resistance, it identified several alternatives approaches that may be used. One of these elements was the re-evaluation of bacteriophages as a means to treat, and potentially to prevent, bacterial infections. However, it was noted that certain aspects of phage-based intervention were not sufficiently developed to meet the regulatory expectations of the United States Food and Drug Administration (FDA) and proposed areas that would need standardization and demonstration of value for this method to become acceptable. To explore this concept more fully, NIAID held two widely attended and productive workshops—one in 2015 and one in 2017 (jointly sponsored by FDA)—to assess the progress and challenges in this area. The workshops drew heavily on the practical experience with phage therapy in other parts of the world, most notably in the Republic of Georgia and in Poland, focusing on the types of medical indications of greatest value and on potential models for its use. NIAID has now significantly increased its investments in this area, both through its funding of basic research and its support for the translational aspects that are needed to support product-oriented efforts.

2 Historical Context of Phage Therapy

At the end of the nineteenth century, there was new hope that we would soon be able to solve the problems of identifying and treating infections. This optimism was based on several technical and intellectual advances in medicine—(1) we had developed the basic tools for culturing bacteria and proven that, for certain conditions, these pathogens could be causally linked to infectious disease, (2) we had established the vector-borne basis of a disease (yellow fever) and thus we could target mosquito populations to reduce disease burden, and (3) we had shown that inactivated/weakened pathogens and/or their toxins could be used to immunize individuals against infection (i.e., vaccination and serum treatment). With the dawn of this new era of microbiology and medicine, new practices were refined and goals were set to improve human health broadly and to prevent major outbreaks of disease. The exciting demonstration in the mid-1910s of filterable entities that could destroy specific bacteria, i.e., bacteriophages, suggested that an effective method for treating bacterial infections would follow quickly; for an excellent and extensive review of

[1] https://obamawhitehouse.archives.gov/sites/default/files/docs/national_action_plan_for_combating_antibotic-resistant_bacteria.pdf

phage and its early medical applications, the reader is referred elsewhere (Abedon et al. 2011).

One of the most telling features of human awareness is the manner in which new innovations in science and technology are portrayed in popular culture, and this is particularly true for phage therapy. One piece of literature that seemed to capture the hopes and challenges of this new era was *Arrowsmith*, the novel for which Sinclair Lewis was awarded the 1926 Pulitzer Prize. Although it is best known for its social commentary on human tendencies when presented with the prospect of fame and fortune, this novel portrays not only the excitement of discovering previously unknown entities (invisible elements referred to as "the X principle," i.e., phages) and their application to medical need but also the dilemma of how to show unambiguously that it is the treatment which is responsible for the cure. In the novel, the introduction of this new cure to stop an epidemic of plague on a fictitious island comes with a dramatic challenge—to prove the value of this treatment by using a control group of individuals that would not be given the proposed cure. In the end, expediency and ethical considerations prevail and the lives of many are saved; however, the proof of the cure is never established. This "proof of cure" dilemma for phage treatment remains with us 100 years later.

Phages were first used to treat wound infections and are reported to have been used as interventions in disease outbreaks (plague, cholera). Not surprisingly, much of the initial interest in phage products was to support the needs of the military (of the former Soviet Union), where contamination of food and water were common, resulting in gastrointestinal disease (dysentery) and to treat wound infections, which was an urgent need. The apparent safety, stability and portability of such medical remedies were considered crucial to the needs of the soldiers. These early medical applications of phage provide useful insights into the perception of product "value," which is an important aspect of all product development that must be considered. As will be discussed later, it is essential to establish the distinctive attributes and economics associated with a medical product in order to support its adoption and continued use. Versions of these early phage products, which are comprised of complex collections of phage ("cocktails"), continue to be used in the Republic of Georgia for the treatment of gastrointestinal infections ("Intesti-phage") and wounds ("Pyo-phage").

The technological revolution in chemical discovery and synthesis, combined with improvements in isolation and characterization of bacterial pathogens, led to a new generation of medical solutions—antibiotics—that would largely replace bacteriophages as a therapeutic option. The coincident interests of chemical and pharmaceutical industries following World War II led to the creation of several classes of small molecule antibacterial drugs. These could be manufactured from widely available starting materials and then characterized definitively, providing a reliable source of treatment. Perhaps most significantly, the new remedies were *broad-spectrum* antidotes that could treat many types of bacterial infections, which seemed to be a very attractive feature of these drugs. In Western medicine, there had been substantial skepticism about the reliability of phage therapy, and this, coupled with the availability of seemingly superior solutions, led to the virtual abandonment

of phage therapy outside of former Soviet Union (FSU) in favor of these new antibiotic "miracle drugs." Even in the FSU, use of phage waned, although adjunctive approaches, i.e., antibiotics plus phages, were often used to improve medical outcomes.

The perception that disease arising from bacterial infections could always be controlled with antibiotics began to erode in the 1950s, as evidence accumulated that drug-resistant bacteria were emerging. To a scientific world that had yet to fully understand the basis for genetic mutations, this was a disturbing trend, but it was hoped that future generations of drugs would become available to overcome this issue. However, drug resistance continued to emerge as a new fact of life, including the resistance of bacteria to many different classes of drugs. Such multidrug-resistant (MDR) bacteria clearly posed a major threat to public health, as evidenced by the establishment of medical treatment facilities, notably the Ludwig Hirszfeld Institute in Poland, that began to accept patients for whom conventional drug therapy was unsuccessful. The inability to treat bacterial infections, even with a wide array of approved medications, ushered in an era of exploratory evaluations of bacteriophage treatment administered under the broad umbrella of "compassionate use," i.e., the use of an experimental procedure when approved treatments are deemed ineffective for a serious, and potentially life-threatening, medical condition. It is against this backdrop—the rise of MDR bacterial pathogens and its impact on future therapeutic management of infection—that renewed interest in bacteriophage treatment occurred.

3 Renewed Interest in Phage-Based Medical Interventions in the United States

In the United States, bacteriophages have played an important role in modern basic science, particularly for a mechanistic understanding of viral replication in host cells. It was well known that phage particles could be manipulated easily and used as tools for molecular biology by virtue of their ability to incorporate fragments of DNA into its particles, enabling "libraries" of genetic elements that could be used to transform bacteria. Outside of such basic research purposes, the product applications of phage were not widely appreciated. In the 1990s and 2000s, phages were increasingly studied as a means to reduce exposure to harmful bacteria associated with food, leading to authorization of phage products by the FDA for food safety applications, eventually to include pathogens such as *Listeria*, *Salmonella*, and *E. coli*; these approvals were an important signal that regulators did not view phage as posing inherent safety risks, at least when taken orally. However, for therapeutic indications, the decades-long use of antibiotics had assured that drugs, and not phage, were to be considered the "standard of care" for bacterial infections. Even with the growing concerns of emerging MDR pathogens, there was little advocacy for their clinical use and doubts that they would ever be adopted by the medical community.

Much of this changed after the publication of NIAID's strategic plan outlining a longer-term vision to address antibacterial resistance.[2] This document, which was followed quickly by announcement of the CARB initiative, expressed an interest in re-evaluating phage and phage-derived products for therapy, as well as considering their broader value in combatting drug-resistant bacteria. This interest was described under the "Innovative Directions" section of the NIAID strategy as having the benefits of specific eradication of bacterial pathogens without the debilitating side effects on the host microbiome, which is a serious concern particularly with very ill patients. Importantly, this section outlined several pragmatic concerns, which were considered critical for its adoption in Western medicine, as follows:

> . . .While phages have been used clinically in some countries for decades, their use in the U.S. has been limited. Process improvements in the production, quality assurance, and validation of these products are required, and carefully controlled clinical studies will be needed to establish efficacy in treating drug-resistant pathogens. . .

Within the NIAID, an interest group on bacteriophage was formed to consider the potential benefits of this alternative approach and to assess management of potential risks for phage and related products. Notable in this effort was the inclusion of colleagues from the FDA's Center for Biologics Evaluation and Research (CBER). This informal relationship was important to the critical thinking needed for this assessment, allowing vigorous discussion and insights in both directions that could be used to support both development strategy for products as well as the regulatory expectations. One early outcome of this group's interactions was the decision to convene a workshop consisting of the leaders in the field; the goals of the meeting would be to inform the scientific community of the current status of phage therapy and to obtain consensus on the challenges and key opportunities for medical intervention.

This initial workshop, "Bacteriophage Therapy: An Alternative Strategy to Combat Drug Resistance," was hosted by NIAID and held in Rockville, MD, in July of 2015.[3] It was attended by members of the commercial, medical, regulatory and public interest sectors and received attention from a scientific publication as signaling a "revival" of interest in this area (Madhusoodanan 2016). Although the workshop included considerable discussion about the reliability of phage therapy and the importance of conducting rigorous clinical studies to demonstrate efficacy of the treatments, there also were several encouraging presentations and audience questions that suggested opportunities for future development and medical use. Presentations at the meeting included historical overviews on medical approaches, ongoing clinical efforts and basic research investigations including some which involved "engineered" bacteriophage that might have specific desirable properties. Some of the themes that were developed at the initial workshop are discussed below.

[2] https://www.niaid.nih.gov/sites/default/files/arstrategicplan2014.pdf

[3] https://web.archive.org/web/20160413022959/https:/respond.niaid.nih.gov/conferences/bacteriophage/Pages/default.aspx

The overview of the efforts at the Hirszfeld Institute in Poland to employ phage therapy for patients with drug-resistant infection offered a compelling argument for its use under "compassionate use" guidelines (referred to as "expanded access" by the FDA). The utility of such treatments over several decades, with more than 1300 patients, was seen as evidence of the potential of phage treatment for mitigating drug-resistant infections of many different types including sepsis, infected ulcers, urinary tract infection (UTI), respiratory infections, and osteomyelitis (Weber-Dąbrowska et al. 2000). The protocol used for treatment required the direct demonstration of phage efficacy against the patient's isolate. Thus, this method constitutes a form of "personalized" medicine that can be tailored to an individual situation, but it is also dependent upon having access to a collection of diverse bacteriophage. At the meeting, and in later discussions, the logistical aspects of such a "center-based" approach were considered. Would such "expanded access" treatment of complicated infections, modeled after those used in Poland and the Republic of Georgia, be a practical solution for most countries? This may be quite difficult if the patients requiring such efforts were in intensive care environments and could not be transported. By contrast, if a "distributed" model were used to make phage available to hospitals, how would this be organized and administered? Would there be master banks of phage for such emergency treatment and, if so, what types of requirements would be placed on the characterization and diversity of phage that would reside in such repositories? The juxtaposition of these alternative models for treatment of MDR bacterial infections underscores the importance of cooperative planning and building of the resources that could be deployed when needed.

Discussion of surgical applications, which was initiated by members of the military science community, suggested an important potential use of phage to reduce the likelihood of post-operative infection. In this case, phage treatment would be done as prophylaxis, rather than therapy, and would likely be included as part of the surgical procedure. This administration of phage could be done by irrigating the wound bed or by incorporation into a "time release" matrix that could be resorbed during the recuperative phase. This would likely be done as an adjunctive treatment, i.e., along with antibiotics. In light of the increased frequency of surgical procedures, many of which are elective (e.g., joint repair, replacements) rather than based on an urgent medical need, such a phage-based application would seem appealing from an operational and economic point of view. The market for such procedures is quite significant and there may be considerable value in a simple procedure that reduces the risk of infection, particularly in settings where MDR pathogens are a potential risk. In cases of traumatic injury and complex wounds that require surgery, there is always a significant risk of infection and, if re-infection occurs, the follow-up procedures (debridement and cleaning of the wound bed prior to its closure) are often difficult and costly. Phages would appear to be a safe option and may have the advantage of replicating locally to clear a post-surgical bacterial infection particularly if it were associated with more complex communities of pathogens (biofilms). However, the choice of phages for such preventative applications would necessarily be very important to ensure coverage against many potential opportunistic pathogens.

Another subject of interest involved the identification of medical conditions, and specific patient populations, that may be associated with hospital-acquired (nosocomial) and community-acquired MDR pathogens. These include intensive care patients that need long-term care and often require respiratory support, individuals with recurrent infections (e.g., of the urinary tract, cystic fibrosis, rhinosinusitis, otitis, etc.) and those with complicated skin and soft tissue infections (burns, diabetic ulcers, osteomyelitis). In some cases, standard of care treatment may not be adequate, either because of highly drug-resistant pathogens, the presence of biofilms or because of the toxicity of the treatments that are required. Prior studies at the Hirszfeld Institute had shown mitigation of drug-resistant disease for a wide variety of medical conditions, including many of those described above; therefore, in such cases adjunctive therapy with phage may provide a useful means to overcome a complicated infection. In addition, simple prophylactic use of phage may be indicated, i.e., to eliminate colonization by drug-resistant bacteria such as vancomycin-resistant *Enterococcus* (VRE) or methicillin-resistant *Staphylococcus* (MRSA), to reduce the risk of exposure to such problematic pathogens. Because immunocompromised individuals constitute a major patient group at risk of such infections, questions were raised about the efficacy of phage in these populations. There was no clear consensus on this question and several participants voiced the view that phage therapy probably provides a means to rapidly reduce pathogen burden, enabling the host immune system to efficiently eradicate the infection. Because of the significant usage of antibiotics in patient populations with impaired immunity (e.g., cancer, organ transplantation patients), if phages were known to be effective in such immunocompromised individuals, this would be of considerable value.

The regulatory pathway for approval of phage therapy products was discussed at the meeting; many of the issues that were highlighted by FDA representatives mirrored the concerns described in the NIAID strategy statement, i.e., the need to characterize the properties and purity of each phage preparation, the consistency of processes for manufacture (so-called Chemistry, Manufacturing and Controls or CMC), assays to measure potency and stability of phage preparations, and clinical studies to show definitive evidence of efficacy, etc. An effort was made to outline the specific phases of clinical study that would be required, with an emphasis on the increasing requirements for quality assurance of product (current Good Manufacturing Practices, or cGMP) in the later stages of such work. In the context of potency and reliability, there also would be a need to demonstrate the value of each phage used as part of the therapeutic phage cocktail and to consider the potential for development of resistance to these phages, this being particularly important if the goal were to treat chronic conditions that require repeated administration of phage. No specific regulatory concerns were voiced about the use of engineered phage, per se, except to note that such genetic modifications would require adequate description and verification of the stability and safety profile of the phage; this would include evidence of their ability to remain lytic (as opposed to becoming temperate), their potential for DNA transduction and the likelihood of resistance development.

General consideration of the key "drivers" for adoption of phage therapy, beyond its use under expanded access guidelines, focused on the acceptance by medical professionals. The conclusion expressed by infectious disease physicians was, unequivocally, that definitive clinical data was lacking and that it would be required for use. In addition, a strong economic model for business development would be needed that would justify the rigorous and costly development of such products, whether for treatment or prevention of disease. Concerns were also voiced about the relatively sparse understanding of phage biology that exists and that much more funding would be needed to support future medical use. Discussions in this area focused on a lack of fundamental knowledge of the phage that might be used, noting that considerable research would be required to fully characterize the dynamics of phage-bacteria interactions, particularly in a physiologically relevant host environment, before such products could be rationally developed. The consensus view was that more detailed information was needed in several areas—phage bioavailability and clearance under conditions of its medical use, immune tolerance to phage and the basic mechanisms that contribute to development of resistance.

As part of its broader solicitations to study antimicrobial resistance (AMR), NIAID increased its funding of phage-based studies; in 2013 there were no grants for phage therapy and, by 2016, this had increased to nine awards. These research grants covered many aspects of phage therapy including the development of novel engineered phage and phage-like entities, approaches to more efficiently deliver phage to areas of respiratory infection, and several studies with translational implications for specific indications including cystic fibrosis, complicated UTIs, and decolonization of patients at risk of acquiring nosocomial pathogens such as VRE. NIAID also began efforts to support the needs of the phage community by targeting resources toward its animal model testing services, encouraging the creation of detailed product development plans to define the risks and market opportunities for development of specific products, and initiating collaborations to collect phages that may be relevant to high-priority MDR pathogens, e.g., *Klebsiella*, *Acinetobacter*, etc. In addition to such direct financial support, outreach was initiated to engage the phage community through conference presentations and by expanding cross-government efforts to provide timely awareness of current phage-based efforts.

In 2017, a second workshop was held in Rockville, MD, which was jointly sponsored by CBER/FDA and NIAID/NIH.[4] This workshop, "Bacteriophage Therapy: Scientific and Regulatory Issues," was an effort to update the scientific and medical communities on progress in this area and to examine more fully the regulatory aspects that are relevant to medical use of phages. Notably, the workshop provided a tacit acknowledgment by the regulatory agency of the growing interest by phage product developers and the need for general guidance regarding phage-based products; fittingly, the first session was introduced by Dr. Peter Marks, the Director of CBER. Talks included two dramatic case studies of patients who had recently

[4] https://www.fda.gov/BiologicsBloodVaccines/NewsEvents/WorkshopsMeetingsConferences/ucm544294.htm

undergone phage therapy treatment in the United States under expanded access use. One of these involved a patient who recovered, following multiple intravenous doses of phage, after having slipped into a coma due to complications of the infection (Schooley et al. 2017). This case has been the subject of much public attention in the media about the use of phage as an alternative therapy when other options are not possible. The subjects discussed at the meeting covered both clinical goals and public health needs that might be met by use of bacteriophage, as well as new experimental research on phage. Some of the concepts that were discussed are described below.

The medical potential for expanded access use of phage was illustrated in two case studies that were presented. In one case, a complex intraabdominal infection with *Acinetobacter* was not treatable using antibiotics, and the patient's condition deteriorated rapidly, requiring the use of life support and drugs to maintain blood pressure. Outreach to specific members of the phage community (participants in the 2015 workshop) and coordination with FDA enabled a rapid response to this crisis, and the phage preparations that were provided over a period of a few weeks were able to rescue the patient. This heroic effort benefited not just from phage treatment but also from the expert medical care needed to sustain the individual, due to the many complexities of his condition. Some of the lessons learned from this episode included the challenges of assuring phage purity (removal of endotoxins) and the reality of phage resistance to the pathogen, which occurred very quickly. The other example involved a recurrent infection with MDR *Pseudomonas*, which was associated with a coronary bypass surgical procedure (Chan et al. 2018). This infection had proven difficult to treat, with four episodes of sepsis requiring prolonged infusions of antibiotics, and the development of bacterial biofilm in the area of the lesion. Administration of the phage cocktail appeared to provide unique value by efficiently disrupting the biofilm to allow sterilization of the infection. However, perhaps even more significant, from a scientific point of view, was the use of a phage that directly targeted a primary mechanism of antibiotic resistance, a membrane transporter responsible for drug efflux. In this case, any bacterial resistance to the phage necessarily would result in the *restoration of drug sensitivity*. Such a strategy for phage selection, based on the ability to interfere with a drug resistance mechanism, may hold promise as a general approach for development of adjunctive therapies involving phage.

The workshop also included a presentation on the first randomized, blinded multicenter clinical trial involving phage (the Phagoburn trial, supported by European Commission funding), which illustrated the types of challenges that would likely be faced by developers of phage products to be broadly used for a specific medical need. In this case, the medical indication targeted by the trial was severe burns that are infected by the opportunistic pathogens, *Pseudomonas* or *E. coli* (or both, potentially). The need to cover two different pathogens, each requiring a cocktail of phages, imposed a significant burden on the developer to characterize the phage and to demonstrate stability and activity of each component in the treatment. In addition, the need to change the cocktail components, which might be anticipated due to resistance in some isolates, was a central concern to the study's sponsor. This

issue raised the key question of how developers would respond to the need for modification or "evolution" of phage products as conditions change, as well as to anticipate the regulatory requirements for product registration that may apply. By analogy to the substitutions of influenza strains that take place yearly during vaccine development, it seems likely that *replacement* of phages, with ones that are closely related functionally, may be acceptable without the need to file new marketing application. Such allowance of modifications to an approved phage cocktail would be of great consequence to developers, and this issue will likely require formal regulatory guidance in the future.

The many thoughtful presentations by FDA officials at this workshop were crucial for several reasons. They addressed many commonly held notions of product bias against phage products and emphasized that the process for review and approval was fact-based, flexible, and interactive. The talks addressed how specific elements of product applications, which apply to all classes of antibacterial therapeutics, e.g., the spectrum of activity, product purity, consistency of manufacturing process, etc., apply similarly to phage therapy products. Distinctions were made about the requirements that apply if the product's intended use is considered "personalized" (expanded access), as opposed to more widespread use for specific indications. Expectations related to certain preclinical data, e.g., bioavailability/elimination of drug, animal model efficacy studies, as well as regulations for the initiation of clinical studies under an Investigational New Drug (IND) application, were discussed; here again, the overarching theme was that the underlying principles apply similarly to all potential therapeutics. These principles, which include CMC guidance and the adequacy of clinical data to support a particular indication, were framed to allow phage developers to appreciate the generality of FDA regulations. The use of guidelines for "streamlined/adaptive" protocols for clinical studies was also mentioned, which could afford developers additional flexibility in the assessment of potential products for treatment of bacterial infections. Substantial emphasis was placed on communications with regulatory officials prior to initiating phage product development efforts, via pre-IND meetings, at which time specific clarification and advice can be obtained.

Several research themes of the workshop were noteworthy, inasmuch as they focused on key practical challenges to the development of phage products and opportunities for their use. These included detailed studies of the mechanisms by which bacteria may become resistant to phage and the dynamics of those processes, these being essential for directed efforts to engineer phage to circumvent such resistance. Presentations on efforts to engineer bacteriophage or to create phage-like particles with improved properties were also presented. The potential improvements being considered included the expansion of host range to reduce the need for complementary phage in a cocktail and the notion that phage could be modified to deliver specific "cargo" (such as biofilm-degrading enzymes or CRISPR-Cas elements that target drug resistance genes) that would enhance their medical utility. Other topics that were discussed included pragmatic technical issues of selecting phage that could be optimally combined to create cocktails, including methods to automate and accelerate this process using large banks of phage isolates.

Another intriguing talk on *Vibrio cholerae* phages highlighted their evolutionary adaptation to bacterial CRISPR defense mechanisms and the co-opting of these elements for use against the bacterium. That presentation also suggested the potential use of phage cocktails as an intervention strategy during outbreaks of cholera, using a community-based prophylaxis strategy to limit the spread of this highly debilitating disease and to minimize potential social disruption. Such a preventative approach may have significant public health implications, particularly in areas of the world (e.g., South Asia, Africa) that may expect to encounter such outbreaks.

4 Potential Models and Indications for Medical Use of Bacteriophage

Bacteriophage therapy represents only one of many innovative solutions that might be employed in the battle against MDR bacteria. However, there are many reasons why phage would appear to provide a reasonable alternative for future development. They are the product of ~3 billion years of evolution and, as such, have been selected for an important functional endpoint, the killing of bacterial pathogens. They are, by far, the most abundant form of life on earth and this diversity creates an almost limitless supply of candidates for development. The apparent safety of phages, combined with the ability to counteract harmful pathogens without affecting the benefits of our commensal bacteria, would also seem to be extremely valuable. However, the exquisite specificity of phage for their prey could also be problematic since they may be ineffective against even closely related isolates of a particular pathogen. This "narrow host range" challenge will need to be addressed by creating well-defined cocktails of naturally occurring phage or, perhaps, by iterative selection or engineering of phage to increase their host range. It is very likely that resistance to phages will continue to be an ongoing concern, as this reflects the very nature of their co-evolutionary relationship with bacteria. Strategies that bias the selection of therapeutic phages toward those whose receptors contribute to bacterial virulence, or to mechanisms conferring drug resistance, may be purposeful approaches to counteract such an intrinsic biological tendency to respond to selective pressure.

It will be important to prioritize development of potential phage-based interventions based on the likelihood of their impact on, and adoption by, the infectious disease community. As underscored by the examples presented in both of the NIAID workshops, a strong case can be made for use of phage under "expanded access," at least for otherwise untreatable drug-resistant infections. To address such a need in a comprehensive way, it will be necessary to produce and qualify phages that address targets of highest concern, e.g., pathogenic *E. coli*, *Pseudomonas*, *Klebsiella*, *Acinetobacter*, MRSA, etc. Such an effort to create a therapeutic phage repository will need significant resources, and will likely require funding and sponsorship from governmental bodies, as well as input from regulators on the requirements for inclusion. As discussed previously, there are two general models that are possible for treatment of such at-risk patient populations. One can imagine creating centers that specialize in the treatment of drug-resistant bacterial

infections, similar to the efforts of the Hirszfeld Institute. Such centers would have expertise necessary for the microbiological identification of appropriate phage, in addition to experience in formulating and administering them. Broadly applicable research protocols could be established that would ensure timely inclusion of such patients and standardized procedures for maintaining records of phage treatments; such a research protocol has been in place for studies at the Hirszfeld Institute since 2009.[5] One potential drawback is that such "centers" may be limited to treating those infections which do not require urgent medical action, since travel to the facility would be a considerable risk for patients who have true health emergencies. An alternative model would be to create a repository resource that can provide qualified phages directly to hospital pharmacies, either "on demand" or as part of a process to create an inventory of phage for pathogens that are viewed as being relevant to that medical facility. Such a "distributed" model may be more adaptable to the needs of critically ill individuals and the challenges of meeting urgent medical needs; however, it may also require training in the appropriate selection, formulation and administration of phage cocktails by medical staff and oversight on monitoring of resistance development. It is unclear how such a dedicated phage repository would need to be managed, in order to address such emergency situations, and what types of technical support would be required for use of phage therapy. Because of the relatively rare instances for such use, approval of individual clinical protocols (investigational use under expanded access) would likely be on a case-by-case basis and require specific justifications.

Beyond the expanded access use described above, there are several important medical indications for infection that would appear plausible as priorities for phage therapy. At the outset, it is essential to carefully define attainable goals for treatment (or prevention) because of the significant regulatory burden that is required for product approval by regulatory agencies. To be considered viable candidates, the unique advantages of the product over current interventions must be demonstrated, along with assurances that they can be produced consistently and used reliably for the particular indication. Based on consensus views of the phage community at the workshops, and as evidenced by the initial efforts of several phage therapy enterprises, a list of indications can be compiled for such bacteriophage products. As of the writing of this chapter, a number of these have been initiated or are in the patient recruitment phase; the details of these clinical trials and their design can be accessed at www.clinicaltrials.gov by entering the appropriate clinical trial "identifier." In the United States, most clinical trial efforts related to phage products will require standard IND filings and review by FDA, although it may be possible to shorten this process by seeking "accelerated approval" for a serious condition that is considered to be an "unmet medical need." This latter process relies heavily on "surrogate endpoints," i.e., markers that can be correlated with a desired clinical benefit, but that do not directly demonstrate such benefit. The sponsor is nevertheless

[5]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT00945087

required to carry out studies to show that such benefit occurs (so-called Phase 4 trials).

Two indications that seem particularly apropos involve the topical administration of phages to treat wound infections and the oral administration of phage for certain gastrointestinal disorders; this is perhaps not too surprising since these were among the first uses of phage, historically speaking. An initial safety study for topical treatment of *Staphylococcus aureus* infections, co-sponsored by the Walter Reed Army Institute of Research and private enterprise (AmpliPhi Biosciences), was completed in 2016[6] and this product is now being made available by the company for use under expanded access, as would be the case for drug-resistant forms such as MRSA.[7] In addition, recruitment is underway for clinical safety studies to evaluate the oral administration of phage that target a type of *E. coli* which has been associated with the debilitating inflammatory responses seen in Crohn's disease.[8] This effort, involving a commercial collaborator (Intralytix), may be able to leverage the specificity of phage for a subset of bacteria that populate the gut, and may have exciting implications for targeted therapeutic intervention in the gut microflora. Interestingly, a talk in the 2105 workshop, by a representative of the Bill and Melinda Gates Foundation, had discussed a Grand Challenges project that sought to use phage as a strategy for "re-modeling" of the gut microbiome.[9] That effort hoped to improve the health of children suffering from a chronic condition of gut inflammation brought about by malnutrition; it was envisioned that such phage manipulation might be used to restore functional stability of the gut microbiome and enable defense against enteric pathogens.

Urinary tract infections (UTIs) may constitute another indication for which phage may be useful. Recently, a blinded, randomized clinical study was completed in which phages were evaluated for their ability to reduce bacteriuria in patients who were scheduled to have transurethral prostate resection;[10] a synopsis of this study has been published (Leitner et al. 2017). This effort, a collaboration between a European medical center and the Eliava Institute in the Republic of Georgia, utilized intravesical instillation of phage directly into the bladder. This procedure is noteworthy because, in contrast to parenteral routes of administration in which the medication rapidly becomes available systemically, instillation may reduce access of the phage to the circulation and thereby reduce the rate of clearance from the body. Such a "sequestration" approach may improve its ability of phage to eradicate an infection efficiently by remaining in the local region where the pathogen is present. Complicated UTIs, which usually are associated with functional abnormalities in bladder function and/or the presence of drug-resistant pathogens,

[6]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT02757755

[7]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT03395769

[8]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT03808103

[9]https://gcgh.grandchallenges.org/challenge/addressing-newborn-and-infant-gut-health-through-bacteriophage-mediated-microbiome-0

[10]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT03140085

may be actually be a preferred indication for use of phage therapy inasmuch as the standard of care antibiotic treatments may be ineffective, thus encouraging phage use to reduce pathogen burden and resolve the infection.

Complicated skin and soft tissue infections (SSTIs), also referred to as "acute bacterial skin and skin structure infections" (ABSSSI), are a major medical concern, arising from a number of sources—burns, skin ulcers, and abscesses which inevitably damage underlying soft tissue—and these are most often treated systemically with broad-spectrum antibiotics because of their potential for severe outcomes. Under such circumstances, the pathogen(s) is often unknown and an empiric drug regimen is prescribed. The lack of definitive pathogen identification poses challenges to the use of phage for such indications; however, it may be worth considering the use of a broad collection of phages (similar to Pyo-phage preparations used in the Republic of Georgia) as a mitigation strategy for pathogens that may be drug-resistant. Improvements in the method of administration may be needed to ensure that these phages are present for extended periods of time and development of "time release" formulations, which can be directly applied directly to the wound bed, may be warranted. Some types of SSTIs, notably those associated with diabetes (i.e., foot ulcers), may be particularly suitable for phage therapy, in part because of the reduced peripheral circulation often seen in more advanced diabetic conditions; the relatively poor clearance in the extremity may enable a sustained availability of the phage if applied locally. Currently, parenteral antibiotic treatment of moderate to severe diabetic foot ulcers is recommended until there is evidence of clearance of the infection; however, this may not allow for full resolution (healing) of the ulcer due to possible re-infection. A very significant and predictable market exists for such an indication (~15% of diabetics eventually develop foot ulcers), and the complications may be severe, resulting in progressive morbidity including osteomyelitis and the need to amputate. A clinical study involving the topical application of phage for such conditions has been registered in Europe but has not yet begun patient recruitment.[11] The commercial collaborator for this effort, Pherecydes Pharma, was responsible for conducting the previously mentioned Phagoburn clinical trial efforts on burn-related infections, which was initially a collaborative effort that was driven by European military science interests.[12] Recently, the results of the Phagoburn trial were published (Jault et al. 2019) and the findings were disappointing, with phage treatment being somewhat less effective than the active comparator used in that study. Although there may be understandable reasons for this outcome, it nevertheless highlights the concerns that have surrounded phage-based approaches for decades. A useful synopsis of this trial is also given in another chapter in this book (see chapter by Gabard J, Jault P. "*How to achieve a good clinical trial*?", pp. . .).

Phage therapy has been proposed for treatment of recurrent respiratory tract infections including difficult-to-manage conditions such as chronic rhinosinusitis

[11]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT02664740

[12]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT02116010

and cystic fibrosis (CF), which requires periodic reduction of bacterial burden. The pathogen most commonly associated with cystic fibrosis infections is *Pseudomonas*, although *Staphylococcus* is often seen in younger children. The progressive decline in pulmonary function in CF patients can be life-threatening and is exacerbated by recurrent infection, requiring rounds of antibiotic treatment that are usually administered by inhalation. Because this disease is rare, potential therapeutics have been given "orphan drug" status by the FDA which entitles a drug sponsor to certain financial incentives for their development. Respiratory conditions such as CF and chronic sinusitis are significant medical problems that often necessitate long-term antibiotic use, making them susceptible to emergence of drug-resistant pathogens for which phage therapy may be particularly useful. However, there may be challenges both in the formulation of the phage product for inhalational use and in the reliability of the product, since long-term use is likely to lead to phage resistance and require selection of new phage to support its continued use. Efforts to develop phage treatment for *Pseudomonas* infections have been initiated and this product may be obtained from the company (AmpliPhi Biosciences) for investigational use.[13]

Phage treatment is most often discussed in the context of treatment of infection but there may also be value in preventative phage approaches, either for situations where antibiotic use is discouraged (e.g., due to tissue toxicity or debilitating effects on gut microbiome) or because of unique properties afforded by phages, such as their effectiveness in resolving biofilms. As highlighted during the workshops, prophylactic use during surgical procedures may be advantageous and it is conceivable that developers of surgical devices would see this as being a useful strategy for limiting post-operative complications. Infections that occur after surgery involving prosthetic devices, which include joint replacement/repair or vascular grafts, are relatively rare but they have very significant consequences including the potential for septicemia. These infections often involve the formation of biofilms, such as those generated by mucoid forms of *Pseudomonas*, and are very hard to eradicate. This may lead to repeated febrile episodes and a need to restart antibiotic therapy with the patient. Phages appear to be generally effective in disrupting biofilms and thus may be suitable for such indications. Formulations of phage that are imbedded in prosthetic materials (e.g., hydrogel coating of fabrics used in grafts) may also enhance the effectiveness by making phage available over longer periods of time.

Hospital environments, particularly those which support intensive care of patients, are associated with nosocomial are to monitor and mitigate the potential for infection. The sources of such problems are varied and complex involving physical contact of patients with healthcare workers, over-prescription of antibiotics, and contamination of the many devices that are essential for patient care such as ventilators and catheters. For individuals requiring long-term antibiotic therapy, the risk of enteric colonization by drug-resistant pathogenic bacteria is increased and certain infections may occur, notably VRE or those involving cephalosporin- or carbapenem-resistant Enterobacteriaceae. These infections are usually manageable,

[13]Details available at www.clinicaltrials.gov; clinicaltrials.gov identifier NCT03395743

but it may be possible to limit their occurrence, and the potential for their dissemination, by oral administration of phages that are specific to these pathogens. Phage may also be useful to reduce the carriage and shedding of MRSA from the nares, as is often done using antibiotics such as mupirocin, the current standard of care for such pathogen decolonization procedures. There is growing concern that alternative treatments for decolonizing *Staphylococcus* may soon be needed because of increasing bacterial resistance to mupirocin. Such efforts to reduce the incidence of healthcare-associated infection using preventative measures are sometimes undervalued; however, the impact may be of such significance that financial incentives are warranted to encourage improvements in control measures (Drohan et al. 2019). Because a variety of decolonization approaches are already in use, such phage-based infection control efforts would require strong evidence to show that they provide the additional value to hospital management that is needed for their procurement and adoption.

As mentioned earlier, phage may have played a role in the blunting of outbreaks of highly contagious diseases such as cholera, despite rigorously controlled studies to prove this claim. Famously, there have been assertions that individuals from areas where such outbreaks occur periodically, such as India, could be protected by ingesting water that contained bactericidal activity against *Vibrio cholerae* (Hankin 1896); it has been speculated that such activity was due to bacteriophage. As presented at the 2017 phage therapy workshop, a prevention strategy has been proposed to treat affected communities with oral formulations of cholera phage during an outbreak in order to assess the value of such intervention in reducing outbreak severity. In light of the public health impact of cholera, which is often devastating both to individual health and to the socio-economic dynamic of the region, such an effort may be worthwhile. As a result, a detailed product development strategy has been considered for this purpose at NIAID, in keeping with its desire to improve health in parts of the world that may benefit from such approaches.

5 Prospects for the Use of Bacteriophages as Medical Interventions

The renewed interest in bacteriophage-based medical interventions in the United States has been driven primarily by increasing concerns about bacterial drug resistance and the need to seek alternatives to current classes of antibiotics. To date, there are no phage products that have received regulatory approval for medical needs, although FDA has granted approval for several food safety applications. Therefore, the greatest challenge to their viability as products for specific indications is to demonstrate that such products can be shown to be safe, efficacious and reliable based on evidence obtained in clinically relevant settings. This is the same set of challenges that exist for any new treatment or preventative measure. There is good reason to believe that safety issues are unlikely, based on decades of use and numerous studies where this has been evaluated. Efficacy, which has been shown in many individual cases of personalized treatment, has yet to be rigorously proven

for a well-defined product when used for a specific indication and clinical studies that are statistically powered are required to show this unambiguously.

Several intrinsic challenges that are posed by the use of bacteriophage must be addressed. For instance, a characteristic that is often touted as a benefit—that of the exquisite selectivity of phage, which reduces the "off-target" effects on commensal bacteria—also conveys a major challenge for its practical use, namely the ability to ensure effectiveness across disparate isolates of the same pathogen. As described earlier in this chapter, the use of multi-component cocktails that are screened against large banks of pathogen or careful engineering of phage to increase the phage's host range may be needed to circumvent this issue. Intimately connected to this issue is the reality of resistance to phage by the pathogen, an inevitable consequence of their co-evolutionary relationship. It is therefore likely that resistance development and characterization of this process will be among the chief regulatory concerns. To address the problem of resistance, strategic approaches in selecting phage may be needed, perhaps by targeting those phage receptors for which mutations would attenuate the virulence of the pathogen. Similarly, to address reliability concerns, consideration should be given to selection of phage that utilize different receptors and/or mechanisms to disable the host; identification of such "functional complementarity" strategies for phage cocktail components would be highly desirable. In addition, the processes used for manufacture and quality assurance will require considerable standardization and refinement in order to meet regulatory expectations for product consistency.

It is also important to appreciate the context in which phage products will be evaluated clinically. Current best practices in medicine will ethically require that phage be evaluated for treatment of an infection as an *adjunctive approach*, i.e., in the presence of antibiotics. This could create significant challenges in showing that phage provide additional value and may require careful selection of patient populations in which phage use may offer unique advantages (e.g., biofilm eradication, localized application to diabetic ulcers). The rationale and design of clinical trials needed to demonstrate and objectively measure such benefit (the primary and secondary endpoints of the study) will be critical, and developers are strongly encouraged to consult with FDA early in the process. Agreement on what constitutes an "adequate and well-controlled" investigational study should be the overarching objective of such pre-IND discussions.

Unfortunately, the economics of anti-infective solutions also represents a major hurdle, as the "value proposition" for compensating developers still remains a very difficult argument to make. In spite of the urgent need for future antibacterial medications, i.e., to avert a frightening scenario in which MDR bacterial infections are commonplace, the prices of these new medications are still expected to be very low. A number of funding efforts, both in the United States and elsewhere, have been launched to underwrite the development of new therapies; however, these by themselves may not be enough. Acknowledgment of this growing concern may ultimately lead to fundamental changes in the policies, which have existed to support healthcare over the past few decades, and spur new thinking about tangible incentives for development. It is noteworthy that this general notion of incentives

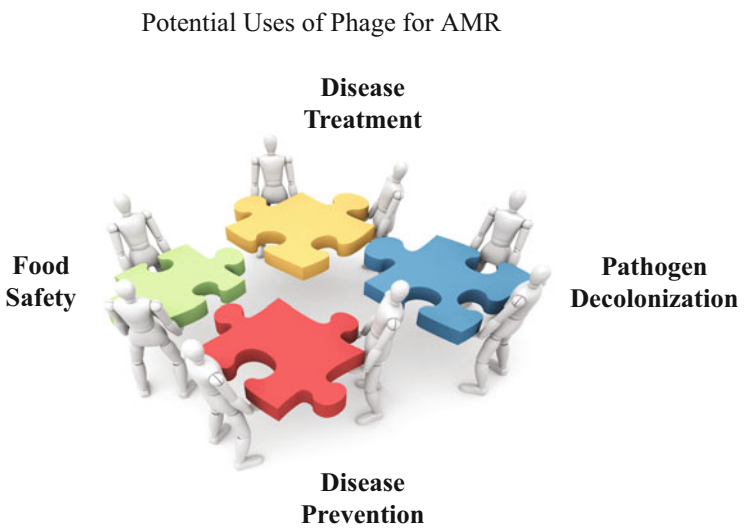

Fig. 1 The big picture: how might bacteriophage be used to combat drug-resistant pathogens?

was proposed recently, following a comprehensive analysis of the economic impact of MDR pathogens—the so-called O'Neill report.[14] Because of the extreme consequences of drug-resistant pathogens on society and its economic productivity (estimated to be $100 trillion by 2050), a series of recommendations have been made to set aside funds specifically for the development of new medications and other interventional agents to address this problem. This fund could be used to defray the development costs and to alleviate the perpetual concern about a "poor return on investment" that commercial enterprises must consider. This type of trust fund would seem warranted, providing an "insurance policy" against the continued erosion of current medical resources in the battle against drug resistance. Phage may be one of many strategic approaches that are needed to reverse the trend toward drug-resistant bacterial pathogens.

Adoption of phage-based interventions by the infectious disease community, as well as by the ecosystem of healthcare in which it is practiced, is likely to represent another major challenge at least in the United States. Practices for empiric prescription of drugs that have existed for decades and are unlikely to stop; however, the willingness of physicians to consider phage as an alternative remedy has already begun. As pathogens become more difficult to treat, it is likely that practitioners and hospital management will see phage as being useful, particularly for specific medical indications and environmental risks (e.g., device decontamination, patient decolonization). While phages are not a panacea for treatment and prevention of bacterial infections, it may be useful to view them as a collection of solutions that are potentially valuable as part of the larger picture of antibiotic resistance (Fig. 1).

[14] https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf

6 Concluding Thoughts

Bacteriophage may provide an important tool in our collection of medical resources to combat drug-resistant infections, but there is still much that will need to be done to assure the efficacy and reliability of these products for specific medical indications. It is also worth considering that, beyond their uses for medical treatment and prevention, phages may also play a broader role in maintaining a robust state of health. The growing interest in the role of commensal organisms (i.e., the human microbiome) in health and disease could provide insights into the natural relationship that humans have with phage. This may involve a delicate balance of phage modulation of our own bacterial communities, which are likely to change under conditions of stress and disease. Studies have shown that phages are inhabitants of the intestinal microflora and are enriched on mucosal surfaces (Barr et al. 2013). These findings suggest that phages may have a role in maintaining a healthy microbiome and potentially play a part in the surveillance of pathogens or conditions that lead to disease. Basic research will be important in defining the extent to which bacteriophages are natural allies in our health and in the repertoire of innate defense mechanisms that we possess. If we understand the roles that bacteriophage may play in these processes, new concepts may emerge that will also enable us to fully utilize their potential as medical interventions in the future.

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Phage Therapy in Orthopaedic Implant-Associated Infections

Paweł Rogóż, Derek F. Amanatullah, Ryszard Międzybrodzki, Robert Manasherob, Nina V. Tikunova, Beata Weber-Dąbrowska, Wojciech Fortuna, Sławomir Letkiewicz, and Andrzej Górski

P. Rogóż
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

D. F. Amanatullah · R. Manasherob
Department of Orthopaedic Surgery, Stanford Hospital and Clinics, Redwood City, CA, USA

R. Międzybrodzki (✉) · A. Górski
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Department of Clinical Immunology, Transplantation Institute, Medical University of Warsaw, Warsaw, Poland
e-mail: ryszard.miedzybrodzki@hirszfeld.pl

N. V. Tikunova
Institute of Chemical Biology and Fundamental Medicine, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russian Federation

B. Weber-Dąbrowska
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Bacteriophage Laboratory, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

W. Fortuna
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Regenerative and Functional Neurosurgery Unit, Department of Neurosurgery, Wroclaw Medical University, Wrocław, Poland

S. Letkiewicz
Phage Therapy Unit, Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

Jan Długosz University in Częstochowa, Częstochowa, Poland

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1 Introduction

Implant-associated infections (IAIs) remain a significant issue in orthopaedic surgery. IAI is the leading cause of implant failure after total knee arthroplasty and the third leading cause of failure after total hip arthroplasty in the United States (Ulrich et al. 2008). The cost of revision surgery for IAI in total hip and knee arthroplasty exceeds $25,000 per case and is expected to exceed a total of $1.62 billion by 2020 (Kurtz et al. 2012). IAI is not just associated with significant cost but also an 11% mortality, over fivefold that of uninfected arthroplasty patients, at 1 year (Zmistowski et al. 2013). IAI occurs in other orthopaedic fields that utilize instrumentation (e.g., traumatology, spine surgery) and other non-orthopaedic fields that utilize implants (e.g., cardiac pacers and breast reconstruction implants). Open and especially high-energy fractures have an increased risk of developing an infection when compared to closed fractures (Kortram et al. 2017). They become an even bigger problem in the third world where industrialization and urbanization have grown in a nearly logarithmic fashion (Gosselin et al. 2009; Stewart et al. 2013). This has led to a dramatic increase in motor vehicle- and motorcycle-related injuries to near epidemic proportion with many fractures being open, high-energy injuries (WHO 2018).

Hence, antibiotics and chemotherapeutics to manage IAI have blossomed over the last 75 years. Initially these agents were considered to be "miracles" that had a profound impact on the management of orthopaedic infection. Over time the use of these agents came at a price. Antibiotic resistance developed rapidly in the most prevalent orthopaedic pathogens, *Staphylococcus aureus* and coagulase-negative *Staphylococci* (CNS), and represents an emerging crisis (CDC 2013; CDDEP 2015; WHO 2015; Matsunaga and Hayakawa 2018; Li and Webster 2018). There is a clear push from government health agencies for better antibiotic stewardship in clinical practice (CDC 2013). The widespread use of such agents in both livestock and fisheries for food combined with the greater use clinically in humans has led to ever-escalating resistant microbial strains that were previously sensitive to common antibiotics. According to the independent review on antimicrobial resistance led by Jim O'Neill (2016) commissioned by the UK Prime Minister, by the year 2050, the leading cause of death from disease worldwide will be due to antibiotic-resistant infections surpassing cancer, diabetes, dysentery, and trauma. This would lead to global cumulative costs estimated at 100 trillion dollars in decreased economic output and productivity (O'Neill 2016).

The need for developing effective and viable alternatives to current chemotherapeutic antimicrobials is evident (Rios et al. 2016; European Commission 2017). Bacteriophages, which are naturally occurring viruses that specifically target and kill bacteria, are considered as a reliable alternative product (O'Neill 2016). A pipeline portfolio review from 2016 commissioned by the Wellcome Trust and jointly funded by the Department of Health (England) includes the use of bacteriophages and anticipates registration of the first phage preparations as early as 2023 (Czaplewski et al. 2016). Therefore, we will discuss the factors which may influence the efficacy of bacteriophage in IAI and the current state of progress toward the clinical application of phage therapy in IAI.

2 Some Bacteriophage Features in the View of Treatment of Implant-Associated Infections

Bacteriophage uses a unique mechanism to kill bacteria, even bacteria that are highly resistant to antibiotics such as methicillin-resistant (MRSA) and vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-resistant enterococci (VRE), extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*, or multidrug-resistant *Klebsiella pneumoniae* carbapenemase (KPC)-producing isolates as it was shown in many in vitro and in vivo studies (Yilmaz et al. 2013; Lehman et al. 2019; Biswas et al. 2002; Matsuzaki et al. 2003; Wang et al. 2006a, b; Cheng et al. 2017; Cao et al. 2015; D'Andrea et al. 2017). Recently, a few case reports in humans on the application of phage therapy in treatment of colistin-only-sensitive *Pseudomonas aeruginosa* and multidrug-resistant *Acinetobacter baumannii* and *K. pneumoniae* infections were presented (Jennes et al. 2017; Schooley et al. 2017; Nir-Paz et al. 2019). Bacteriophage-encoded enzymes not only lyse bacterial cells, but some also have depolymerase activity which disrupts biofilm, a slimy coating that significantly complicates the treatment of IAI (Gbejuade et al. 2015; Morgenstern et al. 2016; Pires et al. 2016, 2017). Morris et al. (2019) described in a recent in vitro study that their cocktail of bacteriophages was able to significantly lower the *S. aureus* biomass on porous titanium implants and the viability of biofilm-embedded bacteria.

It seems that phage-antibiotic synergy (described in detail in this book in the chapter by Knezevic and Sabo) could be used to enhance the efficacy of phage therapy (Torres-Barcelo et al. 2016; Kamal and Dennis 2015). This was demonstrated in vivo by Yilmaz et al. (2013) in a rat model of IAI. This model comprised plastic tibial implants with a pre-established MRSA or with *P. aeruginosa* biofilm and injection of bacterial suspension into the medullary canal. After an infection was induced, the rats were treated with staphylococcal Sb-1 and *P. aeruginosa* PAT14 phages from the George Eliava Institute of Bacteriophages, Microbiology and Virology, Tbilisi, Georgia (direct percutaneous injections into the site of the opening in the medullary canal, once daily for three consecutive days), with antibiotics (14 days of intraperitoneal administration of 20 mg/kg/day teicoplanin in the MRSA group or combined 120 mg/kg/day imipenem/cilastatin and 25 mg/kg/day amikacin in the *P. aeruginosa* group). Significant reduction of bacterial load at the site of infection by the phage only was observed in *P. aeruginosa*-infected animals (this effect was less than 1 log). There was no advantage of bacteriophage application alone over antibiotic treatment both in the MRSA and *P. aeruginosa* groups. Interestingly, the combination of phage with an appropriate antibiotic regimen significantly decreased MRSA biofilm formation (complete eradication of biofilm was observed) compared to the control as well as the bacteriophage- or antibiotic-only treated groups.

Phage-antibiotic antagonism should also be taken into consideration. Chaudhry et al. (2017) reported possible in vitro antagonism between a mixture of two *P. aeruginosa* phages and high-dose tobramycin. They observed that high levels of this antibiotic decreased *P. aeruginosa* PA14 biofilm density more than its eight times lower levels. Upon combination with bacteriophage, the efficacy of the high-dose tobramycin was even lower than that of high-dose tobramycin alone. This

might be explained by a reduction in bacterial density lowering the rate of bacteriophage replication or an antibiotic-dependent inhibition of bacteriophage replication because no increase in the phage titer was observed in this case. Significant reduction of bacterial load in biofilm and an increase in phage titer when high dose of tobramycin was added to the culture 24 h after the phages seem to confirm this hypothesis. This suggests that concurrent bacteriophage-antibiotic treatment may benefit from a delay in the administration of antibiotics.

Another problem of phage therapy is the phage resistance (Smith and Huggins 1983; Levin and Bull 2004; Gill and Hyman 2010) which was described in detail in this book in the chapter by McCallin and Oechslin. In brief, bacteria develop different antiviral mechanisms in the presence of phages that include preventing the phage adsorption, blocking bacteriophage nucleic acid penetration, or degrading its nucleic acid (Labrie et al. 2010). Mutations of phage receptors are the most frequent mechanism of phage resistance (Lindberg 1973; Heller 1992; Labrie et al. 2010). According to analysis done by Międzybrodzki et al. (2012), resistance of a targeted bacterium to a phage occurred in 17% of patients ($n = 53$) subjected to experimental phage therapy for a chronic *S. aureus* infection, in 37% of patients ($n = 14$) infected with *P. aeruginosa*, in 43% of patients ($n = 14$) infected with *E. faecalis*, and in 86% of patients ($n = 11$) infected with *E. coli*. This could be one of the causes of the treatment failure in cases in which the use of another active phage from the phage bank was not possible. Clinical phage resistance may result not only from mutation (like in animal models where infection is induced by a single bacterial strain) but also from selection of insensitive bacterial strain ("hidden" in the infection site and not identified by microbiological culture before treatment or derived from environment as new infection) that filled the niche after treatment of the targeted bacterial strain.

Phage resistance may reduce bacterial virulence (León and Bastías 2015; Oechslin 2018). Capparelli et al. (2010) showed that acquisition of phage resistance by *S. aureus* A172 resulted in reduced growth rate, under-expression of numerous genes, production of capsular polysaccharide, loss of the phage adsorption site (terminal GlcNA), and changes in teichoic acid structure when compared to the parent A170 strain. Some *Vibrio cholerae* O1 El Tor mutants which have spontaneous resistance to temperate K139 phage synthesize incomplete lipopolysaccharide (LPS) molecules with defective O1 antigen, whereas complete O1 antigen serves as a specific K139 phage receptor (Nesper et al. 2000). Such change may be associated with decreased bacterial ability to colonize the mouse small intestine. Gu et al. (2012) reported four phage-resistant variants of *K. pneumoniae* K7 characterized by reduced exponential growth and a 10,000-fold reduction in lethal dose of bacteria after its intraperitoneal injection in mice.

A bacteriophage cocktail is used to increase the antibacterial spectrum of a clinical preparation. Besides, it may also significantly reduce the risk of emergence of phage-resistant mutants. The "step-by-step" (SBS) method (Gu et al. 2012) of bacteriophage isolation led to a cocktail of three *K. pneumoniae* phages which effectively protected mice against *K. pneumoniae* K7-induced bacteremia challenge model as well as significantly reduced the appearance of the phage-resistant mutants

in vitro when compared to a single *K. pneumoniae* phage. Recently, Lehman et al. (2019) published results of preclinical studies of their AB-SA01 cocktail composed of three staphylococcal bacteriophages. The frequency of spontaneous induction of resistance to this cocktail was no greater than 3×10^{-9} which is more frequent for daptomycin and linezolid but less frequent than rifampicin. Interestingly Oechslin et al. (2017) observed that a cocktail of two *P. aeruginosa* phages infused into the superior vena cava combined with intravenous ciprofloxacin was highly effective in killing *P. aeruginosa* aortic vegetations in rats. This was accompanied by the lack of emergence of phage-resistant *P. aeruginosa* mutants. On the other hand, phage-resistant *P. aeruginosa* mutants that emerged in vitro at a rate of 10^{-7} when using a fibrin clot model had altered in vivo infectivity and were characterized by mutations in genes involved in pilus motility or lipopolysaccharide synthesis, as well as increased sensitivity to ciprofloxacin in one case. This probably may explain the outstanding in vivo result.

Bacteriophages are the most abundant organisms on Earth, and it is believed that all bacterial strains have at least one phage to target it (Keen 2015). According to Ormälä and Jalasvuori (2013), "Even if continuous use of phages forced a bacterial population to become permanently resistant to specific phage cocktails, biogeography studies of phage infection patterns suggest that new infectious phages will nevertheless be available." Therefore, isolation of phages from the environment against major bacterial pathogens should be easier than searching for new chemotherapeutic antimicrobials (Chan et al. 2013; Weber-Dąbrowska et al. 2016). Besides, it is also possible to expand the bacteriophage host range experimentally (Mapes et al. 2016; Burrowes et al. 2019) or by genetic modification (Ando et al. 2015; Goren et al. 2015; Dedrick et al. 2019). Because phage therapy has no specific regulations and it is still considered an experimental approach in many countries, concept and guidelines for magistral phage production for its use in an emerging application in cases of a new multidrug-resistant bacteria have been recently proposed (Pirnay et al. 2018). However, patient-specific phage therapy requires time to prepare, making broad-spectrum phage cocktails an important and emerging first-line therapeutic agent (Baker et al. 2018).

3 Bacteriophages and the Host Immune Response

Although bacteriophages are generally considered not pathogenic to mammalian cells, they are able to induce a humoral immune response in a mammalian host. The appearance of anti-phage antibodies remains a critical issue, because they can influence the effect of phage therapy. Anti-phage antibodies are detected using ELISA or indirectly by examination of the specific neutralizing serum effect on a bacteriophage (Łusiak-Szelachowska et al. 2014; Żaczek et al. 2016). A significant increase in serum neutralizing activity during phage therapy occurs when applying phages orally, topically, orally and topically, and/or rectally. Oral administration prompted the lowest level of anti-phage antibodies. Upon termination of phage therapy, the anti-phage serum activity is maintained or gradually decreases

indicating the presence of a circulating antibody. The use of phage cocktail is much more likely to induce a strong serum anti-phage activity in patients. Interestingly, high anti-phage activity does not exclude a beneficial therapeutic effect especially during local bacteriophage administration (Łusiak-Szelachowska et al. 2017). Moreover, anti-phage activity might suggest a functional recovery of the immune system and its emerging reactivity against the infecting bacteria (Górski et al. 2016).

Results of studies conducted by our group from the Hirszfeld Institute of Immunology and Experimental Therapy, PAS, in Wrocław strongly suggest potential anti-inflammatory effect of bacteriophages independent of their known antimicrobial action (Górski et al. 2016). Some patients treated with bacteriophages in the Phage Therapy Unit (PTU) in Wroclaw have a significant decrease in markers of inflammation (e.g., C-reactive protein (CRP)) even in the absence of bacterial eradication. These observations are consistent with the results presented by Międzybrodzki et al. (2008), showing an anti-inflammatory effect of both bacterial specific and non-specific bacteriophages dependent on the ability of the phage to inhibit reactive oxygen species production by granulocytes stimulated with bacteria or lipopolysaccharide. Besides, T4 phage inhibits in vitro activation of nuclear factor-κB (involved in the regulation of the expression of many pro-inflammatory cytokines and adhesion molecules) in endothelial and epithelial cells preventing the effects of the herpes simplex virus type 1 (HSV-1) and reduces cellular infiltration in allogeneic mouse skin transplantation (Górski et al. 2006). Recombinant gp12 protein, an adhesin from T4 phage short tail fiber, administered intraperitoneally to mice may weaken the production of interleukin-1 and interleukin-6 as well as reduce cell infiltration of internal organs in response to stimulation with lipopolysaccharide (Miernikiewicz et al. 2016). Van Belleghem et al. (2017) indicated that bacteriophages may activate genes encoding different cytokines and other anti- and pro-inflammatory mediators (including interleukin-10—a strong anti-inflammatory mediator) in human peripheral blood mononuclear cells. The authors emphasized, based on the analysis of potential interrelationship between these factors, that the prevailing effect of bacteriophage is to reduce inflammation. The anti-inflammatory effects of T4 phage were confirmed in a mouse model of rheumatoid arthritis (Międzybrodzki et al. 2017a, b). Hence, phage therapy may play a role in treatment of IAI where decreasing of edema alone can bring significant clinical and symptomatic improvement. These anti-inflammatory effects could be responsible for the clinical results observed with phage therapy in the absence of pathogen eradication (Międzybrodzki et al. 2012). Roach et al. (2017) suggest synergy between the host immune system and bacteriophages. They showed using an in silico murine model of acute pneumonia induced by *P. aeruginosa* that neutrophil function is essential for successful phage therapy due to the role of neutrophils in controlling the emergence of phage-resistant bacterial strains (Roach et al. 2017). Importantly, neither bacteriophage preparations in vitro (Kurzępa-Skaradzinska et al. 2013) nor phage therapy in humans (Jończyk-Matysiak et al. 2015) influence intracellular killing of bacteria by neutrophils or monocytes.

4 Bacteriophage Delivery

Due to possible bacteriophage replication in the site of infection, phage therapy is considered self-sustaining and self-limiting treatment. Hence, bacteriophage pharmacokinetics, although not well studied, are substantially different from antibiotics. Generally, it is believed that bacteriophage penetration into tissue is quite good when bacteriophage isolates are administered via the blood (Dąbrowska 2019). However, there are no available data on bacteriophage penetration to the bone marrow or bacteriophage distribution on implants which is of importance for treatment of IAI and osteomyelitis. Preliminary data obtained in rats suggest that staphylococcal phages A5/80 and A3/R from the collection of the Hirszfeld Institute as well as *E. coli* T1, T4, and T7 phages reach the bone marrow within 30 min of intraperitoneal delivery (Rogóż et al.—unpublished data). It is estimated that dose-limiting phage detection is 10^6 PFU/rat.

Phage application topically, directly into the site of infection, in theory seems to eliminate the risk related to the anti-phage antibody formation and poor penetration, but it does not guarantee delivery of phage to all the pathogenic bacteria hidden in the soft tissues. Delivery of phage via fistular lavage could reach additional bacteria via deeper penetration from the wound potentially into the blood stream.

Oral administration subjects the bacteriophage to acidic environment of the stomach (Międzybrodzki et al. 2017b). This problem may be resolved by the use of gastric juice neutralizer, proton pomp inhibitor, or H_2 receptor antagonist. However, it does not guarantee success because phages may also differ in their ability to penetrate through the intestinal mucosa as was shown in mice for therapeutic A5/80 (penetrating) and T4 (not penetrating) phages belonging to *Myoviridae* (Międzybrodzki et al. 2017b).

Recently Samokhin et al. (2018) published their results on placing bacteriophages into bone cement. They characterized the in vitro antibacterial effect of two lytic bacteriophages (*S. aureus* phage Ph20 and *P. aeruginosa* phage Ph57). After ex tempore phage impregnation in poly(methyl methacrylate) during the polymerization process at +6 °C to +25 °C, Ph20 lost its effective titer within 6 days, while Ph57 was retained for at least 13 days. Although this study has some limitations (e.g., the authors did not test the mechanical strength characteristics of the used polymer carrier and one of the bacteriophages turned to be very sensitive to experimental conditions), it presents an interesting method which might be applied in IAI if confirmed by further preclinical and clinical studies.

Kaur et el. (2014) investigated bacteriophage delivery by coating orthopaedic grade Kirschner wires (K-wires) with 1.5×10^9 PFU/mL lytic *S. aureus* phage MR-5 in (hydroxypropyl)methyl cellulose (HPMC) gel. Although phage titer dropped by 3 log in the coating process, its stability on the coated wires was acceptable during the next 20 days, and its elution from gel was steady for 48 h. They observed a 3 log reduction in the adhesion of *S. aureus* 43300 to the wires with MR-5 when compared to uncoated wires at 48 h. The combination of the MR-5 and 5% linezolid in HPMC reduced bacterial adhesion by 4 log and inhibited the rate of phage resistance when compared to MR-5 or linezolid alone.

5 Bacteriophage Use in Orthopaedic Practice Including Treatment of Implant-Associated Infections

5.1 Reports from Georgia, Russia, and France

Although phage therapy was extensively used in the Republic of Georgia and the Soviet Union since World War II, there is little information in English language on their results of the bacteriophage application in orthopaedic infections. Some (often very general) reports are available in the monograph by the Eliava Institute presenting results of phage therapy published mainly in the Russian and Georgian literature (Chanishvili et al. 2009). The authors summarize Morozov et al. results published in 1946, obtained in a group of 15 patients suffering from osteomyelitis due to post-operative and amputation wound infections who were administered bacteriophage preparations locally as liquid applications (bandages soaked with bacteriophage mixtures changed every 2–3 days or bathing). In three cases with osteomyelitis, bacteriophages were applied into the wound in 0.7% agar to extend their circulation for a week. After 2–3 weeks of phage therapy, complete cure in 13 of 15 treated patients was observed. According to another report by Nikolaeva, published in 1974, on the use of polyvalent staphylococcal phage in Gorky (at present: Nizhny Novogorod) and Moscow clinics, in Russia, fistular lavage with bacteriophage was an important supplement to surgical removal of the sequestrum in cases of osteomyelitis. Matusis et al. (1974) from the Institute of Traumatology and Orthopedics in Gorky evaluated patients with ununited fractures and festering stumps complicated with osteomyelitis caused by *Staphylococcus* in 93% of cases. Phage therapy and/or systemic antibiotics were applied in conjunction with orthopaedic surgery using a modified method of Ilizarov compression-distraction osteosynthesis for pseudo-arthrosis and osteomyelitis (Barbarossa et al. 2001; Plakseychuk et al. 2002; McNally et al. 2017). Bacteriophage preparations produced by the Tbilisi Institute of Vaccine and Sera (former name of the George Eliava Institute of Bacteriophages, Microbiology and Virology) active against 72% of isolated bacterial strains were used. In patients receiving phage topically and/or intramuscularly ($n = 53$) or those receiving topical bacteriophage combined with antibiotic ($n = 12$), healing of all stumps was observed within 3 months, and consolidation was achieved by 9 months. In comparison group of patients subjected to surgery with systemic antibiotic treatment ($n = 24$), only 62.5% were cured (healing of stumps was prolonged even by 12 months, whereas consolidation was achieved by 13 months).

An interesting non-randomized pilot study on the use of combined phage/antibiotic therapy of IAI has been carried out in Novosibirsk Research Institute of Traumatology and Orthopaedics in Russia (Samokhin et al. 2016). It included 28 patients after total hip arthroplasty complicated by mono- or polymicrobial infection mainly caused by staphylococci (*S. epidermidis* and *S. aureus*). All patients underwent one-stage cement revision hip re-replacement with simultaneous treatment with phage preparations and antibiotics (a prospective study group of 12 patients) or antibiotics only (a retrospective control group of 16 patients). Bacteriophage cocktails produced by the Russian Federal State Unitary Enterprise "Scientific and Production Association for Immunological Preparations—Microgen" (https://www.microgen.ru/en/) were

administered through surgical drains in a volume of 20 mL for 10 days following surgery. Before administration, phage preparations were selected individually, in accordance with their activity against pathogenic bacterial strains isolated from the patients. Patients from the study group were prescribed antibiotic therapy for up to 12 weeks in the postoperative period (1 g of vancomycin twice a day and 2 g of cefazolin three times a day intravenously for 2 weeks followed by an oral treatment with (1) 500 mg of ciprofloxacin twice a day for 3 weeks combined with 450 mg of rifampicin twice a day for 10 weeks or (2) 480 mg of trimethoprim/sulfamethoxazole twice a day for 1 month and then 100 mg of doxycycline twice a day for 8 weeks). During hospital stay, patients from the control group received different targeted antibiotics intravenously (e.g., cefazolin, ciprofloxacin, vancomycin, daptomycin, and rifampicin) for 2–4 weeks in mono- or combined therapy. After discharge, the treatment was continued orally with 0.5 g of ciprofloxacin twice a day for 3–5 weeks or 0.3 g of rifampicin once a day for 3 months. An increase of CRP and erythrocyte sedimentation ratio (ESR) values was observed in both groups 2–3 days after the surgery. On days 10–14, CRP value decreased significantly even to the level much lower than before surgery, whereas decrease in ESR values was not so evident. Median (as well as the first and the third quartiles) CRP values (in mg/mL) were as follows in the study group and the control group, respectively: before surgery, 27.29 (20.50, 38.82) and 35.5 (14.90, 50.33); 2–3 days after surgery, 94.15 (46.22–160.24) and 51.5 (31.77–112.90); 7 days after surgery, 38.92 (17.80–54.79) and 23.72 (16.70–51.52); and 10–14 days after surgery, 14.79 (10.87–26.22) and 12.73 (9.00–31.97). After treatment, only one case of re-infection was recorded in the study group (8.3%), whereas four such cases were documented in the control group (25%). The small sample size and lack of a statistical difference between these two groups should be noted.

There is also some experience of the compassionate phage therapy applications in treatment of osteoarticular infections in France (Patey et al. 2018). The authors present a summary of good clinical outcomes in 15 patients mainly treated at the Villeneuve-Saint-Georges Hospital in France. Nine of these patients suffered from osteoarticular infections including three cases of *P. aeruginosa* or *S. aureus* IAI. Commercial phage preparations from Georgia or Russia were applied directly via articular injection, lavage of the infection site during surgery, or an indwelling catheter after surgery. In all cases, concomitant antibiotic therapy was instituted as well. This resulted in complete cure in seven patients with osteoarticular infections including one with IAI. In the two other IAI cases, another bacteria was isolated, or several fistulas were closed on antibiotics, and stable suppression was achieved.

Recently, Ferry et al. (2018) described a case of an 80-year-old woman with obesity, type 2 diabetes, and chronic kidney failure who was subjected to a debridement, antibiotic, and implant retention (DAIR) procedure combined with topical bacteriophage application due to relapsing postoperative *S. aureus* IAI. In the past, she was subjected to the first- and second-stage prosthesis exchange and three surgical debridements for this infection. Because microbiological analysis revealed that she was infected with multidrug-resistant *P. aeruginosa* and methicillin-sensitive *S. aureus* (MSSA), purified phage preparations containing three *P. aeruginosa* phages (with confirmed activity against the isolated strain) and

three *S. aureus* phages (selected based on their broad lytic spectrum because an original MSSA was not preserved for phage typing) were provided by Pherecydes Pharma. They were mixed and injected into the joint (each phage at a dose of 10^{10} PFU in mixture of saline and Dulbecco's phosphate-buffered saline) just before its closure after finishing DAIR procedure. Results of culture of perioperative swabs did not confirm *P. aeruginosa* infection, while MSSA was still present, and it was retrospectively confirmed to be sensitive against two of three staphylococcal phages applied. Moreover, *Enterococcus faecalis* and *Staphylococcus lugdunensis* (unsensitive to any of staphylococcal bacteriophages used) were also detected. The patient was additionally treated with daptomycin within the first 3 months following surgery, with amoxicillin and clindamycin for the next 3 months, and then only with amoxicillin to suppress the growth of *E. faecalis* and *S. lugdunensis*. Due to acute hip infection with *Citrobacter koseri*, a new debridement was performed with subsequent ciprofloxacin administration for 2 months. The authors concluded that their salvage treatment was safe and there were no clinical signs of infection at the end of 18-month follow-up. However, due to application of the intensive antibiotic treatment (the patient was receiving amoxicillin till the end of the follow-up), it is difficult to assess the influence of phage therapy on the final clinical results obtained in this case.

In 2014, the Pherecydes Pharma lunched a collaborative research project for the development of an innovative phage therapy treatment against bone/joint and diabetic foot ulcer infections (PHOSA) (Pherecydes Pharma 2019a). It was aimed to develop, in a preclinical model, the best cocktail of precisely characterized *S. aureus* bacteriophages against bacterial strains causing such infections and to establish a process of its production according to current pharmaceutical standards. The project was completed in 2017, and the company announced their PHOSAClin project to evaluate the safety and efficacy of these bacteriophages in a clinical study (Pherecydes Pharma 2019b).

5.2 Experience from the Phage Therapy Unit in Wrocław, Poland

The Hirszfeld Institute in Wrocław produces phage preparations for phage therapy since the late 1960s. These preparations were applied during the treatment of hundreds of patients in Polish hospitals, university clinics, and ambulatory care outside the Institute. A few reports summarizing overall results of this treatment were published by professor Ślopek. One of them summarizes overall results of this treatment conducted between 1981 and 1986 based on the survey forms provided to the Hirszfeld Institute by physicians conducting phage therapy (Ślopek et al. 1987). They reported very encouraging data on general efficacy of oral and/or topical phage therapy to treat orthopaedic infections (disease groups denoted as XIII-16, XIII-17, and XVII-21 in Ślopek et al. 1987). The majority of cases were staphylococcal infections. Ninety-two percent of patients, including 13% of cases with outstanding effect manifesting by complete recovery, out of 100 patients (some of them were treated with concomitant antibiotic) had a positive response to phage therapy.

Since 2005, patients admitted to the Hirszfeld Institute Phage Therapy Unit receive an adapted phage therapy protocol compliant with current legal regulations and approval of an independent bioethics committee (Międzybrodzki et al. 2012). This protocol enables access to outpatient phage therapy to Polish and foreign patients suffering from chronic bacterial infections where antibiotics have failed or are contraindicated. The PTU had performed two retrospective analyses of 305 adult patients, in total, admitted for phage therapy between 2008 and 2010 (Międzybrodzki et al. 2012) and between 2011 and 2013 (Międzybrodzki et al. 2018). A seven-point scale (from A to G) was elaborated to assess phage therapy results and compare them between study groups (Międzybrodzki et al. 2012). Seventy-six of these patients (21 women, 55 men, median age 55 years, range 22–88 years) with orthopaedic infections, in majority with IAI, are presented in Table 1. Their predominant pathogen was *S. aureus*, and 67 cases were treated with strain-specific staphylococcal phage lysate alone. They received those phage preparations orally or topically via fistular irrigation and/or wet compresses on the external orifice of the fistula (median cumulative treatment duration was 47 days, range 16–209 days). Good response to treatment considered as a pathogen eradication, a good recovery, good clinical result, or clinical improvement was observed in 43.4% of cases. The rate of fully recovered patients and/or those with pathogen eradicated was 14.5%. In 16 cases, an antibacterial agent (mainly antibiotic) was applied during treatment. If these cases are excluded, the rate of patients with a good clinical response was reduced by 2.6%. Although the rate of pathogen eradication and/or recovery was doubled in patients who received antibacterial in comparison to the phage-only treated group, this difference was not statistically different (analysis was performed using a

Table 1 Results of phage therapy in patients with orthopaedic infections admitted for phage therapy at the Hirszfeld Institute Phage Therapy Unit in 2008–2013

	All cases ($n = 76$)		No antibacterial[a] used during treatment ($n = 59$)		Antibacterial[a] used during treatment ($n = 17$)		Patients with PJI ($n = 13$)	
Category of response to treatment	*n*	%	*n*	%	*n*	%	%	*n*
A—pathogen eradication and/or recovery	1	14.5	7	11.9	4	23.5	3	23.1
B—good clinical result	5	6.6	4	6.8	1	5.9	2	15.4
C—clinical improvement	17	22.4	13	22.0	4	23.5	2	15.5
D—questionable clinical improvement	9	11.8	6	10.2	3	17.6	1	7.7
E—transient clinical improvement	13	17.1	11	18.6	2	11.8	1	7.7
F—no response to treatment	18	23.7	16	27.1	2	11.8	3	32.1
G—clinical deterioration	3	3.9	2	3.4	1	5.9	1	7.7
Good response (total A–C):	**33**	**43.4**	**24**	**40.7**	**9**	**52.9**	**7**	**53.8**
Inadequate response (total D–G):	**43**	**56.6**	**35**	**59.3**	**8**	**47.1**	**6**	**46.2**

No statistically significant differences between analyzed groups were found

[a]Includes antibiotics, chemotherapeutics, disinfectants, and other potential antibacterial agents

Chi-square test). Within a subgroup of 13 patients with PJI (4 cases received antibiotic treatment over the course of phage treatment), good clinical response was achieved in 7 cases (53.8%), whereas pathogen eradication and/or recovery was achieved in 23.1% of cases (2 cases received also antibiotic).

In November 2017, a group of patients with chronic bacterial bone, joint, soft tissue, or periprosthetic infections receiving experimental phage therapy according to the protocol described by Międzybrodzki et al. (2012) were interviewed by phone. Patients were asked to assess the result of phage therapy at treatment termination, 1–12 months later, and at the time of the interview (current status), as well as to express their overall satisfaction with phage therapy (Table 2). Information was collected from 33 subjects who completed phage therapy between 15 months and 7 years ago. The majority of patients used bacteriophage preparations topically. Two patients used the preparations orally. Ten patients were treated for free as a part of "Phages from POIG" program (supported by the European Regional Development Fund within the Operational Program Innovative Economy, 2007–2013, Priority axis 1. Research and Development of Modern Technologies, Measure 1.3 Support for R&D projects for entrepreneurs carried out by scientific entities, Submeasure 1.3.1 Development projects as project No. POIG 01.03.01-02-003/08 entitled "Optimization of the production and characterization of bacteriophage preparations for therapeutic use"). Cumulative treatment time was from 3 to 168 days (median 51 days). Most of them ($n = 23$, 70%) were infected with *S. aureus*. The rest of the cases were infected with *P. aeruginosa*, *S. lugdunensis*, *E. coli*, *Serratia marcescens*, and *Proteus mirabilis* or had a polymicrobial infection. Patient's response to treatment at treatment termination was assessed by a physician according to the scale described previously by Międzybrodzki et al. (2012). Good response to treatment was observed in 12 patients immediately after completing phage therapy (data available for 31 patients), while eradication or healing was observed in 4 cases. During the following 12 months, 8 of 26 responders still reported a good result with phage therapy with eradication or healing in all 8 cases. Interestingly, 21 patients assessed their phage therapy result as good, and 17 reported eradication or healing at the time of the interview. Although the majority (79%) of responders applied another treatment during follow-up, 12 patients linked their current good clinical state to phage therapy. Twenty-two patients confirmed that they were satisfied with phage therapy (13 of them were very satisfied). None of the 33 interviewed persons reported adverse reactions that could be directly related to the application of phage therapy.

Representative examples for effective treatment of IAI with phage therapy after a lack of response to standard therapy are presented below.

Case 1

A 58-year-old male was referred for phage therapy due to staphylococcal infection and inflammatory infiltration in area of the right hip. He underwent a right total hip arthroplasty 2 years prior for osteoarthritis of the hip. The patient was treated by a rheumatologist for over 15 years with nonsteroidal anti-inflammatory drugs. He has a past medical history of hepatitis B infection (HBsAg, negative, anti-HBc, positive),

Table 2 Long-term results of the application of phage therapy in patients with chronic bacterial infections

	Results of treatment at TT[a] (physician's opinion)[b]		Results of treatment—patient's opinion on therapy[b]						Overall patient's satisfaction[b]	
			Results at TT		1–12 months post TT		Present status			
Score	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
A	4	12.1	5	15.2	6	18.2	17	51.5	13	39.4
B	4	12.1	3	9.1	1	3.0	2	6.1	–	–
C	4	12.1	2	6.1	1	3.0	2	6.1	9	27.3
D	6	18.2	–	–	–	–	–	–	–	–
E	4	12.1	1	3.0	1	3.0	1	3.0	–	–
F	9	27.3	16	48.5	15	45.5	7	21.2	2	6.1
G	0	0.0	1	3.0	2	6.1	3	9.1	6	18.2
A–C	**12**	**36.4**	**10**	**30.3**	**8**	**24.2**	**21**	**63.6**	**22**	**66.7**

Legend:

Physician's opinion:	*Patient's opinion:*	*Patient's satisfaction:*
A—pathogen eradication and/or recovery B—good clinical result C—clinical improvement D—questionable clinical improvement E—transient clinical improvement F—no response to treatment G—clinical deterioration A–C—*good response*	A—recovery/wound healing B—significant clinical improvement (almost complete resolution of symptoms) C—moderate clinical improvement (partial relief of symptoms) E—transient clinical improvement (recurrence of infection after the period of improvement) F—no improvement G—deterioration A–C—*a good treatment result was obtained*	A—very satisfied C—satisfied or moderately satisfied F—lack of opinion G—dissatisfied A–C—*satisfied*

[a]TT—treatment termination

[b]Overall 33 patients were interviewed. In some cases, information on treatment results was not unavailable for one category

hepatic steatosis with normal liver function tests, and benign prostatic hypertrophy treated with doxazosin. Three months earlier, he underwent a right hip revision with heterotopic ossification excision and removal of an inflammatory focus with hip lavage and drainage. At this time, his hip cultures grew MSSA from an intraoperative swab, and the patient was started on intravenous ceftriaxone and vancomycin.

Just before starting phage therapy, there was no discharge from the wound (Fig. 1a), but the patient still complained about the severe pain in the right hip while walking, standing, and sleeping. His ESR was 95 mm/h and serum CRP was 11.0 mg/mL. He applied phage lysate containing a matched staphylococcal phage (in a titer no less than 10^6 PFU/mL) from the Hirszfeld Institute collection. He administered the matched phage orally, 10 mL, three times daily, and topically to the fistula lavage via wet compresses on the wound, two times daily, for 28 days. After the 1st week of phage use, the patient reported significant reduction of hip pain.

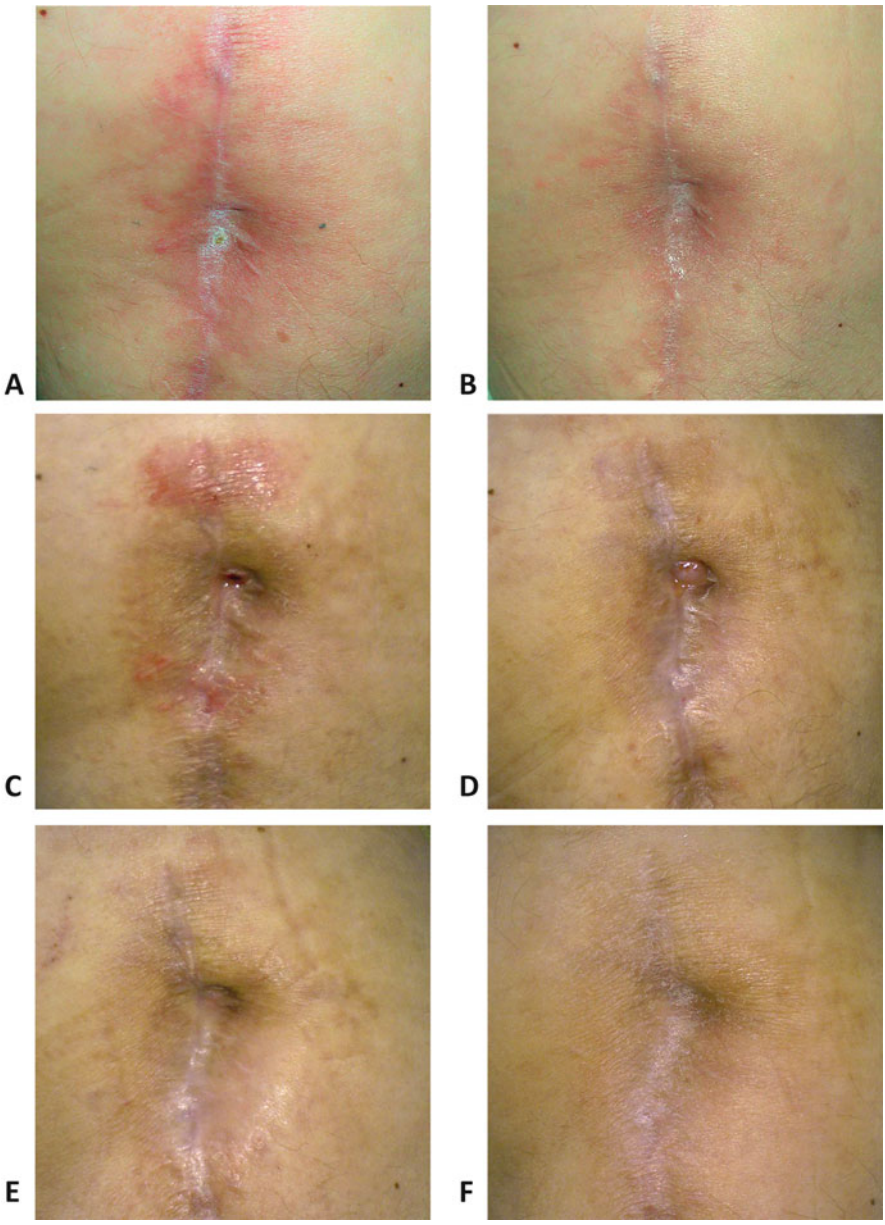

Fig. 1 Postoperative sinus track healing: (**a**) at the onset of the first phage therapy (PT) cycle, (**b**) at the end of the first PT cycle (4 weeks), (**c**) at the onset of the second PT cycle (16 weeks after the end of the first cycle), (**d**) 10 days after the onset of the second PT cycle, (**e**) 3 weeks after the onset of the second PT cycle, and (**f**) 11 weeks after completion of the second PT cycle

However, at the end of the first treatment cycle, his pain increased to the same level as before, and his ESR decreased to 78 mm/h, whereas CRP increased to 18.8 mg/mL. The sinus track was healed (Fig. 1b), and it was not possible to collect a sample for bacterial culture. Phage therapy was not continued due to lack of clinical improvement.

Eleven months later, due to progressive restriction of mobility in the hip joint and heterotopic ossifications of the hip joint, the patient underwent revision surgery with excision of heterotopic ossification, drainage, implantation of gentamicin-impregnated collagen sponge, and intravenous ceftriaxone and amikacin. Despite this treatment, after 5 months, he presented symptoms of periprosthetic infection and was referred for a second round of phage therapy. On admission he was walking on crutches and still complained about a severe hip pain which required diclofenac. His physical examination showed the presence of a sinus track in the vicinity of the postoperative wound on the right hip (Fig. 1c). Microbiological examination of purulent discharge from it confirmed a continued MSSA infection. His ESR was 100 mm/h and CRP was 14.37 mg/mL. He was qualified to the second treatment cycle with phage lysate containing a matched staphylococcal phage (liz/80, in a titer no less than 10^6 PFU/mL) administered at a dose of 10 mL orally, three times daily, and topically to the sinus track with wet compresses on the wound, two times daily. One week later, he observed significant decrease of pain and wound discharge (Fig. 1d). After another 2 weeks, the sinus track closed (Fig. 1e). Topical phage application was stopped on day 26 after starting the second treatment cycle due to the complete wound healing (Fig. 1f). At that time the patient reported no pain with walking. ESR decreased to 74 mm/h, but CRP increased to 20.3 mg/mL, so oral phage application was continued up to 55 days. At the end of treatment, the patient did not require to use analgesic and was able to begin therapeutic rehabilitation. His ESR dropped to 35 mm/h and CRP to 13.43 mg/mL, and they were stable during the next 7 months of follow-up observation. Control skin swabs from the healed wound region showed only the presence of coagulase-negative staphylococci.

Case 2

A 54-year-old male suffered a comminuted right calcaneus fracture as well as compression fracture of L3–L4 vertebral bodies due to a fall from height. He underwent surgical fixation including open reduction of the subtalar joint, stabilization via open reduction and internal fixation using a calcaneal Locking Compression Plate (LPC), and plaster cast immobilization of the ankle (Fig. 2a). His spinal fractures were treated non-operatively with a Jewett brace. He was administered broad-spectrum empiric antibiotics. Two months later, the surgery was complicated by an infection with *Staphylococcus aureus* and *Finegoldia magna* which was treated with wound debridement and administration of targeted antibiotic. After 1 months, an aggravation of the inflammatory process in the wound and enlargement of the necrotic focus with exposure of the hardware were observed (Fig. 2b). Therefore, the hardware was removed, and the patient was subjected to targeted antibiotic treatment (complicated by maculopapular rash) against pathogens (not specified in patient's documentation) isolated from the intraoperative swab at the time of hardware removal. Then he was subjected to hyperbaric oxygen therapy

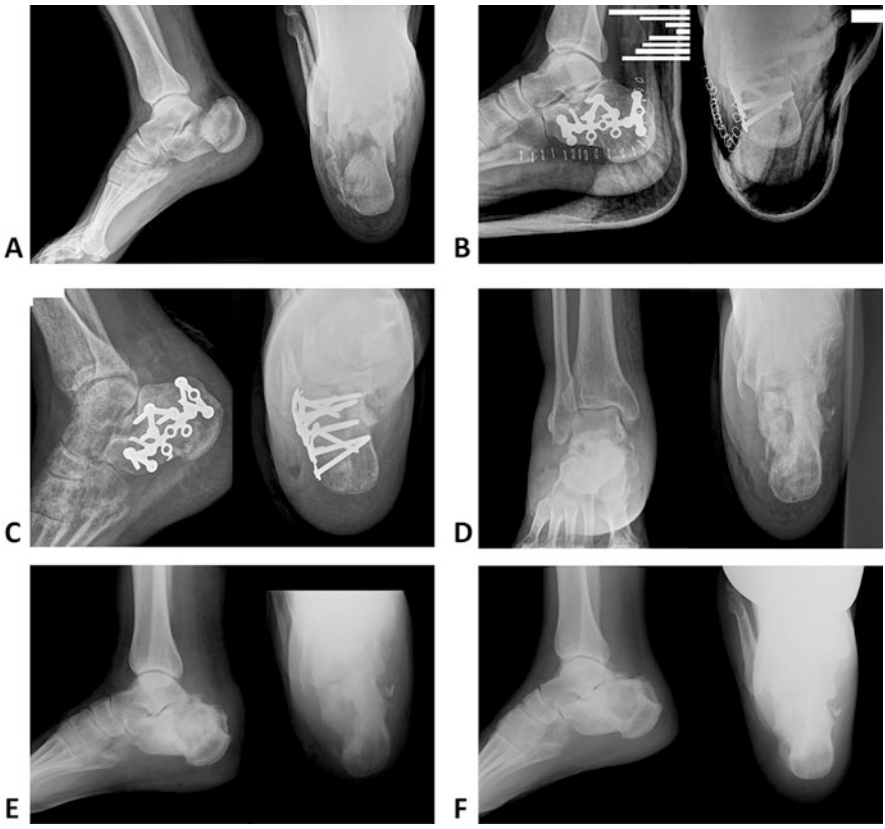

Fig. 2 Lateral and Broden's radiographic views of the right calcaneus demonstrating (**a**) a comminuted fracture to the right calcaneus (**b**) treated in a plaster cast after surgical fixation of the calcaneal fracture. (**c**) Diffuse bone atrophy at the calcaneus, talus, tarsal, and metatarsal bones as well as the tibia in the setting of soft tissue edema 4 months after the injury. (**d**) Removal of hardware 12 months after the injury. (**e**) Multiple areas of sclerosis and oval thinning bone with the external bone fragments and edema of the soft tissues 17 months after the injury (1 month before beginning the PT). (**f**) Stabilization of the calcaneus structure with partial regression of the previously described pathological changes and complete regression of the soft tissue edema even months after completion PT (28 months after the injury)

(30 sessions). Four months later, he was admitted to a hospital due to scattered erythema on the trunk and limbs and ulceration formed on the right shank infected with *Pseudomonas aeruginosa* which was accompanied by acute eczema. He received topical and systemic antibacterial and anti-allergic treatment including targeted antibiotics and H_1-antihistamine. After the next 3 months, the heel was still inflamed (Fig. 2c) and infected with *S. aureus* and *P. aeruginosa* (as confirmed by pre- and intraoperative swabs), and so the patient was subjected to another debridement surgery with implantation of garamycine sponge and targeted antibiotic course. However, the healing process was complicated by inflammatory reaction and

seropurulent discharge, so debridement and implantation of another garamycine sponge were repeated 2 months later. Because *P. aeruginosa* and *S. agalactiae* group B were isolated from the intraoperative swab and *E. faecalis* was isolated from the wound on the ankle, he got combined antibiotic treatment (amoxicillin/clavulanic acid and ciprofloxacin). He also underwent the second course of hyperbaric oxygen therapy (30 sessions).

Despite this intensive standard treatment, the patient still presented symptoms of inflammation of the right calcaneus in the form of a small sinus track in the area of the right lateral malleolus. MSSA was isolated from the discharge (Fig. 2d). His ESR was 17 mm/h and his CRP was 1.0 mg/mL. He was qualified for experimental PT and was administered MS-1 phage cocktail lysate (containing an equal mixture of staphylococcal A5/80, P4/6409, and 676/Z phages from the Hirszfeld Institute collection, each in a titer no less than 5×10^5 PFU/mL) prepared at the Institute of Biotechnology, Sera and Vaccines BIOMED S.A. in Cracow, Poland, to flush the fistula and to cover it with wet dressings for 30 min three times daily. Swabs of the sinus track were taken just before starting the phage therapy and on day 19 of treatment confirmed co-infection with *Streptococcus pyogenes* group A, and there was no anti-streptococcal phages in the initial cocktail; the patient was also administered intramuscular benzylpenicillin between days 23 and 30 of initiating phage application and orally administered 1.5 million units of phenoxymethylpenicillin twice daily between days 36 and 50 of initiating phage application. On days 33 and 51, MSSA was isolated from the fistula. On day 66, PT was stopped since the sinus track was closed for the first time since 1.5 half year. Control swab taken 9 days later showed only presence of commensal skin flora—*Staphylococcus haemolyticus*. No symptoms of the recurrence of the inflammatory process and infection were reported during next 5 years of follow-up (Fig. 2e). The patient still complains only on temporary pain of the heel during the first minutes of walking after awakening.

6 Conclusions

Phage therapy is still an untapped approach to augment the current standards of care enabling eradication or durable suppression of IAI. The results are promising, although they are not derived from standard clinical trials. However, they may confirm general (Międzybrodzki et al. 2012) and remote (as presented here) safety of clinical use of phages. Despite the fact that phage therapy requires more basic and clinical data, phage therapy is an emerging new modality for the treatment of orthopaedic and non-orthopaedic IAI which could be a good model for future clinical trials of phage therapy. This is especially true in the era of multidrug-resistant bacteria and a clear push from national governments as well as global health agencies to improve antibiotic stewardship in clinical practice.

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The Use of Bacteriophages in Animal Health and Food Protection

Katarzyna Kosznik-Kwaśnicka, Gracja Topka, Aleksandra Dydecka, Agnieszka Necel, Bożena Nejman-Faleńczyk, Sylwia Bloch, Grzegorz Węgrzyn, and Alicja Węgrzyn

1 Introduction

The therapeutic use of bacteriophages, called phage therapy, is most often considered in the light of human medicine. However, application of bacteriophages in veterinary medicine is also important, and in fact, this method is perhaps better developed there than in human medicine. Regulations on the use of bacteriophages to treat animals are less restrictive relative to those on their medical use; thus, it is easier to test efficacy and mechanisms of phage therapy in infections of animals and humans. This chapter is focused on development of phage therapy for animals, including animal breeding, aviculture, and aquaculture. Moreover, the use of phages in food protection will also be discussed briefly, as will be methods for phage isolation, propagation, purification, and administration.

2 Phage Therapy in Animals

During the recent years, the whole world is facing the problem of infectious diseases related to animals that pose a risk to human and animal health (Gupta et al. 2017). This could be caused by various factors, in particular such as rapidly increasing

Katarzyna Kosznik-Kwaśnicka and Gracja Topka contributed equally to this chapter.

K. Kosznik-Kwaśnicka · A. Węgrzyn
Department of Molecular Biology, Faculty of Biology, University of Gdańsk, Gdańsk, Poland
e-mail: katarzyna.kwasnicka@biol.ug.edu.pl; alicja.wegrzyn@biol.ug.edu.pl

G. Topka · A. Dydecka · A. Necel · B. Nejman-Faleńczyk · S. Bloch · G. Węgrzyn (✉)
Laboratory of Molecular Biology, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Gdańsk, Poland
e-mail: gracja.topka@phdstud.ug.edu.pl; aleksandra.dydecka@phdstud.ug.edu.pl; agnieszka.necel@phdstud.ug.edu.pl; bozena.nejman@biol.ug.edu.pl; sylwia.bloch@biol.ug.edu.pl; grzegorz.wegrzyn@biol.ug.edu.pl

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emergence of antibiotic-resistant bacteria worldwide (Bengtsson and Greko 2014; Ventola 2015; Carvalho et al. 2017). With the development of agriculture, resistance to antibiotics spread quickly because their overuse and misuse allow the selection of antibiotic-resistant bacteria (WHO 2014; Ventola 2015). Furthermore, the use of antibiotics as a preventative measure has become increasingly common. What is particularly worrying is that in some countries, farmers add antibiotics to animal feed in order to enhance animal productivity and quality of meat. The European Union and several other developed countries have implemented policies to reduce the use of antibiotics, recognizing its role in the rise of antibiotic resistance. Since 2006, the use of antibiotics as growth promoters in animal feeds is forbidden in the European Union (Wegener 2003; Castanon 2007; EU regulation No 470/2009; Millet and Maertens 2011). However, the problem of bacterial infections is not restricted to animal breeding. It is found also in aviculture and aquaculture. This problem, and the possibility to solve it by the use of bacteriophages that are therapeutic agents, is discussed in subsequent sections.

2.1 Animal Breeding

In this subsection, we focused on animal breeding and more specifically on animal husbandry. This chapter shows the current state of knowledge about phage therapy in livestock: cattle (dairy cattle and beef cattle), swine, sheep, and horses.

2.1.1 Phage Therapy in Farm Animals: Overview

Infectious diseases of farm animals are a major global threat to public health, animal health, and welfare (Tomley and Shirley 2009). For this reason, researchers focused on reaching for a new approach in combating bacterial infections. Recent studies indicated that bacteriophages are becoming increasingly attractive for antibacterial therapy, especially for treating various infectious diseases of farm animals and controlling foodborne pathogens (Kazi and Annapure 2016; Lin et al. 2017). Advantages of phage therapy over the use of antibiotics can be their ubiquitous nature, specificity, prevalence in the biosphere, replication at the site of infection, and low inherent toxicity of phages, which makes them a safe technology to control animal diseases (Loc-Carillo and Abedon 2011; Colavecchio and Goodridge 2017). Therefore, phages are being considered valuable antibacterial means, and they give the opportunity to reduce the current use of antibiotics in agriculture, increasing animal productivity, improving their health, and providing environmental protection (Carvalho et al. 2017; Svircev et al. 2018).

2.1.2 Phage Therapy in Cattle

Bovine Mastitis

Bovine mastitis is a common disease and one of the most relevant bovine pathologies. Indeed, according to the data available, it is the most costly disease in the global dairy industry, due to losses (a reduction of output due to mastitis) and expenses (related to

infection prevention) (Hogeveen et al. 2011; Fan et al. 2016; Gomes et al. 2016). For example, in the USA, total loss amount is estimated to be 2 billion dollars per year which gives 140–280 dollars per cow (Sordillo and Streicher 2002). Recent research that took place recently in Sweden showed that the loss caused by one case of mastitis clinica was estimated at US$735 (Hultgren and Svensson 2009). There are numerous etiological factors associated with bovine mastitis clinica. About 137 microbial species, subspecies, and serovars are isolated from the bovine mammary gland (Watts 1988; Sharif et al. 2009). The most important pathogens causing contagious mastitis in cattle are *Staphylococcus aureus* (Gill et al. 2006a; Boss et al. 2016), *Escherichia coli*, and *Streptococcus uberis* (Bradley 2002; Barrett et al. 2005). The abovementioned problem of antibiotic resistance has resulted in dramatic situation that many commonly used antibiotics are ineffective. Therefore phage therapy seems to be a promising alternative. In recent years, researchers tried numerous attempts to control bovine mastitis clinica using phages (Schmelcher et al. 2015; Fan et al. 2016; Porter et al. 2016).

Mastitis Caused by *Staphylococcus*

As mentioned earlier, *Staphylococcus aureus* is one of the most important pathogens causing mastitis (Boss et al. 2016). Bovine mastitis caused by *S. aureus* is especially difficult to fight due to its resistance to antibiotic treatment and its propensity to recur chronically (Gill et al. 2006a). The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection in dairy animals is especially dangerous (Wang et al. 2015). *S. aureus* also shows high resistance against penicillin, tetracycline, ampicillin, and amoxicillin (Jamali et al. 2014; Szweda et al. 2014).

Some studies have demonstrated two different approaches to investigate the efficacy of phages in treatment of *Staphylococcus aureus* infection (Fan et al. 2016). In the first approach, their results indicated that bacteriophage IME-SA1 could eliminate or reduce the level of *S. aureus* and, thus, had a potential to its use in treatment of infections caused by this bacterium. In this research, a group of 100 *S. aureus* strains isolated from swine, poultry, and cows were tested (including MRSA strains). Phage IME-SA1, reported by Fan et al. (2016), displayed lytic activity against 35% of the *S. aureus* isolates. The second approach in the course of their research was to clone and express recombinant phage endolysin Trx-SA1 from this phage. Such recombinant endolysin displayed lytic activity against 43% of the *S. aureus* isolates. In the next step, they used recombinant endolysin Trx-SA1 to treat mastitis. Research has been carried out on four dairy cows with mild clinical mastitis. Each udder quarter was treated with endolysin (intramammary infusion of 20 mg of recombinant endolysin per day). They determined that three udders were infected with *Staphylococcus aureus*, one by *Escherichia coli*, and one by *Streptococcus agalactiae*. During this experiment, they observed changes of pathogens' levels and somatic cells' count in milk samples after treatment of bovine mastitis (samples are taken for 3 days). The experiment performed in this study demonstrated reductions in pathogen levels and somatic cell count (SCC) in milk from the udder quarters with *S. aureus* mastitis, while *E. coli* infection was not treated successfully. Experiments performed by Fan et al. (2016) indicate that phage IME-SA1 and

recombinant endolysin Trx-SA1 might be an alternative treatment strategy for mastitis caused by *S. aureus*.

In another study, performed by Gill et al. (2006a), the efficacy of phages in treatment of established bovine *S. aureus* intramammary infection has been determined. In this experiment, 24 infected cows were treated for a 5-day course with 10-ml intramammary infusions with lytic *S. aureus* bacteriophage K (1.25×10^{10} PFU/ml) or saline as a negative control. These results showed that phage treatment was able to induce a heightened immune response as exhibited by an increase in the SCC of treated udders. On the other hand, the cure rate was 3 of 18 quarters (16.7%) in the phage-treated group, which was not observed in any control samples (Gill et al. 2006a; Basdew and Laing 2011). Summarizing this work, although the phage-treated group was not significantly improved compared with the control group, obtained results are promising. Another study performed by the same group showed that there are several limiting factors in mastitis phage therapy. The main problem is whey protein binding to the bacterial surface, disturbing phage attachment, so phage administration requires further studies and optimization before use (Gill et al. 2006a, b; O'Flaherty et al. 2009; Fan et al. 2016).

Mastitis Caused by *Streptococcus*

Streptococci belong to the most frequently isolated bacterial species from mastitis cases in cow (Keefe 1997; Bradley 2002). Among the streptococci that cause mastitis, there are *Streptococcus uberis*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae* (Calvinho et al. 1998; Lammers et al. 2001; Notcovich et al. 2016). Studies indicated that the main causes of mastitis in dairy cows were *S. agalactiae* and *S. dysgalactiae*. Recent work, however, indicated that another bacterial species, *S. uberis*, shows up with increasing frequency in mastitis infections (Barrett et al. 2005; Petersson-Wolfe and Currin 2012; Collado et al. 2016; Notcovich et al. 2016).

Endolysins of phages λSA2 and B30 were reported by Schmelcher et al. (2015). In this work, they evaluated therapeutic potential of two lysins against mastitis caused by streptococci. Endolysin activity was tested in milk using commercial whole-fat ultra-high-temperature (UHT) sterilized milk. Samples of milk were infected by *S. dysgalactiae*, *S agalactiae*, or *S. uberis*. In the next step, purified enzyme was added, and samples were taken immediately before and immediately after the addition, as well as 1, 2, and 3 h after infection. In this work, they demonstrated activities of B30 and λSA2 lysins in cow milk against representative strains from the three most relevant mastitis-causing streptococcal species. They observed that λSA2 lysin was characterized by its high activity in milk against *Streptococcus dysgalactiae* (reduction of CFU/ml by 3.5 log units at 100 μg/ml), *Streptococcus uberis* (4 log), and *Streptococcus agalactiae* (2 log), whereas the B30 lysin was less effective. In summary, the B30 lysine exhibited significantly lower activity than λSA2 against all tested species (Schmelcher et al. 2015). Analyzing the results for λSA2, the next step should be experiments with cows.

Mastitis Caused by *Escherichia coli*

Strains of *Escherichia coli* belonging to environmental pathogens commonly cause bovine mastitis. Inflammation of the mammary gland caused by *E. coli* and coliform bacteria is named "coli mastitis," and it is a common and often fatal disease in lactating dairy cows (Hogan and Smith 2003; Malinowski and Gajewski 2009; Hagiwara et al. 2014). According to the available data, only two antibacterial agents have beneficial impacts in the treatment of *E. coli* mastitis. These are fluoroquinolones and cephalosporins. They belong to very important medicines whose use in animals should be heavily restricted and based on bacteriological diagnosis (Suojala et al. 2013). Therefore, it seems necessary to look for new ways to treat mastitis caused by *E. coli*, for example, phage therapy.

Suojala et al. (2013) conducted a study in which they focused on potential use of bacteriophages in preventing *Escherichia coli* mastitis on dairies. They used one phage cocktail consisting of four bacteriophages and tested it on strains from two distinct geographical regions (*E. coli* isolates from dairy cows in Washington State and from New York State). The use of phage cocktail inhibited growth of 58% of the Washington State isolates and 54% of isolates from New York State. These results show that test cocktail had a relatively wide spectrum of action against strains from two distinct regions. These tests were performed on samples of raw milk. They also performed an experiment involving the use of cocktails and bovine mammary epithelial cells. The experiments showed that pretreatment of cell cultures with the phage cocktail substantially reduced adhesion and survival of *E. coli* compared with controls (Suojala et al. 2013).

Bovine Diarrhea

Diarrhea is a commonly occurring disease in calves that causes major losses in dairy animal husbandry because of high calf mortality and morbidity (Anand et al. 2015). According to the report of the 2007 National Animal Health Monitoring System for US dairy, half of the deaths among calves was caused by diarrhea. This is the main reason of productivity and economic loss to cattle producers throughout the world (Cho and Yoon 2014; Muktar et al. 2015). The data shows that enterotoxigenic *E. coli* (in particular *Escherichia coli* ETEC K99+) belongs to the most common reasons that cause diarrhea in beef and dairy calves in a few days after birth (Moxley and Smith 2010; Anand et al. 2016). In 2006, in Norway, losses were estimated to be about US$10 million (where calf production is 284,000 heads per year).

Anand et al. (2016) isolated and characterized a new bacteriophage VTCCBPA9 with a broad host spectrum which showed bactericidal activity against calf diarrheal isolates of *Escherichia coli* in vitro. In this study, they used *Escherichia coli* ETEC isolated from diarrheal bovine calves and determined biological activity of the bacteriophage VTCCBPA9 against these pathogens. The results indicated that bacteriophage VTCCBPA9 showed bactericidal activity against 47.3% (62/131) *E. coli* isolates (also ETEC strains). Most importantly, promising activity effect against ETEC pathogens suggested the use of this virus in phage therapy as a tool against resistant pathogens.

In other studies, performed by Smith and Huggins (1983), it was also shown that phages can be effectively used in treating experimental *Escherichia coli* diarrhea in calves. They tested a cocktail of two bacteriophages B44/1 and B44/2 against *E. coli* B44 (enteropathogenic *Escherichia coli* O9:K30,99)-caused diarrhea. Calves treated by phage mixture had much lower numbers of *E. coli* B44 in their alimentary tract than untreated calves.

E. coli O157:H7 Infection

Escherichia coli O157:H7 is a meaningful human pathogen that resides in healthy cattle and other ruminants and is not a pathogen in these animals (Jeong et al. 2011). Dairy cattle have been identified as the main reservoir of *E. coli* O157:H7 (Wang et al. 1996). Transmission to humans occurs most frequently through eating raw or undercooked beef or drinking raw milk or water while less frequently through contact with manure or animals (Johnson et al. 2008; Ferens and Hovde 2011). Infection with *E. coli* O157:H7 can cause bloody diarrhea, hemorrhagic colitis, hemolytic uremic syndrome (HUS), thrombocytopenic purpura, and death (Griffin and Tauxe 1991). Phage therapy can be a good way to control infections in livestock and can help in protection of people against *E. coli* O157:H7 infection.

Niu et al. (2008) tested 4 bacteriophages against 422 STEC O157:H7 isolates (297 bovine; 125 human). They determined the host range and lytic capabilities of phages rV5, wV7, wV8, and wV11 against a collection of STEC O157:H7 in an in vitro experiment. Phage wV7 lysed all human and bovine isolates, phage rV5 lysed 342 isolates, wV11 lysed 321 isolates, and wV8 lysed 407 of the 422 isolates. These results indicate that tested bacteriophages have the ability to lyse all human and bovine isolates but each of them has a different host range. Analyzing these results, it is recommended to make a phage cocktail and next to try to use it in efficacious on-farm therapy (Niu et al. 2008).

Promising results are demonstrated by Waddell et al. (2000). Their experiment showed successful elimination of *E. coli* O157:H7 in experimentally inoculated (10^9 CFU) calves through the oral administration of 10^{11} PFU of a cocktail of six phages on days −7, −6, −1, 0, and 1 post-inoculation with pathogenic *E. coli* O157:H7 (phage cocktail was added to the milk). The results indicated that the use of multiple doses of phage cocktail is very important in effective phage therapy (Waddell et al. 2000; Zhang et al. 2015a, b).

2.1.3 Phage Therapy in Sheep

Phage therapy in sheep focuses mainly on treatment of infections caused by *E. coli* O157:H7. Interesting results were observed in the work presented by Raya et al. (2006). These researchers have isolated and characterized a new bacteriophage CEV1, efficiently infecting *E. coli* O157:H7. In vitro experiments showed that bacteriophage CEV1 is able to efficiently infect 90% (17/19) of tested *E. coli* O157:H7 strains. In the next step, they focused on in vivo experiments. Studies involved eight sheep (four treated and four control). Tested sheep were inoculated with $\sim 10^9$ CFU/sheep of novobiocin-resistant *E. coli* O157:H7 EDL 933. Then, after 3 days, half of the tested group received either a single oral dose of CEV1

($\sim 10^{11}$ PFU). In order to take samples, after 2 days, animals were humanely euthanized. It was observed that the level of O157:H7 was reduced 2–3 log units in the ceca and rectums of CEV1-treated sheep compared to control. These promising results suggest that treatment with CEV1 may be important element in an approach for reduction of *E. coli* O157:H7 levels in animals (Raya et al. 2006).

In another study performed by the same group, sheep were infected with *E. coli* O157:H7 and treated with a cocktail of two phages, CEV1 and CEV2. In this experiment, three groups of sheep were employed, each group containing four animals. Eight sheep were free of *E. coli* O157:H7-infecting phage and were divided into two groups (1 and 2). The last group (3) contained sheep that were natural carriers of phage CEV. Their data showed that a cocktail of two phages (CEV2 and CEV1) was more effective (>99.9% reduction) than the use of only CEV1 (~99%) compared to the control (group of sheep untreated and free of *E. coli* O157:H7-infecting phage). According to these results, it seems to be a better solution to use phage cocktail in phage therapy for farm animals instead of one single phage. Interestingly, they have also observed that sheep naturally carrying CEV2 and untreated by phage cocktail had the lowest level of tested pathogens (~99.99% reduction) (Raya et al. 2006, 2011).

2.1.4 Phage Therapy in Pigs

Pig Diarrhea

One of the first studies on the efficacy of phages in treatment of piglet diarrhea was demonstrated by Smith and Huggins (1983). They investigated the efficacy of a two-phage mixture (B44/1 and B44/2) against infection induced by enteropathogenic strain of *Escherichia coli* O9:K30,99, called B44, in neonatal pigs. In this experiment, 14 piglets were used which were inoculated orally about 6 h after birth. At a predetermined time after infection, piglet was given 10^{10} PFU of P433 phage by inoculation. Half of the tested pigs were treated by cocktail of two phages at the onset of diarrhea, 13–16 h after infection. None of the treated pigs died, and if there was diarrhea, it was mild. Another half remained untreated, and in those pigs, severe diarrhea developed (four died after 26–65 h). In an in vitro experiment, both phages showed a high capacity to lyse bacteria. A mixture of two phages, P433/1 and P433/2, and phage P433/1 alone cured diarrhea, caused in piglets by strain of *E. coli* P433 (Smith and Huggins 1983; Johnson et al. 2008; Zhang et al. 2015b).

Escherichia coli causing postweaning diarrhea (PWD) is an important cause of death in weaned pigs and occurs widely throughout the world (Fairbrother et al. 2005). PWD is considered a very serious disease affecting pigs during the first 2 weeks after weaning. This disease is revealed by severe diarrhea, dehydration, growth retardation in surviving piglets, and even death. PWD is responsible for economic losses due to mortality, morbidity, and costs of treatment (Rhouma et al. 2017). Colonization factors (CFs) and enterotoxins differentiate ETEC from other categories of diarrheagenic *E. coli*. The main factors of colonization are fimbriae; in the case of pigs, the most frequently encountered fimbrial adhesins are F4 (Dubreuil 2017). Experiments performed by Jamalludeen et al. (2007) focused on phages that

might be used in prevention and treatment of porcine postweaning diarrhea due to O149 enterotoxigenic *E. coli* (ETEC). In their research, they focused mainly on O149:H10:F4, because this is the dominant ETEC serotype. They isolated and characterized nine phages against ETEC. Six of these phages (GJ1–GJ6) lysed O149:H10:F4 ETEC, and their effectiveness was 99–100% of 85 O149:H10:F4 ETEC strains, and three phages (GJ7–GJ9) lysed O149:H43:F4 ETEC with efficiency reaching 86–98% of 42 O149:H43 ETEC strains. These results provide a basis for the use of these bacteriophages in therapy of O149 ETEC infections in weaned pigs (Jamalludeen et al. 2007; Johnson et al. 2008). In another study, also performed by Jamalludeen et al. (2009), they used previously isolated bacteriophages for prophylaxis and treatment of experimental infection of pigs caused by O149:H10:F4 enterotoxigenic *Escherichia coli* (Johnson et al. 2008; Jamalludeen et al. 2009). In this experiment, phages were administered orally shortly after the challenge, and for therapeutic use, they were given 24 h after the challenge, following the onset of diarrhea. During tests, they focused their attention on several parameters: weight change, duration of diarrhea, and severity of diarrhea. Generally, this work indicated that the isolated phages were effective in moderating the course of experimental O149:H10:F4 ETEC diarrhea in weaned pigs when given prophylactically or therapeutically.

E. coli O157:H7 and *Salmonella* Infection

Phage therapy was also used to combat infection in pigs caused by *E. coli* O157:H7 (Morita et al. 2002; Jamalludeen et al. 2007) or *Salmonella* (Lee and Harris 2001; Switt et al. 2013). Nowadays, the pig industry should reduce its antibiotic use; therefore treatment with bacteriophages might pose an effective alternative. As we know, most strains of *E. coli* are harmless for host animals and live in a symbiotic way. However, there are reports pointing that swine have the potential to harbor EHEC that infect humans (Nakazawa et al. 1999; Callaway et al. 2004). In some cases, these bacteria can cause severe illness (diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome) or even death in humans, for example, *E. coli* O157:H7 (Attar et al. 1998). Cornick and Helgerson (2004) also proved that swine do not have an innate resistance to colonization by *E. coli* O157:H7 and pigs infected with *E. coli* O157:H7 transmitted the microorganism to healthy pigs.

2.1.5 Phage Therapy in Equine

The available data shows that research on phage therapy in equine are focused on in vitro experiments. Anand et al. (2015) isolated and characterized lytic bacteriophage BPA6 against a pathogenic strain of *Aeromonas hydrophila*. These pathogens have been isolated from feces of normal horses (6.4%) but in some cases are responsible for pathological processes in equine, mainly septic arthritis, enteritis, and reproductive disorders (Igbinosa et al. 2012; Anand et al. 2015). Isolated bacteriophages displayed lytic activity against 8/14 (57.1%) of the *Aeromonas* spp. isolates. These results indicate that lytic bacteriophage BPA6 can be a potential tool against *Aeromonas hydrophila* pathogens and could be used as biocontrol agent in equine environment.

2.2 Aviculture

2.2.1 Current Challenges of Poultry Industry: Bacteria, Antibiotic Use, and Drug Resistance

Aviculture is currently the basis of the world's protein production as consumption of poultry meat has been growing for the last 50 years (Clavijo and Florez 2018, Table 1, Fig. 1). It is estimated that poultry production may reach 130 million tons of meat by 2020 (OECD). To meet the demands of growing world's population, breeders focused on such traits as fast growth, breast meat yield, and efficiency of feed conversion rates (Fadiel et al. 2005; Borda-Molina et al. 2018). Furthermore, chicken feed is heavily supplemented with amino acids, vitamins, enzymes, and probiotics in order to improve growth performance (Borda-Molina et al. 2018). Until recently, the use of growth-promoting antibiotic (GPA) was also allowed in aviculture (FDA 2000). Subtherapeutic concentrations of antibiotics increased animal production by stabilizing the gut microbiome and allowing the bird to obtain more nutrients from the diet (Dibner and Richards 2005; Lu et al. 2008; Díaz-Sánchez et al. 2015). However, this practice was shown to allow foodborne pathogens to develop antibiotic resistance (Singer and Hofacre 2006; Diarra et al. 2010; Singh et al. 2010; Schwaiger et al. 2012; Mehdi et al. 2018).

Several species of bacteria capable of causing foodborne illnesses in humans are commonly present in chicken intestine, most importantly *Campylobacter* and *Salmonella* (Atterbury et al. 2007; Oakley et al. 2014). *Campylobacter* is not considered to be pathogenic in avian hosts (Stern et al. 1995; Lee and Newell 2006). *Salmonella enterica* is generally believed to be a minor taxon in chicken gut, sporadic in distribution, and present only temporary (Liljebjelke et al. 2005; Oakley et al. 2014). This species can cause disease in chickens, depending on type of serovar and health condition of the bird (Hernandez et al. 2012; Clavijo and Florez 2018). Most human cases of salmonellosis and campylobacteriosis are caused by consumption of contaminated meat. Contamination is usually a result of a carcass coming into contact with feces of an infected animal (Wegener et al. 2003; Capparelli et al. 2010).

Furthermore, *Salmonella enterica* serotype Gallinarum and certain strains of *Escherichia coli* can cause severe infections in chickens (Dho-Moulin and Fairbrother 1999). Colibacillosis, caused by *E. coli*, is a severe respiratory and systemic infection of farmed poultry. Signs of colibacillosis are respiratory distress, reduced appetite, and poor growth. Postmortem foamy exudate and caseous exudate are observed in bird's air sac (Dho-Moulin and Fairbrother 1999; Knobl et al. 2001). *Salmonella enterica* serotype Gallinarum is a pathogen responsible for fowl typhoid (FT), a disease characterized by acute systemic infection with mortality reaching up to 80% of birds affected (Kwon et al. 2010; Filho et al. 2016). Both these diseases are responsible for heavy economic losses in the industry (Oliveira et al. 2009; Kumari et al. 2013).

Because of the development of drug resistance in bacteria (Liljebjelke et al. 2017; Nhung et al. 2017; Mehdi et al. 2018), and due to the fact that antibiotics may have a negative impact on the environment (Gonzalez Ronquillo and Angeles Hernandez 2015) and consumers health (Chan 1999; Kummerer 2009; Mehdi et al. 2018), the

EU banned the use of antibiotics as growth promoters in 2006 (EU regulation No 470/2009), and FDA enforced limitation on use of antibiotics in food animals (FDA 2005, 2012; Díaz-Sánchez et al. 2015). Therefore, there is a need to find alternative ways to prevent diseases in avian farms and contamination of food products, and alternative ways of fighting bacteria are being researched.

2.2.2 Phage Therapy in Aviculture: Experiments on Bird Models

The most common bacteria responsible for foodborne infection in humans derived from poultry are *Salmonella*, *Campylobacter*, and *Escherichia coli*. A considerable percentage of isolates are resistant to antibiotics, due to their misuse, discussed in the previous paragraph (Wernicki et al. 2017; EFSA and ECDC Report 2015). The use of bacteriophages to eliminate those pathogens seems promising as phages are present in every ecosystem, and thus they are easy to obtain, and they are more specific than antibiotics (Brussow and Kutter 2005; Loc-Carillo and Abedon 2011). Furthermore, since the use of phages in treatment of infections caused by multidrug-resistant bacteria in humans has a high success rate (Weber-Dąbrowska et al. 2000; Kittler et al. 2017; Lin et al. 2017; LaVergne et al. 2018) and phages are effectively being used in food safety (Gracia et al. 2008; Sillankorva et al. 2012; El-Shibiny et al. 2017), their application in disease prevention of poultry seems to be a logical consequence.

Salmonellosis

Salmonellosis is one of the diseases that is most commonly associated with contaminated poultry meat and eggs. *Salmonella enterica* is divided into over 2500 serovars, with different level of pathogenicity (Gal-Mor et al. 2014). In case of many *Salmonella* serotypes, birds often act as a carrier, without developing illness symptoms. There are however serovars, like *S.* Gallinarum and *S.* Pullorum, that can be a cause of serious infections in bird (Dho-Moulin and Fairbrother 1999; Lim et al. 2011; Tie et al. 2018). The most common serotypes responsible for the disease in humans are *S.* Typhimurium and *S.* Enteritidis. Other serotypes vary from one continent to another and even from country to country (Tindall et al. 2005; Feasey et al. 2012; Gal-Mor et al. 2014).

Use of phages against *Salmonella* proved to be effective on a number of occasions. 100% efficacy in eliminating *S.* Enteritidis strains from the tonsils of quails was reported by Ahmadi et al. (2016). Birds were given orally 100 ml of phage suspension (10^9–10^{10} PFU/ml) for 3 days, and the therapeutic effect was visible within first 6 h after experimental infection (Ahmadi et al. 2016). Single dose of 10^{11} phage particles administrated orally decreased the occurrence of *S.* Enteritidis in chickens by 3.5 log units (Fiorentin et al. 2005). Positive effect of phage administration as feed additive was observed in combating infections induced by *S.* Gallinarum in flocks of laying hens. The use of bacteriophages led to a drop in mortality from 30 to 5% (Lim et al. 2011). Phage YSP2 was reported by Tie et al. (2018) to have a therapeutic potential against diarrhea in chickens caused by *S.* Pullorum as the dose of 10^{10} PFU/ml reduced mortality in chickens from 50 to around 20%. However, the phage was reported to be less effective in treatment of *S.* Pullorum infection than furazolidone

(Tie et al. 2018). Some studies suggested that phages may be used in combined treatment with other preparations, such as probiotics. A mixture of three phages applied together with probiotic to combat *S.* Typhimurium infections in broilers indicated strong synergistic antibacterial effect (Torro et al. 2005). In another study, simultaneous application of three phages by aerosol spray (two doses at 6 days of age) and probiotics (single dose at 1 day of age) reduced intestinal colonization with *S.* Enteritidis (Borie et al. 2009).

However, the effectiveness of phage therapy may strongly depend on a number of factors such as the serotype of *Salmonella* causing infection, individual properties of a phage, adaptive mechanisms of the bacteria, treatment schedule, and phage dose (Capparelli et al. 2010; Bardina et al. 2012; Wernicki et al. 2017). Some studies reported only short-term effectiveness of phage therapy due to development of resistance to the bacteriophage by the bacteria (Andreatti Filho et al. 2007; Capparelli et al. 2010). On the other hand, in some cases, resistance acquisition resulted in the loss of virulence of *Salmonella* (Capparelli et al. 2010). In some cases, the treatment proved to be ineffective in reducing Salmonella colonization of birds; however bacterial isolates were determined to be phage susceptible and have not yet developed the resistance. These results suggest that there may be limiting parameters other than resistance (Hurley et al. 2008). Experiments performed by Bardina et al. (2012) focused on the impact of phage administration schedule in reducing colonization of poultry. Chickens were divided into three groups. One received a single dose of phage 8 h prior to the infection with *S.* Typhimurium, and the other received three doses (4 h prior and then at 7th and 10th day after the infection). The third group received treatment simultaneously with bacterial inoculation and at 6, 24, and 30 h after the infection. Even though concentration of *S.* Typhimurium dropped in groups 2 and 3, only in group 3 a significant decrease in mortality was observed (from 100 to 50%). In cases of groups 1 and 2, all chickens eventually died, though it occurred later than in the case of untreated chickens (Bardina et al. 2012).

Campylobacteriosis

Campylobacteriosis, caused by *Campylobacter jejuni* and *Campylobacter coli*, is currently the most common foodborne disease. These bacteria constitute a larger portion of the bacteria colonizing the gastrointestinal tract in poultry (up to 80%) (Friedman et al. 2000), and it is estimated that even up to 85% of processed meat may be contaminated with *Campylobacter* bacteria (Firlieyanti et al. 2016). One of the first studies on the efficacy of phages in treatment of *Campylobacter jejuni* colonization of poultry showed an immediate reduction of CFU counts in chicks receiving oral treatment immediately after bacterial inoculation. In case of adult birds, colonization by *C. jejuni* was inhibited, by 2 and later by 1 log unit in broiler ceca. Unfortunately, phage administration prior to bacterial inoculation did not prevent colonization. However, the study has shown that it may delay the spread of bacteria (Wagenaar et al. 2005). Similar results were observed when a suspension of phages infecting *C. jejuni* and *C. coli* was added to chicken water or feed. Administration of phages caused a 2 $\log_{10}$ CFU/g decrease in colonization, and the effect was maintained for over a week. However, preventive treatment again did

not stop colonization and only delayed it (Carvalho et al. 2010). In another study, a considerable but short-termed reduction in CFU was obtained in the intestines of birds infected with *C. jejuni* and then treated with phage cocktail consisting of two phages. Best results were obtained when bacteriophages were given to birds at final concentration of 10^7–10^9 phage particles (Loc Carillo et al. 2005). Similar results were obtained in two other studies involving infections with *C. jejuni* and *C. coli* (Atterbury et al. 2005; El-Shibiny et al. 2009). Firlieyanti et al. (2016) performed an experiment involving the use of phages on chicken liver. They observed that reduction in viable count of *C. jejuni* was modest and ranged between 0.2 and 0.7 $\log_{10}$ CFU/g (Firlieyanti et al. 2016). In regard to resistant development, it was observed by different research groups that the level of phage resistance of *Campylobacter* is rather low, reaching about 4% (Loc Carillo et al. 2005) or 13% (Carvalho et al. 2010). However, different results were presented by another research group. Fisher et al. (2013) conducted a study focusing on comparison of development of bacterial resistance to single phage and phage cocktail against *C. jejuni*. In all three trials involving broiler chicken, the level of phage-resistant *C. jejuni* reached from 43 up to 90%. The use of phage cocktail did not prevent bacteria from developing the resistance, but delayed and lowered the emergence of resistant isolates. However, they have also observed that even though phage-resistant bacteria emerged, the level of colonization was lower than in non-treated birds (Fisher et al. 2013). It was therefore speculated that development of resistance may reduce colonization capability of bacteria (Loc Carillo et al. 2005; Fisher et al. 2013).

Colibacillosis

One of the first studies involving the use of phages against colibacillosis in chicken focused on sepsis, which untreated results in 100% mortality. Intramuscular injection of phages at doses of 10^6 and 10^4 PFU/ml was shown to completely eliminate mortality of chickens with sepsis caused by *E. coli* (Barrow et al. 1998). However, since colibacillosis is a disease that develops primarily in bird's air sack (Dho-Moulin and Fairbrother 1999), phage therapy of avian pathogenic *E. coli* focuses mostly on using aerosol and direct application to bird's air sac. Phage mixture applied to air sac of 7-day-old birds was able to reduce mortality by 50%. In chicks (1–3 days of age), the use of aerosol decreased mortality from 20 to 3%. However, there was no significant difference in case of birds challenged at 8, 10, or 14 days old (Huff et al. 2002a). In another study performed by the same group, chicks were infected with 10^4 CFU/ml of *E. coli* and treated with aerosol containing phages at titers of 10^4 and 10^2 PFU/ml. Use of phages reduced mortality to 35% (10^2 PFU/ml dose) and 0% (10^4 PFU/ml dose). It was also shown that this kind of treatment can be successful when applied in ovo, and its results are comparable to standard treatment using enrofloxacin (Huff et al. 2004, 2009). It was also demonstrated that combined use of enrofloxacin and phage cocktail has a synergistic protective effect in chickens (Huff et al. 2004). Huff et al. (2002b) also suggested that phages distributed in form of aerosol can be used as a preventive measure before possible infection might happen, i.e., before transport. In order to achieve the highest effectiveness, phages should be administrated to birds 1–3 days before being exposed to putative risk factor (Huff

et al. 2002b). Research performed by Oliveira et al. (2009, 2010) addressed the efficacy of phage treatment depending on administration route and phage type and titer used. It was observed that sprayed phages were able to reach the respiratory tract within 3 h after administration. In case of oral administration, phages were able to reach lungs; however, some of the phages were also found in other internal organs, i.e., liver and duodenum. Moreover, intramuscular injection resulted in phage presence in all organs collected (Oliveira et al. 2009). In another experiment, chickens were given a suspension (10^9 PFU/ml) of one of three phages, either by oral application or by spraying directly into the beak. Birds were infected with pathogenic *E. coli* immediately after phage distribution. One phage in particular, phi F78E, administered both orally and by spray, resulted on average in a 25% decrease in mortality (Oliveira et al. 2010). Therefore, spray and oral administration are recommended in order to control respiratory infections caused by *E. coli* (Oliveira et al. 2009, 2010). Skaradzińska et al. (2017) have also performed in vitro tests on *E. coli* carrying plasmid encoding resistance to β-lactam antibiotics isolated from turkey farms. This research group found that phages isolated from litter samples were effective against antibiotic-resistant strains of *E. coli* isolated from turkey farms in Poland. However, experiments analyzing effectivity of those phages in vivo still need to be performed (Skaradzińska et al. 2017). It was also shown that phages can be effectively used as a means of protection against colibacillosis by spraying the chicken pens. 200 ml of phage suspension at a titer of 8×10^8 on the litter and surface of the pen reduced mortality of 3-week-old broilers (El-Gohary et al. 2014). It is therefore suggested that use of phage suspension to spray chicken pens may be an effective way to prevent *E. coli*-associated diseases in chicken (Oliveira et al. 2010; El-Gohary et al. 2014; Wernicki et al. 2017).

Other Diseases

Phage therapy was also used to combat Gram-positive bacteria found in poultry: *Clostridium perfringens* and *Listeria monocytogenes* (Wernicki et al. 2017). While in case of *L. monocytogenes* research mainly focus on the use of phage cocktails on processed meat (Housby and Mann 2009; Bigot et al. 2011), there have been a few studies conducted on chicken model regarding phage therapy against *C. perfringens*. Miller et al. (2010) showed that phage treatment of chicken infected with *C. perfringens* was even more effective in reducing mortality than commonly used vaccine against this bacterium. In another study, a combined treatment of a phage cocktail and endolysins was applied. Combination of phages and murein hydrolase enhanced the performance of a phage, and lytic effect was observed against all ($n = 51$) strains tested (Zimmer et al. 2002a, b). However, there are still very few studies regarding phage therapy against *C. perfringens*, and more data is needed to fully evaluate its performance (Wernicki et al. 2017).

2.2.3 Phage Therapy in Aviculture: Applications to Market

Due to EU policy regarding the use of bacteriophages in disease prevention, there is currently no phage-based product to be used in aviculture in counties that are EU members (Debarbieux et al. 2016). However, Proteon Pharmaceuticals, a Poland-

based company, released phage cocktail that is commercially available in Russia and Ukraine. The product, BAFASAL®, can be used as feed or water additive and is a mixture of phages infecting some of the most common *Salmonella enterica* serovars, including Enteritidis, Typhi, Paratyphi, Typhimurium, Brandenburg, and Hadar (Wójcik et al. 2015). Tests performed on a total number of 220 broilers showed a significant decrease in the number of *Salmonella* Enteritidis in gastrointestinal track of chickens (Proteon Pharmaceuticals report). BAFASAL® was also recently registered in the USA and is currently under the review by EU commissions.

2.2.4 Prospects and Challenges of Phage Therapy in Aviculture

Data presented by many research groups all around the world show that phage therapy may be a potential means for prevention against pathogenic colonization of birds (Wernicki et al. 2017). Phages were found to be effective in reducing mortality in bird cases of colibacillosis (Barrow et al. 1998; Huff et al. 2002a, b; Oliveira et al. 2009, 2010). These viruses lower the rate of colonization of bird's gastrointestinal tract with *Campylobacter* and *Salmonella* (Fiorentin et al. 2005; Loc Carillo et al. 2005; Atterbury et al. 2005; El-Shibiny et al. 2009; Ahmadi et al. 2016) and prevent the birds from developing systemic illnesses caused by some *Salmonella* serotypes (Lim et al. 2011; Tie et al. 2018). However, use of phage therapy as a widespread means for prevention of diseases is still under debate. One of the problems is the fact that even though phages reduced bacterial count in bird's gastrointestinal tract, in some cases, re-emergence of bacteria was observed after a few days (Wagenaar et al. 2005; Fisher et al. 2013). The results also seemed to depend strongly on the type of phage, dose, and time of administration (Oliveira et al. 2009; Capparelli et al. 2010; Bardina et al. 2012). Therefore, more research is needed in order to determine a procedure that will bring the best possible results. There is also more research needed on phage resistance development and phage-bacteria coevolution. Understanding those mechanisms may help in phage applications in the future.

Phage development, properties, and genetic material need to be analyzed in depth before viruses can be used in phage therapy. This procedure is time-consuming, and not all isolated phages fulfil necessary requirements (Zhang et al. 2013; Lee et al. 2016; Skaradzińska et al. 2017). Furthermore, phages infecting some of the taxa are harder to isolate than the others. For example, phages infecting *Campylobacter* spp. are often difficult to isolate and to propagate in laboratory environment, and large number of samples need to be analyzed in order to find a suitable phage (Janez and Loc Carillo 2013; Firlieyanti et al. 2016; Sorensen et al. 2017; Gencay et al. 2017). There are also reports showing that the choice of the primary isolation strain may bias the selection of bacteriophages (Sorensen et al. 2015). Therefore, phage cocktail needs to undergo many trials in order to test its efficacy and safety before it can become an actual product.

Furthermore, regulations of the European Union do not fit bacteriophage therapy and use of phages adequately. Therefore, phages cannot be used as a common alternative to antibiotics or other antimicrobial compounds. Until the regulations will not be

adapted, commercially available phage products will most likely not be available in countries that are EU members (Debarbieux et al. 2016; Fauconnier 2017).

2.3 Aquaculture

The Food and Agriculture Organization (FAO 2016) showed that aquaculture is one of the most rapidly growing sectors for animal food production, supporting approximately 50% of the global human fish consumption. Aquaculture continues to grow more rapidly than any other animal food-producing sectors. The growing demand for fish and shellfish as well as the more stringent rules on wild catches has led to increased production in the aquaculture sector (Thompson et al. 2004). Aquaculture is becoming one of the fastest growing productive sectors, providing nearly one-third of the world's seafood supplies (Kramer and Singleton 1992). Unhygienic food practice causes foodborne disease and also can damage, infect, or even kill marine products. It makes huge financial losses (Richards 2014).

Currently, the use of disinfectants and antibiotic on a large scale is very popular to prevent bacterial diseases in marine organisms. This has led to environmental pollution by the remains of antibiotics remaining in seawater and the presence of antibiotic-resistant bacteria (Kalatzis et al. 2018). In fact, in the marine environment, the majority (90%) of bacterial strains are resistant to more than one antibiotic, and 20% are resistant to at least five antibiotics (Lagana et al. 2011). Therefore, alternative strategies to the use of antibiotics should be developed to combat problems associated with treatment and prevention of diseases in aquaculture (Weber-Dąbrowska et al. 2016). Phage therapy may be a promising alternative for this, but its use in aquaculture requires a detailed observation of the seasonal dynamics of the total bacterial communities, the microbiological water quality, and the presence of pathogenic bacteria in the water system (Pereira et al. 2011). All-year observations have shown the higher complexity of the whole bacterial structure and the emergence of new populations of the main pathogenic bacteria of fish during the warm season, especially in the spring (Pereira et al. 2011).

2.3.1 Pathogenic Bacteria in Aquaculture

There are two groups of bacteria which contaminate aquaculture products: naturally occurring in the aquatic environment (e.g., *Clostridium botulinum*, *Listeria monocytogenes*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio cholerae*) and introduced from outside by animal waste, sewage, or industrial sources (e.g., *Enterobacteriaceae* such as *Escherichia coli*, *Shigella*, and *Salmonella*) (Fukuda et al. 1996; Nakai et al. 1999; Nakai and Park 2002). The biggest threats to fish are vibriosis and photobacteriosis—fish disease caused by local bacterial species from the genera *Photobacterium*, *P. damselae*, and *Vibrio*, *V. alginolyticus*, *V. vulnificus*, *V. salmonicida*, and *V. parahaemolyticus* (Moriarty 1998; Defoirdt et al. 2007). These diseases can cause significant mortality in fish, reaching values of up to 100% in infected facilities. Significant numbers of those bacteria remain on the skin of marine organisms and may harm consumer's health also (Kalatzis et al. 2018).

2.3.2 The Use of Bacteriophages in the Treatment and Prevention of Infections in Aquaculture

Phage therapy may be a promising strategy for controlling diseases in aquaculture (Subharthi 2015). The available literature indicates that phages have been successfully used to control pathogenic bacteria in water environment (Matsuzaki et al. 2005; Kalatzis et al. 2018; Wu et al. 1981; Pal et al. 2016; Skurnik and Strauch 2006). Bacteriophages were first used to treat infections of *Aeromonas hydrophila* in eel's redfin. After 3-h infection of phage AH1, the *A. hydrophila* had completely lost its infectivity and mortality (Wu et al. 1981). Next, phage therapy was used in yellowtail in the aquatic culture in Japan against *Lactococcus garvieae* in 1999 (Nakai et al. 1999). Since then, many scientists want to find more and more phages against pathogenic bacteria that infect marine organisms.

Phage Therapy in Fish

The use of phages to prevent fish infection is well documented (Silva et al. 2014b; Pal et al. 2016; Nakai et al. 1999; Stevenson and Airdrie 1984; Wu et al. 1981; Park and Nakai 2003; Nakai and Park 2002). Several groups demonstrated therapeutic efficacy of phage therapy against infectious diseases caused by *Pseudomonas plecoglossicida*, *Enterococcus seriolicida*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Photobacterium damselae*, and *Lactococcus garvieae*. These infections affect marine fish, such as seabream (*Sparus aurata*), Atlantic salmon (*Salmo salar*), and rainbow trout (*Oncorhynchus mykiss*) (Higuera et al. 2013; Nakai et al. 1999; Park et al. 2000; Park and Nakai 2003; Almeida et al. 2009; Gudding and Van Muiswinkel 2013).

Nakai and Park described the successful use of phages against *Enterococcus seriolicida* infection of yellowtail and *Pseudomonas plecoglossicida* infection of ayu (Nakai and Park 2002; Park and Nakai 2003). Both bacterial species are typical opportunistic pathogens because they are ubiquitous in fish and their culture environments (Nakai and Park 2002). In recent years, various groups have paid attention to the infection caused by *Flavobacterium*. Madsen et al. (2013) have shown that phage FpV-9 protected fish against *Flavobacterium psychrophilum*, the Gram-negative fish pathogen responsible for rainbow trout fry syndrome (RTFS) in salmonid hatcheries worldwide. Another group found FCP1 phage isolated from fish farm, active against *Flavobacterium columnare* bacteria, which causes cottonmouth disease in fish (Prasad et al. 2011).

Phage Therapy in Seafood

Seafood is also exposed to contamination with bacteria. Most of oysters or shrimp produced in Australia are distributed live and are frequently eaten unclean or raw or lightly cooked (Mohamed et al. 2003). Hence, human pathogens may not be removed and can be eaten with food, causing various human diseases. Thus, pathogenic bacteria such as *Escherichia coli*, *Campylobacter*, *Staphylococcus*, and *Salmonella* species can be easily transferred (Hatha et al. 2005; Pal et al. 2016).

These bacteria may cause severe infections, leading to a relatively high level of morbidity and mortality. One of them, caused by enterotoxigenic *E. coli*, was reported in sushi restaurants in Nevada (USA) in 2004, where 130 patients

developed severe symptoms like diarrhea or vomiting (Jain et al. 2008). The consumption of butterfly shrimp and oysters was identified as the most likely vehicle of infection. Le et al. (2017) used the bacteriophage cocktail in controlling *Escherichia coli* strains and *Salmonella enterica* contaminants of the edible oysters. The used phages (five different *E. coli*-specific phages from *Siphoviridae* family and a *Salmonella*-specific phage from *Tectiviridae* family) resulted in significant decrease of the number of these bacteria on edible oysters (Le et al. 2017). Therefore, phage treatment might be an effective tool to ensure safety of aquaculture produce.

Phage Therapy in Coral Reefs

One of the most diverse and important water ecosystems on earth is coral reefs (Bryant et al. 1998). However, infectious diseases contribute to a decrease in their quantity (Kerri et al. 2004; Doss et al. 2017). Thus, phage therapy was also used against pathogens in corals, such as *Thalassomonas loyana* which cause bleaching and white plague-like disease. Phage BA3 inhibited this infection and transmission of this disease from one coral to the others (Efrony et al. 2009; Barash et al. 2005; Thompson et al. 2006). The growth of bacteria *Vibrio coralliilyticus*, causing the tissue lysis of the coral, was also inhibited with the use of specific bacteriophages (Ben-Haim et al. 2003).

2.3.3 Vibrios in Aquaculture

One of the main threats to marine organisms is vibriosis, caused by bacteria of the *Vibrio* genus (Goulden et al. 2012; Schiewe et al. 1981; Toranzo et al. 2005). The main factors causing epidemics are *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, and *V. splendidus* (Thompson et al. 2005; Seed et al. 2014; Kalatzis et al. 2018; Plaza et al. 2018). Diseases caused by vibriosis, including early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND), contribute to losses in the aquaculture industry (Kalatzis et al. 2018). Many previous reports indicated that phage therapy is an effective treatment against vibriosis disease.

Bacteria of the genus *Vibrio* usually enter larval-rearing water through live feeds, such as *Artemia* and rotifers (Kalatzis et al. 2016). Live foods are able to swim in water column and are available to fish and shellfish larvae thereby Rasool et al. 2014). Live feed, like *Artemia*, can also accumulate bacteria from the water column and can transfer the pathogenic and resistant strain into the hatchery (Maleknejad et al. 2014). Therefore, phage therapy is also helpful in this case, to control the number of *Vibrio* bacteria in the live feeds prior to their introduction in the hatchery system. One of the main pathogenic species in larval rearing is a ubiquitous bacterium *V. alginolyticus*. Kalatzis et al. (2016) isolated two novel broad host range lytic bacteriophages φSt2 and φGrn1 from this bacterium. These viruses are able to infect all host strains and also *V. harveyi* and *V. parahaemolyticus* species (Kalatzis et al. 2016; Andrews and Harris 2000). Similar research has been carried out during the production of fish larvae of zebrafish—*Danio rerio*—experimentally exposed to *V. anguillarum* (Cantas et al. 2012; Silva et al. 2014b; Higuera et al. 2013).

It was found that phages prevented the infection by vibrios without affecting the beneficial bacterial community.

Additionally, some bacteria, such as luminous *Vibrio harveyi*, cause serious mortalities. Four bacteriophages were isolated, three from oyster tissue and one from shrimp hatchery water. The bacteriophage treatment resulted in over 85% survival of *Penaeus monodon* larvae infected with *V. harveyi*, suggesting that bacteriophage therapy would be an effective alternative to antibiotics in shrimp hatcheries (Karunasagar et al. 2007; Vinod et al. 2006).

Wang et al. (2017a, b) demonstrated that two *Siphoviridae* phages can eliminate *V. harveyi* strains infecting abalone (*Haliotis laevigata*). The effect of phage therapy on vibriosis in Atlantic salmon (*Salmo salar*) was also tested. The bacteriophage CHOED was found, which provided 100% protection of the fish against *V. anguillarum*, when MOI 1 and MOI 10 were use. What is important, untreated fish suffered over 90% mortality (Higuera et al. 2013).

Infections by *Vibrio* have been observed also in the sea cucumber (*Apostichopus japonicus*)—marine animals which are used for food. *A. japonicus* was cultivated on a commercial scale in northern China, where production reached 5865 tons in 2002. However, the rapid expansion of the aquaculture by *Vibrio* species contributes to economic loss. It was demonstrated that phage isolated from the raw sewage obtained from the drain pipes from the local hatchery of sea cucumber controls infections caused by *V. cyclitrophicus*, *Vibrio alginolyticus*, and *Vibrio splendidus* (Li et al. 2016a–c; Zhang et al. 2015a).

The team from Malaysia found a novel vibrio phage (VpKK5), from *Siphoviridae* family, that was lysing the *V. parahaemolyticus* strain, pathogenic to shrimp and tropical cultured marine finfish. The KVP40 phage is also worth attention (Lai et al. 2016). Matsuzaki et al. (1992) showed that this myovirus has a broad host range, which may mean that a number of different *Vibrio* species have a receptor for the phage in common. This phage is able to infect several strains of different *Vibrio*: *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, *V. anguillarum*, *V. splendidus*, *V. mimicus*, *V. natriegens*, and *V. fluvialis*.

However, most of the presented reports focused on the isolation and characterization of phages capable of reducing the pathogenic bacteria in aquaculture only in vitro. So, it is necessary to carry out more in vivo tests to fully prove the advantages of using phage therapy. Thanks that it will be possible to produce and approve phage-based preparations against infections in aquaculture. At present, such therapeutics are not available.

2.3.4 Phages Delivery Method

Phages have different abilities to maintain their lytic potential against pathogens and to reach target organs of adequate density. Hence, the phage delivery methods are of vital importance for a successful therapy. According to the available literature, it is known that phages can be administered in three different ways: parenteral delivery, oral administration, and immersion in a bath containing phages.

Intramuscular injection of phage (parenteral delivery) has proven to be one of the most successful delivery methods in animal studies, because the phages can

immediately reach the systemic circulation (Ryan et al. 2011). It was reported that bacteriophages could be detected in the fish tissues for several days after administration (Nakai and Park 2002; Madsen et al. 2013). For example, the results obtained by Prasad et al. (2011) suggest that intramuscularly route of phage introduction resulted in reduction of clinical symptoms and a better lytic impact on bacterium, relative to other delivery methods. What is important, to spread phages to all the internal organs via the circulatory system, a high phage concentration is necessary. However, this method is labor intensive, including work with small animals, and may be rather stressful for water organisms (Christiansen et al. 2014; Kalatzis et al. 2018).

Orally administered phage through phage-impregnated food allows continuous supply with a large amount of marine organisms (Oliveira et al. 2012). The main problem with this method is phage stability in the highly acidic and proteolytically active environment of the stomach. Research performed by Christiansen et al. (2014) demonstrated that orally administered phages can penetrate the intestinal wall and be absorbed into the circulatory system. Phages were detected in the kidney, spleen, and brain after the application. This method resulted in constant, high abundance of phages in the fish organs for several weeks (Christiansen et al. 2014). Additionally, studies with the use of goldfish have shown that phages are capable of penetrating the intestinal wall (Kawato and Nakai 2012). Phage-coated feed has been successfully used also for treating *Pseudomonas plecoglossicida* infections in ayu and *F. columnare* infections in catfish (Park and Nakai 2003; Nakai and Park 2002; Prasad et al. 2011). Furthermore, Christiansen et al. (2014) reported that continuous delivery of the feed pellets coated with phage FpV-9 is a successful method for prevention of rainbow trout fry syndrome caused by *F. psychrophilum.*

The conditions in gastrointestinal tract are unfavorable for phages, which can affect the phage viability (Christiansen et al. 2014). During the phage delivery by oral route, the problem is phage stability in the highly acidic and proteolytically active environment of the stomach, because each phage can have different sensitivity to pH (Kim et al. 2010; Ryan et al. 2011; Bucking and Wood 2009).

It was found that in aquaculture, oral administration is the most cost-effective delivery method for immunization due to low cost and less stress to fish (Yasumoto et al. 2006; Nakai and Park 2002; Park et al. 2000; Martínez-Díaz and Hipólito-Morales 2013).

In fish larviculture, supplementation of phages by oral administration or parenteral delivery is difficult. Therefore, viruses must be directly released into the culture water (Silva et al. 2014b). The survival of phages in these conditions must be high. The fish larvae are immersed in a bath containing a high titer of phage. Thanks that phages can reach the specific site of infection. Additionally, marine fish species drink water to maintain their internal ionic balance, and therefore, phages present in the water have the opportunity to encounter pathogenic bacteria for which the infection route is through the fish intestinal mucosa (Christiansen et al. 2014). This can be exemplified by research conducted by Silva et al. (2014b) with the use the phage VP-2 against *V. anguillarum* in fish larviculture. This phage is able to survive for long time (at least 5 months) in marine water. Additionally, due to releasing this virus directly into the water, phage can control the bacterial colonization not only

inside fish but also on fish larvae skin (Silva et al. 2014b; Weber et al. 2010). Therefore, this method allows to simultaneously reduce pathogens within the animal and in its immediate environment (Richards 2014).

The choice of the appropriate and the most successful method of phage administration in aquaculture depends on the conditions prevailing in aquaculture and should be adapted to each organism and pathogen separately. New dimensions in phage application in aquaculture may be phage cocktails. For example, Chan et al. (2013) and Defoirdt et al. (2007) have observed that a mixture of different phages, i.e., phage cocktails, is more effective for treatment of bacterial infections. Thus, the spectrum of virus activity is expanded and overcomes the resistance to adherence of phage by the pathogenic host bacteria (Le et al. 2017). Hence, mixtures or cocktails of phages may be useful to prevent the development of phage-resistant pathogens in aquaculture.

2.3.5 Concerns About Phage in Aquaculture

The increasing temperature in the oceans, and the fatal effects of vibriosis and other pathogenic bacteria on aquaculture, causes that using the phages to control this problem is very important. Phage therapy has many advantages. The most important are fast phage isolation procedures and reasonably inexpensive methods which are both environment- and consumer-friendly. However, phages have also the potential risk factors, like dispersal of unwanted genes or effects on fish microbiota (Kalatzis et al. 2018). A serious threat to marine organisms can also be endotoxins. They might be potentially toxic for fish or shellfish (Opal 2010; Boratyński et al. 2004).

Another problem with using the phages in aquaculture might be the immune response. In water organisms, such as fish, the immune system may contribute to phage decay (Pirisi 2000). In available literature, there is little information about production of antibodies after phage delivery in aquaculture (Oliveira et al. 2012). This problem was addressed in only a few studies. When phages were used against infection in yellowtail (Nakai et al. 1999) and in ayu (Park and Nakai 2003), no bacteriophage-neutralizing antibodies were detected.

On the other hand, fish larvae do not have the ability to develop specific immunity (Vadstein 1997); thus, the immune system cannot remove the phage particles from circulatory system (Duckworth and Gulig 2002). Similarly, corals have no adaptive immune system; therefore, the use of phages against corals pathogens is practical (Kerri et al. 2004; Efrony et al. 2009; Nair et al. 2005).

For other organisms living in water, like sea cucumbers and other echinoderms, phagocytes and bulbous cells are the major line of defense against pathogens. Nitric oxide synthase and acid phosphatase are the main defense enzymes of these cells. They change when the organism is infected with pathogens. Li et al. (2016b, c) demonstrated that feeding phages of infected sea cucumbers provoked partial immune response, but there is no effect on the normal growth of sea cucumbers (Dolmatova et al. 2004; Li et al. 2016b, c).

In summary, all these reports demonstrated that it is necessary to select the appropriate phage to perform effective phage therapy. Latent period of phages, burst size, lytic potential, phage time of survival in the water, host range of the

phage, and efficiency of bacterial inactivation are important when phages are selected. In aquaculture, the greatest success of therapy can be achieved by using phages which withstand various environmental stresses.

2.3.6 Summary of Phage Therapy in Aquaculture

Increase of aquaculture production causes the emergence of more and more cases of bacterial infections. This causes significant economic losses for the industry. Microbial diseases have caused mass mortality of fish and other marine organisms (Kalatzis et al. 2018). The increasing use of antibiotics has led to severe negative side effects, like the selection of resistant bacterial strains. Nowadays, several environment-friendly prophylactic methods must be developed to control diseases and to maintain healthy microbial environment in aquaculture systems. The alternative approach may be using lytic phages for the treatment or prophylaxis of bacterial infectious diseases.

The available literature reported that bacteriophages have the therapeutic potential in the control of bacterial disease for fish, finfish (Park et al., 2000; Nakai and Park 2002; Park and Nakai 2003), and also seafood, like prawns (Vinod et al. 2006; Karunasagar et al. 2007), oysters, or shrimp (Le et al. 2017). Viruses also have been used against bacterial infections of coral reefs with promising results (Efrony et al. 2009).

It has been documented that in many tested cases, phage therapy is cost-effective, eco-friendly, and safe for aquacultured species and for animals. It provides the same or better protection of infected marine organism than antibiotics. This is exemplified by phage trials in a commercial shrimp hatchery using two lytic *Vibrio harveyi*-specific broad host range bacteriophages. Phage application caused about 87% shrimp survival, while in antibiotic-treated (oxytetracycline and kanamycin) shrimp, the survival was 67% (Karunasagar et al. 2007). Another group presented the results with the use of lytic bacteriophages against pathogenic *Vibrio splendidus* strains in *Apostichopus japonicus*. They reported that 18% animals survived the infection when using the control diet and 82% survived after antibiotic-supplemented or phage cocktail-supplemented diet (Li et al. 2016b, c).

Therefore, phage therapy seems to be a promising alternative to the use of antibiotics in aquaculture. This approach is important in production processes to obtain products with reduced bacterial loads or to limit current pathogenic bacteria in water. Additionally, prophylactic using of phages can improve microbiological water quality. This is a successful method to control pathogenic bacteria in aquaculture.

2.4 Brief View of Methods for Phage Preparation

2.4.1 Phage Isolation

The prevalence of bacteriophages in environment is a great advantage of using phages against bacteria over other antimicrobial agents. Every environmental sample containing pathogens in which we are interested in presumably contains a phage

(or phages) that can infect and lyse bacteria. Due to high concentration of microorganisms, the most common source of bacteriophages is urban sewage (Jurczak-Kurek et al. 2016; Li et al. 2016a; Switt et al. 2013; Abatángelo et al. 2017), but phages can also be easily isolated from rivers, from wastewater of clinics and hospitals, or directly from organisms (Bachrach et al. 2003; Merabishvili et al. 2012; Bhetwal et al. 2017).

Phage isolation is usually simple, quick, and inexpensive in comparison to other antimicrobials (Skurnik et al. 2007; Loc-Carillo and Abedon 2011). There are a lot of methods to acquire bacterial viruses, but all of them are based on the similar pattern (Gill and Hyman 2010). The most direct way is sterilization of environmental sample to remove cellular microorganisms, by using centrifugation or filtering through the membrane filter. In the most cases, sterilized sample is added directly to host strain (s) and plated by double agar overlay plaque assay to estimate the appearance of plaques (Kropinski et al. 2009). Spot assay also can be used, but it may overestimate both the overall virulence and the host range (Mirzaei and Nilsson 2015). In the next step, single plaque of isolated phage should be transferred into liquid medium (Mattila et al. 2015). To improve the visibility of phage plaques, the use of sublethal doses of antibiotics is suggested especially in the case of environmental samples (Los et al. 2008; Santos et al. 2009; Kaur et al. 2012). Before plating, samples can be concentrated by precipitation with polyethylene glycol (PEG), super-speed centrifugation, tangential flow filtration (TFF), or even organic flocculation with skimmed milk (SMF), though each of these methods may influence the carriage or survival of the phage (Calgua et al. 2008; Gill and Hyman 2010; Castro-Mejía et al. 2015; Hjelmsø et al. 2017). Samples may also be enriched by culturing in the presence of one or more of the desired bacterial hosts. It allows initially small population of the phages to propagate until they reach a concentration which is easily detected by standard methods. Enrichment can be carried out by adding a sterilized liquid to a rapidly growing host culture and incubating for the time depending on the growth rate of the host. The raw sample may be added to the host culture, or the host culture may be diluted into a volume of environmental sample in such a way that host is numerically dominant in the culture (Gill and Hyman 2010). Many phages require ions such as Ca^{2+} or Mg^{2+} for attachment or intracellular growth; thus, it is important to include 1–2 mM Ca^{2+} in all the media (Van Twest and Kropinski 2009). To provide that phage isolated for therapeutic usage is able to lyse the pathogenic strain of interest, isolation should be conducted with using the same bacterial strain.

2.4.2 Phage Characterization

All isolated phages must be characterized to confirm the potential for their use in phage therapy. The first step is examining the ability to lyse other bacterial strains which were not used in the isolation process. The desired range of hosts depends on the purpose of use; like in intestinal infections, a usage of narrow host range phages is recommended to protect commensal bacteria. Describing a host range may also test if this phage can be used to treat infections with other pathogenic strains or to find the bacterial strain in which phage develops more efficiently making it easier to find the most effective procedure.

There are also some developmental features which have to be tested: adsorption rate, latent period, burst size, and morphology of plaques. Determination of phage adsorption kinetics begins with mixing phage with bacteria culture in appropriate medium and then checking free phage loss, infected-bacteria gain, or uninfected-bacteria loss over time (Hyman and Abedon 2009). The latent period is the time interval between phage adsorption and releasing the phage progeny from lysed bacteria. Measurement of phage latent period duration may be conducted by detecting the released virions or survived bacteria. It is also important to determine a burst size which represents the number of phage progeny. Both latent period and burst size can be examined in one-step growth experiment which was described in detail by Hyman and Abedon (2009). In all these experiments, it is important to use multiplicity of infection (MOI) less than 1 to prevent multiple adsorption, lysis from without, or bacteria adsorption capacity limits (Delbrück 1940; Hyman and Abedon 2009; Abedon 2011). Based on plaque morphology, we can point out phages with lytic activity. In phage therapy, temperate phages should be avoided, due to possibility of containing genes which alter the phenotype, encoding toxins or virulence factors, and ability of such phages to conduct general transduction (Scott et al. 2008; Chen and Novick 2009; Lang et al. 2012; Goh et al. 2013; Fortier and Sekulovic 2013; Navarro-Garcia 2014). Temperate phages form turbid plaques in contrast to lytic phages which form clear plaques without halo (Abedon and Yin 2009). It should be concerned that plaque morphology depends on many factors like growth phase of the bacterial host or diffusion of virions in agar plate; hence, the "lifestyle" of the phage should be also validated through genome analysis (Gill and Hyman 2010).

Current technology of DNA sequencing and the low cost of this process made phage genomic analysis easier and more accessible. Genome sequencing and characterization of each isolated phage can show the presence of virulence, antibiotic resistance, or lysogenic genes which helps to exclude phages non-usable for therapy. Nowadays, there are a lot of computational tools to predict function of viral genes or for phage identification or classification, e.g., Unipro UGENE, GLIMMER, GeneMark, or RAST (Fancello et al. 2012; Lobanova et al. 2017; Aziz et al. 2018; McNair et al. 2018; Tithi et al. 2018).

2.4.3 Phage Purification

Bacteriophages are isolated using bacteria culture, making them contaminated with unwanted culture compounds, e.g., toxins and other immunomodulators. The most basic method for purifying phage lysate is low-speed centrifugation and then filtration through membrane filter (see Sect. 2.4.1), but these preparations can induce few side effects when administrated in phage therapy (Sulakvelidze and Kutter 2005). Most protocols of purification are focused on segregation from the lipopolysaccharide (LPS), a component of Gram-negative bacterial cell membranes which is known to be an endotoxin (Cavaillon 2018). The most typical phage purification method for small-scale preparation is high-speed centrifugation in a cesium chloride gradient (Boulanger 2009; Nasukawa et al. 2017). However, this method requires long time and expensive and specialized equipment, and it is limited

by the size of probe (Gill and Hyman 2010). Moreover, some phages can be instable in the high osmotic environment (Carlson 2005). Endotoxins can also be removed by extraction with organic solvents based on the fact that phages retained in the aqueous phase, while endotoxin accumulates in the organic solvent (Szermer-Olearnik and Boratyński 2015). Another alternative method is using anion-exchange chromatography using large pore size monolithic anion exchangers and chromatography system. Sponge-like structure of columns provides a large surface area for binding and thus improving accessibility of viruses (Oksanen et al. 2012). Advances of using this method are high resolving power, high capacity, simplicity, and controllability, making this technique suitable for processing large volumes. The first protocol for this method with cellulose as an adsorbent (ECTEOLA columns) was proposed in 1957 by Creaser and Taussig (1957). Recently most of procedures are conducted with commercially available monoliths, e.g., Convective Interaction Media® monoliths or SepFast™ Supor Q with high efficiency (Kramberger et al. 2010; Monjezi et al. 2010; Adriaenssens et al. 2012; Liu et al. 2012). Another method without mechanical purification is enzymatic inactivation of the endotoxin using alkaline phosphatase (Bentala et al. 2002). However, the treatment of the phage preparations with alkaline phosphatase may have low endotoxin removal efficiency and a negative impact on the number of infectious phage (Van Belleghem et al. 2017).

2.4.4 Phage Stabilization and Formation

Phages may be unstable in aqueous solutions due to the fact that their building blocks are proteins (Chi et al., 2003), and storage method should be adapted to the phage biology and properties. The most frequently used and efficient long-term storage way of preparations is cooling (4 °C) and freezing (–20 °C, –80 °C) (Fortier and Moineau 2009). Methods with the use of lower temperatures may require addition of cryoprotectors that increase phage stability in water solutions of various compounds, like glycerol, which stiffen the structure of proteins and inhibit their aggregation. It has been used many times to preserve liquid preparations with high survival of phage (Nyiendo et al. 1974; Mendez et al. 2002), but individual cases show that this method may change their activity and titer (Clark 1962).

The preservative compounds are also used in another storage method—lyophilization (Clark and Geary 1973; Puapermpoonsiri et al. 2010). Freeze-drying is a low-temperature dehydration process based on freezing the product, lowering pressure, and ice sublimation. Lyophilization is characterized by high effectiveness for the long-term preservation of bacterial cells, stability of lyophilized preparations at room temperature, and easy transportation of phages prepared this way (Fortier and Moineau 2009). The disadvantage of this method may be the reduction in phage titer as a consequence of the freeze-drying procedure itself (Clark 1962; Ackermann et al. 2004; Dini and de Urraza 2013; Merabishvili et al. 2013). Important factors decreasing a number of survival phages are osmotic stress and ability of phage aggregation (Puapermpoonsiri et al. 2010; Louesdon et al. 2014). Despite that, phage titers of freeze-dried preparations can be stable in long-term storage, even for 2 years when stored refrigerated (Clark 1962). Merabishvili et al. (2013) tested the influence of six preservative compounds on stability of *Staphylococcus aureus* phage ISP after

freeze-drying pointing on sucrose and trehalose to be the most effective protectant in this case and that the effectiveness of stabilization depends on protectant concentration (Merabishvili et al. 2013). Some papers pointed on addition of specific particles to the phage solution, including sodium glutamate, gelatine, peptone, casein, or skimmed milk. This may increase viability of phage after lyophilization (Steele et al. 1969; Engel et al. 1974; Puapermpoonsiri et al. 2010).

Additional procedure is spray drying in which liquid preparation is atomized and converted into mist and then contacted with a hot dry gas inside a drying chamber. In this process, solvent is quickly evaporating causing formation of insoluble compounds which are phage and excipients in the form of powder. The most commonly used protectant in this procedure is trehalose, but also usage of lactose, leucine, glucose, sucrose, or mannitol was reported (Matinkhoo et al. 2011; Vandenheuvel et al. 2013; Leung et al. 2016). This method has also been shown to result in loss of phage titer due to sensitivity to thermal and shear stress (Leung et al. 2016).

Phages can be also stabilized by encapsulation in protective particles, by coating with polymers or lipids, or incorporated into the droplets (Malik et al. 2017). Most of phage encapsulation methods consist of the process of emulsification, followed by solvent removal. Emulsion can be water-in-oil (Surh et al. 2007; Kim et al. 2015), oil-in-water (Esteban et al. 2014), or water-in-oil-in-water (Surh et al. 2007; Wang and Nitin 2014; Rios et al. 2018). Droplets that contain phages can be also produced by extrusion, mostly followed with gelation process, ionotropic gelation, heating, or covalent cross-linking (Dini et al. 2012; Ma et al. 2012; Gul and Dervisoglu 2017). There are many polymers which have been used in phage encapsulation process. The most frequently used is alginate (Colom et al. 2017; Ahmadi et al. 2018; Cortés et al. 2018) due to low toxicity and immunogenicity of this protectant (Lee and Mooney 2012). Coating with alginate may require a calcium carbonate as a cross-linking agent (Colom et al. 2017). Another polymers used in this method are agarose (Bean et al. 2014), chitosan which shows muco-penetrative properties that may increase residence time in gastrointestinal tract (Bernkop-Schnürch et al. 1998; Takeuchi et al. 2005), or whey protein (Vonasek et al. 2014). Phages can also be encapsulated in synthetic polymers, like poly(lactic-*co*-glycolic acid) (PLGA) (Puapermpoonsiri et al. 2010), poly(*N*-isopropylacrylamide) (Hathaway et al. 2015), or polymethyl methacrylate (Stanford et al. 2010). Carrier which is encapsulating the phage to protect against storage conditions should also be chosen based on the form of phage application, for example, to help to deliver phage directly to the site of infection. Encapsulated phages released may be induced by polymer solvation (Korehei and Kadla 2014) or enzyme-driven degradation (Bean et al. 2014). Carriers may be also designed to respond to specific pH, like in gastrointestinal tract (McConnell et al. 2008; Stanford et al. 2010). To close emulsion in nanofibers, electrospinning is usually used. This method is based on drawing a charged solution of polymer and phage onto a grounded electrode during solvent evaporation (Korehei and Kadla 2013; Cheng et al. 2018). Protection of phages in this procedure may be provided by encapsulating them in fibers, like polyethylene oxide, cellulose diacetate (Korehei and Kadla 2014), or polyvinyl alcohol (Sarhan and Azzazy 2017).

Recently, some researchers are focusing on liposomes as a potential phage protectant, due to their high biocompatibility and ability to enhance stability and availability of carried particles (Torchilin 2005; Swaminathan and Ehrhardt 2012). Particular liposome features, like size, charge, lamellarity, and surface modifications, play a crucial role in stability and achieving phage destination (Eloy et al. 2014). Usually, liposome encapsulation is conducted by the thin-film hydration method (Angelova and Dimitrov 1986). It is based on dissolving lipids, like cholesterol and phosphatidylcholine, in organic solvent, usually chloroform, followed by evaporation of the solvent in a vacuum. In the next step, created film is rehydrated, causing formation of multilamellar liposomes (Colom et al. 2017). Liposomes may be manipulated by extrusion through porous membranes to achieve smaller size (Nieth et al. 2015; Zhang 2017). For creation of giant unilamellar vesicles (GUV), electroformation (Angelova et al. 2018) can be used, as well as rapid solvent exchange (Buboltz and Feigenson 1999) or gel-assisted formation which is based on rehydrating of film on polyvinyl alcohol gels instead of using rehydratation buffer (Weinberger et al. 2013). Liposomes with addition of charge inducer, for example, stearylamine, may protect against liposome aggregation and increase interaction with the mucus, improving potential for intestinal infection treatment (Hua et al. 2015; Singla et al. 2016). Moreover, functioning of liposomes may be expanded by modifications, like adding specific ligands (e.g., antibodies) for targeted delivery (Koning et al. 2002), polymers with hydrophilic properties to increase phage preparation circulation (Wang et al. 2013), or markers for tracking the liposomes (Urakami et al. 2009).

Isolated phages can also be immobilized on different kinds of surfaces, for protection against biofilm formation on medical devices, or antimicrobial dressing for biomedical use (Nogueira et al. 2017; Maszewska et al. 2018). Virus particles may be immobilized by passive adsorption. This process may cause a poor orientation of phage tails needed to interact with pathogen cells decreasing phage activity (Bennett et al. 1997). In other studies, phages were immobilized by chemical biotinylation on streptavidin-coated surfaces (Gervais et al. 2007) or covalent attaching on polyethylene and polyhydroxyalkanoate surface (Pearson et al. 2013; Wang et al. 2016), getting proper orientation of phage particles.

2.4.5 Phage Administration

Phage preparations for therapy purpose contain either only one phage or a mixture of phages in the form of a cocktail. The latter type may prevent from cross-resistance leaving bacteria resistant to one phage sensitive to another. For cocktail preparation, employment of phages using different receptors for binding a host is suggested (Gill and Hyman 2010).

The route of phage product administration depends strongly on the site of infection. For fighting pulmonary infection, phages may be administrated by nebulization of aerosol (Borie et al. 2009; Cooper et al. 2013). Gastrointestinal tract infections may be treated by oral application of phages using tablets or liquid solution (Sulakvelidze et al. 2001) or by rectal application (Sheng et al. 2006; Rozema et al. 2009; Wang et al. 2017a, b). For dermatological purposes, phages can be administered in the form of cream, lotions, or ointments (Brown et al. 2016,

2018). Phage particles may be applied directly in the wound through injection into the wound (McVay et al. 2007; Chhibber et al. 2018) or soaked bandages (Miao et al. 2011; Sarhan and Azzazy 2017). Nowadays, phages are also considered as an antibacterial agent in dental infections to treat caries and infection of root canal. For this purpose, usage of mouth wash, mouth rinse, topical gel, toothpaste, tooth powder, and slow-release implant would be proper ways of phage application (Norris 1990; Delisle 2004). Moreover, different products may occur in the form of nasal and ear drops or throat, fistulas and abscesses rinses to fight local infections.

2.5 Phage-Based Products Against Pathogenic Bacteria in Food

Year 2006 was a crucial year in the history of the use of bacteriophages in prevention of bacterial diseases. The US Food and Drug Administration and US Department of Agriculture have approved several bacteriophage products to be used for food protection against *Listeria monocytogenes*. LMP-102 (now ListShield™, Intralytix Inc.) was approved for treating of poultry and meat products, while LISTEX (Micreos) was approved to be used on cheese. A year later the same product was approved for use in all food products (US FDA/CFSAN: Agency Response Letters No. 000198 and No. 000298). Since then Intralytix has come up with two phage preparations targeting *L. monocytogenes*: ListShield™ and ListPhage™. ListPhage™ is an antimicrobial preparation for controlling *L. monocytogenes* in pet food (Intralytix Inc. ListPhage™ product description), while ListShield™ is designed to protect food products such as meat, poultry, cheese, and processed and fresh fruits and vegetables against *L. monocytogenes* contamination (Intralytix Inc. ListShield™ product description). Another product, designed to fight against *L. monocytogenes* in food, is already mentioned above, LISTEX (Micreos).

Studies addressing the effectiveness of phage-based products in reducing *L. monocytogenes* on food ready-to-eat beef and turkey showed that the presence of phage resulted in lower *L. monocytogenes* numbers of about 2 log CFU/cm^2 over a 28-day storage period at 4 °C in comparison to an untreated control. In this study, sliced meat cores stored at 4 and 10 °C were inoculated with *L. monocytogenes* to result in a surface contamination level of 10^3 CFU/cm^2. Phage preparation was then applied at 10^7 PFU/cm^2, and samples were taken at regular time intervals during product's shelf-life to enumerate viable *L. monocytogenes*. For meat stored at 10 °C, cell numbers of phage-treated samples remained below those of the untreated control only during the first 14 days of the experiment. The experiments also showed that phage can be used in combination with chemical antimicrobials to enhance the safety of meats and other food products (Chibeu et al. 2013). Other studies determined that LISTEX™ solution was able to reduce *L. monocytogenes* by 1.5–3 log within 24 h after applications in case of use on salmon fillets (Soni and Nannapaneni 2010) and sashimi (Migueis et al. 2017). Silva et al. (2014a) showed that the treatment with phage (8.3×10^7 PFU/g)-contaminated (10^5 CFU/g) cheeses caused an immediate drop in bacterial CFU by 2 log units compared to the control. However, after 7 days under refrigeration, bacterial reduction reached approximately 1 log unit. The

statistical analysis showed a significant difference ($p < 0.05$) between treated samples (at both 0 and 7 days) and control (Silva et al. 2014a). Furthermore, LISTEX™ is claimed to reduce *L. monocytogenes* up to 2 log in frozen vegetables (carrots) if contamination occurs before freezing and in the case of contamination happening after defrosting (carrots and beans) (Micreos Food Safety BV, LISTEX™ product description and data sheet).

After successful introduction of phage preparations for food protection to market, the number of products available is growing. Preparations against *Salmonella enterica* and *Escherichia coli* soon followed first preparations against *L. monocytogenes*.

There are currently three phage-based products for food protection against *Salmonella enterica* currently available on the market. SalmoFresh™ and SalmoLyse® are preparations produced by Intralytix Inc. SalmoFresh™ is designed to protect meat, especially poultry, sea food, fish, fruits, vegetables, and packed food from *S. enterica* contamination. Producers declare that phages contained in this preparation are active against the following serovars of *S. enterica*: Typhimurium, Enteritidis, Heidelberg, Newport, Hadar, Thompson, Kentucky, Georgia, Grampian, Agona, Senftenberg, Alachua, Infantis, Reading, and Schwarzengrund. The product has been approved to use in the USA, Canada, and Israel (Intralytix Inc. SalmoFresh™ product description). SalmoLyse® is used for controlling *Salmonella enterica* in pet food. It contains six phages that are able to infect the same *S. enterica* serovars as SalmoFresh™ (Intralytix Inc. SalmoLyse™ product description). The study by Heyse et al. (2015) showed that the cocktail was able to lyse 930 *Salmonella enterica* strains representing 44 serovars. In experiments involving dried pet food, it showed that treatment (dose $\geq 2.5 \pm 1.5 \times 10^6$ PFU/g) of feed after its contamination with various *S. enterica* serovars was able to reduce the count of *Salmonella* within 60 min (Heyse et al. 2015). Another study showed that raw pet food ingredients (like chicken, tuna, or turkey) treated with two concentrations of SalmoLyse® ($2–4 \times 10^6$ PFU/g and 9×10^6 PFU/g) showed significantly reduced (up to 92%) *Salmonella* contamination in comparison to control experiments. It was also determined that no side effects were observed in cats and dogs eating phage-treated food (Soffer et al. 2016). Salmonellex™ is produced by European-based company Micreos. It has been approved for clean label processing in the USA, EU, Canada, Australia, New Zealand, Israel, and Switzerland. The producers claim it reduces number of pathogens by 1–3 log (Micreos Food Safety BV, Salmonellex™ product description). Yeh et al. (2018) compared the effectivity of phage preparation (at final concentration of 10^9 PFU/ml), UV light, and organic acids on *Salmonella* populations in ground beef. The study determined that individual applications of phage preparation and UV light decreased *Salmonella* population approximately 1 log CFU/g, while combined applications of phage and UV provided a decrease of 2 log CFU/g (Yeh et al. 2018). This study suggests that in order to increase effectivity of phages, a combine treatment of food should be applied.

One of the main products against *Escherichia coli* is EcoShield™ (Intralytix Inc.). It is a commercially available product (approved by the FDA in 2011), composed of three lytic phages active against pathogenic strains of *E. coli* O157: H7. EcoShield™ is designed to protect various foods, including ground beef and

lettuce (Intralytix Inc. EcoShield ™ product description). Some studies showed that application of this phage mixture has proved to eliminate from 94 to 100% of *E. coli* O157:H7 and 87% in lettuce after a 5-min contact time (Carter et al. 2012; Sillankorva et al. 2012). Carter et al. (2012) demonstrated that EcoShield™ was a very effective product against *E. coli* O157:H7, but it did not protect food from recontamination.

Finalyse (Passport Food Safety Solutions) is another phage-based product specific for *E. coli* O157:H7 and other STEC pathogens. Finalyse is a mixture of naturally occurring phages, and it is sprayed on cattle to effectively reduce *E. coli* levels prior to entering the beef packing facility (Sillankorva et al. 2012). The producers claim it reduces the number of pathogens by $\geq$1 log after 1-h phage incubation (Passport Food Safety Solutions, Finalyse product description).

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Combining Bacteriophages with Other Antibacterial Agents to Combat Bacteria

Petar Knezevic and Verica Aleksic Sabo

1 Introduction

Antimicrobial resistance in the postantibiotic era revived interest in various alternative antibacterial agents that effectively control bacterial growth. In the focus of the academic community from that aspect are plant products, such as components of essential oils and plant extracts, animal antimicrobial peptides, as well as bacteripohages and their lytic enzymes.

2 Discovery of Bacteriophages and Antibiotics

Bacteriophages are viruses that infect bacteria and were independently discovered a century ago by a French-Canadian microbiologist Félix d'Hérelle (1917) and British physician Frederic Twort (1915). On September 15, 1917, d'Herelle presented to the Académie des Sciences by Dr. Emile Roux the invisible microbial antagonist of dysentery bacillus and introduced it as "bacteriophage" into the scientific community (d'Hérelle 1917). He also proposed to use these entities as antibacterial agents and successfully applied an oral phage preparation to treat bacterial dysentery (d'Hérelle 1919). Following this example, during the 1920s and 1930s several institutes in various locations preformed similar experiments and were controlled by d'Herelle in order to ensure the produced phage quality. In the Eastern Europe, Georgian scientist Georgi Eliava with help of Felix d'Herelle established Tbilisi Institute of Bacteriophages, Microbiology and Virology (Dublanchet and Bourne 2007). Besides this institute, several commercial laboratories and companies in France, Germany, and the United States produced phage preparations (Gratia 1922; Pockels

P. Knezevic (✉) · V. Aleksic Sabo
Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Novi Sad, Serbia
e-mail: petar.knezevic@dbe.uns.ac.rs

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1927; Straub and Appelbaum 1932; Straub and Rakieten 1932). However, the bacteriophage approach has undergone the mixed success at the time because of poor basic understanding of phage biology, lack of data from clinical trials, and general overexpectations.

The situation was dramatically changed when the first antibiotic penicillin from fungi of genus *Penicillium* was discovered by Scottish scientist Alexander Fleming in 1928 and purified by Florey and Chain in 1942 (Tan and Tatsumura 2015). Waxman (1947) introduced the term "antibiotic" in order to denote natural products of microorganisms that inhibit other microorganisms. Many antibiotics have been further chemically modified to expand their activity spectrum and to make molecules more stable under certain conditions. Rolinson (1979) denoted them as semi-synthetic antibiotics (e.g., amoxicillin). Finally, many other conventionally used antibacterial agents are called antibiotics, although they are neither natural microbial products nor modified natural compounds, but are exclusively obtained by chemical synthesis. These agents are called chemotherapeutics. Ehrlich (1910) discovered the first one: it was Salvarsan used to combat *Treponema palidum*. Thus, the term "chemotherapeutic" was originally introduced to describe a synthetic compound intended for bacterial growth control. Some agents today are called "antibiotics" since they are applied as antibacterial therapeutics, although according to the definition they are chemotherapeutics, such as fluoroquinolones. The confusion started when term "chemotherapy" has been widely used for anticancer therapy. Moreover, some natural products are called chemotherapeutics because of their application in anticancer treatment; for example, mitomycin C is obtained from bacterium *Streptomyces caespitosus* (Tomasz 1995). To avoid confusion, the term "chemical agent" will be used as common name for these agents, while terms "antibiotic," "semisynthetic antibiotic," and "chemotherapeutic" will be used as originally introduced by Waxman, Rolinson, and Ehrlich, respectively.

Following broad-range antibiotics appearance after the World War II, in Western Europe started a new era, so-called the golden age of antibiotics. Until the 1980s antibiotics era has experienced an extraordinary expansion in the treatment of diseases for which bacteria are etiological agents (Dublanchet and Bourne 2007), causing the phage therapy in the Western countries to be forgotten. Meanwhile, during 1960s and 1970s when antibiotics were at their peak in the West, phages remained a standard part of the healthcare systems in the USSR despite the wide acceptance of other antibacterial agents. One of the best-known centers for bacteriophage study and production of therapeutic phage preparations is the institute in Tbilisi, which from its founding till 1990s provided the entire Soviet Union with phage preparations against various infections. Since 1990s and the collapse of the USSR, the institute operates under the name of its founder, Eliava (Kutateladze and Adamia 2010). Another famous center with extended phage therapy experience in European Union is Ludwik Hirszfeld Institute of Immunology and Experimental Therapy of the Polish Academy of Sciences (Górski et al. 2009).

Nowadays, with emergence of antibiotic-resistant bacteria, phage therapy once again finds its part as a potential complementary or alternative way to treat or prevent various infective diseases. The main problem is a lack of evidence-based studies using modern standards as well as the lack of an adapted regulatory framework

(Verbeken et al. 2007; De Vos and Pirnay 2015). Besides great interest in the potential for phage therapy, there are some unsolved issues regarding its safety, raising the concerns of its actual utility (Loc-Carrillo and Abedon 2011; Lu and Koeris 2011). However, novel antibacterial agents do not represent the only solution for preventing rapid increase of drug-resistant bacteria. An effective solution could be novel approaches, i.e., strategies that include combinations of different antibacterial agents. Besides possible combinations of two or more conventional antimicrobial agents, conventional antimicrobials with other non-antibiotic drugs (e.g., some antipsychotic and anti-inflammatory drugs improve the antibiotic efficacy in vitro) and with plant bioactive compounds or bacteriophages should be (re) considered. Besides the more efficient bacterial killing with combine agents, the approach should prolong the emergence of antibiotic-resistant strains, although do not offer a permanent solution for problem of spreading bacterial resistance to chemical antimicrobial agents. Furthermore, many currently used antibiotics are toxic, are teratogenic, or cause reactions of hypersensitivity in therapeutic concentrations, and combined therapy with phages can decrease effective antibiotic concentrations. Finally, such therapeutic approach could contribute to easier commercialization of phage-based therapeutics.

In the light of re-born bacteriophage application, possible efficient combinations of different phages and chemical antibacterial agents are considered in this chapter.

3 Bacteriophages vs. Chemical Antibacterial Agents

There are several crucial differences between chemical agents and bacteriophages, when they are consider as antibacterial agents, summarized in Table 1.

As mentioned previously, chemical antibacterial agents are natural, semisynthetic, or synthetic compounds with determined chemical formulas. On the contrary, bacteriophages are microorganisms that consist of proteins and nucleic acids, originating exclusively from nature, where they are very abundant (approx. 10^{30} on the Earth) (Suttle 2005). In this regard, detection or synthesis of new chemical antibacterial agents is a time-consuming process and may take several years to accomplish, while new phage strains or even species can be easily isolated by standard procedures. Similarly, production of chemical antimicrobials is usually expensive and complex, while phage production is relatively cheap and simple. In the light of phage and chemical agent different nature, Kutateladze and Adamia (2010) suggested that phages as therapeutic remedies should not be regulated in accordance with the standards applied to antibiotics or phytopharmaceuticals but to regulate the phage preparations as "biological preparations" rather than "pharmaceuticals." Just to mention, the specific nature of phages, i.e., the fact that they are viruses along with the prejudice that they are "Stalin's cure" probably affected their current official approval and commercialization as antibacterials.

Chemical antimicrobials and phages differ in mode of action and activity range. Most conventional antibacterial agents show bacteriostatic, bactericidal, or bacteriolytic activity, depending on chemical class and applied concentration, while

Table 1 Comparison of chemical antibacterial agents and obligatory lytic bacteriophages

Characteristic	Chemical antibacterial agents	Obligatory lytic bacteriophages
Nature	• Chemically defined molecules	• Biological agents, i.e., bacterial viruses containing proteins and nucleic acid
Origin	• Natural, semisynthetic, and synthetic agents; ecologically unacceptable	• Natural agents (extremely common in the environment); ecologically acceptable
Mode of action	• Bactericidal, bacteriostatic, or bacteriolytic effect	• Bacteriolytic effect
Activity spectra	• All conventional chemical agents target more than one bacterial species and many have very broad activity spectra • Non-selective action affects the patient's microbiota • Determination of bacterial etiological agent is unnecessary for broad-spectrum antibiotics but an antibiogram is preferred)	• Narrow lytic spectra, usually specific for several strains of one bacterial species • The high specificity for pathogen; do not affect microbiota • Determination of bacterial etiological agent is necessary and determination of phage lytic efficacy is preferred
Side effects	• Multiple side effects (allergies, secondary infections, etc.)	• Humans are constantly exposed to phages without known consequences
Resistance emergence	• De novo resistance emerges less frequently • Resistance to antibiotics is not limited to targeted bacteria and spread by horizontal gene transfer	• Resistance is frequent • Phage-resistant bacteria remain susceptible to other phages having a similar host range • Rapid bacteriophage evolution, along with bacterial host cell
Pharmacokinetics	• Do not necessarily concentrate at the site of infection and then metabolized and eliminated from the body	• Multiplication at the site of the infection until there are no more bacteria and then they are eliminated by mononuclear phagocytic system ("intelligent" drug)
Mode of application	• Repeated doses are needed to eradicate bacteria; intravenous, intramuscular, topical, per os application	• Initial dose increases exponentially at the site of infection, so in many cases only one dose is needed; topical or per os application
Production	• Production is expensive and complex	• Production is low-cost and relatively simple
Discovery	• Development of a new chemical antimicrobial agent is a time-consuming process and may take several years to accomplish	• Phage isolation from the environment is relatively easy and fast process
Public attitude	• Widely accepted and applied	• Sporadically applied; there are many prejudices

obligatory lytic bacteriophages, used in phage therapy, exclusively act as bacteriolytic agents. Bacteriophages have usually narrow activity range, mainly being active against some strains of one species (Ross et al. 2016), while chemical agents have broad host range, frequently having effect against both Gram-positive and Gram-negative bacteria. The narrow host range seems to be a shortfall of bacteriophages, but it is also a desirable characteristic when stability of normal microbial communities of human body is considered, particularly gut and vaginal. Resistant cells to phages appear more frequently than to chemical agents, which is one of the main shortfalls of bacteriophage application. However, there are some hypermutable strains that can obtain resistance to chemical agents with rate similar as for bacteriophages (Auerbach et al. 2015). Although phage-resistant bacteria relatively rapidly evolve (Bohannan and Lenski 2000; Lenski and Levin 1985), the bacterial resistance mechanisms to chemical antimicrobials and phages differ substantially (Labrie et al. 2010) and developed resistance to phages is not spread by horizontal gene transfer, in comparison with chemical antimicrobial resistance. Finally, bacteriophages as viruses can also mutate along with phage-resistant bacteria, adopting their lytic spectra to new hosts (Kysela and Turner 2007).

Phages and chemical antibacterial agents have different pharmacology, i.e., pharmacodynamics—drugs impact on a body/microorganisms and pharmacokinetics—body's impact on a drug (Abedon and Thomas-Abedon 2010; Nilsson 2014). The phages are self-amplifying agents usually efficient in one dose and kill target bacterium, while chemical agents should be applied repeatedly, based on time or concentration-dependent antibacterial activity and kill bacteria indiscriminately. Their pharmacokinetics is also different: bacteriophages concentrate at the site of infection and are eliminated naturally from organism by mononuclear phagocytic system, urine, and feces, after disappearing of the targeted bacterial host cells from the site of infection. On the other hand, chemical agents do not concentrate at the infection site and metabolized prior to elimination.

It is clear that both phages and antibiotics possess certain advantages as antibacterial agents; thus one of the approaches to combat multidrug-resistant bacteria is to combine chemical and biological antibacterial agents.

4 Historical Examination of Interaction Among Chemical Antibacterial Agents and Phages

At the beginning of the twentieth century, when Felix d'Herelle has discovered phages, their application as antibacterial agents was a very attractive research field. Although discovery of penicillin dramatically decreased interest in phage therapy, some researchers still conducted these experiments even during 1940s and later.

In the context of antibiotic and phage interaction, the first experiments were conducted to examine antiviral effects of some antibiotics. For instance, it has been proven that tyrothricin from *Aneurinibacillus migulanus* (formerly *B. brevis*) and actinomycin A from soil *Streptomyces antibioticus* inactivate some phages (Neter 1942). Penatin from *Penicillium* sp. is proven to inactivate phages, decrease

plaque number and virion adsorption to cells, but phages still were able to cause lysis of bacterial host cells (Anderson 1943). Similarly, bacteriostatic concentrations of streptomycin delays phage lysis of staphylococci (Edlinger and Faguet 1950) and subinhibitory concentration of terramycin (oxytetracycline) inhibits cell lysis by phages (Edlinger and Faguet 1951). The same was confirmed for aureomycin (chlortetracycline) with *E. coli* and T3 phage (Altenbern 1953), while subinhibitory concentrations of chloromycetin (chloramphenicol) against T1 phage infecting *E. coli* allow lysis but reduce virion yield (Bozeman et al. 1954); the similar effect of chloromycetin was observed for *S. aureus* and its phages (Edlinger 1951); etc. Jones (1945) examined effect of phage combination with clavacin (mycotoxin patulin), streptothricin (antibiotic from *Streptomyces fradiae*), streptomycin, actinomycin, and penicillin against *E. coli* and *S. aureus*. Interestingly, the author found that streptothricin, streptomycin, and clavacin cause inactivation of various phages in bacteria-free filtrates (streptomycin irreversibly), whereas penicillin and actinomycin were without effect. All these findings rather indicate an adverse effect of antibiotics on phage multiplication, than on possible synergy.

The first observation of antibiotic-phage combination decrease of bacterial growth was reported by Neter and Clark (1944). They showed that exposure of bacteriophage to 2000 U of penicillin for 18 h did not affect the lytic activity of the bacteriophage and additive effect was observed when combination of bacteriophage and 0.2 U of penicillin was used against *S. aureus*.

The first set of detailed experiments clearly describing phage-antibiotic synergism was carried out by Himmelweit (1945), who after simultaneous application of phage and penicillin against *S. aureus*, drew the following conclusions: (1) penicillin does not affect the multiplication of *Staphylococcus* phage K, acting on staphylococcus S3K, nor does it interfere with the lethal and lytic action of this phage; (2) *Staphylococcus* phage K and penicillin together produce more rapid killing and lysis of staphylococcus S3K than either alone; and (3) the acceleration in the rate of lysis by bacteriophage is particularly with low concentrations of penicillin. Similar was observed by Nicolle and Faguet (1947), as well as Rountree (1947). Later, Yamagami and Endo (1969) observed that UV irradiation and subinhibitory concentrations of mitomycin C enlarge T4 plaque size and related it with bacterial cell filamentation and shortening of the phage latent period. However, the significance of these findings remained unrecognized until beginning of the twenty-first century.

Interestingly, during 1970s and 1980s there are several reports on interference of phages and antibiotics, used to elucidate phage biology and phage replication cycle characteristic. Using phage-antimicrobial combination it was proved that functional *E. coli* DNA gyrase is required for multiplication of some phages. For instance, replication of T5 was inhibited by novobiocin, coumermycin A, and nalidixic acid that interfere with bacterial DNA gyrase (Constantinou et al. 1986). Similarly, Bacillus phage SPO1 and PBS2 were inhibited by novobiocin and nalidixic acid that affect the enzyme functionality (Price and Fogt 1973; Alonso et al. 1981). Besides the DNA gyrase activity, novobiocin shuts off synthesis of early and late RNAs, diminishing transcription and thus phage production (Sarachu et al. 1980).

Bacterial transcriptional inhibitors, such as rifampin from *Streptomyces mediterranei*, streptolydigin from *S. lydigus*, and actinomycin D from *S. parvulus* also impair gene transcription of some *Bacillus*-specific phages (Dosmar et al. 1977; Osburne and Sonenshein 1980).

5 Reviving Idea of Phage Synergistic Combination with Other Antibacterial Agents

In the twenty-first century, first experiment on chemotherapeutic (enrofloxacin) and bacteriophages against *E. coli* was performed in vivo, using chickens as models (Huff et al. 2004). Inoculation of 10^4 CFU/mL in chicken air sac caused mortality in 68% of animals (Fig. 1). When enrofloxacin was added in water (50 ppm, 7 days), mortality was decreased to 3%, while mortality after one intramuscular injection of phage (unknown family, 10^9 PFU/mL) was 15%. However, when both agents were combined simultaneously, mortality was not recorded. Independently, or inspired by these results, many authors have examined the synergy among conventional antimicrobials and bacteriophages.

6 Phage Interaction with Chemical Antibacterial Agents

Among the first experiments conducted in order to examine phage and chemical agents interaction was conducted by Comeau et al. in 2007. They observed that sublethal concentrations of certain antibiotics can substantially stimulate the host

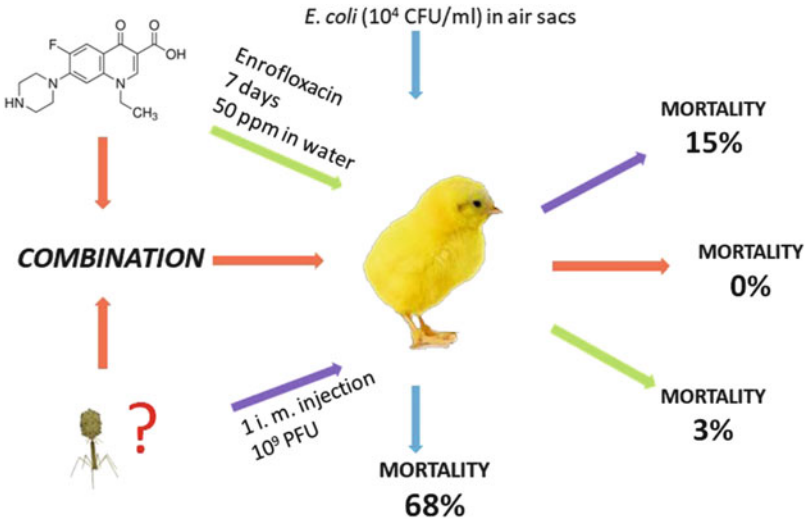

Fig. 1 The first in vivo experiment indicating chemical agent-phage synergy (Huff et al. 2004)

bacterial cell's production of some virulent phage and the combination was later applied to more efficiently decrease cell densities than either treatment alone. The authors defined the phenomenon of the synergy as "phage-antibiotic synergy" or PAS. However, in the experiments, for instance, ciprofloxacin was used, which is a synthetic agent. Thus, it seems that definition of the phenomenon as PAS is confusing: respecting the definitions of antibiotic, semisynthetic antibiotic, and chemotherapeutic (see Sect. 2) and avoiding confusion, here will be used term "chemical agent-phage synergy" or CAPS.

The CAPS is usually examined against planktonic cells but also against cells embedded in extracellular matrix, i.e., against biofilms (Table 2). It is well documented that bacterial biofilm mode of growth enhances resistance to antibacterial agents, making bacterial cells even 100–1000 times more resistant to antibiotics (Mah and O'Toole 2001) and 150–3000 times more resistant to disinfectants (Patel 2005). Biofilm occurrence during infection represents a limiting factor for therapeutic success, because the bacterial cells within biofilm are protected with matrix from physical, chemical, and biological stress (Hassan et al. 2011). Bacteriophages' anti-biofilm activity essentially has been shown to decrease the biomass and not to eradicate biofilm (e.g., Knezevic and Petrovic 2008; Knezevic et al. 2011; Fong et al. 2017, etc.), which raised a concern. Consequently, some authors have investigated the role of bacteriophages in combination with chemical agents to restore antibacterial activity. Thus, particular attention in this chapter will be paid to CAPS against bacterial biofilms.

6.1 Phage Interaction with Cell Wall Synthesis Inhibitors

More than half a century after Himmelweit observation of penicillin-phage K synergy (Himmelweit 1945), other beta-lactams have been examined from this aspect. Semisynthetic antibiotics also act synergistically with phages, for instance, sub-MIC of ampicillin (8 μg/mL) and a siphovirus TH (MOI = 1) reduced a number of planktonic cells of *P. mirabilis* for 5 logs (Yazdi et al. 2018). *P. mirabilis* biofilm removal for approx. 93% after 24 h was obtained with a high dose of ampicillin and larger number of phages (246 μg/mL and MOI = 100, respectively); ampicillin alone reduced biofilm for 44% and phages for 70%. The similar phenomenon has been observed for amoxicillin with a phage against *K. pneumoniae* planktonic cells and biofilms, where biofilm biomass was reduced for 3–4 log with ampicillin-phage combination (256 μg/mL and MOI = 0.01, respectively) as compared to ~2 log reduction when biofilm was exposed to bacteriophage alone (Bedi et al. 2009). Carbenicillin, a carboxypenicillin, exhibited synergy in combination with *P. aeruginosa* podovirus LKD16 (Torres-Barcelo et al. 2016).

Synergy was observed for first-generation cephalosporin cefazolin and phage SATA-8505 combination that reduced *S. aureus* biofilm for 3 logs, but only when treatment with phage preceded the antibiotic (Kumaran et al. 2018). While phage combination with second-generation cephalosporins has not been examined, the third-generation cephalosporins have been examined in the greatest extent. It was

Table 2 Combined effect of phages or their endolysins with various antibacterial agents

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
Staphylococcus aureus (S3K)	K (*Myoviridae*)	Penicillin	Synergy	Himmelweit (1945)
Escherichia coli (serotype 02)	SPR02 and DAF6 (unknown)	Enrofloxacin	Synergy	Huff et al. (2004)
Pseudomonas aeruginosa strains: (PAO1) (PAK)	Filamentous phages (*Inoviridae*): Pf3 Pf1	Tetracycline Gentamicin	Synergy Synergy	Hagens et al. (2006)
E. coli (MFP)	ΦMFP (*Siphoviridae*)	Cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefixime) Aztreonam Gentamicin, tetracycline Quinolones, mitomycin C	Synergy Synergy No synergy Synergy	Comeau et al. (2007)
S. aureus (Sa9)	Φ35 and Φ88 (unknown)	Nisin	Synergy followed by cross-resistance	Martinez et al. (2008)
Klebsiella pneumoniae (B5055)	Specific bacteriophage	Amoxicillin	Synergy	Bedi et al. (2009)
K. pneumoniae (B5055)	KPO1K2 (*Podoviridae*)	Ciprofloxacin	SYNERGY	Verma et al. (2010)
S. aureus (D43-a)	SAP-26 (*Siphoviridae*)	Rifampicin Azithromycin Vancomycin	30% biofilm cells alive 40% biofilm cells alive 60% biofilm cells alive	Rahman et al. (2011)
S. aureus (ATCC27700)	SA5 from Eliava preparation (*Myoviridae*)	Gentamicin	Synergy	Kirby (2012)
E. coli (ATCC11303)	T4 (*Myoviridae*)	Cefotaxime	Synergy in biofilm control	Ryan et al. (2012)
Pseudomonas fluorescens (SBW25 and a hypermutator strain mutS^{-})	SBW25ϕ2 (unknown)	Rifampicin	WT rif-resistant strains reverted to a rif-sensitive phenotype in the presence of phages	Escobar-Páramo et al. (2012)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
P. aeruginosa strains: (PA-4 U) (M2) (ATCC9027)	Pseudomonas phages: Delta (*Podoviridae)* 001A (*Siphoviridae)* Sigma-1 (*Siphoviridae)*	Gentamicin, Ciprofloxacin, polymyxin Ceftriaxone Ceftriaxone Ceftriaxone	No synergy No synergy No synergy Synergy with Sigma-1	Knezevic et al. (2013)
P. aeruginosa (PAO1)	LUZ7 (*Podoviridae)*	Streptomycin	Synergy	Torres-Barcelo et al. (2014)
P. aeruginosa (PAO1) *E. coli* B (ATCC11303)	BP-1 (unknown) T4 (*Myoviridae*)	Tobramycin Tobramycin	No synergy in biofilm reduction Synergy in biofilm reduction	Coulter et al. (2014)
Burkholderia cepacia	KS12 and KS14 (My*oviridae*)	Ciprofloxacin Tetracycline Minocycline Levofloxacin Ceftazidime Meropenem	Synergy; confirmed in vivo on *G. mellonella* model Synergy; confirmed in vivo on *G. mellonella* model No synergy No synergy No synergy Synergy; confirmed in vivo on *G. mellonella* model	Kamal and Dennis (2014)
S. aureus MDR (SA4 and SA7)	Φ (unknown)	Gentamicin Vancomycin Tetracycline	Synergy Synergy Synergy	Ali et al., (2015)
S. aureus (KACC 13236)	SA11 *(Siphoviridae)*	Ciprofloxacin	Synergy	Jo et al. (2016)
P. aeruginosa (PAO1)	Pyobacteriophage (phage cocktail)	Imipenem	Synergy	Papukashvili et al. (2016)
P. aeruginosa (various strains)	3 phages	Amikacin Meropenem	Synergy in 87% stains; for biofilm in 50% Synergy in 73% strains; for biofilm in 14%	Nouraldin et al. (2016)
E. coli (MG1655)	P1 (*Myoviridae*)	Ciprofloxacin	Lysogenization by bacteriophage P1 renders *E. coli* more sensitive to ciprofloxacin	Ronayne et al. (2016)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
P. aeruginosa (PAO1)	LKD16 (*Podoviridae*)	Carbenicillin Gentamicin Trimethoprim	Synergy Synergy Synergy	Torres-Barcelo et al. (2016)
P. aeruginosa (PA14)	NP1 (*Siphoviridae*) and NP3 (*Myoviridae*)	Ceftazidime Ciprofloxacin Colistin Gentamicin Tobramycin	Synergy against biofilm Synergy or facilitation against biofilm No synergy against biofilm No synergy against biofilm Facilitation against biofilm	Chaudhry et al. (2017)
P. aeruginosa (CHA)	phage cocktail PP1131	Ciprofloxacin Meropenem	Synergy in vitro and in vivo (endocarditis model) Synergy in vitro and in vivo (endocarditis model)	Oechslin et al. (2017)
Proteus mirabilis from UTI	TH (*Siphoviridae*)	Ampicillin	Synergy against planktonic cells and biofilm	Yazdi et al. (2018)
S.aureus (ATCC 35556)	SATA-8505 *(Myoviridae)*	Cefazolin Vancomycin Dicloxacillin Tetracycline Linezolid	Synergy Synergy Additive Additive Additive	Kumaran et al. (2018)
Acinetobacter baumannii MDR	KARL-1 *(Myoviridae)*	Meropenem Ciprofloxacin Colistin	Synergy No synergy Additive	Jansen et al. (2018)
P. aeruginosa (FADD1-PA001, JIP865, 20844n/m(s))	PEV20 (*Podoviridae*)	Ciprofloxacin Amikcin Colistin Aztreonam	Synergy, also after nebulization (except strain 20844n/m(s)) Synergy (except strain 20844n/m(s)) Synergy (except strain 20844n/m(s)) No synergy	Lin et al. (2018)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
S.aureus (Newman)	A PYO phage from Georgian PYO cocktail (*Myoviridae*)	Gentamicin Oxacillin Vancomycin Tetracycline ciprofloxacin Daptomycin Erythromycin Linezolid Rifampicin	10xMIC decrease phage density Modest phage growth at 10xMIC Antagonism at 10xMIC Synergy at 2xMIC; antagonism at 10xMIC Synergy at 2xMIC; 10xMIC prevented phage growth Modest phage growth at 10xMIC 10xMIC decrease phage density 10xMIC decrease phage density No synergy	Dickey and Perrot (2019)
P. aeruginosa (ATCC 39018)	RNA phage (unkonown)	Chlorine (sodium hypochlorite)	Synergy	Zhang and Hu (2013)
K. pneumoniae (B5055; O1: K2)	KPO1K2 (*Podoviridae)*	Iron antagonizing molecule $CoSO_4$	Synergy	Chhibber et al. (2013)
K. pneumoniae (B5055) and *P. aeruginosa* (PAO1); mixed-species biofilm	Klebsiella phage KPO1K2 and Pseudomonas phage Pa29 (*Podoviridae)*	Xylitol	Synergy	Chhibber et al. (2015)
E. coli (CECT 434)	CEB_EC3a (S*iphoviridae)*	Two Portuguese honeys	Synergy or additive	Oliveira et al. (2017)
P. aeruginosa (E2005-A)	ΦE2005-A (unknown)	*E. coli* HU2117 (beneficial strain; preformed biofilm on urinary catheters)	Synergy in decrease of *P. aeruginosa* adhesion	Liao et al. (2012)

(continued)

Table 2 (continued)

Bacterial species (strain)	Bacteriophage (family) or endolysin	Agent in combination	Effect	Reference
S. aureus (ATCC 13301 and CCARM 3080)	SA11 (*Siphoviridae*)	Probiotic strain *Lactobacillus rhamnosus* GG	Synergy	Woo and Ahn (2014)
Streptococcus pneumoniae (DCC1490, DCC1476, DCC1420, 8249)	Endolysin Cpl-1	Penicillin Gentamicin Levofloxacin Azithromycin	Synergy (depending on strain) Synergy (depending on strain) No synergy No synergy	Djurkovic et al. (2005)
S. aureus, clinical strains	Chimeolysin P16–17	Gentamicin	Augmentation	Manoharadas et al. (2009)
S. aureus (MRSA)	Chimeolysin ClyS	Oxacillin	Synergy	Daniel et al. (2010)
S. aureus (various MRSA strains)	Endolysin CF-301	Vancomycin Daptomycin	Synergy	Schuch et al. (2014)
S. pneumoniae	Endolysins Pal and Cpl-1	Endolysins Pal and Cpl-1	Synergy	Loeffler and Fischetti (2003)
S. pneumoniae (susceptible and MDR strains)	Endolysin Cpl-1 and Pal	Major pneumococcal autolysin LytA	Synergy	Rodríguez-Cerrato et al. (2007)
S. aureus (MRSA)	Endolysin LysK	Lysostaphin, *Staphylococcus simulans* bacteriocin	Synergy	Becker et al. (2008)
S. aureus (Sa9)	Endolysin LysH5	Nisin	Synergy	García et al. (2010)
A. baumannii (various strains)	Atilysin Arrt-175	Ciprofloxacin Tobramycin	No synergy No synergy	Defraine et al. (2016)
S. aureus (ATCC B1707, LAC, Newman, ATCC 29213)	Endolysin SAL200	Nafcillin, cefazolin Vancomycin	Synergy or indifferent effect Synergy or indifferent effect	Kim et al. (2018a)
S. pneumoniae (MDR)	Endolysin Cpl-711	Amoxicillin Cefotaxime Levofloxacin Vancomycin	Synergy Synergy No synergy No synergy	Letrado et al. (2018)

proven that cefotaxime, ceftazidime, cefixime, or ceftriaxone combined with phage ΦMFP act synergistically against *E. coli*, observed as plaque enlargement and phage titer increase (Comeau et al. 2007). Other studies confirmed these findings: cefotaxim-myovirus T4 combination showed synergy against *E. coli* biofilm (Ryan et al. 2012), ceftriaxone-siphovirus Sigma-1 against *P. aeruginosa* planktonic cells, as well as ceftazidime in combination with myovirus NP3 or siphovirus NP1 against *P. aeruginosa* biofilms (Chaudhry et al. 2017). Only two reports indicated lack of phage-beta lactam synergy: in the first dicloxacillin was combined with phage SATA-8505 against *S. aureus*, showing additive effect (Kumaran et al. 2018), and in the second report lack of synergy was observed in combination of third-generation cephalosporin ceftazidime and myoviruses KS12 and KS14 against *Burkholderia cepacia* (Kamal and Dennis 2014).

Carbapenems also were successfully combined with bacteriophages: imipenem synergistically caused dispersal of *P. aeruginosa* biofilm when combined with a commercial Pyobacteriophage preparation (Tbilisi, Georgia) (Papukashvili et al. 2016), while meropenem showed synergy against this bacterium when combined with a phage cocktail PP1131 in in vitro fibrin clots (Oechslin et al. 2017). The same was confirmed for meropenem and selected specific phages against *P. aeruginosa* both planktonic cells and biofilms (Nouraldin et al. 2016). Meropenem (>128 μg/mL) in combination with a myovirus KARL-1 (MOI = 10^{-1}) showed synergy with a complete clearance of *A. baumannii* liquid culture (Jansen et al. 2018). Interestingly, while combination ceftazidime-myoviruses KS12 and KS14 was not promising against *B. cepacia*, the same phages with meropenem showed synergy even in vivo using *Galleria mellonella* larvae model (Kamal and Dennis 2014).

Interaction of monobactam aztreonam and siphovirus ΦMFP against *E. coli* also was characterized as synergistic (Comeau et al. 2007). Thus, almost all current data show that phage-beta-lactam combinations have a great therapeutic potential.

Vancomycin is a glycopeptide antibiotic produced by *Amycolatopsis orientalis* that inhibits cell wall synthesis of susceptible bacteria (Watanakunakorn 1984). When it was combined with specific phages against *S. aureus* the synergy was recorded (Ali et al. 2015; Kumaran et al. 2018). High doses of vancomycin can antagonize phage multiplication, as shown for 10xMIC and a myovirus against *S. aureus* (Dickey and Perrot 2019).

This indicates that not only beta-lactams but also glycopeptide antibiotics are promising from the aspect of application, indicating generally a good combining potential of antimicrobials that inhibit cell wall synthesis with bacteriophages.

6.2 Phage Interaction with Antimicrobials that Disrupt Bacterial Cell Membranes

Phage combination with agents that affect bacterial cell membrane integrity has not been widely examined. Colistin and polymyxin B, cationic cyclic polypeptide antibiotics produced by *Bacillus* sp., disrupt cell membranes and are active against both Gram-positive and Gram-negative bacteria (Gupta et al. 2009). When they are

combined with bacteriophages, a lack of synergy was observed, and interactions were additive or indifferent. It was proven for combination of polymyxin B and siphovirus Sigma-1 or podoviruses Delta and 001A against *P. aeruginosa* planktonic cells (Knezevic et al. 2013); colistin and a siphovirus NP1 or myovirus NP3 against *P. aeruginosa* biofilm (Chaudhry et al. 2017); and colistin combined with a myovirus KARL-1 against *Acinetobacter baumannii* (Jansen et al. 2018). Similar was confirmed for non-therapeutic surfactants, such as sodium-dodecyl sulfate and bile salts that reduce phages' detrimental effect on bacterial growth (Scanlan et al. 2017). However, Lin et al. (2018) determined synergy between podovirus PEV20 and colistin against *P. aeruginosa* strains FADD1-PA001 and JIP865, but not against 20844n/m(s).

Another polypeptide antibiotic nisin from *Lactococcus lactis* is active against Gram-positive bacteria, generating pores in cell membrane but also interrupting cell wall biosynthesis through specific lipid II interaction (Prince et al. 2016). Leverentz et al. (2003) conducted an interesting research with nisin and phages against *L. monocytogenes* on honeydew melon and apple slices. On honeydew melons, bacterial populations treated with phages LM-103 and LMP-102 (1×10^7 PFU) and nisin at 200 and 400 IU were reduced for 1.5 and 2.5 logs, respectively, and on apple 0.6 and 0.3 logs, respectively, in comparison with bacterial number when only nisin was applied. A similar experiment was performed against *L. monocytogenes* on ready-to-eat pork ham slices—nisin (50 μg/L) and phage P100 (1.5×10^7 UFP/mL) in combination had a small anti-listeria effect at the beginning of the experiment, indicating antagonism between these agents. However, at 72 h, almost 3 log cycles of reduction were observed in the number of viable bacterial cells (Figueiredo and Almeida 2017). Nisin combined with phages Φ35 and Φ88 acts better against *S. aureus* in pasteurized milk (Martinez et al. 2008). Although decrease of bacterial CFU in comparison to more active agent (phages) was less than 2 logs, the interaction was described as synergistic (but it was rather additive). The experiments showed that nisin-adapted cells seriously compromised bacteriophage activity, changing adsorption and plaquing, while phage-resistant mutants were still sensitive to nisin. This finding suggests that cross-resistance between phages and this polypeptide antibiotic can be developed during time.

The available results indicate that phage combination with agents active against cell membrane integrity, except nisin, is not a good option from the aspect of potential application.

6.3 Phage Interaction with Antimicrobials that Inhibit DNA Replication

Antimicrobials that specifically bind to DNA gyrase and inhibit DNA synthesis were frequently included in contemporary studies of interactions with phages. However, ciprofloxacin was almost exclusively examined from this aspect, and in many studies a synergy with phages has been confirmed: with a siphovirus ΦMFP against *E. coli* (Comeau et al. 2007); with a podovirus KPO1K2 against *K. pneumoniae* biofilm

(Verma et al. 2010); with two myoviruses KS12 and KS14 against *B. cepacia* (Kamal and Dennis 2014); with a phage SA11 against *S. aureus* (Jo et al. 2016); NP1 siphovirus and NP3 miovirus against *P. aeruginosa* biofilm (Chaudhry et al. 2017); with myovirus P1 against *E. coli* (Ronayne et al. 2016); and with a phage cocktail PP1131 (Oechslin et al. 2017) or a nebulized phage PEV20 against *P. aeruginosa* (Lin et al. 2018). Although the results seem consistent, there are several reports indicating lack of synergy. For instance, ciprofloxacin was not successfully combined with siphoviruses and podoviruses against *P. aeruginosa* (Knezevic et al. 2013), as neither a myovirus KARL-1 against *A. baumannii* (Jansen et al. 2018). In addition, one more fluoroquinolone—levofloxacin—was unsuccessfully combined with *B. cepacia* myoviruses (Kamal and Dennis 2014).

Interestingly, Lu and Collins (2009) engineered a bacteriophage to overexpress proteins and to attack gene networks that are not directly targeted by antibiotics, showing that suppression of the SOS network in *E. coli* using engineered bacteriophage in vitro enhances killing with quinolones by several orders of magnitude and in vivo significantly increases survival of infected mice. They demonstrated that engineered bacteriophage enhanced the killing of antibiotic-resistant bacteria, persister cells, and biofilm cells; reduced the number of antibiotic-resistant bacteria that arise from an antibiotic-treated population; acted as a strong adjuvant for other bactericidal antibiotics (e.g., aminoglycosides and β-lactams); and furthermore reported that engineering bacteriophage to target non-SOS gene networks and to overexpress multiple factors also can produce effective antibiotic adjuvants.

Mitomycin C, used for therapy of some types of cancers and frequently applied to induce lytic cycle of temperate phages (Raya and H'bert 2009), kills bacteria through a growth-independent mechanism in contrast to most other antibiotics. It is passively transported and bioreductively activated, leading to spontaneous cross-linking of DNA (Kwan et al. 2015). Even this agent with a specific mode of action gives CAPS when combined with a siphovirus against *E. coli* (Comeau et al. 2007).

Considering available data, it is obvious that DNA replication inhibitors along with beta-lactams have a great potential in CAPS-based therapy.

6.4 Phage Interaction with Antimicrobials that Inhibit Transcription

Among antibiotics that inhibit RNA synthesis, only rifampicin (or rifampin) was examined in combination with bacteriophages. This antibiotic obtained from *Amycolatopsis rifamycinica* specifically binds to bacterial RNA polymerase, preventing transcription and consequently protein expression (Wehrli 1983). The augmentation of biofilm removal (with approx. 30% of survived cells) was confirmed for combination rifampicin-siphovirus SAP-26 against *S. aureus* (Rahman et al. 2011). Escobar-Páramo et al. (2012) examined resistance appearance of wild type and hypermutator strain of *P. fluorescens* in presence of phage SBW25ϕ2 and rifampicin. They noticed that the evolutionary response of populations under different treatments varied depending on the order in which the antimicrobials were added

and whether the bacterium was a hypermutator. In the study, wild-type rifampicin-resistant populations involved in biofilm formation often reverted to rifampicin sensitive when stresses were added sequentially. In contrast, when the mortality agents were applied simultaneously, phage populations frequently went extinct and the bacteria evolved antibiotic resistance. These authors concluded that evolutionary response to the combined effects of antibiotic and phages is difficult to predict, depending to some extent on the order in which the antimicrobial agents are added, and on bacterial mutation rates, where these two factors determine the way genetic diversity is created and maintained in bacterial populations during the process of adaptation. Similar study was carried out using *S. aureus* and a myovirus—regardless to mode of application (simultaneous or phage first and rifampicin second), antibiotic efficacy was not increased, but the phage completely prevented the ascent of rifampicin-resistant bacteria (Dickey and Perrot 2019).

The results of transcription inhibitor combination with phages are limited and although are not very promising, the further studies are needed.

6.5 Phage Interaction with Antimicrobials that Inhibit Translation

Phages have been combined so far with antibacterial agents that inhibit protein synthesis, but most studies were conducted using tetracyclines and aminoglycosides, while other groups were examined scarcely or neglected (e.g., amphenicols, linezolid, macolides).

6.5.1 Phage Interaction with Tetracyclines

The tetracycline is an antibiotic first isolated from *Streptomyces aureofaciens* that expresses its antibacterial activity binding to 30S ribosomal subunit. The tetracycline gives synergy with phage Φ against *S. aureus* (Ali et al. 2015) or with myoviruses KS12 and KS14 against *B. cepacia* (Kamal and Dennis 2014). Interestingly, the same *B. cepacia* phages did not act synergistically with a semisynthetic tetracycline, minocycline. The synergy was confirmed against *S. aureus* with a myovirus at 2xMIC, while 10xMIC was antagonistic (Dickey and Perrot 2019). However, a lack of interaction was observed by Comeau et al. (2007) in combination with siphovirus ΦMFP against *E. coli*, while in combination with SATA-8505 against *S. aureus* only additive effect was observed (Kumaran et al. 2018). The tetracycline derivatives glycylcyclines and fluorocyclines have not yet been examined from this aspect.

6.5.2 Phage Interaction with Aminoglycosides

Aminoglycosides are a large group of antibiotics obtained from *Streptomyces* sp. or *Micromonospora* sp. that binds to 30S ribosomal subunit, and the most frequently gentamicin is combined in in vitro studies with phages. This antibiotic was combined with *S. aureus* (Ali et al. 2015) or *P. aeruginosa* (Torres-Barcelo et al. 2016) specific phages and showed synergistic interactions.

Due to increased complexity of the pharmacodynamics in phage-antibiotic combinations, Kirby (2012) used the continuous culture system for *Staphylococcus aureus* to simulate the pharmacokinetics of periodic antibiotic dosing alone and in combination with lytic phage, while the further evaluation of the conditions governing the observed pharmacodynamics was analyzed using computer model representation of the system. This study proves that treatment with gentamicin induced a population of cells with a strong aggregation phenotype, and consequently an increased ability to form biofilm, but also induced increase of susceptibility to the phage action. This dual treatment with gentamicin and phage resulted in lower final cell densities than either treatment alone, and unlike in the phage-only treatment, phage-resistant isolates were not detected in the dual treatment. According to the results of this experimental approach, dual therapy can be more efficient than single therapy, particularly if there is an overlap in the physiological pathways targeted by the individual agents.

However, some studies showed lack of gentamicin-phage synergy: with phage ΦMFP against *E. coli* (Comeau et al. 2007) or sipho- and myoviruses of *P. aeruginosa* (Chaudhry et al. 2017).

Other aminoglycosides have been examined sporadically: streptomycin showed synergy with podovirus LUZ24 against *P. aeruginosa* (Torres-Barcelo et al. 2014), while amikacin gave synergy with podovirus PEV20 against some *P. aeruginosa* strains (Lin et al. 2018). Nouraldin et al. (2016) reported that an amikacin-phage combination eradicated biofilm in 50% of the *P. aeruginosa* isolates, while the meropenem-phage combination eradicated biofilm in only 14% of the strains. In the contrary to these results, Sillankorva et al. (2012) showed that the amikacin-phage combination for control *P. aeruginosa* biofilms was characterized as antagonistic. Similarly, tobramycin and T4 myovirus were successfully combined against *E. coli,* while significant enhancement activity of tobramycin-PB-1 phage combination against *P. aeruginosa* biofilm was not observed (Coulter et al. 2014). However, authors recorded significant reduction of the emergence of antibiotic- and phage-resistant cells in both *E. coli* and *P. aeruginosa* biofilms. For some tobramycin-phage combination the effect was only facilitative, such as in combination with phages NP1 (*Siphoviridae*) and NP3 (*Myoviridae*) against *P. aeruginosa*.

6.5.3 Phage Interaction with Other Translation Inhibitors

The linezolid is a synthetic agent from oxazolidinone group of antimicrobials that binds to the 50S subunit of the prokaryotic ribosomes, preventing formation of the initiation complex for protein synthesis (Livermore 2003). When combined with SATA-8505 phage against *S. aureus*, it showed additive effect (Kumaran et al. 2018). A macrolide azithromycin efficiently was combined with siphovius SAP-26 against *S. aureus* biofilm and after the treatment, only 40% of cells were alive (Rahman et al. 2011).

The results on therapeutic application of phages with translation inhibitors seem inconsistent and probably depend on host-antibiotic-phage system and mode of agents' application. Thus, further research is needed to elucidate CAPS with protein synthesis inhibitors.

6.6 Phage Interaction with Other Antibacterial Agents

Bacteriophages have been successfully combined so far with some inorganic agents. For instance, chlorine (210 mg/L) and RNA phages (3×10^7 PFU/mL) reduced biofilm growth for 94% and removed 88% of already formed *P. aeruginosa* biofilm (Zhang and Hu 2013). Similarly, cobalt (II) sulfate ($CoSO_4$) that antagonizes iron, in combination with a depolymerase-producing podovirus KPO1K2 synergistically inhibit formation of *K. pneumoniae* biofilms (Chhibber et al. 2013). Xylitol, a 5-carbon polyol sugar alcohol commonly used as a non-carcinogenic sweetener with anti-biofilm properties (Nayak et al. 2014), can be successfully combined with podoviruses KPO1K2 and a non-depolymerase-producing Pa-29 against multi-species biofilm developed by *P. aeruginosa* and *K. pneumoniae* (Chhibber et al. 2015). Interestingly, *E. coli* biofilm was successfully controlled by combining two Portuguese honeys with depolymerase-producing podovirus EC3a with recorded synergistic and additive effects (Oliveira et al. 2017).

Particularly interesting are studies that examined phage combination with other biological agents. For instance, the combined effect of probiotic *Lactobacillus rhamnosus* and siphovirus SA11 against *S. aureus* under the simulated intestinal conditions seems promising (Woo and Ahn 2014). The bacteriophage SA11 combined with the probiotic effectively reduced *S. aureus* number for 4 logs with relative decrease of virulence factor expression (adhesion- and efflux-related genes), supporting the application of bacteriophage to control the ingested antibiotic-resistant foodborne pathogens. Potential application of biological agents in biofilm control can be reflected not only in the treatment of the already established biofilms, but also in prevention of its formation on biotic and abiotic surfaces, especially in hospitals. Liao et al. (2012) showed that the combination of phages with a pre-established biofilm of *E. coli* HU2117 (a benign and potentially protective strain without P-fimbriae) was synergistic in preventing urinary catheter colonization by *P. aeruginosa.* Such pre-treatment of catheters decreased *Pseudomonas aeruginosa* adherence for approx. 4 logs, and neither *E. coli* nor phage alone generated significant decreases.

7 Combination of Bacteriophage Enzymes and Chemical Antimicrobials

A novel class of antibacterial agents derived from bacteriophages are (endo)lysins. These lytic enzymes are produced by progeny bacteriophages at the end of their replication cycle to degrade bacterial cell wall and liberate new virions. A typical endolysin contains C-terminal catalytic domain (CD) responsible for cell wall enzymatic degradation and N-terminal cell wall binding domain (CBD) responsible for substrate recognition (Yang et al. 2014). According to Fischetti (2008), lysins are defined as hydrolytic enzymes affecting bacterial cell wall (i.e., peptidoglycan) that selectively and rapidly kill ($\geq$3 log CFU in 30 min) specific Gram-positive bacteria, providing a targeted therapeutic approach with minimal impact on unrelated

commensal microbiota. The antibacterial activity of exogenously applied lysin is typically limited to Gram-positive bacteria, i.e., organisms without an outer membrane or surface lipids and waxes. Although endolysins have broader activity spectra than phages, their properties can be further improved by combining CD and CBD from various phages. These engineered chimeric lysins, named chimeolysins, usually possess extended bacteriolytic activity against various Gram-positive bacteria (Dong et al. 2015). Finally, the activity of endolysins has been extended to Gram-negative bacteria, by fusing endolysins with signal peptides that allow penetration through outer membrane of Gram-negative bacteria (i.e., artilysins) (Lai et al. 2011; Lukacik et al. 2012; Briers et al. 2011).

Although phage lysins and their products are very active against bacteria, some studies showed that they can be more active in combination with antibiotics. Application of antibiotics that inhibit cell wall synthesis theoretically could influence the effectiveness of lysins, as they can allow the unhindered passage of lysins to peptidoglycan, especially having in mind that the lysins are proteins ($\geq$25 kDa in size) with larger size than antibiotics (0.3–1.6 kDa in size) (Fischetti et al. 2006). The first such synergy was observed by Djurkovic et al. (2005)—they reported a synergistic effect of bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin against *Streptococcus pneumoniae*, while such effect was recorded neither with levofloxacin nor azithromycin, suggesting that observed synergistic effect is antibiotic-specific. These authors presumed that the increased access of the enzymes to their respective cleavage sites or the enhanced destructive effect of a two-dimensional digestion in the three-dimensional peptidoglycan was responsible for the observed synergy. Recently, in another study, combinations of CF-301 lysin with vancomycin or daptomycin synergized in vitro and significantly increased survival of mice in staphylococcal-induced bacteremia compared to treatment with antibiotics alone (Schuch et al. 2014). These authors confirmed the superiority of CF-301 combinations with antibiotics in 26 independent bacteremia studies, proving that combinations of CF-301 and antibiotics represent an attractive alternative to antibiotic monotherapies currently used to treat *S. aureus* bacteremia. Similar was confirmed against *S. aureus* when lysine LysH5 was combined with nisin (García et al. 2010), Sal200 with nafcillin and vancomycin (Kim et al. 2018a), as well as when LysK was combined with a bacteriocin lysostaphin from *Staphylococcus simulans* (Becker et al. 2008). Lysins also showed synergistic action when combined mutually (e.g., Pal and Cpl-1 against *Streptococcus pneumoniae*) (Loeffler and Fischetti 2003) or with *S. pneumoniae* autolysin LytA (Rodríguez-Cerrato et al. 2007). Interestingly, Letrado et al. (2018) examined combinations of endolysin Cpl-711 with various antibacterial chemical agents, and confirmed synergy with beta-lactams amoxicillin and cefotaxime, but not with fluoroquinolone levofloxacin and glycopeptide vancomycin (additive effect), indicating that synergy between endolysins and chemical agents, even when they inhibit cell wall synthesis, is not an universal phenomenon. Chimeolysins also show synergistic interaction with antibiotics—e.g., combination of beta-lactam oxacillin with ClyS, obtained by fusion of Staphylococcus phage Twort lysin CD with CBD from phiNM3 phage lysin, synergistically kill MRSA (Daniel et al. 2010). The activity of a chimeolysin P16–17, composed of N-terminal domain of the Staphylococcus phage P16 endolysin and

C-terminal domain of P17 minor coat protein, is augmented by gentamicin (Manoharadas et al. 2009). Besides combinations of lysins with conventional antimicrobials, other outer membrane-permeabilizing agents also were tested. In their study, Briers et al. (2011) showed that ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) was the most suitable component to combine with endolysin EL188, as *P. aeruginosa* growth has been reduced up to 4 logs for 30 min.

The combined therapy of artilisyn Art-175 and ciprofloxacin or tobramycin against *A. baumannii* resulted in an improved antibacterial effect for both mixtures over that obtained with conventional antibiotic monotherapy, but no significant improvement compared to results obtained with Art-175 alone (Defraine et al. 2016). Since Art-175 kills the bacterial cells very efficiently, the authors explained lack of synergy by the fact that neither ciprofloxacin nor tobramycin can exert its action.

These in vitro studies demonstrate that bacteriophage-encoded endolysin combination with certain antibacterial agents is promising and that co-administration approach can overcome some disadvantages of phage therapy, such as phage narrow activity spectra.

Another interesting group of phage-encoded enzymes, which are part of virions, are exopolysaccharide depolymerases. Using the combinations of phages and antimicrobials (amoxicillin and ciprofloxacin) the eradication of *K. pneumoniae* biofilms was successful, and it was explained by presence of phage-encoded depolymerase with polysaccharide-degrading activity, which allowed the passage for the antibiotic to the biofilm cells (Bedi et al. 2009; Verma et al. 2010). Thus, bacteriophage virions can possess enzymes that dissolve the biofilm matrix or induce corresponding enzyme production by the host bacterium. This was reported for clinical mucoid *P. aeruginosa* strains from CF sputum, where bacteriophage-derived enzyme was able to depolymerize the alginic acid of *P. aeruginosa* (Hanlon 2007; Glonti et al. 2010). The enzyme alginase produced by bacteriophages could have the potential to improve the condition of CF patients by facilitating the expectoration of sputum, accelerating phagocytic uptake of bacteria, and perturbing bacterial growth in biofilms. The ability of bacteriophages to weaken and/or even destroy the biofilm matrix can also enable or improve the penetration of other antibacterial agents, which consequently can augment interactions of bacteriophages and conventional antimicrobials. Having in mind that in the most cases bacterium *P. aeruginosa* is important for producing the biofilm matrix within multispecies biofilm, the CAPS effect could also be extended to the other present bacterial species. Although those species might not be targeted by the specific bacteriophage/enzyme, they could become more susceptible to antibiotics, due to matrix destruction. However, to our knowledge, combinations of EPS depolymerases and antibiotics have not yet been examined.

8 Methods for Testing Effectiveness of Phage-Chemical Antimicrobial Combinations In Vitro

The methods used for phage and chemical agent interaction are not defined and thus methodology used in present studies varies significantly. The standardization of methods used for establishing the in vitro synergistic interactions among chemical

agents and phages could save time and resources, contribute to more precise interpretation of data and reproducibility, and finally may influence the greater interest of pharmaceutical companies to invest in order to obtain adequate preparations for the treatment infections caused by drug-resistant bacteria.

To determine synergistic interactions, the broth microdilution limited series method, agar dilution method, or disk diffusion method can be applied (Eliopoulus and Moellering 1996; Doern 2014). The synergy testing by disk agar diffusion methods provides qualitative screening of interactions between two antibacterial agents, but the results are best for studying antagonism. The zone diameters for individual antimicrobial agents of interest are determined using standard techniques and for the estimation of agent combinations use another Mueller-Hinton agar plate and place disks separated by distance equal to sum of the zone radii for each disk when tested alone. After incubation the interface of the zones of inhibition are observed. According to the Eliopoulus and Moellering (1996) synergism shows an enhanced zone, indifference show no change, and antagonism shows an abbreviated zone. However, when phage interaction with chemical antimicrobials is examined by disk diffusion method, phages are usually inoculated with host bacterium and a disc with chemical antimicrobial is placed on the surface of agarized medium. The increase of phage plaque diameter around disc with antimicrobial indicates synergistic activity (Comeau et al. 2007). The disc agar diffusion method provides only qualitative information; therefore, it could be used prior time-kill assay for qualitative screening of interactions. The synergy can be applied to increase plaque size and number for easier phage isolation or plaque enumeration (Los et al. 2008; Santos et al. 2009). The subinhibitory concentration of ampicillin (2.5–3.5 μg/ml) in bottom agar can enhance visibility of extremely small plaques on *E. coli* O157:H^- lawns. The subinhibitory concentrations of penicillin G, ampicillin, cefotaxime, or tetracycline with 5% glycerol in top agar enlarge plaques of a siphovirus PVP-SE1 specific for *Salmonella enterica* serovar Enteritidis (Santos et al. 2009). The best results were obtained with 5% glycerol and 0.5 μg/mL of ampicillin, 0.06 μg/mL of cefotaxime, or 1.5 μg/mL of tetracycline. Some antibiotics, e.g., kanamycin and rifampicin, did not cause this phenomenon.

In general, the most commonly used methods for synergy testing among various chemical agents are time-kill method and checkerboard method, and today there are different experimental designs based on these two methods. These methods for estimation of interactions among antimicrobial agents are in consideration when the predictability of synergism is unknown, as with a new antimicrobial agent, or when the predictability is unreliable because of the development of bacterial resistance or treatment failures.

One of the commonly used methods for determining interactions among antibacterial agents is broth microdilution checkerboard method. This is a two-dimensional, two-agent broth microdilution assay for evaluation of antimicrobial agents' combination against drug-resistant organisms (Verma 2007; Wagner and Ulrich-Merzenich 2009; Doern 2014; Aleksic et al. 2014; Knezevic et al. 2016). This method is based on broth microdilution susceptibility method for evaluation of the bacteriostatic and/or bactericidal activity of antibacterial agents' specific

concentrations in combination at a fixed time. Prerequisite for this assay is estimation of MIC values for each agent, in order to establish the range tested concentrations for antibacterial agents (e.g., usually from 1/32 × MIC to 4 × MIC). The concentrations of one agent in microtiter plate are decreasing horizontally and for another vertically, enabling to test wide range of concentration for two agents in combination. Detected in vitro interactions are calculated and interpreted as synergistic, additive, indifferent, or antagonistic depending on whether the antibacterial activity of agents in the combinations is greater than, equivalent to, or less than the activities of the agents when applied alone. The fractional inhibitory concentration (FIC) of each agent is calculated for each combination of tested agents as follows:

FIC of agent A = (MIC of agent A in combination) / (MIC of agent A alone).

FIC of agent B = (MIC of agent B in combination) / (MIC of agent B alone).

The type of interaction among tested antibacterial agents is determined by summation of FIC values to obtain fractional inhibitory concentration index (FICI):

FICI = FIC of agent A + FIC of agent B.

The summation of two agents' antibacterial activity can be interpreted as synergistic interaction if FICI $\leq$ 0.5, additive interaction if 0.5 $<$ FICI $\leq$ 1, indifferent interaction if 1 $<$ FICI $\leq$ 4, and antagonistic interaction if FICI $>$ 4. These definitions of interaction types differ among investigators, and these here represented are the most commonly used (EUCAST 2000; Schelz et al. 2006; Mulyaningsih et al. 2010; Bassole et al. 2011). Interactions among antibacterial agents also can be represented geometrically with isobolograms. When phage lytic enzymes are tested, this method is very convenient, as enzyme concentration can be expressed in the same units as antibiotics. In the light of bacteriophage combination, checkerboard testing is impossible to perform, as concentration of the phages cannot be expressed in the same units for calculating the FIC index. However, phage MOI can be expressed as protein concentration, which can be useful for PACS examination by checkerboard method.

The time-kill method is used for examining the rate at which concentrations of an antimicrobial agent kill bacteria (Verma 2007; Wagner and Ulrich-Merzenich 2009; Aleksic et al. 2014; Doern 2014; Knezevic et al. 2016). This method can be used to study both the time-dependent and concentration-dependent antibacterial activity of tested antimicrobial agents. It can be used for evaluation of new antibacterial agents and its combinations with other agents, and possibly offer an answer for treatment failure in clinical trials, where bacterial count during time may be crucial for therapy outcome. This method enables varying the time point in which the specific agent will be added (e.g., agents could be added in two or more different time points—simultaneously with phage or after appropriate delay), also the agents' dosage can be varied, and more than two agents could be tested simultaneously. Before performing the time-kill experiment, some parameters must be defined: (1) the MICs for the agents which will be used, (2) other concentrations of antimicrobial agent to test (e.g., usually two and four times MIC), and (3) sample time points, based on agents' mechanism of action and used organism growth rate (usually 0, 4, 8, 24, and 48 h after inoculation, and for agents with fast bactericidal activity, e.g., 1, 2, 4, 6, and 24 h). When bacteriophages are applied as one of the antibacterial agents, multiplicity

of infection (MOI) used in the experiment should be determined according to previously established bacteriolytic efficiency. The general experiment conditions (temperature, incubation period etc.) depend on the used test organism and experimental design. After the establishing the main parameters, the assay is conducted by adding standardized inoculum (5.5×10^5–1×10^6 CFU/mL) into broth media containing different concentrations of an antimicrobial agents, different combination of agents, and also into the broth without agent as a control of bacterial growth. At the previously determined sample time points the number of viable CFU/mL from each test tube are determined by performing serial dilutions and by plating onto nutritive media (preferably Mueller-Hinton agar). The plates are incubated and the results for each treatment and control are plotted versus time, to obtain time-kill curves. The results obtained for two chemical antimicrobials can be applied also for phage-chemical antimicrobial combination: if bacterial count (CFU/ml) decreased by $\geq$2 log for the phage-antibiotic combination compared to the more active single agent, as well as to the initial inoculum titer, the interaction is considered as synergy (Knezevic et al. 2013). Chaudhry et al. (2017) used another approach to estimate CAPS. For calculation, they used the cell density obtained in the control (C; no treatment), and the surviving cell density after treatment with agent A, agent B, and the combination of A and B (S_A, S_B, and S_{AB}). The fraction of cells surviving A is S_A/C, of cells surviving B is S_B/C, etc. For facilitation, the following inequality should be fulfilled: $\log(S_A) - \log(C) > \log(S_{AB})$—logC, i.e., $\log(S_{AB}) - \log(S_A) < 0$ and $\log(S_{AB}) - \log(S_B) < 0$. Thus, no facilitation (hence no synergy either) is when the effect of combined treatment is no better than the effect of the best single treatment. Synergy requires $S_A/C \times S_B/C > S_{AB}/C$, i.e., log(C) – log(SA) – log (SB) + log(SAB) < 0. The authors conservatively applied a 0.05 criterion to both tests for rejection.

The time-kill method is known as labor-intensive, due to determining the CFU number in multiple sampling times for various treatments. At each step in the protocol, plaque assays could be performed using the soft agar overlay method to enumerate phage titers (Kutter and Sulakvelidze 2005). According to the Pillai et al. (2005) the time-kill curves can be considered as a clinically relevant model if the applied concentrations of used agents represent those which can be achieved at the site of an infection. To make results comparable and the most accurate, a time-kill curve method should be standardized and used as a gold standard for further studies of CAPS.

Due to facts that the main challenge for the expansion of phage application is a necessity to perform large-scale clinical trials in accordance with US FDA or European guidelines, which are usually very expensive and take several years, above-described in vitro time-kill method represent a valuable first step in evaluation of CAPS for in vivo studies and further clinical trials.

9 Possible Mechanisms of Chemical Agent-Phage Synergy

The mechanisms that generally lead to pharmacological synergy of conventional antimicrobials and alternative agents imply (1) multi-target effect in which agents target different sites in/on the bacterial cell, (2) pharmacokinetics or

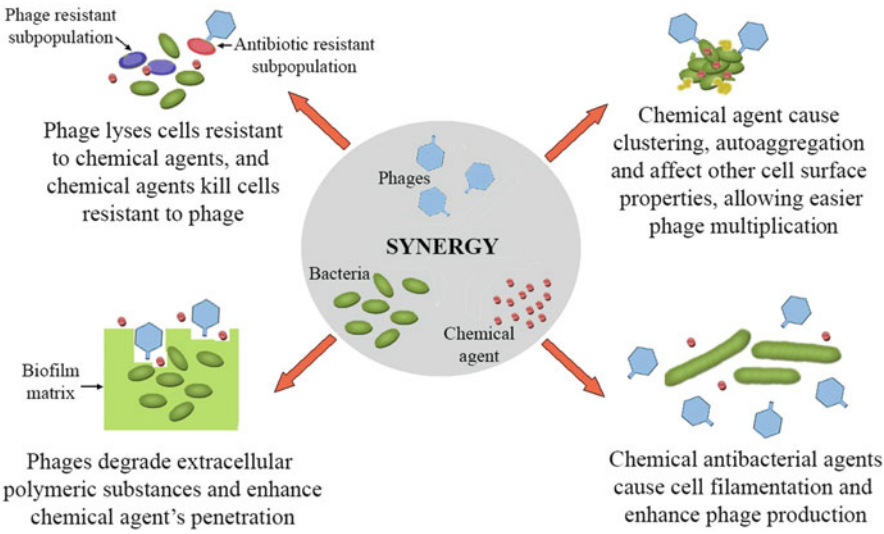

Fig. 2 Possible mechanisms of chemical agent-phage synergy (CAPS)

physicochemical effects (e.g., improvement of solubility or bioavailability), or (3) targeting a specific resistance mechanism of bacteria (Hemaiswarya et al. 2008; Wagner and Ulrich-Merzenich 2009). However, mechanisms of PACS seems to be different and some of them are following: (1) cell elongation/filamentation by antibiotic and subsequent phage multiplication; (2) prevention of phage- and/or antibiotic-resistant mutant occurrence; (3) phage change of cell permeability that allows antibiotic penetration; and (4) antibiotic effect on cell autoagreggation and other cell surface properties (Fig. 2).

Comeau et al. (2007) postulated that cell filamentation is responsible for the phenomenon, as beta-lactams and quinolones caused both filamentation and CAPS in *E. coli* and *Y. enterocolitica,* while gentamicin and tetracycline caused neither filamentation nor CAPS. Since antibiotics trigger SOS response, involvement of this reparative mechanism was examined in the CAPS onset, but it was shown that CAPS is independent from SOS response. The cell filamentation makes larger or altered pools of phage precursor molecules and removes inhibitors of phage assembly, facilitating cell lysis. The antibiotics also may accelerate cell lysis by, for instance, interrupting peptidoglycan synthesis and allowing holins/edolysins to further act in cell destruction. Furthermore, this was supported by an increase of plaque size when phages and antibiotics were combined. Not only the plaque size but also T4 burst size increased from 8 to 80 and 163 PFU mL^{-1} in combination with 1.86×10^{-4} and 7.43×10^{-3} μg mL^{-1} of cefotaxime, respectively (Ryan et al. 2012). Linezolid, tetracycline, and macrolides (telithromycin and clarithromycin) enhance a phage MR5 plaque diameter on MRSA lawns from 1.2 to 3.5 times, with reductions in the time of phage adsorption and the latent period (Kaur et al. 2012). The role of cell filamentation was further proved by Knezevic et al. (2013), indicating that a cell

elongation/filamentation appears to be a necessary but insufficient reason for phage-antibiotic synergy. Namely, both ceftriaxone and ciprofloxacin caused cell enlargement, but only ceftriaxone with a siphovirus gave a synergy, and not with a podovirus. It was also pointed out that some antibiotics can impede phage multiplication, by inhibition of DNA gyrase activity or protein synthesis, disruption of cell membrane integrity, etc., and that synergy may depend on specific phage-host and phage-antibiotic combinations. Importance of phage-host system was further confirmed, as combination of a myovius and ciprofloxacin resulted in a synergy during biofilm formation control (Sagar et al. 2016). Kim et al. (2018b) further contribute to the phenomenon elucidation, as observed that the phage adsorption efficiency was not changed after filamentation; although phage DNA and mRNA production was increased, a limited increase in protein production was noticed. The authors concluded that synergy is a result of a prolonged period of viral particle assembly due to delayed lysis, caused by the increase in the cell surface area and thus shortage of intracellular holins for aggregating and forming holes in the host membrane. Reactive oxygen species (ROS) stress by hydrogen peroxide also led to an increased production of phages, but heat stress does not. According to the current data, the antibiotics that cause cell elongation/filamentation play an important role in CAPS, but it is not the only explanation of synergy appearance, since antibiotics, which do not cause cell filamentation, also give synergy with phages.

The CAPS can be explained by the reduction of number of bacterial mutants resistant to phages and/or antibiotics. Simplifying this model, it can be stated that cells resistant to antibiotic are sensitive to phage and vice versa. Zhang and Buckling (2012), using a kanamycin-phage combination against *P. fluorescens,* observed a dramatical decline in bacterial population survival compared with single agent application, and ascribed this to prevention of bacterial resistance evolution. It is interesting that cells resistant to both agents suffered very large fitness costs. The mutant occurrence prevention during combined treatment can be an explanation for, e.g., a phage and tobramycin synergy (Coulter et al. 2014), since tobramycin does not cause cell filamentation. Using *E. coli* as a model, it was proven that the combined treatment decreased tobramycin-resistant cells for >99.99% and phage-resistant cells for 39%. On contrary, PB1 phage decreased tobramycin-resistant *P. aeruginosa* cells for 60%, while phage resistance was decreased 99%. Oechslin et al. (2017) confirmed that 2.5xMIC of meropenem and ciprofloxacin completely inhibit appearance of phage-resistant mutants. Significant impact of ciprofloxacin-phage (Jo et al. 2016) and linezolid-phage (Kaur et al. 2016) combination against resistance occurrence of *S. aureus* also have been documented. Similarly, when streptomycin and phage are applied, the best synergy was obtained when streptomycin was added 12 h after the bacteriophage treatment. It is interesting to mention that when *P. aeruginosa* was used as a model in CAPS, appearance of phage-resistant bacteria was not only short-term prevented but also long-term, regardless to antibiotics applied. In addition, CAPS had no impact on *P. aeruginosa* virulence, which is promising from the aspect of clinical application (Torres-Barcelo et al. 2016). In accordance with these findings, combined application of phages and rifampicin reduced appearance of antibiotic-resistant mutants, although synergy

was not recorded (Dickey and Perrot 2019). However, the combined treatment does not always lead to mutant occurrence prevention. In a microcosm system with *P. aeruginosa* PAO1 treated in a spatially structured environment with myovirus 14/1 and gentamicin combination, it was observed that phage–antibiotic rapidly lost the efficacy against both planktonic and biofilm populations due to rapid resistance evolution (Moulton-Brown and Friman 2018). Namely, phage selection correlated positively with increase in antibiotic resistance and biofilm growth was favored most in the combination treatment, with a relatively small cost of resistance. The authors concluded that spatial heterogeneity can promote rapid evolution of generalized resistance mechanisms without corresponding increase in phage infectivity, leading to failure of phage–antibiotic treatments in the evolutionary timescale. Still, these results support resistance prevention mechanism, considering that both resistant mutants and lack of synergy was observed in the experimental system.

It is evident that not only the type of antibiotic and phage, but also their order of application influence the final success of combined treatment, supporting the theory of decreased number of mutants. It was shown that when phages are added first, cephazolin and vancomycin more efficiently affect biofilm of *S. aureus* (Kumaran et al. 2018). For eight examined chemical antimicrobials, treatments with phage first, then antibiotic at either 2x or 10xMIC effectively reduced bacterial density (Dickey and Perrot 2019). The chemical antimicrobials at 2xMIC were ineffective against biofilm but effectively reduced the bacterial density of cultures treated previously with phage for 24 h. For some antimicrobials (ciprofloxacin, daptomycin, and linezolid), simultaneous treatment was as effective as sequential treatment, while for gentamicin, oxacillin, vancomycin, tetracycline, and erythromycin, sequential treatment with lower concentrations of antimicrobials (2xMIC instead of 10xMIC) was more effective. Interestingly, there was generally very little change in phage density when antibiotics were added at 2xMIC or 10xMIC, with an exception of gentamicin at 10xMIC that decreased phage number. The authors concluded that when it had a significant effect, phage pre-treatment improved the efficacy of low concentrations of antibiotics, but it decreased the efficacy of high concentrations of antibiotics. The influence of antimicrobials' order of application has also been observed for combination of ciprofloxacin and myovirus ELY-1 against *E. coli* (Lopes et al. 2018). The efficacy of the combined treatment varied with the antibiotic concentration and the time of antibiotic addition, being the best when MIC of ciprofloxacin was added 6 h after phage addition. Such treatment reduced the bacterial density and prevented the emergence of resistant variants for 1–2 logs. It was previously mentioned that CAPS varied depending on the order in which the rifampicin and phages are added and that sequential addition of agents in a biofilm system caused reversion of rifampicin-resistant mutants to sensitive (Escobar-Páramo et al. 2012).

Application of *S. aureus* as a model suggests that combined therapy can be more efficacious if there is an overlap in the physiological pathways targeted by the individual agents (Kirby 2012). For instance, bacteria treated with gentamicin show a strong aggregation phenotype and increased ability to form biofilm, but the aggregators are also more susceptible to phages than the parental cells. Thus, dual

treatment with gentamicin and the phage resulted in lower final cell densities than either treatment alone, and phage-resistant isolates could not be detected in the combined treatment.

According to some data, phages can improve antibiotic penetration into bacterial cells. For instance, besides the cell filamentation and autoaggregation, phage polysaccharide depolymerases can also contribute to the CAPS phenomenon (Sagar et al. 2016). As indicated, some phages possess the depolymerizing enzymes as a part of virions and can break down capsules and slime layers (e.g., *P. aeruginosa* alginate) around bacterial cells or in biofilm matrix (Hughes et al. 1998; Harper et al. 2014). This enzymatic degradation further potentiates antibiotic diffusion and cell penetration (Yan et al. 2013). Besides *Caudovirales*, *Inoviridae* (Pf1 and Pf3) also show CAPS in combination with gentamicin, tetracycline, chloramphenicol, or carbenicillin against PAK *P. aeruginosa* strain, whose cell wall is a barrier for antibiotic penetration (Hagens et al. 2006). The synergy is considered unique, since *Inoviridae* releases from a cell by extrusion, without lysis. It is believed that pores for extrusion allow antibiotic entry into a cell and consequently increase bacterial sensitivity to an antibiotic, leading to the synergism.

In a report of CAPS synergy in biofilm eradication, it was noted that the effectiveness of combined treatment of phages and amikacin on *P. aeruginosa* biofilm can be explained by altered surface charges of phage-resistant phenotypes and disruption of the biofilm matrix induced by some of the phages which can enhance the antibiotic penetration (Nouraldin et al. 2016).

Finally, other possible mechanisms should not be neglected. It has been proven that many prophages exist in sequenced bacterial genomes, as well as (pro)phage genetic elements in various strains (e.g., Knezevic et al. 2015). In this context, Fothergill et al. (2011) reported that the choice of antibiotic could dramatically affect the levels of free *Pseudomonas* phages, where ciprofloxacin and norfloxacin caused a level of phage induction higher than that observed with other CF-relevant antibiotics (tobramycin, colistin, ceftazidime, meropenem) against *P. aeruginosa* Liverpool epidemic strains (LES). Another well-known example is application of mitomycin C for prophage induction (Raya and H'bert 2009). Accordingly, antibiotic application can cause prophage induction from bacterial strains that contain complete prophages, by affecting lysis-lysogeny decision and thus can convert a lysogenic infection into a productive infection, which further contribute to enhanced bacteria lysis.

Probably all mentioned mechanisms play a role in synergy, but potential involvement of other still unrecognized factors or processes in the phenomenon onset should be further defined.

10 Shortfalls of Present Studies

There are several shortfalls of current studies: (1) negligence of bacteriophage biology, (2) existing prophages in bacterial hosts used as models, (3) variation in methodology, and (4) avoidance of CAPS mechanism elucidation.

In many studies, it is not clear which phage is used, as their phenotypic and genotypic characteristics were not examined. Thus, it is not clear whether the used phages are temperate or obligatory lytic. Temperate phages are not appropriate from the aspect of therapy, since they can carry various virulence factors, being able for both specialized and generalized transduction, as well as lysogenic conversion of bacteria, contributing to their virulence (Howard-Varona et al. 2017). For instance, Rahman et al. (2011) used a temperate phage induced from *S. aureus* to control biofilm, combining it with antibiotics. Even some obligatory lytic phages, whose procapsids are filled with DNA by head-full mechanism (e.g., T4), should be avoided in phage therapy, since they are more frequently involved in generalized transduction (Schneider 2017). Fortunately, current progress in the genome sequencing will diminish shortfalls of future studies.

As indicated, many bacteria carry prophages in their genomes, and their destiny during antibiotic action or infection with another bacteriophage is unclear. For example, more than 60% of *P. aeruginosa* strains contain at least one genetic element of Pf1-related phages (fam. *Inoviridae*), including strain K or PAO1 (Knezevic et al. 2015), used in the study of Hagens et al. (2006). The wild strain K is already infected with Pf1 phage, so application of this phage for CAPS in the experiments caused super-infection; similarly, PAO1 is infected with Pf4, so super-infection by Pf3 used by these authors can affect Pf4 phage production and thus cell survival. Furthermore, it was shown that lysogenization by myovirus P1 renders *E. coli* more sensitive to the DNA-damaging antibiotic ciprofloxacin (Ronayne et al. 2016). This implies that further attention should be paid on indigenous prophage destiny when CAPS is considered.

Variation in CAPS determination methodology is significant, so the results are rarely comparable, even if the same model strains, phages, or antibiotics were used. As mentioned above, a time-kill curve method should be a gold standard for CAPS estimation.

Finally, in the presented literature, CAPS mechanisms are not widely discussed, and there is a lack on studies focused on mechanism elucidation. These data can significantly contribute to understanding of CAPS and its better exploitation.

11 Advantages of CAPS Application

The advantages of chemical antimicrobial-phage combinations are numerous. First, it is proven that co-administration of phages and antibiotics could increase the phage efficacy by stimulating increased phage production, as seen in *Salmonella enterica*, *Escherichia coli,* and *Burkholderia cepacia* (Comeau et al. 2007; Kamal and Dennis 2014). Similar to multidrug therapy, combined phage-antibiotic therapy is less likely to fail due to bacterial resistance, because bacteria resistant to one agent still can be sensitive to the second agent and vice versa (Lu and Koeris 2011; Burrowes et al. 2011). Third, it is unlikely for phage- and drug-resistance to be acquired simultaneously, which is noted for some strains that carry multiple drug resistance determinants on mobile genetic elements (Partridge 2011). Fourth, the resistant

bacteria arising from combined phage-antibiotic treatment are usually less virulent comparing to resistant bacteria arising from drug treatment alone (Verma et al. 2009). The CAPS decreases effective antibiotic concentration, so reduces adverse effects of chemical antimicrobial agents, occurring after administration of high doses (Read et al. 2011). The CAPS can re-sensitize multidrug-resistant and pan-drug-resistant strains and thus present a possible treatment of potentially lethal infections. Finally, the synergy appears relatively fast, so in vivo bacterial numbers can be reduced for only several hours to the levels that the immune system can successfully cope with. Despite all these advantages, the clinical studies on phage-antibiotic application have not yet been conducted.

12 Concluding Remarks

According to the current studies, several consistencies related to the chemical agent-phage synergism can be observed: (1) CAPS is a strain-dependent phenomenon, being related to a strain's phage and chemical agent susceptibility; (2) it depends on mechanisms of chemical agent activity (that should not impair phage replication) and order of agents' addition/activity is sometimes crucial; (3) subinhibitory doses of various chemical antimicrobial agents can give synergy with phages, but most frequently the phenomenon is observed with beta-lactams and (fluoro)quinolones; (4) CAPS can be effective against planktonic cells, but also against bacterial biofilms; (5) there are several proposed mechanism of CAPS that probably play a role in the phenomenon; and (6) CAPS offers various advantages over single agent application. Accordingly, future studies should be focused on mechanism elucidation and clinical trials, to apply CAPS as new antibacterial armament.

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Phage Therapy of Infectious Biofilms: Challenges and Strategies

Luís D. R. Melo, Diana Priscila Pires, Rodrigo Monteiro, and Joana Azeredo

1 Introduction

Biofilms, which are microbial aggregates surrounded by polymeric substances, are implicated in many chronic and difficult to treat infectious diseases. In general, a number of pathologies in humans, such as endocarditis, urinary tract infections, burn infections, chronic otitis media, chronic bacterial prostatitis, respiratory infections in cystic fibrosis patients, periodontitis catheters' or other indwelling devices' infections, have been clearly associated with biofilms (Arciola et al. 2018). In a biofilm formation event, microbial cells adhere to abiotic or biotic surfaces, initiating a sessile mode of life characterized by a slow growth and production of polymeric substances. This sessile behaviour conveys several benefits to the microbial population, namely, increased tolerance to adverse environmental conditions and a rational use of nutrients which guaranties the survival of the community. These features explain the persistence and difficult treatment of biofilm-associated infections. Biofilm cells are much more tolerant to antibiotics and host defences compared to their planktonic counterparts (Hoiby et al. 2010). This high tolerance is usually explained by diffusional limitations of the antimicrobials through the exopolymeric matrix, inactivation of the drug by components of the matrix, the slow growth rate of bacterial cells and the stringent response induced by starvation and the presence of persister cells (Hall and Mah 2017; Salisbury et al. 2018).

The high tolerance of biofilms to antibiotics has been a major argument in favour of phage therapy, but also the fact that biofilms potentiate and facilitate antibiotic resistance is also a strong argument. However, in a similar way to what happens with antibiotics, biofilms confer protection to cells against phage predation and aid the proliferation of phage-resistant phenotypes, which may also limit phage therapy against biofilms. Nevertheless, there are numerous in vitro and in vivo studies

L. D. R. Melo · D. P. Pires · R. Monteiro · J. Azeredo (✉)
CEB—Centre of Biological Engineering, LIBRO—Laboratório de Investigação em Biofilmes Rosário Oliveira, University of Minho, Braga, Portugal
e-mail: jazeredo@deb.uminho.pt

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reporting the high efficacy of phages against biofilms (Pires et al. 2017a). Most important of all are the successful clinical outcomes of phage therapy against chronic infections (Kutter et al. 2010). Curiously, the only two European randomized and controlled phase I/II clinical trials of phage therapy reporting positive results were performed against infectious biofilms: chronic *Pseudomonas aeruginosa* otitis (Wright et al. 2009) and *P. aeruginosa* and *Escherichia coli* infected burn wounds (Jault et al. 2019). So, why phage therapy fails against biofilms in some cases while it is so successful in others? There is no straightforward answer to this question. The phage killing efficiency is highly dependent on the phage characteristics, bacterial features, biofilm structure and also the surrounded environment that is, in most cases, very complex. For a good biofilm killing efficacy, the phage should be able to replicate in growth-arrested cells, and penetrate the inner layers of the biofilms (Gonzalez et al. 2018). Structural polysaccharide degrading enzymes displayed by some phages have been pointed to confer phages an advantage against biofilms; however, this has been shown for a limited number of cases (Pires et al. 2016). Phage resistance is also an important feature in biofilm-phage interaction, since most of the in vitro biofilm killing studies reported a biofilm regrowth after six to eight hours of phage interaction (Pires et al. 2017b). This regrowth is associated with the proliferation of phage-resistant variants, which are well-adapted to the sessile mode of life. So, as a general rule, phages using multiple receptors or a cocktail of phages can delay but not prevent the emergence of resistant mutants. At this point, other factors, such as human natural defences, play an important role in infection control. It is therefore very difficult to predict the efficacy of phage therapy against infectious biofilms using in vitro studies. Particularly, when biofilms are formed in artificial conditions that poorly reproduce the in vivo ones (Trivedi et al. 2017). In vitro phage–biofilm interaction studies are useful for an initial screening of phages but do not replace in vivo studies for an accurate efficacy evaluation. There are in vitro models of biofilm formation that better mimic in vivo conditions that should be preferentially used. A special attention should also be given to polymicrobial biofilms, usually responsible for several chronic infections, like chronic wound cystic fibrosis associated lung infections, among others. In fact, only few studies have addressed phage interaction with multi-species biofilms (Pires et al. 2017a).

As mentioned above, biofilms may impose some difficulties for phage interaction, replication and burst from their hosts, and, therefore, combined treatments usually give better results. Combinations of phages with antibiotics, enzymes, antimicrobial peptides or mechanic dispersion are some of the approaches suggested (Fig. 1). Usually, mechanic or enzymatic dispersion of the biofilm helps phage penetration through the biofilm matrix resulting in higher killing efficacies.

2 Methods to Study Phage–Biofilm Interaction

Several methods to analyse and characterize phage–biofilm interactions have been described. These methodologies are focused on the response of biofilm cells to phage application and on the assessment of phage efficacy against the biofilm

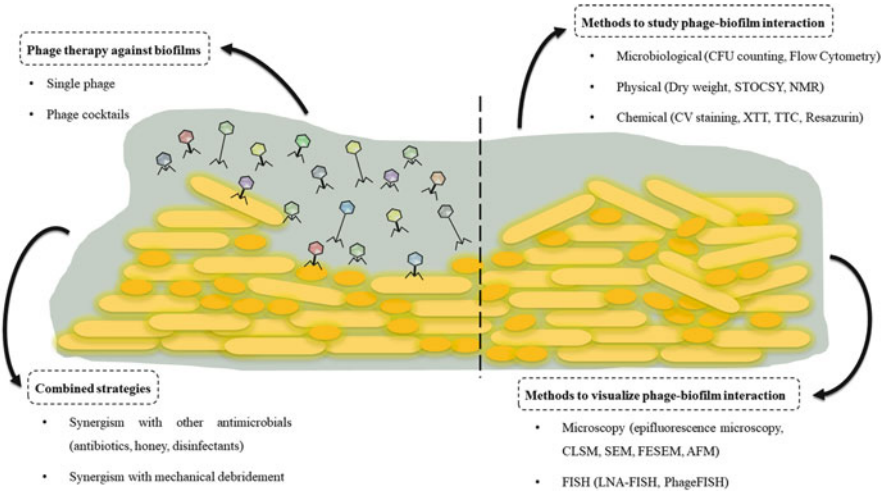

Fig. 1 Schematic representation of strategies to overcome biofilm barriers using phages or combined therapies (left) and methods to study and visualize phage–biofilm interactions (right)

biomass (Fig. 1). The first thing to keep in mind when studying phage–biofilm interactions is the experimental setup where the biofilms are formed.

2.1 In Vitro, Ex Vivo and In Vivo Models of Biofilm Formation

Biofilm control with phages has been widely explored using several in vitro models, such as microtiter plates (Pires et al. 2017b; Latz et al. 2017; Pei and Lamas-Samanamud 2014) and flow reactors (Fu et al. 2010; Melo et al. 2016; Curtin and Donlan 2006). Biofilm formation in microtiter plates is inexpensive and allows high-throughput studies. Therefore, several works have assessed the efficacy of phages against biofilms formed in microtiter plates, namely targeting ESKAPE pathogens (Fu et al. 2010; Verma et al. 2009; Alves et al. 2014; Liu et al. 2016). In the classical procedure, the wells are inoculated with a bacterial cell culture and, at different time points, the wells are emptied and washed to remove planktonic cells and allow the growth of sessile bacteria. The biofilm is then challenged with a phage suspension. The major drawback of this procedure is that the biofilm biomass is composed not only by sessile bacteria but also by deposited or entrapped planktonic cells, which bias the subsequent studies (Azeredo et al. 2017). To overcome this problem, the Calgary Biofilm Device (CBD) was developed. In this device biofilms are formed at the coverlid, composed of pegs that fit into the wells of the microtiter plate containing the growth medium and bacteria (Ceri et al. 1999). The biofilm formed on the pegs does not result from cell sedimentation but only from sessile development. This device has been used in several phage–biofilm interaction studies (Chylkova et al. 2017; Carson et al. 2010; Vandersteegen et al. 2013).

Despite the high reproducibility and multiparametric analysis that the aforementioned models allow to perform, they inefficiently mimic conditions found in clinical, food industry or veterinary environments where biofilms are commonly found. In those real conditions, biofilms are exposed to shear forces under continuous liquid flow. Consequently, the use of more refined dynamic models is necessary in studies assessing the efficacy of anti-biofilm agents, such as phages. Examples are flow cells, drip flow reactors (DFR), modified Robbins devices (MRD) and rotary biofilm devices. A simple dynamic biofilm formation device is the Foley catheter, which is particularly useful to assess biofilms formed on catheters, where the hydrodynamic conditions can be regulated to reproduce the real ones. Different phage–biofilm interaction studies were assessed using these devices, where phages were added to preformed biofilms or incorporated in hydrogel coatings to control or prevent *P. aeruginosa (Fu* et al. 2010*)*, *Staphylococcus epidermidis* (Curtin and Donlan 2006) and *Proteus mirabilis* biofilms (Melo et al. 2016; Nzakizwanayo et al. 2015).

In general, most of the in vitro studies aiming at evaluating phage efficacy against biofilms have reported interesting outcomes: significant reductions of cell number and total biomass of mono or even multi-species biofilms have been observed after phage treatment for a short time period (Pires et al. 2017a; Donlan 2009). However, it remains unclear whether such in vitro biofilm models actually resemble the in vivo biofilms that are commonly responsible for chronic infections. In fact, choosing the right in vitro model may be very challenging since mimicking the complexity of the host environment using the in vitro systems is still an arduous task (Bjarnsholt et al. 2013; Lebeaux et al. 2013). To overcome this issue, several studies have been performed using ex vivo models, which lie in the middle of in vitro and in vivo models, and consist in the use of tissue from a living organism in an artificial environment outside the organism with minimal alterations of natural conditions. Ex vivo models are one step closer to mimic real environments than in vitro models and, at the same time, allow more controlled experimental conditions than in vivo models, becoming an interesting alternative approach to the use of living organisms (Lebeaux et al. 2013). Besides, in vivo models always involve ethical and economic considerations, and their approval is generally time-consuming.

For instance, different models have been used to understand the role of biofilms in wound healing (Brackman and Coenye 2016). As porcine models are considered to be a good representation of human skin and wound healing, Alves et al. developed a high-throughput ex-vivo burn wound model using porcine skin to assess the potential of phages to control *Staphylococcus aureus* biofilms (Alves et al. 2018). Similarly, Milho et al. used a porcine skin model to assess the efficacy of single phages or cocktail of phages to control single species biofilms of *E. coli*, *P. mirabilis*, *Acinetobacter baumannii* and *P. aeruginosa* (Milho et al. 2019). Both studies suggested that ex vivo models can be a valuable approach to evaluate the potential of phage therapy against biofilms formed on wounds. However, the lack of the host response and the short duration of the experiments are still major limitations of ex vivo models. In order to better understand the role of the system/environment around a bacterial infection, in vivo models are the best logical choice, since they

bring the third party involved in the combat of infections, the immune system. So far, several in vivo biofilm models have been described, ranging from invertebrate models, such as *Galleria mellonella*, to mammalian in vivo models, namely mice, rabbits or sheep (Lebeaux et al. 2013; Rumbaugh and Carty 2011). Taking advantage of these models, the potential of phage therapy against bacterial diseases has also been studied in vivo (Latz et al. 2017; Nakai and Park 2002; Singla et al. 2016; McVay et al. 2007; Henry et al. 2013). The mice model of wound infections is, for instance, one of the most commonly used models to assess the efficacy of phages against biofilm infections caused namely by *S. aureus* or *P. aeruginosa (Lebeaux* et al. 2013*)*.

2.2 Assessment of Biofilm Parameters

Different methods can be used to assess the phage killing efficacy. The most widely used technique to determine phage efficacy against biofilms is the determination of the remaining biofilm population by colony forming units (CFU). This is a straightforward technique based on serial dilutions that can be applied in every microbiology lab. This approach has some disadvantages that can lead to a wrong assessment of phage killing efficacy as the number of CFU might not correspond to real amount of viable cells present in the biofilm. In fact, biofilm cells form aggregates that are difficult to disperse even with optimized sonication times preceding the cell plating. Moreover, biofilms are formed by a subpopulation of viable but non-culturable cells (some induced by dormancy), which is usually not detected by CFU technique (Cerca et al. 2011). This limitation can be overcome by flow cytometry combined with bacterial cell staining with viability fluorophores (Cerca et al. 2011). Despite being more expensive and not available at all labs, this technique allows the detection of viable but non-culturable cells, which can be differentiated from dead cells. For example, LIVE/DEAD fluorochromes have been used in *S. epidermidis* biofilms to assess bacterial viability. In addition to a very quick counting of the number of cells, this methodology also allowed evaluating the physiological state of cells (Cerca et al. 2011; Pires and Melo 2018). Recently, the phage–host interaction of *S. epidermidis* phage SEP1 was assessed, using this methodology, in planktonic cells, and it was shown to be a valuable tool to understand the bacterial response to phage infection (Melo et al. 2018).

There are also physical methods that can be used to assess biofilm biomass, namely dry or wet weight measurements. In this way, the efficacy of phages can be determined by the weight difference between an untreated and a phage-treated biofilm. Sillankorva et al. demonstrated that the *Pseudomonas fluorescens* phage phiIBB-PF7A reduced biofilm biomass between 63% and 91% depending on the biofilm age and the conditions under which the biofilm had been formed and phages applied (Sillankorva et al. 2008). However, this method is very time-consuming and has low sensitivity particularly when assessing samples with small weight differences (Azeredo et al. 2017).

More sensitive but also indirect methods of biofilm biomass assessment are those based on nuclear magnetic resonance (NMR) and electrical impedance and have been employed to assess phage–biofilm interactions. Sonkar et al. demonstrated that the transient metabolic flux of phage infection can be measured by the statistical total correlation spectroscopy (STOCSY) and partial least squares discriminant analysis (PLS-DA) applied to NMR with a very high sensitivity (Sonkar et al. 2012). Recently, Gutiérrez et al. suggested the use of an impedance-based method to monitor biofilm formation. The authors used a real time cell analyzer equipment that uses specific microtiter plates coated with gold-microelectrodes to detect cell adherence. This method was validated using a *S. epidermidis* specific phage (Gutierrez et al. 2016).

Chemical methods are indirect methods to measure biofilm constituents and rely on the use of dyes or fluorochromes that can bind or adsorb to biofilm constituents, namely cells or matrix components. Safranin or crystal violet (CV) staining has been widely used to study biofilm communities. With these stainings, the total biofilm biomass can be quantified directly on microtiter plates (Pitts et al. 2003). Several biofilm–phage interaction studies have employed this methodology to measure the extent of biofilm reduction (Naser et al. 2017; Pires et al. 2013). CV staining is versatile, takes into account live and viable but non-culturable cells and is high-throughput. However, it includes some limitations, namely lack of reproducibility, bias of the estimate of sessile development capability, do not allow discrimination of species in polymicrobial communities and, although widespread, there is an absence of a standardized protocol that turns difficult the comparison of results between studies. Because of these limitations there has been some controversy on the use of these approaches (Azeredo et al. 2017). Other colorimetric methodologies have been used to assess the cellular physiology of biofilms. The most routinetly used methods to quantify metabolic activity in biofilms are XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt), TTC (2,3,5-triphenyl-2H-tetrazolium chloride) and Resazurin. XTT and TTC are tetrazolium salts that are cleaved by dehydrogenase enzymes of metabolic active cells in biofilms. Dalmasso et al. have shown that at the higher doses tested, the phage ΦAPCM01 completely inhibited the biofilm metabolic activity, assessed by XTT (Dalmasso et al. 2015). In another study, using TTC, Kim et al. demonstrated that phage PA1Ø significantly inhibited the bacterial growth of all *P. aeruginosa* strains tested (Kim et al. 2012).

The other referred compound, resazurin (also known as Alamar Blue), is a stable redox indicator that is reduced by metabolically active cells (Pettit et al. 2005). This dye offers several advantages in comparison with XTT and TTC, namely, less time-consuming, less expensive and less toxic to cells (Azeredo et al. 2017). Using this approach, Mendes et al. demonstrated that a phage cocktail strongly reduced the cell viability of *S. aureus*, *P. aeruginosa* and *A. baumannii (Mendes* et al. 2014*)*.

2.3 Visualization of Phage–Biofilm Interactions

Phage–biofilm interactions can be observed by a set of imaging modalities. The advantages and disadvantages of each methodology have been widely described elsewhere (Azeredo et al. 2017). The effect of phages on biofilms has been demonstrated by epifluorescence microscopy (Montanez-Izquiredo et al. 2012), confocal laser scanning microscopy (CLSM) (Khalifa et al. 2015), scanning electron microscopy (SEM) (Fu et al. 2010), field emission scanning electron microscopy (Sillankorva et al. 2008) and atomic force microscopy (Dubrovin et al. 2012).

FISH-based methods are elegant ways to visualize phage–host interactions. Phage FISH was performed on *Pseudoalteromonas* cells using polynucleotide probes during the course of phage infection, allowing a single-cell analysis (Allers et al. 2013). Instead of using one 350 bp gene region as polynucleotide probe target, up to 12 regions of 1 phage gene were targeted and consequently, the detection efficiency was greatly increased. The *unk* gene was chosen as target for this approach due to its uniqueness and size (long enough to accommodate twelve 300 bp probes). More recently, locked nucleic acid (LNA) probes were developed to detect *P. aeruginosa* and *A. baumannii* infected cells. These probes targeted the major capsid protein-coding sequences. On biofilms, this technique allowed to discriminate infected cells from non-infected populations and also to assess their spatial distribution (Vilas Boas et al. 2016).

3 Phages for Biofilm Prevention

Microorganisms can quickly colonize and form biofilms in a variety of medical devices, such as catheters or heart valves. This might result in device-related infections that are frequently associated with high morbidity and mortality rates (Fu et al. 2010; Curtin and Donlan 2006). Among other strategies, phages have been proposed to prevent device-associated biofilms and several studies have been conducted so far.

In 2006, Curtin and Donlan proposed an approach consisting on a pretreatment of hydrogel-coated silicone catheters with phages in order to prevent *S. epidermidis* biofilm formation (Curtin and Donlan 2006). The treatment of catheters with phages significantly reduced biofilm cell counts by more than 4-logs during the 24 h exposure period, comparatively to the untreated catheters (Curtin and Donlan 2006). A similar approach was adopted by Fu et al. who studied the effect of a single phage or phage cocktail to prevent *P. aeruginosa* biofilm formation on catheters (Fu et al. 2010). These authors also demonstrated a significant reduction of biofilm formation on phage-treated catheters, highlighting the potential of phages to impair biofilm development of clinically relevant organisms on medical devices. However, it is important to note that phage-resistant biofilm isolates were recovered in this study. Not all studies of phage–biofilm interactions have reported resistance. Nevertheless, it is generally accepted that biofilms regrowth is usually observed at 6 to 8 h post-phage biofilm infection due to the proliferation of phage resistance

variants (Pires et al. 2017b). Resistance is a very important feature that should be taken in consideration when developing a phage product for clinical applications.

In another study, models simulating a complete closed drainage system were applied to evaluate the possibility of phages to prevent catheter blockage. A phage cocktail was applied to prevent or control colonization of catheters. The referred cocktail delayed by threefold the time of blockage on colonized catheters (established infection) and fully prevented blockage on early infection (early-stage infection) (Nzakizwanayo et al. 2015). More recently, Melo et al. used a continuous model on Foley catheters to form *P. mirabilis* biofilms with a flow set to mimic the actual average flow in a catheterized patient. The authors used artificial urine as media to mimic urinary tract infections and a phage cocktail to prevent biofilm formation. After 168 h of flow, a significant reduction in the number biofilm cells was observed in the phage-coated catheters (Melo et al. 2016).

Another study on biofilm prevention with phages was performed by Kelly et al. (Kelly et al. 2012). The goal of this work was to assess the ability of a cocktail of the *S. aureus* phage K and six modified derivatives to prevent biofilm formation on microtiter plates (Kelly et al. 2011, 2012). The simultaneous application of the phage cocktail and *S. aureus* cells resulted in a complete inhibition of biofilm formation during the time course of the experiment (48 h) and curiously, no bacterial resistance towards phages was observed (Kelly et al. 2012). The authors suggested that the phage mixture could be potentially applied on coatings of medical devices to prevent healthcare-associated infections.

4 Phages as Biofilm Control Agents

For years, the therapeutic anti-biofilm potential of phages has been assessed in different in vitro models. However, as previously mentioned, the conditions tested usually do not simulate what happens in reality putting at risk the true application of phages for a particular bacterial infection. As in vivo biofilm models can be challenging to implement, ex vivo models can be used as a closer to reality approach. Some ex vivo models are already well implemented, such as burn wound models from porcine or poultry skin (Alves et al. 2018; Milho et al. 2018, 2019).

Alves et al. used a porcine skin model to evaluate the potential of phages against *S. aureus* biofilms. The authors obtained very promising results achieving a 1.5-log reduction of colony-forming units when the phage treatment was applied and an increase of phage titre 24 h post-inoculation (Alves et al. 2018). Using a similar model, Milho et al. formed biofilms of different microorganisms, and they were further treated with a single phage or a phage cocktail applied 24 h post-inoculation, and the effect was evaluated after 4 h and 24 h of treatment. In *E. coli*, *A. baumannii* and *P. aeruginosa* biofilms, the number of viable cells decreased 2-log after 4 h of infection with a single phage and 1-log when the phage cocktail was applied. At 24 h post-infection, the reductions were identical but with a marginally better reduction using the phage cocktail. On the other hand, on *P. mirabilis* no significant difference was observed with both treatments at any time-point (Milho et al. 2019). In another

study, Milho et al. withdrew the same conclusion, when attempted to minimize *S. enteritidis* colonization on poultry skin surfaces. In order to accomplish this, the authors inoculated the strain at 4 °C and applied the phage PVP-SE2 (Milho et al. 2018). Khalifa et al. carried an unusual ex vivo experiment, where they used a human root canal model to study *Enterococcus faecalis* phage treatment. Phage EFDG1 reduced significantly a 2-week-old biofilm, showing extraordinary potential for phage therapy to control this type of infections. In 3 days, the phage was able to decrease by 2-log bacterial load, and, 7 days post-treatment, the biofilm viable cells decreased 4-log when compared with untreated control (Khalifa et al. 2015).

Regarding biofilm control using in vivo models, there are only few studies described (Table 1). A very promising experiment against chronic lung infections was performed in a murine model and showed that phage therapy could eradicate *P. aeruginosa*. In this study, mice were infected with *P. aeruginosa*, and phage was administered at a MOI of 10 in different time points. In the more prolonged treatments, the phage was not able to eradicate all bacteria as in the shorter treatments. Nevertheless, it revealed high efficacy against well-established 6-day lung infections (Waters et al. 2017).

In other studies, phage therapy was tested against infections associated with medical devices, namely, in biofilms formed in catheters. Basu et al. used an in vivo wound model where biofilm-laden catheter sections of *P. aeruginosa* were implanted in subcutaneous pockets of mice. First, the biofilms were grown in catheter sections for 48 h, and then two catheter sections were implanted in male albino mice. For 10 days, mice were injected daily (into the pocket) with a phage cocktail at a MOI of 10, and after the phage therapy, CFUs and PFUs were assessed. When compared with the baseline, the phage cocktail was able to reduce bacterial load in 2-log, but comparing with the control the reduction was only 1-log. The role of immune system is probably the cause for the differences between control and baseline values and, when the phage was applied, an addictive effect emerged leading to an additional 1-log reduction. Concerning the phage concentration, it has increased 1-log in average showing replication of the phage in the infection site (Basu et al. 2015).

Using a mice wound model, Holguín et al. tested the efficacy of a putative temperate phage, ΦPan70, in the treatment of *P. aeruginosa* biofilms. Mice were infected with bacteria and, in different mice, after 0 min, 45 min, 24 h and 48 h, phages were added and each mouse was monitored during 15 days. When the phages were applied at 0 min and 45 min, despite mice recovery, four and five of the five mice survived, respectively, but it was observed a severe lesion development on the back skin after 8 days that did not disappear after 15 days. Phage administration at 24 h or 48 h showed that four of the five treated mice survived. As with the first two time-points, mice developed the same lesion but in this case they were able to fully recover. A second experiment was developed using a second phage dose administration 48 h after the first one, but only in mice where the first dose was given at 0 min and 45 min. The goal of this experiment was to understand if the lesion chronically developed by mice could be prevented or fully recovered. Results indicated a full recovery of the skin lesion and the health of mice was maintained optimal during the 15 days of monitoring. These experiments revealed that phage

Table 1 Most relevant in vivo studies about phage–biofilm interactions

Bacteria	Phage(s)	Experimental approach	Results	Reference
Single phage/cocktail				
P. aeruginosa	ΦBHU—Cocktail of 11 phages: 49, 61, 83, 89, 98, 2255, 7799, 10858, 10956, 10958, 10976	A phage cocktail was applied on biofilms formed in catheter sections implanted in mice skin.	The phage cocktail treatment at a MOI of 10 was able to reduce bacterial viable cells by 2-log when compared with the baseline, and 1-log regarding the controls. The influence of immune system in in vivo experiments was demonstrated.	Basu et al. (2015)
P. aeruginosa	ΦPan70	A phage treatment was applied to treat a biofilm infection in a mouse model.	Phage treatment resulted in mice survival between 80% and 100%.	Holguin et al. (2015)
P. aeruginosa	Cocktail of six phages PYO2: DEV, E215, E217, PAK_P1, PAK_P4	A phage cocktail was applied in a *Galleria mellonella* model and in a mouse for the treatment of acute respiratory infection.	The phage cocktail resolved pseudomonal acute pneumonia in mice and treated bacteremia in wax moth larvae.	Forti et al. (2018)
P. aeruginosa	Phage cocktail PP1131	Phage cocktail application to treat burn wounds infected by *P. aeruginosa*.	At very low concentrations, PP1131 decreased bacterial loads in burn wounds at a slower pace than the standard treatment.	Jault et al. (2019)
Combined strategies				
P. aeruginosa *S. aureus*	vB_PsaP PAT14 Sb-1	A phage-antibiotic treatment was applied to treat implant-related infections inside rat tibiae.	In the *Pseudomonas* group, the number of bacterial cells was significantly lower when the phage-antibiotic treatment was applied. Despite	Yilmaz et al. (2013)

(continued)

Table 1 (continued)

Bacteria	Phage(s)	Experimental approach	Results	Reference
			the decreased number of CFUs in *S. aureus* biofilms treated with phage or with teicoplanin, the biofilm was completely eradicated when both were applied.	
P. aeruginosa *S. aureus* *A. baumannii*	Cocktail of five phages: F770/05, F510/08, F44/10, F125/10, F1245/05	A phage cocktail was applied after debridement in a rodent and a pig wound infection model	The combination of debridement followed by the application of phage cocktails improved the efficacy of the biofilm removal by reducing bacterial cell counts.	Mendes et al. (2013)
Staphylococcus aureus	Unknown	A phage treatment was applied after debridement against biofilm-infected wounds using a rabbit ear model.	The combination of both treatments significantly improved all measured wound healing parameters and reduced bacterial cell counts (2-log).	Seth et al. (2013)
Staphylococcus aureus	CSTA (Cocktail of *S. aureus*)	EDTA and a phage cocktail were applied (combined or not) in sheep model of sinusitis.	The individual application of EDTA and phage cocktail resulted in significant reductions of biofilm cells. On the other hand, the combination of both treatments didn't show a synergistic effect and longer periods of treatment should be tested.	Drilling et al. (2014)

therapy was able to eradicate *P. aeruginosa* biofilm infection with 80%–100% mice survival and improved health condition (Holguin et al. 2015).

Although not only focused on biofilms, other in vivo phage therapy studies using animal models as well as early work in human patients have demonstrated the huge potential of phages to control a wide range of bacterial infections and no adverse effects have been reported so far (Jault et al. 2019; Forti et al. 2018; Abedon et al. 2011). These kind of experiments using different models allow the development of more advanced work such as humans' trials. In a recently reported trial published by Jault et al., a treatment with a phage cocktail of *P. aeruginosa*-infected burn wounds called PhagoBurn was used in patients. The phage cocktail was applied topically along 7 days in a daily basis, and observations were made at the end of the treatment and after 14 days. Despite the low concentration of phage used in the treatment, the authors reported promising results. The treatment with PhagoBurn was able to reduce two quadrants or more in half of participants (Jault et al. 2019).

5 Combined Strategies to Enhance Phage–Biofilm Interactions

Phages might have a limited action against biofilms. The low metabolic state of biofilm bacteria, the barrier imposed by the biofilm matrix, the inactivation of phages by matrix components and the emergence of resistant variants are some of the obstacles encountered by phages when interacting with biofilms. Combined strategies using different antimicrobials, mechanical debridement or other bacterial cells were developed to overcome these limitations and increase biofilm control (Fig. 1). Table 1 presents the most relevant in vivo studies where phage treatment was complemented with other treatments.

5.1 Phages Combined with Antimicrobials

Phages were already combined with numerous antimicrobials with the intent to obtain a synergistic effect that could improve treatment. Several studies showed this effect when both antibiotics and phages are applied together (Torres-Barcelo and Hochberg 2016). Rahman et al. tested a combined treatment using the temperate phage SAP-26 and three different antimicrobial agents, rifampicin, azithromycin and vancomycin, to control *S. aureus* biofilms. After 24 h of treatment, the best result was obtained using the phage–rifampicin combination, which destroyed the biofilm matrix and highly reduced the number of bacterial cells (Rahman et al. 2011). Similar results were reported by Bedi et al. The authors showed that a combination of a phage with a MOI of 0.01 and amoxicillin at 512 μg/mL resulted in a synergistic effect for the control of *Klebsiella pneumoniae* biofilms. Biofilm bacterial cell counts decreased 2-log more when exposed to both agents than with the antibiotic alone (Bedi et al. 2009). Coulter et al. tested tobramycin in combination with phages to combat *P. aeruginosa* and *E. coli* biofilms. The authors found that phage infection in

combination with tobramycin can significantly reduce the emergence of antibiotic and phage-resistant cells in both *E. coli* and *P. aeruginosa* biofilms; however, the biomass reduction was more effective on *E. coli* biofilms (Coulter et al. 2014).

In another study, Chaundhry et al. reported that the administration order of the phage (NP1 or NP3) and the adjuvant antibiotic have a tremendous impact in the efficiency of the treatment. The authors tested several antibiotics such as ceftazidime, ciprofloxacin, colistin, gentamicin and tobramycin to treat *P. aeruginosa* biofilms formed in vitro and also in a monolayer of human epithelial nasopharyngeal cells. Each antibiotic was tested alone, with the phage simultaneously and after 4 h and 24 h of phage administration. The only significant effect was obtained by adding first the phage and 24 h after gentamicin and tobramycin. In these two conditions, the bacterial load decreased 1.5-log and 2-log, respectively. This study concluded that each phage–antibiotic combination can give different outcomes. Moreover, it was also referred that with some antibiotics a lower dose can be more effective than a higher one and that the administration of phages prior to the antibiotic treatment can enhance the antimicrobial effect (Chaudhry et al. 2017).

Other studies have also reported the in vitro synergistic effect between conventional antibiotics and phages (Verma et al. 2009; Ryan et al. 2012; Verma et al. 2010). There is, however, a lack of studies with animal models showing this synergy. One of the few studies was conducted by Yilmaz et al. who combined phages and antibiotics to control *P. aeruginosa* and *S. aureus* biofilms formed in implant-infection models inside rat tibiae. For the *P. aeruginosa* treatment, a combination of the phage vB_PsaP PAT14 with imipenem plus cilastatin and amikacin was used. For *S. aureus* biofilms, teicoplanin was used as adjuvant of the Sb-1 phage. On both species biofilms, a slight effect was observed when they were treated with phages or antibiotics. On the other hand, the number of bacterial cells was significantly lower when the combination of phage and antibiotics was administered (Yilmaz et al. 2013).

Besides antibiotics, other antimicrobials have also been tested in combination with phages. Zhang et al. combined chlorine with a phage to evaluate *P. aeruginosa* biofilm control. The authors tested the formulation both for biofilm prevention and for biofilm control with very promising results in both scenarios. Their results showed that biofilm formation was almost completely prevented when the combination was applied with chlorine with a minimum of 21 mg/L. As expected, the efficacy dropped for biofilm control experiments, since the biofilms are harder to remove after formation, nevertheless, with high chlorine concentrations, biofilm removal was almost 90% (Zhang and Hu 2013). In another study, Drilling et al. tested the use of ethylenediaminetetraacetic acid (EDTA) to treat *S. aureus* sinusitis in a sheep model. The authors applied a single EDTA treatment, a phage cocktail treatment and a combination of both. An interesting biofilm reduction was observed with single treatments and unlike other studies, this study showed a lack of synergy in the combined treatment (Drilling et al. 2014).

Oliveira and Ribeiro et al. reported the use of honey in combination with phage EC3a for *E. coli* biofilm control. This treatment was applied against 24 h- and 48 h-old biofilms. A large synergistic effect between both antimicrobials was observed,

and the best result was obtained against the younger biofilms with almost 6-log reduction after 12 h of treatment (Oliveira et al. 2017). More recently, *E. coli* and *P. aeruginosa* dual-species biofilms were challenged with honey and a cocktail of two phages. Despite not having a synergistic effect, the authors observed that the combined treatment resulted in a slightly better antibacterial outcome than single treatments (Oliveira et al. 2018).

5.2 Phages Combined with Mechanical Debridement

Mechanical debridement consists in the use of a mechanical force to disturb the biofilm matrix. It can be assumed that debridement enhances phage infection as a consequence of a better phage accessibility to the biofilm cells. Moreover, cells released due to debridement also become more susceptible to phage infection, suggesting that this type of approach can be valuable therapeutically. Mechanical debridement has been tested using in vivo models. For instance, Mendes et al. used a rodent and a porcine wound infection models to assess the efficacy of a phage cocktail treatment on wounds after debridement. In both models, the results were identical, showing a significant reduction of *S. aureus* and *P. aeruginosa* cell counts, but not of *A. baumannii (Mendes* et al. 2013*).* In another study, Seth et al. showed that a phage was not active against intact *S. aureus* biofilms. However, the combination of both treatments significantly improved wound healing parameters, assessed by histological analysis and bacterial killing (Seth et al. 2013).

6 Conclusions

The clinical relevance of bacterial biofilms highlights the importance to develop prophylaxis, diagnosis and control strategies. The use of phage therapy has been gaining importance on the last years and some phage-based products are currently on clinical trials. Nevertheless, there is still a considerable lack of therapeutic options for biofilm-associated infections. Phage efficacy against biofilms has been mainly proved using in vitro models that poorly mimic the in vivo conditions. Besides, the study of phage–biofilm interactions is very dependent on the methodology of biofilm formation, inocula amount, biofilm formation devices, and culture media used among other variables. There is, therefore, a need for standardized protocols and biofilm formation conditions that better mimic real conditions. In vitro phage–biofilm interaction studies are useful for an initial screening of phages, but do not replace in vivo studies for an accurate efficacy evaluation. However, while several in vivo models of biofilm infection have been described so far, there is still a limited knowledge about the therapeutic application of phages in real conditions.

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Part IV

Bacteriophage-Derived Antibacterial Agents

Phage Lysins: Novel Alternative to Antibiotics

Vincent A. Fischetti

1 Background

Viruses that specifically infect bacteria are called bacteriophage (or phage). After replicating inside its bacterial host, the phage is faced with a problem; it needs to efficiently exit the bacterium to disseminate its progeny to begin a new cycle. To solve this, double-stranded deoxyribonucleic acid (DNA) phages have evolved a lytic system to weaken the bacterial cell wall resulting in hypotonic bacterial lysis or "lysis from within."

Phage lytic enzymes or lysins are highly efficient molecules that have been refined over millions of years of evolution to effectively release it progeny phage. These enzymes target the integrity of the cell wall, and are designed to attack one of the five major bonds in the peptidoglycan. With few exceptions (Loessner et al. 1997), lysins do not have signal sequences, so they are not translocated through the cytoplasmic membrane to attack their substrate in the peptidoglycan; this movement is tightly controlled by a second phage gene product in the lytic system, the holin (Wang et al. 2000). During phage development in the infected bacterium, lysin accumulates in the cytoplasm in anticipation of phage maturation. At a genetically specified time, holin molecules are inserted in the cytoplasmic membrane forming patches, ultimately resulting in a hole through localized membrane disruption (Wang et al. 2003). The cytoplasmic lysin is now able to access the peptidoglycan to cleave specific bonds, thereby causing immediate cell lysis as a result of internal turgor

This chapter is an update of the former publication (Fischetti V.A. (2014). Using what phages have evolved to kill pathogenic bacteria. In: Phage therapy: current research and applications. Eds: J. Borysowski, R.Międzybrodzki, A.Górski. Caster Academic Press, Norfolk, UK, pp 331–341. ISBN 978-1-908230-40-9) with the permission of Caister Academic Press.

V. A. Fischetti (✉)
Laboratory of Bacterial Pathogenesis, Rockefeller University, New York, NY, USA
e-mail: vaf@rockefeller.edu

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pressure (which in the gram-positive organism could be as much as 15–25 atm), and the release of progeny phage (Wang et al. 2000). In contrast to large DNA phage, small ribonucleic acid (RNA) and DNA phages use a different release strategy. They call upon a phage-encoded protein to interfere with bacterial host enzymes responsible for peptidoglycan biosynthesis (Bernhardt et al. 2001; Young et al. 2000) resulting in misassembled cell walls and ultimate lysis. Scientists have been aware of the lytic activity of phage for nearly a century, and while whole phage have been used to control infection (Matsuzaki et al. 2005), not until recently have lytic enzymes been exploited for bacterial control in vivo (Fischetti 2018; Loeffler et al. 2003; Nelson et al. 2001; Schuch et al. 2002). One of the main reasons that such an approach is only now even being seriously considered, is the sharp increase in antibiotic resistance among pathogenic bacteria. Current data indicate that lysins work best against gram-positive bacteria, since they are able to make direct contact with the cell wall carbohydrates for binding and peptidoglycan cleavage when added externally, whereas the outer membrane of gram-negative bacteria limits this direct interaction. Recently, however, lysins which are able to both disrupt the outer membrane of gram-negative bacteria and cleave the peptidoglycan have been identified (Lai et al. 2011; Lood et al. 2015). Lysins, termed artilysins, have also been engineered to penetrate the outer membrane to kill gram-negative bacteria (Briers et al. 2014). However, in nearly all cases, gram-negative lysins have low or no activity in the presence of serum.

Bacterial lysis can also occur through the addition of an external agent resulting in what is termed "lysis from without." This lytic event can arise either through the exposure of the bacteria to a high multiplicity of infection (MOI) of phage (Abedon 2011; Delbruck 1940) or the exposure of the bacteria to phage-derived or other wall-degrading lysins. These events do not occur to any great extent if at all in nature, but are the result of phage or lysin manipulation in the laboratory. Both events utilize enzymes that cleave the peptidoglycan; in the case of whole phage, lytic enzymes are part of the phage tail structure used to locally degrade the peptidoglycan of either gram-positive or gram-negative bacteria, allowing entry of phage DNA (or RNA in the case of RNA phage) to the cell. Lysis through lytic enzymes may occur in nature if the lysins used by the phage to release progeny happen to interact with sensitive bacteria in the vicinity of the lysing bacteria. But this would be a rare event, since lysins have evolved binding domains to retain the free lysin on the cell wall debris of the lysing bacteria. In general, lysis from without is more likely to occur with gram-positive bacteria since their cell wall peptidoglycan is more directly exposed on the cell surface than gram-negative bacteria, that are protected by an outer membrane. Because both lytic events are rapid and efficient, the power of these enzymes could be harnessed to control bacterial pathogens.

Most human infections (viral or bacterial) begin at a mucous membrane site (upper and lower respiratory, intestinal, urogenital, and ocular). Furthermore, these human mucous membranes are the reservoir (and sometimes the only reservoir) for many pathogenic bacteria found in the environment (i.e., pneumococci, staphylococci, streptococci, hemophilus) some of which are resistant to current antibiotics. In most instances, it is this mucosal reservoir that is the focus of infection in the population

(Coello et al. 1994; de Lencastre et al. 1999; Eiff et al. 2001). To date, except for polysporin and mupirocin ointments, which are the most widely used topically, there are no anti-infectives that are designed to control colonizing pathogenic bacteria on mucous membranes (Hudson and Hay 1976); we usually must wait for infection to occur before treating. Because of the fear of increasing the resistance problem, antibiotics are not indicated to control the carrier state of disease bacteria. It is acknowledged, however, that by reducing or eliminating this human reservoir of pathogens in the community and controlled environments (i.e., hospitals and nursing homes), the incidence of disease will be markedly reduced (Eiff et al. 2001; Hudson and Hay 1976). Toward this goal, an effective use for lysins is to prevent infection by safely and specifically destroying disease bacteria on mucous membranes. For example, based on extensive animal results, lysins specific for *S. pneumoniae* (Loeffler et al. 2001), *S. pyogenes* (Nelson et al. 2001), and *S. aureus* (Gilmer et al. 2013) may be used nasally and/or orally to control these organisms in the community as well as in nursing homes and hospitals to prevent or markedly reduce serious infections caused by these colonizing bacteria.

Like naturally occurring antibiotics, which are used by bacteria to control the organisms around them in the soil environment, phage lysins are the culmination of millions of years of evolution by the bacteriophage in their association with bacteria. Specific lysins have now been identified and purified that are able to kill specific gram-positive bacteria seconds after contact (Loeffler et al. 2001; Nelson et al. 2001). For example, nanogram quantities of lysin could reduce 10^7 *S. pyogenes* by >6-log minutes after enzyme addition. No known biological compounds, except chemical agents, kill bacteria this quickly. Because of their highly effective activity against bacteria for the control of disease, the term "enzybiotics" was coined (Nelson et al. 2001) to describe these novel anti-infectives.

2 Structural Characteristics of Gram-Positive Lysins

Lysins from DNA phage that infect gram-positive bacteria are generally between 25 and 40 kDa in size except the PlyC lysin from the C1 phage of group C streptococci, which is 114 kDa. PlyC is unique because it is composed of two separate gene products, PlyCA and PlyCB. Based on biochemical and biophysical studies, the catalytically active PlyC holoenzyme is composed of eight PlyCB subunits for each PlyCA (Nelson et al. 2003). The eight PlyCB subunits spontaneously form a donut-like structure onto which the two PlyCA catalytic domains are non-covalently attached (McGowan et al. 2012). A feature of all other gram-positive phage lysins is their two-domain structure (Fig. 1). With rare exceptions (Diaz et al. 1990; Garcia et al. 1990), the N-terminal domain contains the catalytic activity of the enzyme. This activity may be either: i) an endo-β-N-acetylglucosaminidase or N-acetylmuramidase (lysozymes), both of which act on the glycan moiety of the wall peptidoglycan, ii) an endopeptidase which cleaves the stem peptide moiety, iii) an N-acetylmuramoyl-L-alanine amidase (or amidase), which hydrolyzes the amide bond connecting the glycan strand and peptide moieties (Loessner 2005; Young 1992), or an

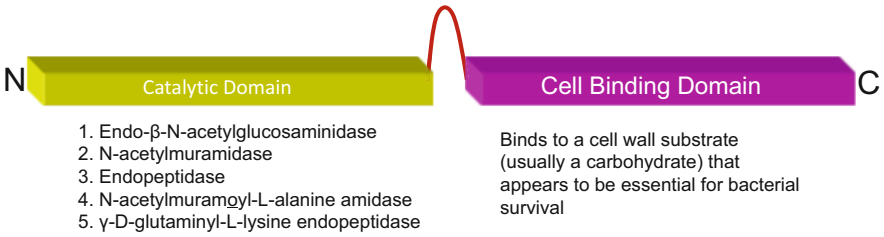

Fig. 1 Basic structure of phage lysins. In general, lysins range between 25 and 40 kDa in size and have a domain structure. The N-terminal domain is invariably the catalytic domain which cleaves one of the five major bonds in the peptidoglycan, and the C-terminal domain binds to a carbohydrate determinant in the cell wall

endopeptidase that specifically cleaves the cross-bridge. Recently a phage lysin with γ-D-glutaminyl-L-lysine endopeptidase activity has also been reported (Pritchard et al. 2007), but these enzymes are not as prevalent as the others. In some cases, particularly in staphylococcal phage and some streptococcal species of phage, two and even three different catalytic domains may be linked to a single binding domain (Cheng et al. 2005; Navarre et al. 1999). The C-terminal cell-binding domain (termed the CBD) on the other hand binds to a specific substrate (usually carbohydrate) found in the cell wall of the host bacterium (Garcia et al. 1988; Lopez et al. 1992, 1997). Efficient cleavage generally requires that the binding domain bind to its cell wall substrate, offering some degree of specificity to the enzyme since these binding substrates are only found in enzyme-sensitive bacteria. However, catalytic domains alone are able to cleave the peptidoglycan in some cases.

The first complete crystal structure for the free and choline-bound states of the Cpl-1 lytic enzyme, which lyses pneumococci, has been published (Hermoso et al. 2003). As suspected, the data suggest that choline recognition by the choline-binding domain of Cpl-1 may allow the catalytic domain to be properly oriented for efficient cleavage. An interesting feature of this lysin is its hairpin conformation suggesting that the two domains interact with each other prior to the interaction of the binding domain with its substrate in the bacterial cell wall (in this case choline). A second lysin, PlyL, encoded by a lysogen in the *Bacillus anthracis* genome, and a third, Ply21, from *B. cereus* phage TP21 have similar characteristics (Low et al. 2005). Their data suggest that the structure of PlyL and Ply21 are also in a hairpin conformation where the C-terminal domain blocks the activity of the catalytic domain through intramolecular interactions that are reversed when the C-terminal domain binds to its substrate in the cell wall freeing the catalytic domain to cleave its substrate. This suggests that lysins are autoregulated so that they are unable to interact with substrates in the cytoplasm that are precursors for cell wall assembly, but function only when they pass through the hole in the membrane to their peptidoglycan substrate. Such an inactivated enzyme would also be unable to kill potential host bacteria nearby if released from a lysed organism, thus preventing its competition with released phage.

When the sequences between lytic enzymes of the same enzyme class (i.e., amidases) are compared, high sequence homology is seen within the N-terminal catalytic region and very little homology in the C-terminal cell-binding region, suggesting that lysins are each tailored for a specific species. It seemed counterintuitive that the phage would design a lysin that was uniquely lethal for its host organism; however, as we learned more about how these enzymes and how they function, a possible reason for this specificity became apparent. As mentioned above, because of their specificity, enzymes that spill after cell lysis have a good chance of killing potential bacterial hosts in the vicinity of the released phage progeny therefore competing for phage survival. Thus, in addition to autoregulating the lysin's activity before contacting its substrate in the peptidoglycan, we believe that the gram-positive lysins have evolved binding domains that bind to their cell wall substrate at a high affinity (Loessner et al. 2002) to limit the release of free enzyme after lysis, so the enzyme remains bound to cell wall debris. This binding substrate in the cell wall appears to be unique with certain species. This does not appear to be the case for lysins produced by gram-negative phage. Thus, in two ways gram-positive phages protect the activity and release of active lysins to prevent competition with free phage.

Because of their domain structure, it seemed plausible that different enzyme domains could be swapped resulting in lysins with different bacterial and catalytic specificities. This was actually accomplished by early detailed studies of Garcia and colleagues (Garcia et al. 1990; Weiss et al. 1999), in which the catalytic domains of lytic enzymes from *S. pneumoniae* phage could be swapped resulting in a new enzyme having the same binding domain for pneumococci, but able to cleave a different bond in the peptidoglycan. In addition, DNA mutagenesis has been used to create lysins with higher antibacterial activity (Cheng and Fischetti 2007). These manipulations allow for enormous potential in creating designer enzymes with high specificity and equally high cleavage potential. In recent years, this idea has been capitalized upon and lysins have been engineered to achieve certain characteristics not present in native lysins (Daniel et al. 2010; Dong et al. 2015; Donovan et al. 2006). For a more complete review of the subject, please see Yang et al. (2014).

3 Structural Characteristics of Gram-Negative Lysins

At this time, only a few lysins from gram-negative phage have been characterized (Briers et al. 2011; Huang et al. 2014; Junn et al. 2005; Lai et al. 2011; Larpin et al. 2018; Lood et al. 2015). One would think that since phage lytic enzymes function from the inside to access and cleave the peptidoglycan, that lysins from both gram-positive and gram-negative bacteria would be the same, but they are largely different. Generally speaking most gram-negative lysins are lysozymes, i.e., muramidases or N-acetylmuramide glycanhydrolase without true binding domains as see with gram-positive lysins. The complexity of gram-negative lysins became apparent when Lood et al. (2015) combined the induced phage from several strains of *A. baumannii*, shotgun cloned their DNA, and determined which clones exhibited lytic activity.

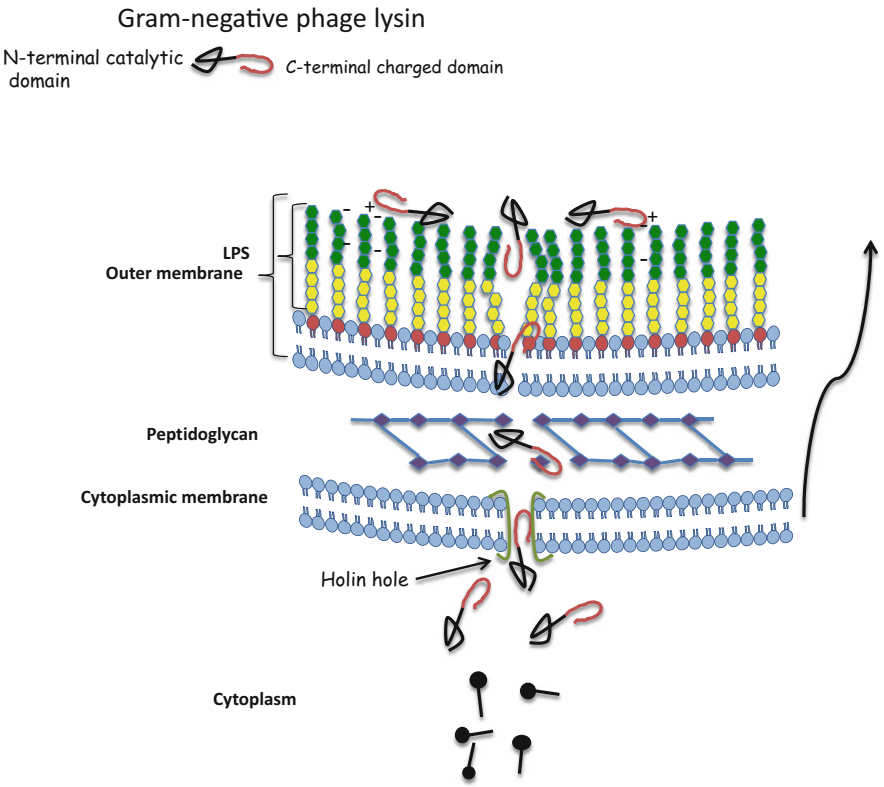

Fig. 2 Proposed model of the action of gram-negative lysins. Based on current data (Lood et al. 2015; Thandar et al. 2016), many gram-negative lysins have a lysozyme catalytic domain and a positively charged domain at the C-terminus. In this model, the two domains are represented in black and orange, respectively. As the lysin passes through the holin-induced hole in the cytoplasmic membrane, it interacts with the peptidoglycan, thereby cleaving the glycosidic bonds with the lysozyme domain. The lysin then interacts with the outer membrane where the positively charged domain destabilizes this region resulting in phage release. The positively charged domain would continue to interact with the negatively charged LPS preventing release of the lysin in the environment which could affect nearby bacterial hosts

When the inserts of several lytic clones were sequenced they found that the lysins fell into three distinct structural groups: (1) a TIGR02594 domain, (2) a catalytic domain and a binding domain like gram-positive lysins, and (3) a lysozyme domain. Both the TIGR02594 and lysozyme domains were flanked on either both or one side with a short positively charged domain, not previously reported. Since gram-negative bacteria have a lower internal turgor pressure (~3–5 atm) than their gram-positive counterparts (~15–25 atm), there may not be sufficient pressure to disrupt the integrity of the outer membrane after the bonds in the peptidoglycan have been cleaved. Thus, data suggests that the lysin's structural organization functions to both cleave the peptidoglycan with its enzymatic domain and disrupt the outer

membrane with the charged domain, resulting in more efficient lysis and phage release (Thandar et al. 2016) (Fig. 2).

4 Mechanism of Action

When examined by thin section electron microscopy, it seems obvious that lysins exert their lethal effects by forming holes in the cell wall through peptidoglycan digestion. The high internal turgor pressure of gram-positive bacterial cells is controlled by the highly cross-linked and thick peptidoglycan. Any disruption in the wall's integrity would result in the extrusion of the cytoplasmic membrane and ultimate hypotonic lysis (Fig. 3). Catalytically, a single enzyme molecule should be sufficient to cleave an adequate number of bonds to kill an organism; however, it is uncertain at this time whether this theoretical limit is possible. The reason comes from the work of Loessner (Loessner et al. 2002), showing that a listeria phage enzyme had a binding affinity approaching that of an IgG molecule for its wall substrate. This suggests that each lysin molecule could be a single-use enzyme, requiring several enzymes to effectively cleave enough bonds in a localized area of the cell to cause a lytic event. It should be noted that lysins from gram-positive phage closely resemble fungal cellulases, which are similarly constructed with both cell

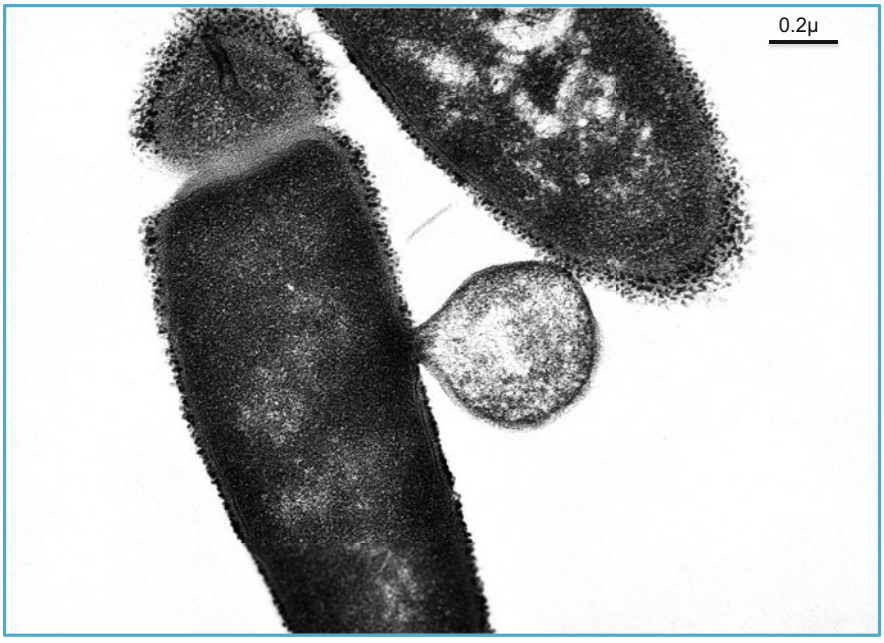

Fig. 3 Electron microscopy of lysin treated bacilli. One minute after treatment of *B. cereus* with lysin, membrane extrusion is observed prior to lysis and ultimate death of the bacterium

wall binding and catalytic domains joined by a flexible linker. It is unknown at this time whether lysins, like cellulases, use their binding domains to "bind and slide" across the wall peptidoglycan as they cleave (Jervis et al. 1997; Payne et al. 2015) or are simply enzymes that stay substrate-bound and are only able to cleave adjacent bonds, i.e., "one use enzymes."

5 Lysin Efficacy

In general, lysins only kill the species (or subspecies) of bacteria from which they were produced. For instance, enzymes produced from streptococcal phage kill certain streptococci, and enzymes produced by pneumococcal phage kill pneumococci (Loeffler et al. 2001; Nelson et al. 2001). Specifically, a lysin from the C1 streptococcal phage (PlyC) will kill group C streptococci, as well as groups A and E streptococci, the bovine pathogen *S. uberis* and the horse pathogen, *S. equi*, with essentially no effect on other gram-positive bacteria, particularly the commensal streptococci normally found in the oral cavity of humans. Unlike antibiotics, which are usually broad spectrum and kill many different bacteria found in the human body (some of which are beneficial), lysins which kill only the disease organism with little to no effect on the normal human bacterial flora may be identified. One of the most specific lysins reported is the lysin for *B. anthracis* (PlyG); this enzyme only kills *B. anthracis* and rare but unique *B. cereus* strains (Schuch et al. 2002). Another highly specific lysin is a chimeric lysin for staphylococci called ClyS (Daniel et al. 2010). Because ClyS is an endopeptidase that cleaves the peptidoglycan cross-bridge, and only staphylococci have polyglycine in their cross-bridge, this enzyme was shown to have lytic activity on all staphylococci and no other species of bacteria tested (Daniel et al. 2010).

In some cases, however, phage enzymes may be identified with broad lytic activity. For example, an enterococcal phage lysin PlyV12 has been reported to not only kill enterococci but also a number of other gram-positive pathogens such as *S. pyogene*s, group B streptococci, and *Staphylococcus aureus*, making it one of the broadest acting lysins identified (Yoong et al. 2004). However, its activity for these other pathogens was somewhat lower than for enterococci. Similar broad activity was seen for an enzyme called PlySs2 isolated from a *S. suis* phage (Gilmer et al. 2013) with strong activity against a number of other pathogens such as *S. aureus*, *S. suis*, *S. pyogenes*, *group B streptococci*, and *S. pseudintermedius*.

6 Lysin Synergy with Other Lysins and Antibiotics

Several lysins have been identified from pneumococcal bacteriophage which are classified into two groups: amidases and lysozymes. Exposure of pneumococci to either of these enzymes leads to efficient lysis. Both classes of enzymes have very different N-terminal catalytic domains but share a similar C-terminal choline-binding domain. When these enzymes were tested to determine whether their

simultaneous use is competitive or synergistic the results clearly showed that they are synergistic (Loeffler and Fischetti 2003). The same is true for endolysins directed to streptococci responsible for mastitis (Schmelcher et al. 2015). In vivo, the combination of two lysins with different peptidoglycan specificities was found to be more effective in protecting against disease than each of the single enzymes (Jado et al. 2003; Loeffler and Fischetti 2003; Schmelcher et al. 2015). Thus, in addition to more effective killing, the application of two different lysins may significantly retard the emergence of enzyme-resistant mutants.

When the pneumococcal lysin Cpl-1 was used in combination with certain antibiotics, a similar synergistic effect was seen. Cpl-1 and gentamicin were found to be increasingly synergistic in killing pneumococci with a decreasing penicillin MIC, while Cpl-1 and penicillin showed synergy against an extremely penicillin-resistant strain (Djurkovic et al. 2005). Synergy was also observed with staphylococcal-specific lysins and antibiotics with MRSA both in vitro (Fig. 3) (Daniel et al. 2010) and in vivo (Daniel et al. 2010; Rashel et al. 2007). A reason for this effect may be partially explained by the finding that the cell wall of MRSA is less cross-linked than MSSA (Trotonda et al. 2009), allowing small quantities of lysin to have a more dramatic effect on cell wall integrity. Thus, the right combination of enzyme and antibiotic could help in the control of antibiotic-resistant bacteria as well as reinstate the use of certain antibiotics for which resistance has been established.

7 Use of Lysins in Biological Fluids

Based on published findings, lysins from phage that infect gram-positive bacteria generally work in most, if not all bodily fluids: serum (Schmelcher et al. 2015; Witzenrath et al. 2009), saliva (Nelson et al. 2001), intestinal (Wang et al. 2015), and cerebral spinal fluid (Grandgirard et al. 2008). However, it is now clear that lysins from phage that infect gram-negative bacteria as well as Artilysins are unable to function effectively in the presence of serum (Briers et al. 2014; Larpin et al. 2018; Lood et al. 2015), perhaps as a result of binding to serum proteins by the positively charged region at the ends of these molecules (Thandar et al. 2016). Thus, until gram-negative lysins are engineered to avoid these interactions, they may be limited to topical use.

8 Animal Models of Infection

Animal models of mucosal colonization were used to test the capacity of gram-positive lysins to kill organisms on these surfaces, perhaps one of the more important uses for these enzymes. An oral colonization model was developed for *S. pyogenes* (Nelson et al. 2001), a nasal model for pneumococci (Loeffler et al. 2001), and a vaginal model for group B streptococci (Cheng et al. 2005). In all three cases, when the animals were colonized with their respective bacteria and treated with a single dose of lysin, specific for the colonizing organism, these organisms were reduced by

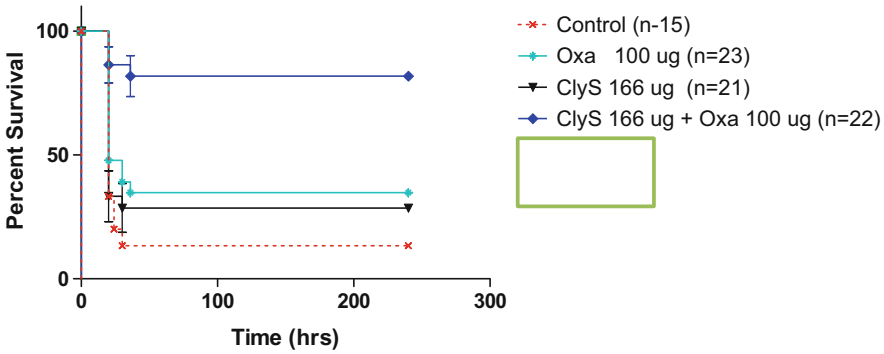

Fig. 4 Synergistic effects of ClyS and oxacillin protected mice from MRSA septicemia-induced death. Mice were intraperitoneally injected with ~5 × 10^5 CFU of MRSA strain MW2 in 5% mucin. Three hours postinfection, mice received an IP injection of a suboptimal concentration of ClyS (166 μg) or 20 mM phosphate buffer along with an IM injection of oxacillin (100 μg) or saline control. Mice were monitored for survival for 10 days, and the results of 5 independent experiments were combined and plotted in a Kaplan Meier survival curve

several logs (and in some cases below the detection limit of the assay) when tested again 2–4 h after lysin treatment. These results lend support to the idea that such enzymes may be used in specific high-risk populations to control the reservoir of pathogenic bacteria and thus control disease. A perfect example is the prevention of secondary infections after influenza. Recent studies reveal that 50–90% of deaths resulting from influenza are due to a secondary infection caused predominantly by *S. pneumoniae*, *S. aureus*, and *S. pyogenes* in that order (Brundage and Shanks 2007; Morens et al. 2009). Reducing the carriage of these mucosal pathogens during flu season would have a significant impact on disease.

Similar to other proteins delivered intravenously to animals and humans, lysins have a short half-life ($T^{½} = 15–20$ min) (Loeffler et al. 2003). However, the action of lysins for bacteria is so rapid that this may be sufficient time to observe a therapeutic effect (Jado et al. 2003; Loeffler et al. 2003) (Fig. 4). Mice intravenously infected with type 14 *S. pneumoniae* and treated 1 h later with a single bolus of 2.0 mg of Cpl-1 survived through the 48 h endpoint, whereas the median survival time of buffer-treated mice was only 25 h, and only 20% survival at 48 h. Blood and organ cultures of the euthanized surviving mice showed that only one Cpl-1-treated animal was totally free of infection at 48 h, suggesting that multiple enzyme doses or a constant infusion of enzyme would be required to eliminate the organisms completely in this application. Similar results were obtained when animals were infected and treated intraperitoneally with lysin (Jado et al. 2003; Rashel et al. 2007). Because of lysin's short half-life, it may be necessary to modify the lysins with polyethylene glycol or the Fc region of IgG, to extend its residence time in vivo to several hours (Walsh et al. 2003). In recent studies, phage lysins have also been shown to be successful in the treatment of meningitis by adding the lysin directly into the brain intrathecally (Grandgirard et al. 2008) and endocarditis by delivering

the lysin intravenously by constant IV infusion (Entenza et al. 2005). Both these applications would also benefit from modified long-acting lysins.

Important lysins with respect to infection control are those directed to *Staphylococcus aureus* (Clyne et al. 1992; O'Flaherty et al. 2005; Rashel et al. 2007; Sass and Bierbaum 2007; Sonstein et al. 1971). However, in some cases, these enzymes exhibited low activity or were difficult to produce in large quantities. In one publication (Rashel et al. 2007), a staphylococcal enzyme was described that could be easily produced recombinantly and had a significant lethal effect on methicillin-resistant *Staphylococcus aureus* (MRSA) both in vitro and in a mouse model. In animal experiments, the authors showed that the enzymes may be used to decolonize staphylococci from the nose of the mice as well as protect the animals from an intraperitoneal challenge with MRSA. However, in the latter experiments, the best protection was observed only if the lysin was added up to 30 min after the MRSA. Very similar results were published recently using a lysin termed LysGH15 (Gu et al. 2011). In a more recent publication, a chimera was produced linking the catalytic domain of the Twort phage lysin with the binding domain of a PhiNM3 lysin (Daniel et al. 2010). This chimera had eliminated many of the bad features of native staphylococcal phage lysins in its activity and production.

The crucial challenge for lysins would be to determine whether they are able to cure an established infection. To approach this, a mouse pneumonia model was developed in which mice were transnasally infected with pneumococci and treated with Cpl-1 by repeated intraperitoneal injections after infection was established (Witzenrath et al. 2009). From a variety of clinical measurements, as well as morphologic changes in the lungs, it was shown that at 24 h mice suffered from severe pneumonia. When treatment was initiated at 24 h and every 12 h thereafter, 100% of the mice survived otherwise fatal pneumonia and showed rapid recovery. Cpl-1 dramatically reduced pulmonary bacterial counts and prevented bacteremia. A similar result was seen in a pneumococcal bacteremia model in which mice were pretreated intravenously (IV) with 10^8 pneumococci. Mice with established bacteremia in 18 h were treated IV with a single dose of the pneumococcal-specific lysin Cpl-1. Mice beld 15 min and 120 min after treatment showed no bacterial counts in the blood (below detectible limits) (Fig. 5).

9 Bacterial Resistance to Lysins

Though several attempts have been made, thus far bacteria that are resistant to the lytic action of native lysins have not been reported. In experiments similar to those designed to reveal antibiotic resistance, lysin resistance has not been identified. For example, exposure of bacteria grown on agar plates in the presence of low concentrations of lysin did not lead to the recovery of resistant strains even after more than 40 cycles. Organisms in colonies isolated at the periphery of a clear lytic zone created by a 10 μL drop of dilute lysin on a lawn of bacteria always resulted in enzyme-sensitive bacteria. Enzyme-resistant bacteria could also not be identified after >10 cycles of bacterial exposure to low concentrations of lysin (from 5 to 20 units) in liquid culture (Loeffler et al. 2001; Schuch et al. 2002). These results

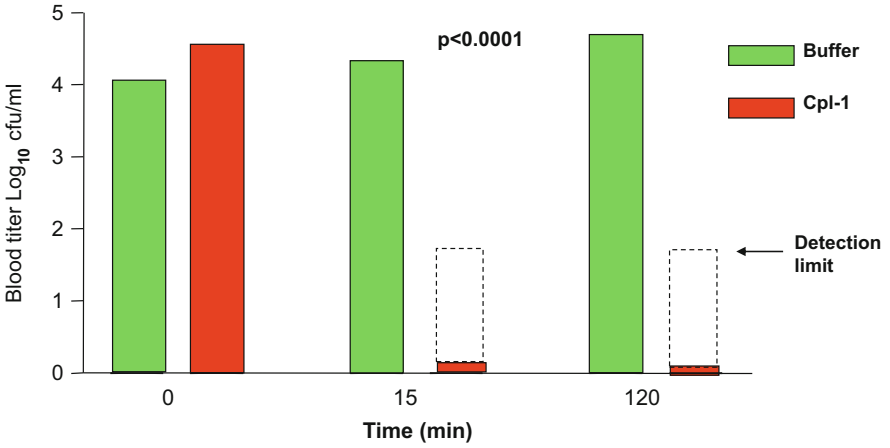

Fig. 5 Survival of bacteremic mice after treatment with Cpl-1. Mice pretreated IV with 10^8 pneumococci were bled 18 h later to determine the quantity of bacteria in the blood. Mice were then treated IV with a single 2 mg dose of Cpl-1, and groups were euthanized 15 and 120 min after treatment to determine remaining pneumococci. A single dose reduced the bacterial burden below detectible limit

may be explained perhaps by the fact that the cell wall receptor for the pneumococcal lysin Cpl-1 is choline (Garcia et al. 1983), a molecule that is essential for pneumococcal viability, and the receptor for the PlyG lysin, specific for *B. anthracis*, is the wall neutral polysaccharide, also essential for viability (Schuch et al. 2013). It is possible that lack of resistance is linked to the evolution of the binding domain, which is designed to prevent lysin spill during lysis. Since lysin spill after lysis would compromise the survival of the phage progeny, lysin binding domains evolved to bind to cell wall substrates that the bacteria could not change easily, ultimately targeting essential wall components. Because of this, resistance would be a very rare event, rarer than the frequency of antibiotic resistance.

10 Identifying and Isolating New Lysins

Gram-Positive Lysins There are a few ways in which lysins may be identified. The first and simplest is to go to the prokaryote sequence database and identify the genome sequence of the organism you are interested in killing with a lysin. Identify the phage lysin in a lysogen, synthesize the gene for that lysin and place it into an expression vector for production. If no genome sequence is available, or there are no lysogens in that species (which is rare), you will need to identify a phage from the environment for that species/organism, produce a high titer stock of the phage, shotgun clone its DNA, and identify lytic activity by overlaying the plated clones with the phage-sensitive bacterium. In this case, a lysin is identified for the organism or species that the phage infects. A more general way of isolating lysins is through

functional metagenomic analysis (Schmitz et al. 2008). This technique uses random environmental phage populations processed for metagenomic analysis. The twist here is to add an amplification step and an expression step to express and produce the products of the isolated lysin genes. This approach has the potential of identifying novel lysins with powerful biotechnological value. Another approach, which combines both the general and specific approaches mentioned above, is to exploit the lysogens in a host genome. This approach, termed multigenomics, identifies the lysin genes in the lysogens within many strains of the same species. In this case the DNA from tens to hundreds of strains of the same species are pooled and processed as in the metagenomic analysis, except here the lysins are from a single species (Schmitz et al. 2011). A related approach to multigenomics is to induce the phage from the various strains (10–50 is best) of a species with mitomycin, UV light, or some other inducing agent, pool the phage, extract the DNA, and shotgun clone to select for lysin-producing clones (Lood et al. 2015).

Gram-Negative Lysins Several lysins for gram-negative bacteria have been identified using some of the same methods used to isolate enzymes for gram-positive bacteria described above (Lai et al. 2011; Larpin et al. 2018; Lood et al. 2015).

11 Clinical Development

The need for antibiotic alternatives had been building over the last few decades and decisions need to be made regarding new therapies to combat antibiotic-resistant bacteria. In a recent review of possible antibiotic alternatives, Czaplewski et al. examined those therapies having the best probability of satisfying this need in the shortest time. They concluded, "On the basis of a combination of high clinical impact and high technical feasibility, the approaches anticipated to have the greatest potential to provide alternatives to antibiotics were phage lysins as therapeutics, vaccines as prophylactics, antibodies as prophylactics, and probiotics as treatments. . ." (Czaplewski et al. 2016).

Two companies, ContraFect and Intron Biotechnology, were the first to be in phase 1 human clinical trials in 2015 using different lysins against *S. aureus* (Czaplewski et al. 2016). In 2018, only ContraFect was actively in phase 2, testing the treatment of *S. aureus* bacteremia and endocarditis in hospitals worldwide with their lysin CF-301, (published as PlySs2 (Gilmer et al. 2013)). The results of that trial were released in early 2019. The results revealed that patients with MRSA bacteremia exhibited a 42% improvement over standard of care antibiotic treatment. Follow up studies on these patients showed that those treated with the combination of standard of care drugs plus lysin discharged from the hospital earlier and had fewer hospital requiring relapses than patients treated with standard of care drugs alone.

12 Concluding Remarks

Lysins are a new biologic to control bacterial pathogens, particularly those found on the human mucosal surface. For the first time, we may be able to specifically kill pathogens on mucous membranes without affecting the surrounding normal flora, thus reducing a significant pathogen reservoir in the population. Since this capability has not been previously available, its acceptance may not be immediate. Nevertheless, like vaccines, we should be striving to developing methods to prevent rather than treat infection. Whenever there is a need to kill bacteria, and contact can be made with the organism, lysins may be freely utilized. Such enzymes will be of direct benefit in environments where antibiotic-resistant gram-positive and now gram-negative pathogens are a serious problem, such as hospitals, day care centers, nursing homes, and the environment. The lysins isolated thus far are remarkably heat stable (up to 60 °C) and are relatively easy to produce in a purified state and in large quantities, making them amenable to these applications. The challenge for the future is to use this basic strategy and improve upon it, as was the case for second- and third-generation antibiotics. Protein engineering, domain swapping, and gene shuffling all could lead to better lytic enzymes to control bacterial pathogens in a variety of environments. Since it is estimated that there are 10^{31} phage on earth, the potential to identify new lytic enzymes as well as those that kill gram-negative bacteria in serum is enormous. Perhaps, someday phage lytic enzymes will be an essential component in our armamentarium against pathogenic bacteria.

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Part V

Phage Therapy: Regulatory and Ethical Aspects

Regulatory Considerations for Bacteriophage Therapy Products

Roger D. Plaut and Scott Stibitz

1 Background

The definition of the term "drug" in section 201(g)(1) of the US Food Drug and Cosmetic (FD&C) Act includes, among other things, "articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease…" and "articles (other than food) intended to affect the structure or any function of the body…" (Title 21 U.S. Code 2017). Biological products subject to licensure under section 351 of the Public Health Service Act fit within the drug definition under the FD&C Act. A biological product is "a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein (except any chemically synthesized polypeptide), or analogous product, … applicable to the prevention, treatment, or cure of a disease or condition of human beings" (Title 42 U. S. Code 2017). Thus, bacteriophage products intended to treat or prevent disease are considered biological products and drugs and are regulated by the Center for Biologics Evaluation and Research (CBER) at the Food and Drug Administration (FDA). Within CBER, product reviewers in the Division of Bacterial, Parasitic, and Allergenic Products in the Office of Vaccines Research and Review are responsible for reviewing the chemistry, manufacturing, and controls (CMC) information in applications for bacteriophage products. These product reviewers are part of a multidisciplinary team, including medical officers, toxicologists, statisticians, and regulatory project managers who review the original investigational new drug application (IND) and any subsequent amendments.

R. D. Plaut (✉) · S. Stibitz
Center for Biologics Evaluation and Research, US Food and Drug Administration, Silver Spring, MD, USA
e-mail: Roger.Plaut@fda.hhs.gov

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2 Regulatory Oversight of Biological Products

Regulations applicable to investigational biological products that meet the FD&C Act's definition of a "drug" can be found at 21 CFR Part 312 (Code of Federal Regulations Title 21, Part 312 2018). With certain limited exemptions (see 21 CFR 312.2(b)), these regulations require that clinical investigations in which a drug is administered to human subjects be conducted under an IND. Submission of an IND application provides the regulatory oversight for a sponsor to use an investigational product in humans. Although a sponsor's goal may be to develop a product for marketing in the United States, IND applications are also required for sponsors who intend to conduct human studies using unlicensed biological products for research purposes only (U.S. Department of Health and Human Services 2013).

Product development for biologics occurs in stages. To facilitate preliminary interactions with the Agency, CBER recently instituted the Initial Targeted Engagement for Regulatory Advice on CBER Products (INTERACT) meeting program. Under this program, a sponsor can meet with CBER informally before requesting a pre-IND meeting, to "obtain initial, nonbinding advice from FDA regarding chemistry, manufacturing and controls, pharmacology/toxicology, and/or clinical aspects of the development program" (U.S. Department of Health and Human Services 2018).

Prior to submitting an IND, a sponsor may choose to request a pre-IND meeting. In this formal process, a sponsor can initiate interaction with the FDA to discuss data needed to support first in-human testing, initial clinical trial design, regulatory requirements for demonstrations of safety and efficacy, and "CMC issues as they relate to the safety of an investigational new drug proposed for use in initial clinical studies" (U.S. Department of Health and Human Services 2001). The FDA highly recommends that a sponsor request a pre-IND meeting, to avoid unnecessary studies, to ensure that studies are designed optimally, and to minimize the likelihood of a study being placed on clinical hold (Code of Federal Regulations Title 21, Sec. 312.42 2018). Prior to the pre-IND meeting, the sponsor poses questions for CBER and provides a description of the sponsor's plans for the IND submission (Shapiro 2002). The questions should be as precise and comprehensive as possible, and the information provided should be sufficient to enable CBER reviewers to effectively address the sponsor's questions. CBER will assemble a multidisciplinary team, including product, clinical, toxicology (if needed), and statistical (if needed) reviewers, to provide responses to the sponsor's questions in advance of the meeting.

In the IND stage, human trials are conducted in sequentially larger numbers of study subjects. Phase 1 studies may focus solely on the safety of study participants treated with the investigational product; however, safety assessment remains a primary focus throughout the product development process. Phase 2 studies may be designed to find the optimal dose and/or duration of treatment and to provide some evidence of effectiveness. Data from Phase 2 studies can help inform the design of Phase 3 studies, which are intended to gather the additional information about effectiveness and safety that is needed to evaluate the overall benefit-risk relationship of the drug and provide an adequate basis for physician labeling. Manufacturing methods may be refined throughout the development process, but

by the end of Phase 3, the sponsor must have demonstrated an ability to consistently manufacture the drug product. Similarly, development and refinement of crucial assays advance throughout the IND stages. Assays that serve to provide assurance of drug substance or drug product quality, potency, or purity should be fully validated prior to conduct of Phase 3 studies, and assays that serve as clinical tests of critical study outcomes should be fully validated prior to their use in Phase 3 studies.

In a guidance document issued in July 2008 (U.S. Department of Health and Human Services 2008b), the FDA provided information regarding current good manufacturing practices (CGMP) for Phase 1 investigational drugs. Notably, the guidance states that some manufacturing controls and the extent of manufacturing controls needed to achieve product quality differ not only between investigational and commercial manufacture, but also among the phases of clinical trials.

In a biologics license application (BLA) submission, a sponsor submits, for in-depth FDA review, a comprehensive package of data to support licensure of the biologic. The FDA also conducts inspections of the manufacturing facilities, as warranted. For a biological product to be licensed, an applicant must demonstrate that the product meets standards designed to assure the "continued safety, purity and potency" of the product. In this context, potency has long been interpreted to include effectiveness (Code of Federal Regulations Title 21, Sec. 600.3 2018). The safety database and the types of evidence required to demonstrate effectiveness will depend on the proposed indication and the nature of the product and will be informed by discussions between the applicant and the Agency. After a BLA is approved, the applicant may submit supplements to the BLA, with data, to describe and support post-approval changes in manufacture, equipment, or facilities and/or to support changes in dosing or indication.

In addition to the INTERACT and pre-IND meetings described above, during the IND stage, sponsors typically meet with CBER at the end of Phase 2 and prior to submitting a BLA. Guidance regarding these interactions with the agency is available (U.S. Department of Health and Human Services 2001).

3 Chemistry, Manufacturing, and Controls for Bacteriophage Products

CBER regulates a wide variety of products, and guidance documents regarding development of biologics for human use are publicly available (U.S. Department of Health and Human Services 2008a). However, development of bacteriophage products presents some unique CMC considerations, which are further described below.

For most bacterial species, many bacteriophages in the environment can infect them; thus, the potential supply of naturally occurring bacteriophages for a given species may be quite large. However, the extent of naturally occurring bacteriophage diversity varies among bacterial species. In addition, the details of the multiple modes of interaction between any given bacteriophage and bacterial host pair are unique. Therefore, drawing general conclusions about such interactions based on

knowledge of a limited number of bacteriophage–host pairs is problematic. For this reason, the IND should include sufficient information to assure the proper identification, quality, purity, and strength of the bacteriophage preparation intended to be used in the proposed human clinical trials.

Although some bacteriophages are capable of infecting multiple species of bacteria (Greene and Goldberg 1985; Harshey 1988; Yarmolinsky and Sternberg 1988; Jensen et al. 1998; Pantucek et al. 1998; Khan et al. 2002; Sullivan et al. 2003; O'Flaherty et al. 2005; El-Arabi et al. 2013; Khan Mirzaei and Nilsson 2015), bacteriophages are generally considered to be very specific. This specificity is often considered to be a beneficial aspect of bacteriophage therapy. Less disruption of the native microbiota is expected than with the use of broad-spectrum antibiotics. However, this aspect of bacteriophage biology complicates the treatment of bacterial infections with bacteriophages in that effective treatment is expected to necessitate prior identification of the infecting species and may require an assessment of the activity of the bacteriophage preparation against the patient's bacterial isolate(s). The specificity of bacteriophages is most often attributed to bacteriophage–receptor interactions. However, internal cellular functions in bacteria, such as gene expression and DNA replication, can also affect the host ranges of bacteriophages (DeWyngaert and Hinkle 1979; Qimron et al. 2006; James et al. 2010; Azam et al. 2018).

A sponsor can benefit from investigating the host range of the bacteriophage(s) in a product under development. This information can inform decisions regarding whether to include multiple bacteriophages in the product and, if so, which bacteriophages should be chosen.

Bacteriophages can be immunogenic, and an adaptive response to bacteriophage treatment is likely. The human immune response may limit the length of time over which the treatment would be expected to remain efficacious, although few studies of this issue have been published. In addition, the extent to which potential antibody–bacteriophage complexes or other consequences of the immune response to bacteriophages could represent safety concerns is unclear. The extent of the immune response may depend on the route of administration. Sponsors may wish to include analyses of the occurrence of immune responses to their products, and their effects on the proposed therapy, in animal studies and proposed human clinical trials.

Some bacteriophages are capable of mediating transfer of genes among bacteria. One of the mechanisms by which this can occur is lysogenic conversion, in which integration of the bacteriophage into the genome of the host bacterium provides a potential selective advantage to the host bacterium. For example, some bacteriophages that carry toxin genes are capable of lysogeny, after which the host bacterium is rendered more virulent than it was previously. Bacteriophages that mediate lysogenic conversion are not good candidates for therapeutic use, because they could increase the virulence of bacterial strain(s) carried by the patient. In addition, bacteria that become lysogenic for a given bacteriophage typically also become immune to killing by that bacteriophage, thus reducing its effectiveness.

Transduction is another mechanism by which bacteriophages can mediate genetic transfer. In generalized transduction, segments of host bacterial DNA are randomly packaged into bacteriophage particles. When these transducing particles encounter new bacterial hosts, although no productive infection can occur (because the particles do not carry the full bacteriophage genome), the particles can inject their DNA, which, through homologous recombination, can be incorporated into the genome of the bacterial host. The result is transfer of bacterial DNA from one bacterium to another. Because of the randomness of the bacteriophage packaging process, in generalized transduction, all chromosomal sequences can be transduced with roughly equal frequency.

Generalized transduction can be detected using microbiological or molecular biological tests. In a microbiological transduction assay, a bacteriophage is propagated on a strain carrying a selectable marker. The bacteriophage lysate is then applied to a strain lacking that marker, and the mixture is plated on appropriate selection media. The transduction frequency can be calculated by dividing the number of resulting colonies by the titer of the lysate.

In a molecular biological assay, the presence of transducing particles in a lysate can be detected by using PCR primers specific for bacterial host sequences, such as 16S ribosomal RNA genes (Sander and Schmieger 2001; Beumer and Robinson 2005; Del Casale et al. 2011). In such an experiment, proper controls must be used to establish the sensitivity of the assay. Importantly, methods for sequencing bacteriophage preparations, even using current next-generation sequencing approaches, are unlikely to be sensitive enough to detect transducing particles, which, if present in a preparation, are likely to be present only at a low frequency and will be heterogeneous in sequence; such methods are therefore not appropriate for this purpose.

In specialized transduction, when a prophage excises from the bacterial host genome, DNA adjacent to the prophage may be packaged into bacteriophage particles due to imprecise excision of the prophage. As in generalized transduction, these particles can inject their DNA into new bacterial hosts, and recombination can occur. However, in this case, only sequences near the site of prophage insertion can be packaged and have the potential to be transduced.

Bacteriophages that are capable of generalized or specialized transduction are not ideal candidates for therapeutic use, because of their ability to transfer antibiotic resistance genes or virulence genes from strains used for bacteriophage propagation to strain(s) carried by the patient. Another scenario relates to contexts in which multiple related species or strains of bacteria may coexist, such as in the human gut. If some of these bacteria carry antibiotic resistance or virulence genotypes and others do not, transduction would be undesirable, because it could mediate the inter-strain transfer of such undesirable genotypes and phenotypes.

Notably, the use for therapy of bacteriophages that are not capable of lysogeny addresses the potential for both lysogenic conversion and specialized transduction. As with generalized transduction, the ability of bacteriophages to undergo lysogeny can be evaluated using multiple methods. In a microbiological test, bacteriophage plaques can be examined for turbidity. If the plaques are turbid, lysogeny has probably occurred; however, if the plaques are clear, lysogeny may have occurred

but may not be detectable by eye. If a sterile toothpick or pipette tip is touched to the center of the plaque and used to streak a plate of the appropriate medium, bacteria that grow may be lysogens. These colonies should be re-tested for resistance to the bacteriophage; if resistant, they can be confirmed to be lysogens if the bacteriophage can be detected after spontaneous excision or after induction with mitomycin C or ultraviolet radiation.

Molecular biological methods can also be used to determine whether a bacteriophage is likely to be capable of lysogeny. Bacteriophage DNA can be sequenced and analyzed for the presence of repressor genes, integrase genes, other homology to known lysogenic bacteriophages, or other indicators of a lysogenic lifestyle. In addition, the genome sequence of a putative lysogen can be analyzed for the presence of a prophage that is absent from the parent strain. This approach will provide solid proof that a given bacteriophage is lysogenic and will also provide the full sequence of the bacteriophage genome.

Considering the biological characteristics of bacteriophages described above, a consensus among scientists in academia, industry, and regulatory agencies has emerged of the appropriate attributes of bacteriophages intended for use in therapy. The bacteriophage genome should not include genes for virulence factors or for antibiotic resistance. Bacteriophages should be non-lysogenic, to avoid the issues of lysogenic conversion and specialized transduction. Ideally, bacteriophages should also be incapable of generalized transduction. If a bacteriophage intended for therapy is found to be transducing, it should be propagated on a strain that is non-pathogenic, lacks determinants of antibiotic resistance that could be easily transduced, and lacks prophages that could reduce the purity of the bacteriophage preparation. In some contexts, this approach to limiting the transfer of undesirable genes may not be sufficient, and a demonstration that the bacteriophages are non-transducing may be required.

To address the IND requirement for sufficient information to assure proper identification, quality, purity, and strength of investigational drugs, sponsors of IND applications for bacteriophage-related products generally provide the following:

- Sources and histories of the bacteriophages
- Whole genome sequences and annotations of the bacteriophages, including analyses for the presence of known virulence factors and antibiotic resistance elements, with explanations of how these analyses were conducted
- Evidence regarding whether the bacteriophages are capable of lysogeny
- Evidence regarding whether the bacteriophages are capable of generalized transduction

In addition, sponsors have provided information regarding the bacterial host strains used to propagate the bacteriophages, including, but not limited to, the following:

- Sources and histories of the host strains
- Whole genome sequences and annotations of the host strains, including analyses for the presence of known virulence factors, antibiotic resistance elements, and prophages, with explanations of how these analyses were conducted

Bacteriophage preparations for therapeutic use should be as pure as possible. Endotoxin levels should be low, although the allowable limits may depend on the route of administration (e.g., topical versus oral versus intravenous). The allowable bioburden of bacteriophage preparations will also depend on the route of administration. Intravenous products, for example, are expected to be sterile.

Research in the field has identified some additional characteristics that could be considered desirable (albeit not required) for bacteriophages intended for therapeutic use. For example, following serial passage in mice, bacteriophage mutants have been isolated that persist longer in the bloodstream (Merril et al. 1996), potentially extending the length of time during which the bacteriophages can encounter their bacterial targets. In addition, some bacteriophages have been found to use virulence factors (Yen and Camilli 2017) or antibiotic resistance proteins (Chan et al. 2016) as receptors; in these cases, bacteria that develop resistance to the bacteriophage via mutation of the receptor become less virulent or more susceptible to antibiotics, either of which is a positive step toward resolution of the infection.

Mixtures of bacteriophages (also called cocktails) have been proposed to be useful for widening the spectrum of treatment, i.e., for use against multiple species of bacteria or multiple strains of a bacterial species. In addition, bacteriophage cocktails are expected to be helpful in reducing the likelihood of emergence of bacterial resistance to the treatment; this approach is similar to the use of multiple anti-infective drugs to treat tuberculosis or HIV. The rationale is that the acquisition of resistance to multiple bacteriophages is expected to be less likely than the acquisition of resistance to a single bacteriophage. This rationale depends on different genetic means of resistance for each of the bacteriophages such that mutation to resistance to one is not mutation to resistance to all. The design of effective cocktails can thus be informed by determination of receptors for candidate bacteriophages and/or by empirical means to examine the ability of different combinations to suppress growth of target strains in vitro (Henry et al. 2012; Estrella et al. 2016; Schooley et al. 2017).

In a cocktail, each bacteriophage should have relevant activity. Potency tests and stability tests should be capable of assessing the quantity of viable bacteriophage particles for each component bacteriophage in the preparation. Importantly, if a mixture is proposed to be modified by adding bacteriophages or replacing one or more of the bacteriophages, adequate CMC information regarding the new bacteriophages should be provided. This requirement exists because, as noted above, the IND regulations require adequate information to assure the proper identification, quality, purity, and strength of the treatment.

4 Expanded Access to Investigational Drugs for Treatment Use

Recent publications have described the use of bacteriophage therapy to treat individual patients in the United States under expanded access provisions (Schooley et al. 2017; Aslam et al. 2018; Chan et al. 2018; Furr et al. 2018; LaVergne et al. 2018). Expanded access to investigational drugs for treatment use facilitates availability of investigational drugs to patients with serious diseases or conditions when there is no comparable or satisfactory alternative to diagnose, monitor, or treat the patient's disease or condition (Code of Federal Regulations Title 21, Part 312, Subpart I 2018). Expanded access refers to use of an investigational drug when the primary purpose is to diagnose, monitor, or treat a patient or patients rather than to obtain the type of information that is generally derived from clinical trials (U.S. Department of Health and Human Services 2016). Because expanded access INDs are not intended for this type of systematic collection of safety or effectiveness data to support licensure, they are not substitutes for adequate and well-controlled clinical trials.

There are three categories of expanded access: (1) for individual patients, including for emergency use; (2) for intermediate-size patient populations (generally smaller than those typical of a treatment IND or treatment protocol); or (3) for widespread treatment use through a treatment IND or treatment protocol (designed for use in larger patient populations). In general, the amount of evidence required to allow treatment to proceed increases as the number of individuals to be treated increases. For all expanded access uses, informed consent of the patient(s) is required, and the FDA must determine that (1) the patient(s) has a serious or immediately life-threatening disease or condition for which no comparable or satisfactory alternative to diagnose, monitor, or treat the disease or condition exists; (2) the potential benefit justifies the potential risks of the treatment use, and the potential risks are not unreasonable in the context of the disease or condition to be treated; and (3) providing the investigational drug for the proposed use will not interfere with the initiation, conduct, or completion of clinical investigations that could support marketing approval of the product for the expanded access use or otherwise compromise the potential development of the expanded access use (Code of Federal Regulations Title 21, Sec. 312.305 2018).

For each category of expanded access, there are two types of regulatory submissions that can be made: (1) an expanded access protocol submitted as a protocol amendment to an existing IND (i.e., an expanded access protocol) or (2) a new IND submission, which is separate and distinct from any existing INDs and is intended only to make a drug available for treatment use (i.e., an expanded access IND).

For individual patient expanded access, the Agency must determine that the patient cannot obtain the product under another IND or protocol. The application can be filed by a treating physician or by the sponsor of a relevant IND. Institutional review board (IRB) approval is required prior to treatment (Code of Federal Regulations Title 21, Sec. 312.310 2018).

An individual patient IND that provides expanded access to an investigational drug for treatment use by a single patient in an emergency situation (i.e., a situation that requires a patient to be treated before a written submission can be made) can be submitted under a new IND (Code of Federal Regulations Title 21, Sec. 312.310 2018). If the treating physician determines that the patient's condition constitutes an emergency that requires the patient to be treated before a written submission can be made, emergency expanded access use may be requested by telephone or other rapid means of communication (U.S. Department of Health and Human Services 2017). The FDA may authorize the emergency use by telephone. In such a case, an expanded access submission must be submitted within 15 working days after FDA's authorization of use, and the IRB must be informed within five working days of emergency use. For an individual patient emergency expanded access IND, the physician sponsor should provide a clinical history of the patient and a treatment plan. For a bacteriophage preparation, information regarding the source of the bacteriophage(s), titer (plaque-forming units [PFU]/mL), endotoxin content, sterility, and results of tests of the activity of the preparation against the bacterial strain (s) infecting the patient (if available) is desirable. This information can be provided through a letter of authorization from the manufacturer, which enables the physician to cross-reference the manufacturer's existing IND or master file for CMC information about the product. The decision whether to allow an emergency IND to proceed will depend on balancing the risks and potential benefits as assessed by the treating physician.

For an intermediate-size patient population expanded access use of an investigational drug, in addition to meeting the criteria in 21 CFR 312.305, the FDA must determine that (1) there is enough evidence that the product is safe at the dose and duration of treatment that is proposed, and (2) there is at least preliminary evidence of effectiveness of the drug, or of a plausible pharmacologic effect of the drug, to make expanded access use a reasonable therapeutic option in the anticipated patient population. If no prior IND exists, the sponsor must apply for one, which will go into effect 30 days after it is received by the FDA, or earlier if the FDA so notifies the sponsor. If there is an existing IND, the sponsor of that IND should apply for an intermediate-size patient population expanded access IND protocol. In such a case, treatment may commence after IRB approval is granted.

Lastly, an expanded access treatment IND or treatment protocol requires that the sponsor be actively pursuing, with due diligence, marketing approval of the drug for the expanded access use. These types of applications are intended for treatment of large (widespread) populations, and they are generally only appropriate when data have already been obtained from Phase 2 or 3 clinical trials. All requirements under 21 CFR 312.305(a) (described above) must be met. Expanded access treatment INDs and treatment protocols go into effect 30 days after receipt by the FDA, or earlier if the FDA so notifies the sponsor.

5 Defined Bacteriophage Products Versus Bacteriophage Banks

Approaches to bacteriophage therapy can be separated into two major categories. First, in an approach involving a defined product, a bacteriophage or a mixture of bacteriophages intended for a specific indication and/or bacterial infection is mass-produced. In a second approach, a bacteriophage bank (a collection of many bacteriophage preparations) is instituted with the intent of providing personalized therapy. In this scenario, the strain infecting the patient is isolated, and in vitro testing is conducted to determine which bacteriophages in the bank are active against the strain. Based on results of the testing, a cocktail may be formulated for the patient. For this scenario, questions remain regarding whether safety and effectiveness information regarding a limited subset of bacteriophages in the bank can be reasonably extrapolated across other bacteriophages in the bank. In addition, it is perhaps even less clear whether for a given bacteriophage, information regarding its use for a particular indication or route of administration can be reasonably extrapolated across different indications or routes of administration. Both approaches to bacteriophage therapy have advantages and disadvantages. Clinical studies performed under IND will provide the information needed to evaluate the safety and effectiveness of these products.

6 Conclusions

Chemistry, manufacturing, and controls information included in an IND application is an important component of successful clinical trials necessary to establish the safety and effectiveness of biological products, including bacteriophage products. The regulatory review process is not an obstacle to the assessment of the safety and effectiveness of bacteriophage therapy. Regulatory agencies, academia, and industry have shared goals and need to cooperate to bring them to fruition. Communication is vital to this cooperative effort. In this spirit, CBER highly recommends that investigators meet with the Agency early in the process of development of bacteriophage products.

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Developing Phages into Medicines for Europe

Eric Pelfrene, Zigmars Sebris, and Marco Cavaleri

1 Introduction

Considering the context of the growing threat of bacterial antimicrobial resistance (AMR) with potentially far-reaching health and economic impacts, bacteriophage therapy has been proposed as one novel strategy in countering this prospect (Czaplewski et al. 2016). Indeed, various historical data sources might suggest that bacteriophages could be safe and efficacious in treating both Gram-positive and Gram-negative bacterial infections, including multi-drug resistant (MDR) organisms (Sulakvelidze et al. 2001). This notion is further supported by more recent experience, such as reports on systemic phage administration in severely ill patients, suffering a difficult-to-treat infection (Jennes et al. 2017; Schooley et al. 2017). Nevertheless, comparative data remain sparse (Rhoads et al. 2009; Wright et al. 2009; Sarker et al. 2016), and convincing evidence from well-designed and rigorously conducted clinical trials is awaited to support introduction of bacteriophages into clinical practice.

Bacteriophages are classified by regulatory authorities as biological substances and fall within the scope of the pharmaceutical legislation (Pelfrene et al. 2016; Reindel and Fiore 2017). Mainly, whole-phage broad cocktails manufactured on an industrial scale may target a single or even multiple species, or conversely, a patient-specific cocktail can be selected from a local phage library (Pirnay et al. 2011). However, the phage concoctions do not easily align with the conventional concept of a medicinal product. Over the past few years, discussions between product developers

E. Pelfrene (✉) · M. Cavaleri
Office of Anti-infectives and Vaccines, Human Medicines Evaluation Division, European Medicines Agency, Amsterdam, The Netherlands
e-mail: eric.pelfrene@ema.europa.eu; marco.cavaleri@ema.europa.eu

Z. Sebris
Regulatory Affairs Office, Human Medicines Evaluation Division, European Medicines Agency, Amsterdam, The Netherlands
e-mail: zigmars.sebris@ema.europa.eu

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and regulatory authorities have intensified on how the pharmaceutical regulation could offer flexibility in outlining the appropriate tests and studies prior to routine acceptance of bacteriophage intervention. In this light, the present contribution reviews the European regulatory requirements for bacteriophage therapeutics, reflects on some of the scientific hurdles and provides thoughts on how the licensing authority could support the specificities of phage therapeutic development.

2 EU Regulatory Framework

Bacteriophages meant for therapeutic administration are governed by the European regulatory framework on medicinal products and more specifically resort as biological products (Directive 2001/83/EC). This framework is applicable to whole phage products, either natural or recombinant, as well as phage-derived products, such as endolysins. It stipulates that for medicinal products "either prepared industrially or manufactured by a method involving an industrial process", a Marketing Authorisation (MA) constitutes a prerequisite. Hence, prior to placing the product on the market, it would be required that besides proving to be safe and efficacious for a given indication, quality can be assured with a manufacturing under current Good Manufacturing Processes (cGMP). The legislation calls also for predetermined "Qualitative and quantitative particulars of all the constituents of the medicinal product", and in case of differences, a separate authorisation may be necessary. Within the current context, it is however appreciated that this would be easier to suit phage-derived proteins and commercially prepared whole phage cocktails, rather than patient-specific concoctions consisting of lytic bacteriophages selected from a local phage library (i.e. local stock containing the active substances).

In absence of a specific quality guideline on bacteriophages, it is advised that the existing guidance pertaining to biotechnology and biological products broadly be followed: bacteriophages (as drug substances) and the final preparation (as drug product) need to be appropriately characterised and include a well-validated production process. Without being exhaustive on these requirements, the following principles apply (Parracho et al. 2012; Pelfrene et al. 2016):

1. Bacterial cell banking systems (cell substrates) should be devoid of prophages and lack antibiotic resistance.
2. Individual bacteriophages should display a lytic activity (i.e. not involving temperate phages) and specifically infect the bacterial isolate.
3. Preparations need to be controlled for impurities (endotoxins, pyrogenic exotoxins, host cell proteins and DNA and residual reagents).
4. Phages need testing for potency and purity (absence of adventitious phages and plasmids, bioburden and sterility).

As limitation to the aforementioned, it is acknowledged that identification and quantification of each individual phage in the Drug Product could prove to be demanding.

Apart from the above, the European Union legislation allows a few exceptions on the requirement to obtain a product licence; e.g. this applies to the magistral formula and the officinal formula, under Article 3 of the Directive (Directive 2001/83/EC), and as well for any advanced therapy medicinal product (ATMP) (EU-Regulation (EC) No 1394/2007), if prepared on a "non-routine" basis according to specific quality standards and meant as a custom-made product within the same EU Member State in a hospital under the exclusive professional responsibility of a medical practitioner. As such, if an applicant would develop a phage product that expresses a recombinant nucleic acid, classification as an ATMP could be sought, with the possibility to obtain such a "Hospital Exemption". Otherwise, specific authorisation requirements could anyway apply for recombinant phage products recognised as ATMP products, including the possibility of a risk-based approach to determine the amount of quality, preclinical and clinical data required in obtaining a MA. However, additionally, recombinant phage therapy would need to conform to the environmental regulation governing the deliberate release of genetically modified organisms (GMO) (Directive 2001/18/EC).

The exemption from EU licensing requirements are foreseen also under Article 83 of Regulation (EC) 726/2004 of the European Parliament and of the Council concerning "compassionate use" (EU-Regulation (EC) 726/2004). Although under remit of the Member States, this allows an unauthorised product to be made available to a group of patients who cannot satisfactorily be treated with currently licensed options and who cannot be enrolled in ongoing clinical trials. Eligibility to take part in such a programme is only possible when the medicinal candidate undergoes clinical trials or a submission has been made by the sponsor to obtain a product licence. As such, it transpires that compassionate use cannot be regarded as a permanent satisfactory regulatory option for phage therapy.

3 Preclinical and Clinical Development

In developing phage therapy, preclinical tests would provide an important contribution to establish proof-of-concept in support of the intended clinical use, including the route of administration, type of infection, whether as adjunctive therapy, concomitantly or sequentially, or for prevention. Pharmacodynamic studies would also contribute to dose selection and to characterise the potential for emergence of resistance. Additional tests to be conducted would mainly cover toxicity and immunogenicity potential. It is however remarked that no standardised methods for in vitro activity and susceptibility are currently available. Hence, as previously advocated by others, an international standard for preclinical effect would be desirable and provide opportunity to establish comparative data (Cooper et al. 2016).

In a classical trial setting, clinical development through stages aims to gather evidence that the therapeutic is safe and efficacious for its intended use in a well-defined patient population. It is however accepted that early phase tests conducted in healthy volunteers will not capture potential outcomes specific to the bacteriophage-bacterial host interaction. Further on, product development would need to establish

the appropriate exposure-response relationship of the therapeutic intervention, explore the role of the immune system in phage removal (including the influence of pre-existing antibodies generated in response to the abundant environmental exposure) and test with an appropriate dosing regimen for safety and efficacy in a large enough group of patients suffering a specified bacterial infection caused by specified species or strains of bacteria. Late stage clinical trials might as well be challenging in avoiding inclusion of a heterogeneous study population (e.g. due to differences in bacterial burden and host immune factors) and the limited host range of bacteriophages may necessitate broad enough cocktails to be effective. With therapeutic use, it is expected that the phage resistance profile of bacteria will evolve, necessitating adjustments to such cocktail composition. Thus, the therapeutic development will need to take account of some unique circumstances. Moreover, the ultimate goal of therapy might be different from increased cure rates as traditionally investigated with standard antibacterial agents and thus could cover other clinical benefits such as time-to-cure, relapse rates or else.

3.1 Clinical Pharmacology

Unlike conventional antimicrobials, whole bacteriophages are large size particles with poor diffusion capacity in nonaqueous media; only a small dose can be administered, with the antibacterial activity fully dependent on generating a "productive infection", i.e. new bacteriophages emerging upon lysis of the host bacteria, leading to their exponential amplification (Marza et al. 2006). This outcome can only hold true if enough bacteriophages can reach the bacterial target in first place, hitting it in a rather direct fashion. As such, they lend themselves ideal to be locally applied at the infection source, although for systemic use, virulent phages (fast producing a great number of progeny) might largely overcome such a limitation (Nilsson 2014). Thus, upon parenteral administration, resultant phage blood concentrations cannot be fully reflective of the activity at the site of infection. Following IV administration, whole bacteriophages are rapidly diluted and cleared from the bloodstream by combined action of the innate and adapted immune system. Although direct removal by the reticuloendothelial system (phagocytes) seems the most important mechanism involved, the potential for generating neutralising antibodies is well recognised (Hodyra-Stefaniak et al. 2015). In this regard, higher anti-phage antibody responses have been observed with the use of cocktails (viz. monovalent therapy) and also with longer treatment duration (Górski et al. 2018). A recently published analysis indicates that a good clinical outcome can nevertheless be expected in those developing high antibody titres during therapy involving oral or local phage administration (Łusiak-Szelachowska et al. 2017). With intravenous use, however, sufficient data are lacking, and a potential detrimental effect caused by high antibody levels cannot be dismissed.

For whole phage therapy, the composition of the cocktail will be critical to its success, and in all likelihood not each individual phage will successfully infect the bacterial target. The aim however is that component strains (ideally infecting via

different receptors) will achieve synergy, resulting in a fast reduction of bacterial density and residual clearance of the bacterial infection by host immunity. Other factors could be considered as well in deciding the composition of the cocktail, such as the capacity to evade phage resistance systems harboured in the host bacteria (Nilsson 2014).

Phage lysins are expected to behave in a more conventional way. To date, research mainly targets elimination of Gram-positive organisms, both systemically and from mucosal surfaces and biofilms (O'Flaherty et al. 2009; Schuch et al. 2017). In this regard, a multicentre, double-blinded, randomised, phase 2 trial has recently commenced in patients with MRSA bacteraemia (including endocarditis) and receiving anti-staphylococcal lysin or placebo, added to standard-of-care antibiotics [ClinTrial.gov NCT03163446] (Fischetti 2018). The development of engineered lysins able to penetrate the outer membrane of Gram-negative bacteria (Briers et al. 2014) may further broaden the appeal for their use as antibacterial products in difficult-to-treat infections.

3.2 Resistance

It has been hypothesised that bacteriophages characterised by fast adsorption rates and large burst size—so ensuring a quick reduction of the bacterial population—would minimise the emergence of resistance during treatment (Nilsson 2014). However, further data are warranted as the emergence of resistance following therapeutic use of phages seems inevitable and accordingly, cocktails will need adaptation at a certain time. Under the current paradigm, the resultant changes in qualitative and quantitative composition of the constituents of the industrial phage cocktail would trigger a rather time-consuming regulatory procedure and, depending on the extent of changes, may even require a new authorisation. As such, it might be desirable to accommodate this requirement for cocktail adaptation by granting a composition change in an expedited manner. Certain precedents exist in the EU pharmaceutical legislation, e.g. for influenza vaccines, for which changes in viral strain antigens are dealt with in an abbreviated fashion (EU-Regulation (EC) No 1234/2008; EMA-Guideline on influenza vaccines 2015). However, in case of bacteriophage therapies, it is still necessary to identify the exact nature of changes that would be required in practice and achieve a scientific consensus on the type and extent of data needed to assure that such changes would not adversely affect the efficacy or safety of the product.

3.3 Indications

Criteria for selecting suitable diseases depend on characteristics of infection, bacteria involved and nature of the bacteriophages themselves (Harper 2018). Use of obligatory lytic bacteriophages with broad strain coverage, limited number of bacterial species causing the pathology and sufficient accessibility of the infection site might increase the likelihood for a successful trial outcome. Hence, topical treatment seems

a most attractive proposition for this technology, e.g. in treatment of otitis media (Wright et al. 2009), diabetic foot ulcer infections (Fish et al. 2016) and as bladder instillation in treatment of urinary tract infection (Leitner et al. 2017). The ability for bacteriophages to disrupt biofilms also counts as an interesting feature to be exploited (Harper et al. 2014; Chan and Abedon 2015). In this sense, Chan et al. (2018) reported on successful phage treatment of a *Pseudomonas aeruginosa* chronically infected aortic graft with associated aorto-cutaneous fistula (Chan et al. 2018). Interestingly, for the case management, the authors indicate leveraging phage-resistance versus antibiotic-sensitivity trade-off, emphasising that understanding of evolutionary biology may help inform future phage therapy strategies. For oral administration—although previously demonstrated as innocuous in healthy volunteers (Bruttin and Brüssow 2005; Sarker et al. 2012; McCallin et al. 2013) and patients alike (Sarker et al. 2016)—a recent murine experiment cautions that orally provided bacteriophages may lead to increased gut permeability and systemic inflammation (Tetz and Tetz 2016). Others however could not confirm such finding in mice and pigs (Hong et al. 2016); moreover Górski's group recently commented upon the role of phages in maintenance of gut immune homeostasis and their capacity to downregulate activation of immune responses, providing an avenue for their therapeutic potential in inflammatory bowel disease and other conditions thought to be influenced by gut microbiota dysbiosis (Górski et al. 2017). This will require further validation through well-designed translational and confirmatory clinical research. Separately, with parenteral administration, concern has been expressed on the potential to invoke a cytokine-mediated inflammatory cascade upon rapid lysis of Gram-negative bacteria (Wittebole et al. 2014), although, in this respect, recently gathered anecdotal evidence suggests no untoward effects (Jennes et al. 2017; Schooley et al. 2017). Neither did comparative in vitro data on endotoxin release in *Escherichia coli* strains subjected to ß-lactam antibiotics, amikacin or bacteriophages cause reason for concern, but the in vivo relevance of these findings remains limited (Dufour et al. 2017). In a mini-review by Speck and Smithyman (2016), the authors comment on previously accumulated experience with intravenous phages administration and regard their use as safe in this manner (Speck and Smithyman 2016).

In summary, site of infection and route of phage administration could prove to be important determinants for efficacy, immune responses and the potential for adverse events. Ultimately, for proposed indications, appropriate evidence on safe and effective use would need to be demonstrated in well-conducted randomised clinical trials. Whether bacteriophages will be given as standalone treatment or as "add-on" to standard antibacterial therapy, will have implication on the study design and the hypotheses to be tested. Importantly, the clinical endpoints in the studies will have to reflect the expected clinical benefit to patients. Additionally, in relation to personalised phage therapy (with specific strains selected upon the infecting bacterial susceptibility), the extent of the safety and efficacy data obtained in such individual patients that would allow for broad generalisation remains to be discussed.

4 Could We Have Phage Products Available as a Standard Healthcare Measure?

As a stage-defined process, development aiming for marketing approval is time-consuming and costly. To date, however, the limited evidence generated to contemporary regulatory norms on safety, tolerability and effective administration of bacteriophages remains a fundamental limitation to its acceptance by the medical community. Thus, in order to propel development of phage treatment, "proof-of-concept" studies, assessing clinical benefit conform to stringent regulatory standards, are foremost required. Encouraging results obtained in small-scale controlled trial setting would certainly help in fostering confidence, inform late-stage trial design and facilitate the discussion on the most suitable regulatory framework for authorisation of bacteriophage products and their variants.

Broad phage cocktails intended for large-scale production could more easily comply with the current legislative requirements (Directive 2001/83/EC), and hence licensing these for treatment use might be a realistic prospect, although the need for cocktail adjustments still require a lengthy variation procedure. Likewise, it is expected that the regulatory framework could readily support the development of phage-derived proteins. However, the EU framework is less conducive in relation to personalised therapy. For the latter, the finished product is comparable to a magistral formula, although the phage library adheres to the concept of an industrial process. In this regard, a "hybrid approach" has been advocated, by which licensing of the active ingredient is deemed paramount, obviating the requirement to grant authorisation to the finished product (Fauconnier 2017). According to such a modus operandi, patient-specific phage therapy would be selected from a local "pre-authorised" phage library (i.e. approval of a European "Biological Master File", presently non-existent). In the advent of evolving bacterial host resistance during the treatment course, a polyphage concoction could then easily be adjusted in a timely and flexible manner by adding newly selected bacteriophages from the prequalified phage stock. The proposition has been advanced that granting an authorisation in such a manner could meet both societal expectations for quality, safety and efficacy and also the practitioners' and patients' needs for customised personalised medicines (Fauconnier 2017). However, this would not obviate the need to develop the standards for approval of individual bacteriophages and depending on the eventual data requirements, it remains to be seen if such approach of assessment at an active substance level (rather than the medicinal product) could bring important benefits and overcome the difficulties related to the need for authorisation of a large number of different bacteriophages.

Further on, initiatives have been taken at national level to support bacteriophage technology; e.g. Belgium recently created a "magistral phage medicines" framework—with bacteriophages conforming to provisions of internal phage monograph for active pharmaceutical ingredients (APIs)—as a pragmatic approach allowing regulatory-compliant use of such treatment for individual patients within its territory (Pirnay et al. 2018). Any developments of the EU-wide regulatory framework for personalised medicinal treatment might in the future result in new approaches in general, and more specifically, for the use of bacteriophages.

Separately, in undertaking the necessary studies, the use of adaptive licensing for phage therapy authorisation has been proposed (Cooper et al. 2016). According to this concept, the need for timely access is balanced with the importance of providing adequate, evolving information on medicines' benefits and risks (Eichler et al. 2015). As such, an initial authorisation of a phage therapy would be granted on the basis of the demonstration of a positive benefit-risk balance in a defined patient population (possibly on basis of surrogate endpoints, e.g. reduction of bacterial load and beneficial effect on inflammatory parameters, with explorative analyses for clinical endpoints, such as symptoms/signs resolution). This would be followed by iterative phases of evidence gathering, including real-world data, and the adaptation of the MA to extend the access of the therapeutic to broader patient populations while gradually refining the knowledge of the benefit-risk balance during the post-authorisation phase. It has been argued by Cooper et al. (2016) that not only pre-made cocktails, but also patient-customised therapy could be eligible for adaptive pathway trials. Use of pre-characterised libraries in formulating the custom cocktail would allow evidence gathering with bacteriophages targeting a specific bacterial pathogen in a defined condition. However, this proposal raises several regulatory concerns and would clash with the first and foremost need for robust clinical evidence on safety and efficacy of phage therapy.

5 Conclusions

Overall, it has become clear that in order to maximise the potential for phage therapy, a pro-active engagement between developers and regulatory authorities is deemed crucial. Informal exchanges on legislative requirements and subsequent formal guidance via established regulatory processes on study design and appropriate tests to be conducted, might provide the best chances to introduce treatment with bacteriophages, if indeed so proven safe and effective for intended indications.

It is recognised that with the threat posed by MDR bacterial pathogens, novel approaches will become necessary. Amongst others, the use of bacteriophages (and derived proteins) is regarded as promising. Spurred by latest state-of-art scientific developments, the regulatory environment for phage therapy is attempting to accommodate this potential treatment option within the parameters of strict safeguards. The regulators eagerly await availability of further data that would facilitate the discussion on how these safeguards can be ensured via appropriate authorisation requirements.

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Phage Therapy in Europe: Regulatory and Intellectual Property Protection Issues

Daniel De Vos, Gilbert Verbeken, Johan Quintens, and Jean-Paul Pirnay

1 Introduction

Five years ago we wrote a similar chapter (De Vos et al. 2014). Since then, there have been some advances in the regulatory and intellectual property protection (IPP) aspects of phage therapy. First, there is an increased awareness by relevant authorities and scientific societies of the potential utility of phage therapy as an alternative or supplement to an increasingly failing antibiotic therapy. With regard to the position of phage therapy in current Western medicine, no paradigm shift has taken place, but we felt that some local advances were worth mentioning. This chapter should therefore be seen as an update of the phage therapy landscape in Europe, with an emphasis on the regulatory and IPP aspects.

Phage therapy is increasingly put on the agenda of a variety of scientific meetings, nationally and internationally. It became also a central discussion topic among different patient interest groups, professional medical associations, health organizations and competent authorities for medicines, all looking for solutions to the emerging antibiotic resistance problems.

D. De Vos (✉) · J.-P. Pirnay
Laboratory for Molecular and Cellular Technology (LabMCT), Queen Astrid Military Hospital, Brussels, Belgium
e-mail: DanielMarie.DeVos@mil.be; Jean-Paul.Pirnay@mil.be

G. Verbeken
Laboratory for Molecular and Cellular Technology (LabMCT), Queen Astrid Military Hospital, Brussels, Belgium

QA/QC and regulatory affairs, Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium
e-mail: Gilbert.Verbeken@mil.be

J. Quintens
Vésale Bioscience, Noville sur Mehaigne, Belgium
e-mail: johan.quintens@vesalepharma.com

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Man has realized that bacteria had evolved to outsmart virtually all the available antibiotics and this while the industrial pipeline for antibacterial agents, in casu antibiotics, had come to a halt (Levy and Marshal 2004; Kumarasamy et al. 2010; Brzuszkiewicz et al. 2011; Bush et al. 2011; Cooper and Shlaes 2011; Leibovici et al. 2012; Karkey et al. 2018; Phoba et al. 2017). This has brought society dangerously close to the situation it was in before the advent of antibiotics, when people died from banal infections. It is actually estimated that in the EU, annually, around 33,000 patients die as a direct consequence of antibiotic-resistant infections (Cassini et al. 2019). Similar numbers are observed in the USA, and the problem is at least as acute in other parts of the world, although exact numbers are not available. Even though, more than 70 years ago, Fleming had more or less predicted the current situation during his Nobel Prize lecture, we were not able to prevent it (Honigsbaum 2018). We were too complacent and overuse and misuse and over-commercialization brought us into this so-called antibiotic crisis (Podolski 2018). It is important that we realize that the phenomenon of antibiotic resistance is not sufficiently understood. Aspects such as optimal antibiotic dosage, the efficacy of short courses versus long courses of antibiotics, the fate of antibiotics in the environment or the utility and effects of antibiotic cycling policies are still being investigated (Möller-Gundersen et al. 2019; Conlon-Bingham et al. 2019; Paterson et al. 2016; Li et al. 2019; Danner et al. 2019; Allen et al. 2010; Renjie et al. 2019; WHO Policy package to combat antimicrobial resistance). The impact of antibiotics on our own microbiota is also not fully understood, but there are clear indications that it is larger than we initially thought (Jernberg et al. 2010). Even though the role of antibiotics in nature has not been thoroughly studied, it is clear that their effect depends on the concentration (hormesis) and type of antibiotic. Baquero and colleagues described the biological phenomenon of antibiotic resistance as a typical emergent characteristic of a dynamic, highly complex and self-organizing system that evolves at the edge of chaos (Martinez and Baquero 2002; Baquero et al. 2003).

The increasing antibiotic resistance problems encouraged several research groups as well as some companies to ‘re-search’ and develop phage therapies. At the end of the nineteenth century, in India, the English bacteriologist Ernest Hankin had detected the antibacterial activity of river water, without knowing about the agent. The idea of phage therapy, i.e. the use of phages to treat bacterial infections, dates back to 1921 when the French-Canadian self-thought scientist Felix d’Herelle cured patients at a children’s hospital in Paris from toxic dysentery by oral phage application. However, phage therapeutic approaches were abandoned in the West when antibiotics emerged as the new ‘wonder drugs’ and elicited Surgeon General William H. Stewart to tell US Congress in 1969 that it was ‘time to close the book on infectious diseases‘ and declare the war against pestilence won (Spellberg and Tyalor-Blake 2013). This implicated that fundamental research into the natural role of antibiotics was not pursued or even initiated. Society became complacent and commerce took over.

Likewise, the role of phages in nature and the potential implication for phage therapy are not sufficiently understood will need to be tackled as soon as possible, on a fundamental as well as on an applied research level. Phages are considered to be the most abundant natural lifelike biological entities on our planet. Like all viruses, phages consist essentially of a genome (DNA or RNA) vehiculated and protected by a capsid,

which consists of proteins. Outside a bacterial cell, this natural biological entity is called a virion that, upon physico-chemical contact with a host bacterial cell, behaves as a genetic replicative parasite, which co-evolves with its host. Knowledge with regard to this co-evolutionary aspect is essential for a long-term sustainable phage therapy concept. The interacting and co-evolving bacterium-phage couplet is engaged in a continuous evolutionary arms race. It basically consists of a continuous emergence and fixation of de novo phage virulence and bacterial host cell-defence traits (Buckling and Rainey 2002; Faruque et al. 2005a, b). Whether phages are to be seen as living organisms or not is still a matter of debate and not entirely without importance for phage therapy (Koonin and Starokadomsky 2017). Considering phage medicines as static molecules or as evolving living entities makes a difference at the regulatory level but has also practical implications, such as how to deal (or not) with the classical requirement for pharmacodynamics and kinetics modelling (Abedon and Thomas-Abedon 2010; Abedon 2014). One thing is sure, viruses replicate and evolve in a Darwinian sense and that they have played, and still play, an important role in the origin and ongoing evolution of life as a whole. This aspect is in line with the 'One Health' approach, which has recently become 'en vogue' (Atlas and Maloy 2014; Kittler et al. 2017). Definitions of life and the structure of the tree of life are still in the centre of scientific debate (Ward 2005; Cleland 2007; Raoult and Forterre 2008; Brüssow 2009; Benner 2010; Tirard et al. 2010; Ma 2016). Of particular interest is the fact that emergence of placental organisms was probably mediated by viruses (Mi et al. 2000; Forterre 2001, 2006). Based on mimivirus research, Raoult and Forterre proposed to conceive a new tree of life model including viruses as 'capsid encoding organisms' versus 'ribosomal encoding organisms' (Raoult and Forterre 2008).

Natural phages are biological entities that play an important role in maintaining equilibriums in bacterial populations of ecological environments, including man. Hence, we should not reduce them to stable drugs, but instead we should exploit the advantages of the evolving bacterium-phage couplet. There are similarities with pre- and probiotics as well as with the faecal microbiota transplantation (FMT) field which are discussed in a recent book by Britton and Canni, entitled *Bugs as Drugs* (Britton and Cani 2018).

Considering the fundamental scientific developments on the nature of phages and the empirical evidence of their therapeutic usefulness, the latter mainly produced in the former Soviet Union, it is clear that therapeutic phages are very different from classical (chemical) drugs such as antibiotics (Chanishvili 2009; Kutateladze and Adamia 2010).

These observations challenge our medicinal product regulatory and developing frameworks, which are not adapted to cater for flexible and sustainable phage therapy approaches. For instance, it is very hard to compile the classical investigational phage medicinal product dossier, which are necessary to set up the well-designed randomized controlled trials (RCTs). These RCTs are dearly and urgently needed to once and for all prove phage therapy efficacy according to Western medicine standards (Verbeken et al. 2007, 2012; Gilmore 2012; Parracho et al. 2012; Brüssow 2012).

More than 100 years after Darwin's 'The origin of species by means of natural selection', we still live in a society that is predominantly based on a mechanistic-deterministic worldview, which hinders the development of evolutionary medicine as

a basic medical science. The implementation of sustainable phage therapy approaches, which take into account the advantages of phages as 'living' and evolving bacterial parasites, for instance, will require adaptations to our current drug development programs and not in the least in the areas of medicinal product (drugs in the USA) regulation and the rules on IPP (Darwin 1968; Corbellini 2008; Williams 2010), two topics that will be discussed more in detail in this chapter.

The focus today is still too much on the immediate effects, which is the result of solving problems by answering 'how' questions with typical mechanistic explanations. Including the 'why' questions, answered with evolutionary/dynamic explanations, would result in longer-term solutions based on more complexity and evolutionary insights (Mayr 2004; Shanks and Pyles 2007; Williams 2010; Valenti 2012).

2 Regulatory Issues

More than 10 years ago, we already realized that the introduction of sustainable and flexible phage therapy approaches in Western medicine would not be possible without an adapted regulatory framework (Verbeken et al. 2007; Fauconnier 2019). Even after the approval by the US Food and Drug Administration (FDA) of the use of phage as antibacterial (preventive) agent in 'the ready-to-eat food' in 2006 the regulatory situation of phage therapy in humans did not concretely evolve (Peek and Reddy 2006). Phages were classified as 'medicinal products' in the EU and as 'drugs' in the USA. In addition, each distinct phage has to be considered as a distinctive medicinal product (drug). Complying with all requirements necessary to develop and market phage therapy medicinal products (PTMPs) is possible in principle, but it is very costly and time-consuming. In addition, considering the continuous evolutionary dynamics between bacteria and phages, it could be that the phage products that are finally placed on the market are (or will soon become) obsolete due to the long developmental times in relation to the divergent evolution of the targeted bacterial populations.

The current medicinal product development and marketing pathway was mainly developed for static chemical drugs like antibiotics and typically consists of the following components:

- Manufacturing according to good manufacturing procedures (GMP)
- Preclinical studies (including in vitro and animal studies on pharmacokinetics, Pharmacodynamics and toxicology
- Phase I–IV clinical trials
- Centralized marketing authorization

That the development and manufacturing of PTMPs according to the established pathway is very costly, time-consuming and does not necessarily result in qualitative and efficient products was demonstrated in the Phagoburn study. Indeed, manufacturing and licensing took up most of the time and budget allocated to the study and the licensed GMP study product's titre decreased dramatically (1000-fold), 15 days after manufacturing (Servick 2016; Jault et al. 2019). Retrospective in vitro analysis showed that some infecting *Pseudomonas aeruginosa* strains were already resistant to the PTMP at the time of application, leading to failed treatments.

In other words, a PTMP containing no less than 12 phages was not enough to target all the *P. aeruginosa* in only a dozen of patients. Notwithstanding these major drawbacks, the PTMP was shown to not elicit serious adverse events and to decrease bacterial burden in burn wounds, but at a slower pace than standard of care (silver sulfadiazine cream) (Jault et al. 2019).

Awaiting the elaboration and implementation of an adapted EU (or global) regulatory framework for sustainable phage therapy approaches, several alternative paths are being evaluated on the national (Member State) level.

2.1 Declaration of Helsinki (Human and Fluss 2001)

Article 37 (Unproven Interventions in Clinical Practice) of the 'Declaration of Helsinki', which was developed for the medical community by the World Medical Association (WMA) (www.wma.net) states:

> In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgment it offers hope of saving life, re-establishing health or alleviating suffering. This intervention should subsequently be made the object of research, designed to evaluate its safety and efficacy. In all cases, new information must be recorded and, where appropriate, made publically available.

So according to this article, and in the countries that ratified the Declaration, some 'desperate' patients could be helped with phage therapy. The Declaration is, however, not legally binding and the implementation and interpretation of this article differ among countries. In Belgium, for instance, 15 patients have been treated under the umbrella of Article 37 (Djebara et al. 2019). One case concerned intravenous bacteriophage monotherapy (without simultaneous antibiotic therapy) against *P. aeruginosa* septicaemia (Jennes et al. 2017).

Even though there are indications that the Declaration of Helsinki allowed for the successful treatment of some desperate patients without adverse events, this collection of very diverse cases (e.g. different indications) in which other antimicrobials were simultaneously applied (with the exception of one case) did not allow for the unambiguous demonstration of phage therapy efficacy.

2.2 Experimental Treatment

In Poland, phage therapy can be applied under a specific 'experimental treatment' regime. At the basis for this framework are the adapted Act of 5 December 1996 on the Medical Profession published in the Polish Law Gazette, 2011, No. 277 item 1634, and Art. 37 of the Declaration of Helsinki. Practically, phage treatment is possible in Poland under certain conditions such as Informed Consent, application by a medical doctor and approval by a bioethics commission, but only when no other potentially effective and proven treatment option is available.

2.3 Compassionate Use

In some countries, the occasional application of phage therapy is possible under the 'compassionate use' regulation. Examples are Australia (Khawaldeh et al. 2011) and France (Patey et al. 2018). Even though the European Medicine's Agency (EMA) has recommendations for the compassionate use (https://www.ema.europa.eu/en/human:regulatory/research-development/compassionate.use), each country there seems to apply its own variant of this treatment option. In general, this regulation authorizes the use of an unauthorized medicine for patients where all existing treatment options have failed and this is only under strict conditions. It can only be applied for products which are tested in clinical trials or are awaiting marketing authorization, and for which there are indications of positive treatment results. Recently, Patey and colleagues (Patey et al. 2018) published a paper describing the compassionate use of phage therapy in osteoarticular infections in France, with mostly positive outcomes. Since 2016, the French 'Agence Nationale de Sécurité du Médicament et les Produits de Santé' (ANSM) is strongly involved as a competent authority. As such they created a specific committee called 'comité scientifique spécialisée temporaire' (CSST) for phage therapy. This committee composed of external and internal experts evaluate each phage therapy request to the ANSM and decide, based on an interdisciplinary analysis, the admissibility of each application. The committee will exist as long as needed.

2.4 Magistral Phage Preparations

Because 'Helsinki' or 'compassionate use' type solutions are logistically demanding and only allow for sporadic applications of phage therapy in desperate cases, they are not the best options to progress to larger-scale applications and to demonstrate the efficacy of phage therapy. Therefore, in Belgium, a phage therapy framework was elaborated within the magistral preparation (compounded prescription drugs in the USA) scheme (Fig. 1) (Pirnay et al. 2018). A magistral preparation is defined as 'any medicinal product prepared in a pharmacy in accordance with a medical prescription for an individual patient' (Article 3 of Directive 2001/83 of the European Parliament and Article 6 quator 3 of the Belgian Medicines law of March 1964). The preparation has to be compounded by a pharmacist from its constituent ingredients in accordance with the pharmaceutical state of the art and on an Medical Doctor's (MD) prescription for a nominated patient. The active pharmaceutical ingredients (APIs) must conform to a specific monograph. Since such an officially published monograph does not exist, neither in the European Pharmacopoeia nor in the National Belgian pharmacopeia, the Minister of Public Health can authorize the use of APIs after a favourable opinion of the national Pharmacopeia Commission. Non-authorized ingredients such as phage APIs are allowed as long as a certificate of analysis, issued by a 'Belgian Approved Laboratory', is provided. Those laboratories are accredited by the Belgian regulatory authorities to perform the quality control for the batch release testing of medical products. In collaboration with the Federal agency for Medicines and Health Products

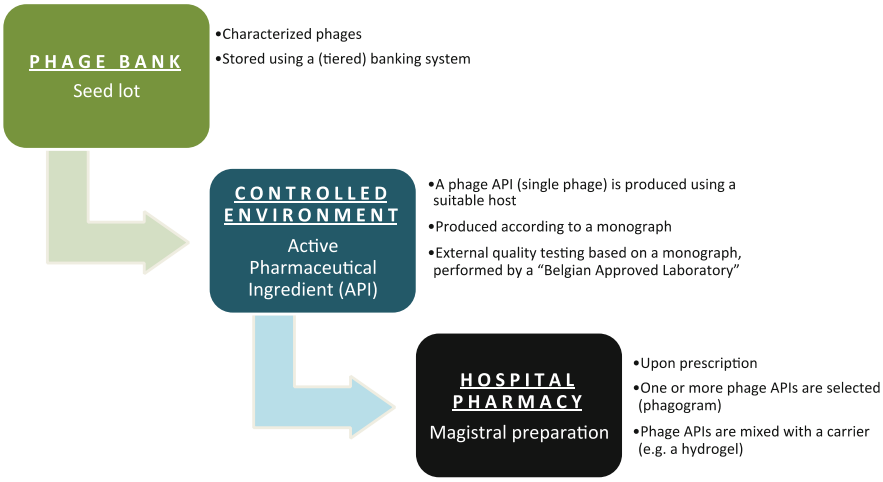

Fig. 1 Schematic overview of the Belgian magistral phage preparation framework (Pirnay et al. 2018)

(FAMHP), the Belgian competent authority for medicines, experts from the Queen Astrid Military hospital in Brussels worked out a specific generic and evolvable supplier monograph. This document describes how phage APIs need to be produced and tested. This phage API monograph received a formal positive advice from the FAMPH on 10 January 2018. This means that, in principle, without any informed consent or other obligation, any medical doctor can prescribe a magistral phage preparation for any requiring patient. To avoid inappropriate and overuse magistral phage preparation can only be made in and by a hospital pharmacist.

As such, some phage magistral preparations were already applied in patients in Belgium and in France. Other countries like Germany and the Netherlands are also considering this approach.

3 Intellectual Property Protection Issues

The fact that no dedicated frame regulating the (re)implementation of phage therapy is lacking hinders the setup of specific large-scale clinical trials and consequently its application in routine daily medicine. But there is another barrier related to Intellectual Property (IP) rights. Principally it is clear that getting patents for phages is not so easy because 'phage therapy' factually is in the public domain since almost a century. There are patents, but how strong they are or will be, in case of 'infringements', is questionable. Investing in therapeutics, in the frame of the actual classic pharmaceutical economic model, is difficult without strong IP. Securing the return on investment, especially at short-term periods, which is the rule, is troublesome and thus classical pharma does not invest (Thiel 2004). The role of IP is essential in all biomedical areas, but science shows an increasing complexity of the field as well as various interrelations

with other disciplines, which globally results in the trend for more personalized medical approaches. This reality forces society to also re-evaluate and redefine the IP field (Aiello et al. 2006; Selgelid 2007; Taubman 2008; Gold et al. 2009; Kapczynski 2009; Van Overwalle 2009, 2010; Kesselheim 2010). An example is the Myriad case around the use of the BRCA1 and BRCA2 genes for diagnosing some breast cancer risks. No concrete advances were noticed in the field during last years (Brower 2012). It could be interesting to discuss the IP issue from a more historical point of view showing the evolution of the field and its deep rooting in the (mechanistic) Industrial Revolution time while our society actually evolved into a more and more complex 'economy of knowledge'. Typical actual example fields are the developments in new bank and insurance systems as well as software developments in the IT world. As such, the biopharmaceutical world is also facing problems that require new and adaptive approaches.

In June 2013, the US Supreme Court judged a landmark ruling declaring that naturally occurring DNA sequences should be ineligible for patents as officially publicly released at the following Internet site, https://supreme.justia.com/cases/federal/us/569/576/. This ruling was remarkable in many ways, recognizing that the art of nature couldn't be patented but belonged to the public domain. In fact, here we are facing the limits of what is patentable and what is not while one of the basic rules or principle in patenting concerns 'what is the difference between a discovery and an invention'. Indeed, already for a long time, there is some controversy in terms of bioethics for what concerns the patentability of genes. However, it draws immediately our attention to a burning question for the biotech industry in formulating 'the boundary' between IP protection on biotechnological inventions and discoveries which belong to nature and so to the common resources. This still remains a troublesome area for the lawmaker and is closely related to bio-ethics, another sensitive subject when discussing about patenting genes or other biological matter which is part of living organisms.

Even within the World Intellectual Property Organization (WIPO), gene patenting remains a controversial issue pointing out some important differences between several areas and levels within the organization. The European Union's 'Biotech Directive' (directive 98/44 EC) allows the patenting of natural occurring genes and even broader of natural biological products as long as they are 'isolated from their natural environment by means of a technical process or being produced by means of such process'. This means a more liberal point of view on gene patenting and the use of living organisms or 'elements from nature'.

But, how is it possible that the lawmaker has such a difficult relationship with biotechnological inventions? Let's first have a look at history of patents, how they originated and how they evolved in time. First of all, patents are made for protecting 'industrial property, procedures and intellectual thinking'. Patents are as old as the fifteenth century but the golden age of the patent race by industry flourished during the Industrial Revolution. Patents were the crucial legal foundation upon which the Industrial Revolution could thrive and develop. The English legal system on patents became the basis of modern patent legislation and not to wonder that the same Industrial Revolution also emerged in the UK and by extension in the British Commonwealth.

From these times it is clear that patent legislation has a focus on Industrial inventions. As the world moves from an economy based on industry to one based on information, protecting other goods and inventions more related to ideas became increasingly important.

Today patent offices meet the challenge of writing patent applications in different fields of modern business where the fundamental patent principles and related rules of patent legislation like the industrialization of an invention are difficult to realize because the claims of the patent are more related to ideas than to physical inventions. Today we speak about patent classes, like software patents where complex algorithms (invention or discovery?) are claimed, or Business method patents including patents about E-commerce, insurance, banking products and systems, among others, where we concretely speak about patenting ideas. These examples are similar to 'biological patents' fields where discussions between patent attorneys, inventors and auditors meet particular difficult questions, because inventions in these fields not always comply with classical patent legislation. It is so that each of these recent fields is developing its own legislative rules, legal history and jurisprudence. Biological patents have in this view a double problematic because they are dealing with living creatures and so belonging to a common property which is nature (and cannot be claimed) and secondly have a strong ethical component related to life and living beings (Taubman 2008; Gold et al. 2009; Leibovici et al. 2012). The latter becoming particularly important when human life is involved.

It is in this ethical context that we have to understand the decision of the Supreme Court that natural gene sequences cannot be patented. This mainly as a consequence of the strong lobby work of the American Medical Association arguing that gene patents inhibit access to genetic testing for patients and hinder research on genetic diseases (https://www.ama-assn.org/delivering-care/gene-patenting).

A first problem to be solved concerns the description of the concerned organism in patents. Biological patents are not new. The first companies patenting microorganisms came from the brewing and baking industry and are as old as the nineteenth century. Patents for new types of yeast were granted in Belgium in 1833 and in Finland in 1843. But a major obstacle in older patents is specifically concerning the very accurate descriptional way of the microorganism. Descriptions were very different from the one patent to the other or even from one country to another. Several national Microbial Type Culture Collections (MTCC) and Gene Banks were recognized by WIPO as International Depository Authorities (IDAs) and have accepted deposits for biological materials, which do not fall within a literal interpretation of 'microorganism'. This led to disputes and lawsuit cases about the ownership of an organism, or the priority of one patent to another.

A milestone in preventing these disputes came in 1980 with the ratification of the Budapest treaty. The Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, in short the 'Budapest Treaty', is an international convention signed in Budapest, Hungary, on 28 April 1977 and entered into force on 9 August 1980. The treaty is administered by the WIPO. Specific information can be found on the WIPO website where the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for Patent

Procedures purposes can be found. Note that very concretely we see here a potential problem in the sense that the organization speaks of 'microorganisms' and thus what about bacteriophages/viruses? It gives however a format for the way a microorganism has to be recorded and identified in a gene bank and is now mandatory for what concerns all patents where microorganisms are concerned. So, now each patent refers to an exact microorganism deposited in a standardized format, which undoubtedly can be identified (and to which also a certain form of ownership or at least discovery can be referred to).

The Treaty however does not define what is meant by 'microorganism', nor to its origin as being biological or synthetic.

The range of materials that can be deposited under the Budapest Treaty includes:

- Cells, for example, bacteria, fungi, eukaryotic cell lines and plant spores
- Genetic vectors (such as plasmids, bacteriophages, viruses) containing a gene, a set of genes or even only DNA fragments
- Organisms used for expression of a gene (making the protein from the DNA)

All states party to the Treaty are obliged to recognize microorganisms deposited as a part of the patent procedure, irrespective of where the depository authority is located.

In practice this means that the requirement to submit microorganisms to each and every national authority in which patent protection is sought no longer exists and previous disputes are avoided.

However, one drawback is that the patent submitter made his microorganism available to the public. Every person, institute or organism can ask a sample of this organism in order to check the patent. This disclosure makes that several companies hesitate to deposit their organisms and refrain from patenting their invention. When a claim concerns a natural occurring microorganism, this makes sense and is a judgment the inventor has to make.

A second important milestone came with the introduction of the TRIPS agreement (Agreement on Trade-Related Aspects of Intellectual Property Rights). This is an International legal agreement made between all the member nations of the World Trade Organization (WTO) setting minimum standards for the regulation by national governments of many forms of Intellectual Property.

TRIPS require nations to provide strong protection for IP as copyrights and patents, recognizes new forms of technology and introduced uniform regulations in all its member states for recognition of such IP rights. So, TRIPS made an end at competing regulations existing in several territories before the agreement. Basic requirements described in TRIPS concerning patents and patentability are, for instance, that patents must be granted for 'inventions' in all 'fields of technology' provided they meet all other patentability requirements and must be enforceable for at least 20 years (Art 33).

Recognizing 'computer programs' as literary work and giving the same protection rights, TRIPS recognized the legal basis for Software and Business Model patents.

An important question for the biotechnology field concerns the relation between TRIPS and biological patents. And a first important question concerns TRIPS and gene patents: does TRIPS allow gene patenting?

Since the USA and Australia have taken legal initiatives to exclude gene sequences from patentability, the question whether TRIPS would allow such a prohibition is legitimate. Article 27 in the TRIPS agreement deals with patentable matter and is in this respect important. The crucial text is as follows:

1. Subject to the provisions of paragraphs 2 and 3, patents shall be available for any inventions, whether products or processes, in all fields of technology, provided that they are new, involve an inventive step and are capable of industrial. Patents shall be available and patent rights enjoyable without discrimination as to the place of invention, the field of technology and whether products are imported or locally produced.
2. Members may exclude from patentability inventions (. . .) to protect public order or morality, including to protect human, animal or plant life or health or to avoid serious prejudice to the environment (. . .).
3. Members may also exclude from patentability: (a) diagnostic, therapeutic and surgical methods for the treatment of humans or animals; (b) plants and animals other than microorganisms, and essentially biological processes for the production of plants or animals other than non-biological and microbiological processes. (. . .)

So, TRIPS itself does not exclude explicitly gene patents, microorganisms or patents on DNA fragments. Recent evolutions made this debate, however, more critical. We now know that a section of a gene/DNA is not an isolated element but has to be seen in the context of a working cell or organism. Identical DNA sequences even can have different functions in different organisms or having multiple functions within the same organism. Patents, however, are not coping with this complexity, and they are dealing with DNA like with chemical entities, claiming all possible activities of a substance at once, as well as the known as the unknown. This has been ground to a lot of criticism arguing that the 'absolute patent production' offered to DNA sequences does not fit the complexity of all possible known and unknown biological functions of a gene. Together with ethical considerations and the difference between inventivity and discovery, this broad scope of DNA patents give rise to significant room for interpretation of the TRIPS agreement and to defend exclusion of DNA/gene patenting in national legislation.

4 Conclusions

The reimplementation of phage therapy is put on the agenda of competent authorities, scientific biomedical societies and patients organizations all over the world. Regulatory wise, the magistral path, as worked out and implemented in Belgium, is a step forward, allowing phage therapy on a routine basis, under the responsibility of medical doctors, accredited laboratories and hospital pharmacists, and facilitated by the authorities. In the initial phase, the production of magistral phage preparation is restricted to hospital pharmacies, to prevent inappropriate and uncontrolled applications and to allow a better overview of phage therapy applications. This pathway is logistically feasible without any, e.g., mandatory informed consents or ethical committee approval and allows for a

wider use of phage therapy. The safety and efficacy of phage magistral preparations can be evaluated in so-called retrospective observational studies. However, if magistral phage preparations are to be used in randomized controlled trials, they need to be GMP certified. Those studies, which should include the elucidation of issues that were historically neglected for antibiotics, are urgently needed to generate a better general understanding of phage therapy and to optimize specific intervention protocols. The growing interest for the magistral phage pathway in several other European countries lets us hope that in the near future, an adequate phage therapy framework will be developed at the EU level, possibly based on the magistral preparation scheme. Fundamental phage research should be performed in parallel to avoid the mistakes we made in the past with antibiotics, i.e. a nonchalant and unlimited use, without prior scientific analysis of the potential consequences. We suggest focusing on sustainable phage therapy applications in accordance with the One Health approach that fits well in the so-called and emerging Darwinian or evolutive medicine's approach (Corbellini 2008; Kittler et al. 2017). The simultaneous use of phages and antibiotics, as suggested by some groups and researchers, might be the best consensus approach to minimize antibiotic resistances as well as the massive emergence of phage resistances among some bacterial populations (Torres-Barcello and Hochberg 2016; Torres-Barcello 2018).

With regard to IPP and return on investment, some changes and adaptations are also mandatory. Society needs industry and vice versa. Like the couple phage-bacterium, it is an evolving couplet.

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Ethics of Phage Therapy

Jan Borysowski and Andrzej Górski

1 Introduction

In general, patients can get access to unauthorized medicinal products either by enrolling in a relevant clinical trial or during compassionate treatment (Caplan and Bateman-House 2015). For a variety of reasons, very few clinical trials of phages have been conducted so far (Henein 2013; Abdelkader et al. 2019). Therefore, currently the primary way of getting access to phage is compassionate use. The overall objectives of clinical trials and compassionate use are completely different. The main goal of a clinical trial is to investigate a medicinal product's pharmacokinetics, safety, and/or efficacy (Nardini 2014). By contrast, compassionate use is a kind of treatment using an unauthorized medicinal product performed to obtain therapeutic benefits in individual patients (Caplan and Bateman-House 2015). In view of this fundamental difference, ethics of clinical trials will differ from ethical considerations for compassionate use.

2 Ethics of Clinical Trials

Since the scale of clinical trials of phages is limited (Henein 2013; Abdelkader et al. 2019), ethics of clinical research will be discussed here very briefly. As mentioned above, the main purpose of a clinical trial is to develop generalizable knowledge about the effects of an investigational medicinal product on the human organism

J. Borysowski (✉)
Department of Clinical Immunology, Medical University of Warsaw, Warsaw, Poland

A. Górski
Department of Clinical Immunology, Medical University of Warsaw, Warsaw, Poland

Laboratory of Bacteriophages, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland

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(Nardini 2014). Subjects who have been enrolled in a trial (either healthy volunteers or patients) may (but also may not) gain direct therapeutic benefits from participation in the trial. As a result of exposing these subjects to risk of harm for the good of future patients, clinical trials have the potential to exploit participants. There are three main safeguards to protect the rights of subjects participating in clinical trials and to prevent their exploitation: (1) legal regulations concerning clinical research; (2) relevant ethical guidelines; (3) bioethics committees (in the USA these are termed institutional review boards—IRBs).

Detailed discussion of legal regulations concerning the conduct of clinical trials and the role of bioethics committees is out of the scope of this chapter. The most important ethical guidelines pertaining to clinical research are contained in the Declaration of Helsinki by the World Medical Association (WMA); in fact, the scope of the Declaration is broader and encompasses medical research involving human subjects including research on identifiable human material and data (World Medical Association 2013). While it is not a legally binding document, since the mid-1960s, when the first version of the Declaration was published, its impact on clinical research has been tremendous. Although the last revisions of the Declaration have been criticized in several respects (Ehni and Wiesing 2019), it remains the reference document containing a set of principles concerning main issues associated with the conduct of clinical trials including, among others, risks, burdens and benefits, trial protocols, role of bioethics committees, informed consent, the use of placebo, post-trial provisions, and dissemination of results (World Medical Association 2013).

In this context, it is worth mentioning also a landmark paper by Emanuel et al. (2000). The purpose of this paper was to formulate main requirements that have to be met clinical research to be ethical. These include (1) social or scientific value, (2) scientific validity, (3) fair subject selection, (4) favorable risk-benefit ratio, (5) independent review, (6) informed consent, and (7) respect for potential and enrolled subjects. According to these authors, meeting all seven requirements is necessary and sufficient to make clinical research ethical (Emanuel et al. 2000).

3 Compassionate Use

3.1 Background

Compassionate use (also termed expanded access) is the therapeutic use of unauthorized (mostly investigational) drugs outside of clinical trials. Compassionate treatment is most often performed in patients with serious including life-threatening diseases in whom all authorized drugs have failed (Caplan and Bateman-House 2015). Interest in compassionate use has substantially grown over recent years; this results from a number of reasons including development of new drugs for serious unmet medical needs, high activity of patient advocacy groups, and growing patient access to data about emerging treatments in Internet sources (Caplan et al. 2018). Consequently, the number of applications for treatment on a compassionate use basis has also increased, at least in the USA (Jarow et al. 2016). Compassionate use of

unauthorized drugs has been reported in a variety of medical specialties including infectious diseases, cardiology, hematology, oncology, pulmonology, neurology, psychiatry, transplantology, ophthalmology, dermatology, nephrology, gastroenterology, and rheumatology (Jarow et al. 2016).

3.2 Compassionate Use: Legal Regulations in the European Union and the USA

In the European Union, compassionate use programs involving groups of patients are regulated by Article 83 (1) of Regulation (EC) No 726/2004 of the European Parliament and of the Council (European Parliament 2004a). According to this article, compassionate treatment can be performed in patients with a chronically or seriously debilitating disease or a life-threatening disease of patients who cannot be treated satisfactorily with an authorized medicinal product. The medicinal product to be used must be either the subject of an application for a centralized marketing authorization or be undergoing clinical trials. In addition, many European countries have introduced specific regulations concerning compassionate use (Balasubramanian et al. 2016). Moreover, Art. 5 of Directive 2001/83/EC of the European Parliament and of the Council, amended by Art. 1 of Directive 2004/27/EC of the European Parliament and of the Council, permits use of unauthorized medicinal products in single patients under direct responsibility of a healthcare professional (European Parliament 2004b).

In the USA, compassionate use (most often referred to as expanded access) is overseen by the FDA, and the main requirements for compassionate use include the following: (1) a serious or immediately life-threatening disease with no comparable or satisfactory alternative therapy, (2) the potential benefits justify the potential risks and the potential risks are not unreasonable in the context of the disease, and (3) there is no threat to clinical trials to support marketing approval of the expanded access use. Moreover, informed consent of the patient and the institutional review board (IRB) approval are required (Electronic Code of Federal Regulations 2009).

3.3 Phage Therapy on a Compassionate Use Basis: Ethical Considerations

The four main principles of medical ethics include non-maleficence, beneficence, respect for the patient's autonomy, and justice (Beauchamp and Childress 2012). Therefore, the key question in the discussion about the ethics of phage therapy is whether such therapy is consistent with these values.

According to the principle of non-maleficence, a doctor should do no harm to his/her patients. Thus, the main question that needs to be asked is whether phage therapy is safe? The gold standard of studies to evaluate the safety of drugs are randomized controlled trials (Nardini 2014). While the first trials generally showed a favorable safety profile of phage, their number has been very small (Vandenheuvel et al. 2015). However, one should take into account also an important role of

uncontrolled (observational) studies as a means of evaluation of the safety of drugs (Sawchik et al. 2018). This is very important in the context of phage therapy which has a very long history, with a great number of papers reporting safety of phages found in observational studies (Henein 2013). Moreover, some phage-based products have been granted GRAS (generally recognized as safe) status and have been approved as food additives (Moye et al. 2018). Thus, at the current state of research, phages are generally considered safe and in our opinion phage therapy may be deemed reasonably consistent with the principle of non-maleficence.

The second main principle of medical ethics is beneficence, which means that a doctor should act for the patient's benefit, making efforts to improve his/her state of health, to alleviate suffering, etc. (Beauchamp and Childress 2012). Accordingly, one could ask what particular benefits can be brought by phage therapy. Unfortunately, no randomized Phase 3 trials to evaluate the efficacy of phages have been conducted yet (Vandenheuvel et al. 2015). Thus, at the current state of research the effectiveness of phage therapy cannot be verified based on standards of clinical research. However, there are numerous papers reporting successful use of phage therapy in uncontrolled studies (Kortright et al. 2019). For instance, at our center, we found a good response to phage therapy in 61 out of 157 patients (39.9%). While in 92 patients (60.1%) the response was inadequate, one should keep in mind that all these patients have ran out of available antibiotics (Międzybrodzki et al. 2012). Thus, phage therapy can be effective in a significant percentage of patients who cannot be treated satisfactorily with any antibiotic.

In one of our studies, we found that phages can exert anti-inflammatory effects which are independent from a reduction in bacterial titer (Miedzybrodzki et al. 2009). These effects are very beneficial given a very important role of inflammatory process during bacterial infection. Moreover, phages can inhibit infection of target cells by some pathogenic viruses (Górski et al. 2019). Therefore, while the primary goal of phage therapy is of course direct elimination of bacteria, the use of phages can also result in other beneficial effects. Thus, while the efficacy of phage therapy still awaits confirmation by large randomized trials, in our view the body of data about the beneficial effects of phages is sufficient to assume that phage therapy can be considered reasonably consistent with the principle of beneficence.

The third main principle of medical ethics is justice (Beauchamp and Childress 2012). In the context of compassionate use, the key problem relevant to justice is how to ensure fair patient selection for therapy. Important guidelines that could aid doctors in that regard were recently published by the Compassionate Use Advisory Committee (CompAC), an independent committee established at a major academic center to help a pharmaceutical company to handle requests for use of its investigational drugs in a fair and transparent way (Caplan et al. 2018). Detailed discussion of the data published by the CompAC is out of scope of this chapter. In brief, fairness in patient selection was achieved by creating rapid response to all applications, developing a single route of entry for applications, independence from a drug's manufacturer, evaluation of each application based on uniform information, and blinding of the committee members to some important data which might cause bias, especially names of patients along with their race, gender and ethnicity, names of doctors, and countries of origin. The CompAC also recommended that the drug's manufacturer

ensure that the company's international websites are easy to navigate for each person seeking information about compassionate use. Moreover, the committee developed and employed a set of specific criteria (largely clinical and to a lesser extent social) to select patients (Caplan et al. 2018).

The fourth main principle of medical ethics is the respect for the patient's autonomy (Beauchamp and Childress 2012). In the context of compassionate use, the main concern relevant to the idea of autonomy is whether patients can make truly informed choices regarding the treatment? Obtaining informed consent for experimental treatments can be difficult for two main reasons (Borysowski et al. 2017). First, the access of patients to data about the safety and the efficacy of investigational drugs may be limited. Second, patients with serious diseases who cannot be treated satisfactorily with approved drugs may be in a difficult situation and may overestimate potential benefits of the treatment and underestimate risks. Obviously, these concerns may apply to phage therapy as well since the safety and efficacy of phages have not yet been shown based on contemporary standards of clinical research. However, we believe that these concerns might be resolved by a doctor taking every effort to fully inform the patient about different aspects of the treatment. In particular, the patient should be informed about experimental nature of phage therapy and how it relates to potential benefits and risks.

3.4 Ethics Codes and Guidelines and Compassionate Use

An important question is whether doctors who would like to use phage therapy on a compassionate use basis have any guidelines to aid in the ethical performance of experimental treatments. Since there are no guidelines specifically devoted to phage therapy, in this section we will present guidance relevant to experimental treatments in general.

In a recent paper we discussed relevant guidelines contained in different international and national codes of biomedical research and medical ethics (Borysowski et al. 2019). We found that the main international code that contains guidance pertaining to compassionate use is the Declaration of Helsinki. It is an unexpected finding because the Declaration is a code of biomedical research rather than of medical ethics (while compassionate use is a kind of treatment and not research). Relevant guidelines are contained in Paragraph 37 of the Declaration, section "Unproven Interventions in Clinical Practice" (World Medical Association 2013). According to this paragraph, the doctor has the right to use an unproven treatment in cases when proven interventions do not exist or have been ineffective. Prior to use of an unproven treatment, the doctor should seek expert advice and obtain informed consent from the patient. An unproven treatment may be employed when it offers hope of saving the life of the patient, re-establishing his/her health, or alleviating suffering. Par. 37 of the Declaration of Helsinki also stresses that the safety and the efficacy of the unproven treatment should be investigated. Moreover, it obliges the doctor to record all data about the use of the unproven treatment; where applicable, these data should be disseminated (World Medical Association 2013).

We also evaluated whether the WMA International Code of Medical Ethics (ICME) contains any guidance about compassionate treatments (Borysowski et al. 2019). According to one of the main duties of doctors listed in this code, "a physician shall act in the patient's best interest when providing medical care" (World Medical Association 2006). Unfortunately, this statement is rather ambiguous in the context of discussion about compassionate use. On the one hand, when all authorized drugs have failed, a doctor may try to use an unauthorized drug as the therapeutic "last resort". However, when the effectiveness and safety of this drug are uncertain, can the doctor confidently state that he/she is acting indeed in the patient's best interest? In some cases, unproven drugs could actually deteriorate the patient's health (Borysowski et al. 2019). Except for this rather general guideline, the ICME does not contain any articles specifically devoted to the use of unproven treatments (World Medical Association 2006).

4 Concluding Remarks

In practice, most patients with antibiotic-resistant infections get access to phage therapy on a compassionate use basis. Therefore, the key question in the discussion about ethics of phage therapy is whether such treatment is consistent with main values of medical ethics. In our view, at the current state of research phage therapy seems to be reasonably consistent with the principles of non-maleficence and beneficence. Moreover, every effort should be undertaken to help patients to make informed choices about the treatment. Another important problem is to ensure fair patient selection.

Antibiotic resistance of bacteria itself is a problem which has some important ethical aspects (Littmann et al. 2015). Detailed discussion of these is out of scope of this chapter. However, we fully agree with A. Henein who suggested that, in view of the current scale of antibiotic resistance, not pursuing phage therapy might be considered an unethical action (Henein 2013). Phage therapy is one of the most promising methods of combatting antibiotic-resistant bacteria and many patients with otherwise untreatable infections might benefit from its use.

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