



Phage therapy: An old concept with new perspectives

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ABSTRACT

The ever-increasing bacterial resistance to antibiotics and the growing number of severe, hard-to-treat infections have driven international studies into a race to develop alternative, less conventional methods of treatment. One such antibacterial strategy has been around for a relatively long time, just recently resparking scientific interest: it is called *phage therapy*. In the fight against multidrug-resistant bacterial infections, this method employs one of the most abundant organisms on the planet Earth—bacteriophages—a type of intriguing, spiderlike viruses which infect bacteria. The newest research in the field of phage therapy and its applications in medicine suggests that this method indeed has the potential to become an alternative form of treatment for patients suffering from various diseases, as it turns out, not only bacterial infections. However, there is still much to be learnt about phage biology, its impact on the human body, and its interactions with bacteria. Nonetheless, in the face of a significant global health threat, the time has come to reexamine this almost-century-old medical strategy.

INTRODUCTION

Antibiotics, since their discovery, have remained humanity's most important weapon against bacterial infections, saving thousands of lives every day. Regrettably, modern medicine is now facing a significant threat to global public health—bacteria change in response to antibiotics, becoming progressively unaffected by them. Antibiotic resistance happens when microorganisms develop a way to defeat the drug which is supposed to eradicate them or hinder their multiplication. In consequence, a growing number of infections become harder to treat. Healthcare providers and scientists are concerned that, according to biostatistics, multidrug-resistant bacteria-related infections are rising to a menacing level and the bacterial defense mechanisms are spreading fast, emerging in every part of the world. Not only does antibiotic resistance entail higher medical costs and longer hospitalizations, but it leads,

first and foremost, to increased mortality amongst humans as well as animals. In addition, resistance to antibiotics is highly accelerated by overuse and misuse of these drugs—in some countries, e.g., in China, antibiotics are dispensed without prescriptions and are sold as *over-the-counter* medicines (Gong *et al.*, 2020).

In the fight against harmful bacteria, an unlikely ally was found quite a long time ago—viruses called *bacteriophages*. These viruses are the natural enemies of bacteria, and they may soon become an alternative to antibiotics in the treatment of multidrug-resistant bacterial infections. The practice of phage therapy has been around for almost a century. It is not a new discovery; it was merely set aside in favor of antibiotics, which turned out to be a cheaper and less complicated form of therapy. Yet, the modern-day decline in the effectiveness of antibiotics has shed new light on phages and their bacteria-destroying abilities. Nonetheless, antibiotics have set a health standard that the world has become quite dependent on and which would be detrimental to lose. Fortunately, current data suggests that phage therapy has a solid potential to be utilized as an alternative method or as a supplement to antibiotic treatment. This work aims to present the newest medical research on phage therapy, with a hint about its history.

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ANTIBIOTICS: DEVELOPMENT, MECHANISMS OF ACTION, AND THE GROWING NUMBER OF DRAWBACKS

In consonance with historical background, the name *antibiotic* comes from classical Greek words—*anti* meaning *against* and *bios* meaning *life*. As a general rule, this term refers to a low-molecular-weight antiliving substance or compound which is active against microorganisms. Taking into consideration the effect on bacteria, the antibiotic mechanism of action focuses on killing them (bactericidal properties) or inhibiting their growth, thus preventing multiplication (bacteriostatic properties).

The start of the modern antibiotic era is most commonly associated with the scientific research of Alexander Fleming who discovered penicillin in 1928. At this point, it should be highlighted that human exposure to antibiotics happened long before their contemporary development. For instance, small traces of tetracycline have been found in skeletal remains of people from ancient Sudanese Nubia (350–550 CE) (Aminov, 2010). Shlaes (2010) underlines that most of the antibiotics available today, including penicillin, erythromycin, and tetracycline and all their relatives, started as byproducts of the metabolism of microorganisms living in soil, on plants, or in our oceans.

Only a small fraction of antibiotics are chemically synthesized; a vast majority of them are still microorganism-produced or semisynthetic derivatives thereof. The stupendous success of antibiotics in the management of infectious, life-threatening diseases has led to the worldwide distribution of these medicines. They are used not only in the treatment of humans but also as a preventive measure in livestock farming. Moreover, they are also utilized against pathogens invading different types of plants.

Antimicrobial agents hinder the growth of microorganisms by inhibiting the molecular action of an enzyme or a nucleic acid—a significant point for the multiplication of the cells. Essentially, an antibiotic molecule binds to a selected, specific site on the target macromolecule, producing a certain complex which renders the molecule unable to fulfill its evolutionary function (Calhoun *et al.*, 2021). The evaluation of a new antibiotic most commonly follows a fixed hierarchy of procedures. First, a new compound/substance is clinically tested against bacterial strains, many of which are equipped with resistance to prior generations of antimicrobial agents. If a sufficient potency against the strains is found, then the compound may be tested on animals, infected with specific bacteria, to evaluate its protective and/or curative features. Later on, the antibiotic may be compared with standard medicines used against these bacterial infections (with sensitive and resistant pathogens). If the new antimicrobial proves its efficiency, it may become a development candidate (Walsh, 2003). The susceptibility of a bacterial isolate to a particular antibiotic is quantified by the *minimum inhibitory concentration* (MIC) and the *minimum bactericidal concentration* (MBC). The MIC indicator measures the lowest dose of antibiotic that is still able to prevent bacterial growth while MBC stands for the minimum concentration required to destroy the bacterium (Hauser, 2018).

One method used to classify antibiotics takes into account their effect on the growth and survival of the bacteria. As mentioned before, in this classification, two major groups

exist: antibiotics of bactericidal traits (they eliminate bacteria, e.g., penicillin) and agents of bacteriostatic properties (they work by inhibition of bacterial proliferation, e.g., chloramphenicol) (Baquero and Levin, 2021; Maffioli, 2013). Another classification is based on the targets present in the cells of microbes, with which antibiotics interact, to hold back bacterial growth. Six major classes can be distinguished: antibiotics that stop bacterial cell wall synthesis, those that disrupt the cellular membrane, agents that inhibit the synthesis of metabolites, those that cease DNA synthesis (replication), antibiotics that hinder RNA synthesis (transcription), and those that inhibit the synthesis of protein (translation) (Bhattacharjee, 2016).

The β -lactam antibiotics, including penicillin and cephalosporins, suppress the synthesis of a polymer called peptidoglycan (murein), which leads to the damage of bacterial cell wall—it happens due to the fact that peptidoglycan serves as its primary component. Notable members of the β -lactam class of antibiotics are carbapenems, which are highly effective and used for the treatment of severe infections, often as almost-last resort medicines. Other useful β -lactams are monobactams, effective against Pseudomonadaceae. Tetracyclines, wide-spectrum drugs, are produced by different strains of *Streptomyces*, and they work by suppressing the synthesis of ribosomal proteins in bacteria (Chukwudi, 2016). Owing to this effect being reversible, tetracyclines were sorted into the bacteriostatic group of antimicrobial agents. Aminoglycoside antibiotics, such as streptomycin and gentamicin, particularly efficient against Gram-negative bacteria, have exhibited a bactericidal effect by irrevocably disturbing protein synthesis through interaction with ribosomes. However, they were shown to be nephro- and ototoxic (Krause *et al.*, 2016). Macrolides (erythromycin, clarithromycin, and roxithromycin) are bacteriostatic and act as protein biosynthesis inhibitors. Polypeptides (actinomycin, bacitracin) operate by permeabilizing bacterial cell membrane or by neutralizing its toxicity. Glycopeptide antibiotics (vancomycin, ramoplanin) act through inhibition of peptidoglycan synthesis—they form a complex with the terminal d-alanyl-d-alanyl group of the intermediates. Vancomycin, produced by *Amycolatopsis orientalis*, is commonly used against methicillin-resistant *Staphylococcus aureus* (MRSA) (Jeffres, 2017). Antitumor antibiotics, such as dactinomycin, act on DNA replication via different mechanisms. Lincosamides, e.g., clindamycin, interfere with the synthesis of protein biomolecules, thus preventing bacterial proliferation, and they are mostly utilized against Gram-positive bacteria (Schwarz *et al.*, 2016). Quinolone antibiotics, the most famous being ciprofloxacin, are chemotherapeutic drugs. They prevent bacterial DNA from duplicating. Polymyxins, produced by Gram-positive bacteria, are used only when no other medicines prove effective—they are highly neurotoxic and nephrotoxic (Yu *et al.*, 2015).

The severity of infection determines the therapy scheme. In life-threatening, high-risk diseases, such as sepsis, antibiotics that cover a broad spectrum of bacteria are administered, most often via the parenteral route. Modern-day medicine is equipped with laboratory sensitivity testing which measures the susceptibility of bacteria, responsible for the infection, to selected antibiotics—the data is organized in a profile named *antibiogram*. It allowed physicians to move away from empiric therapy, based

on suspicion, toward directed, evidence-based treatment (Boehme *et al.*, 2010).

As it was shown, the term antibiotic applies to a vast range of different drugs, exhibiting various mechanisms of action against microbes. It must be remembered that the mentioned medicaments, especially penicillin, have the potential for antagonistic reaction that can result in far worse consequences than the initial infection. According to data, in the United States, one in five hospitalized patients develop an adverse reaction to antibiotics and nearly the same proportion of medicine-related emergency room visits is due to unpropitious reactions to antibiotics (Hall, 2021).

Antibiotic resistance

Antibiotic resistance remains one of the most concerning global health threats. At the beginning of their history, antibiotic agents were regarded as a sort of *miracle drugs*, which have indeed saved countless lives so far. Now their effectiveness is starting to cease gradually with the emergence of multidrug-resistant bacteria. The Centers for Disease Control and Prevention (2021) defines antibiotic resistance as an event “when germs like bacteria or fungi develop the ability to defeat the drugs designed to kill them”. The levels of resistance vary greatly within bacterial groups. According to published research, about 70% of bacteria in hospitals are resistant to at least one drug commonly used for the treatment of the infections triggered by them. Some microorganisms are resistant to all approved antibiotics (Odonkor and Addo, 2018). There are several different mechanisms behind bacterial resistance to antimicrobial agents. First and foremost, bacteria can be resistant to a certain drug *naturally*. This mechanism is not associated with the drug but rather with the bacteria itself—resistance is caused by its inherent structural or functional characteristics; for example, a microorganism lacks the target of the antibiotic molecule or, as in the case of Gram-negative bacteria, the outer membrane of the cellular wall provides a strong barrier against the drug. *Acquired* drug resistance, on the other hand, forms on the genetic basis—chromosomal or extrachromosomal mutations in response to antibiotics to which the bacteria were previously susceptible. It occurs through all of the paths by which microorganisms acquire genetic material: transformation, transposition, and conjugation. Cross-resistance usually happens when bacteria become resistant to agents that harbor similar mechanisms of action. Multiple-drug-resistant microorganisms are resistant against more than one antimicrobial. Extensively drug-resistant bacteria are resistant to almost all approved antibiotics. Finally, pandrug-resistant bacteria are resistant to all commercially available medicines (Magiorakos *et al.*, 2012).

According to the publication authored by Wanda Reygaert, “An Overview of the Antimicrobial Resistance Mechanisms of Bacteria,” resistance mechanisms can be categorized into four groups: (1) limiting uptake of a drug, (2) modifying a drug target, (3) inactivating a drug, and (4) active drug efflux (Reygaert, 2018). Gram-negative bacteria apply all four main mechanisms in their defense, whereas Gram-positive bacteria mostly limit the uptake of a drug. Infections caused by Gram-negative bacteria pose a greater challenge to public health than those caused by Gram-positive bacteria, due to the fact

that fewer antibiotic classes can penetrate through their intricate membrane barriers.

Key factors affecting the bacterial resistance include a worldwide indiscriminate use of antibiotics, alternations in the treatment made by patients themselves (deliberately going off the medicines when feeling better without finishing the full course or forgetting to take the medication), physicians prescribing broad-spectrum antibiotics instead of narrow-spectrum ones, poor infection control in hospitals, and dramatic overuse of antimicrobial agents in animal breeding (Machowska and Stålsby Lundborg, 2018; Odonkor and Addo, 2018). The bacterial resistance to antibiotics is expected to rise even more. Worldwide consumption of antibiotics rose by almost 40% between the years of 2000 and 2010 (Sutradhar *et al.*, 2021). If this trend continues, human medicine will face a dangerous challenge, similar in its magnitude to the obesity, diabetes, and cardiovascular diseases epidemic.

Alternatives to antibiotics

It must be acknowledged that there are other alternative methods currently being designed to combat the challenging problem of bacterial resistance. One approach may be described as *fighting fire with fire*—it refers to probiotic therapy: the application of helpful bacteria in order to source a therapeutic effect. In general, probiotics are defined by the World Health Organization (WHO) as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Araya *et al.*, 2002). Their mechanisms of action include blockage of adhesion sites, production of inhibitory substances (for instance, the so-called bacteriocins, active against Gram-negative and Gram-positive bacteria), macrophage activation, and regulation of inflammatory cytokines’ release (Silva *et al.*, 2020). Consequentially, some scientists suggest the use of predatory microorganisms, such as *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus*, which prey on Gram-negative bacteria, as potential therapeutic agents (Kadouri *et al.*, 2013). Yet, research in this field is still in its infancy, and further studies are needed to confirm those prospective, beneficial-to-humans attributes of predatory bacteria (Dharani *et al.*, 2018).

By now, human monoclonal antibodies have shown positive effects in anticancer, antiviral, and autoimmune therapy. Antibodies can also bind to and inactivate the pathogens, their virulence factors, and/or toxins. Recently, they have been considered as an alternative to multidrug-resistant bacterial infections as well, although their production is still quite expensive (Zurawski and McLendon, 2020).

Bacterial vaccines have been around for some time, with a great rate of success. *Haemophilus influenzae*, *Mycobacterium tuberculosis*, and *Streptococcus pneumonia* vaccines are often included in national vaccination programs. The investment in vaccines for new targets should continue considering their potential to reduce rates of infection and, consequently, the need for antibiotics.

Antimicrobial peptides (AMPs) destroy pathogens directly or indirectly by modulating the host’s defense mechanisms. Several AMPs are being methodologically evaluated as possible

new anti-infectives. Cationic AMPs interact with negatively charged bacterial cell membranes, change their electrochemical potential, and induce cell membrane damage. Examples of AMPs include bacitracin, enfuvirtide, and daptomycin (Lei *et al.*, 2019).

Listed above were the most common substitutes to the antibiotic treatment. Regrettably, none of them, including bacteriophage therapy, have demonstrated the level of effectiveness comparable to the world gold standard—the aforementioned antibiotics.

The most concerning pathogens

Strains of bacteria, fungi, viruses, and parasites resistant to medications are, quite trivially, called *superbugs* in colloquial language. In 2017, the WHO published a report of *priority pathogens* to guide and force international research and development of new antibiotics (Tacconelli *et al.*, 2018). The critical priority category consists of *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and Enterobacteriaceae. All three of them are resistant to the last-resort antibacterial agents—carbapenem antibiotics. *Acinetobacter baumannii* can cause infections in the urinary tract, blood, and lungs or in wounds. Infections occur mostly within healthcare facilities. Multidrug-resistant *P. aeruginosa* is a Gram-negative bacterium, associated with serious diseases, such as sepsis and severe pneumonia. It poses a notable threat to patients hospitalized with cancer and burns, resulting in 50% mortality and accounting for 10% of all nosocomial infections (Yi-Wei Tang, 2014). Enterobacteriaceae are a large family of bacteria which includes pathogens such as *Salmonella*, *Escherichia coli*, *Shigella*, and *Klebsiella*. Enterobacteriaceae are one of the most common causes of hospital-acquired pneumonia and serve as a major culprit in intra-abdominal infections, e.g., cholecystitis, appendicitis, peritonitis, and septicemia (De Angelis *et al.*, 2020).

A high-priority group, devised by the WHO, includes sequentially the following. The first are *Enterococcus faecium*, vancomycin-resistant opportunistic bacteria accountable for endocarditis and meningitis in newborns. Second is *S. aureus* and its wide range of clinical infections varies between mild skin diseases and respiratory illnesses to toxic shock syndrome. About 30% of humans are colonized by *S. aureus* (Tong *et al.*, 2015). Next, *Helicobacter pylori*, expressing a significant urease activity, plays a crucial role in developing chronic gastritis and peptic ulcer disease in humans as well as gastric lymphoma and gastric carcinoma. Following that, *Campylobacter* has become one of the most notorious foodborne pathogens, most commonly found in poultry. It is responsible for enteritis and gastroenteritis in adults and children. Lastly, *Neisseria gonorrhoeae*-induced infections account for sexually transmitted diseases, most frequently manifesting as cervicitis in women and urethritis in men. The medium-priority group incorporate *S. pneumoniae*—a critical source of community-acquired pneumonia and meningitis in children and the elderly. Next is *H. influenzae* which causes, amongst others, infections in neonates, cellulitis, and acute bacterial meningitis (it must be mentioned that a vaccine exists against this pathogen). Lastly is *Shigella*—most often responsible for dysentery. In light of the aforementioned WHO report, it should be pointed out that Mycobacteriaceae are also regarded as

a global priority. The most notable member of this family is *M. tuberculosis*—an infamous pathogen guilty of being the source of World Health Organization (2021).

Dangerous pathogens adapting to medicines designed to destroy them and irrational antibiotic consumption and misuse are a great recipe for worldwide disaster. Some estimate that if proper actions are not taken immediately, by 2050, the number of people dying from bacterial infections can reach a startling 10 million per year (Goff *et al.*, 2017). Scientists, realizing the biohazard the world is about to face, have been looking for a way out: weaponizing viruses to fight the most difficult bacterial infections. Can bacteriophages become humanity's *superheroes* against *superbugs*?

BACTERIOPHAGES: BIOLOGY AND CLASSIFICATION

The history of phage therapy is said to have started in 1915 with the scientific work of Frederick W. Twort who observed, for the first time, mysterious glassy spots on an agar medium with cultivated *vaccinia virus*. Those spots turned out to be dead bacteria. Sadly, Twort was not able to continue his research due to the onset of World War I. A few years later, microbiologist Félix d'Hérelle, working at the Pasteur Institute in Paris, made similar observations as Twort while investigating a dysentery outbreak. He observed lysis (breaking down of the membrane of a cell) in liquid culture and the formation of aforementioned glassy spots, which dazzled Frederick Twort. The scientist immediately recognized that he was dealing with a new type of virus which he named *bacteriophage*, from the Greek *phagein* meaning to devour.

Bacteriophages, or phages for short, are defined as viruses that infect bacteria. They are one of the most common and highly varied entities on the planet and have been observed in every environment where bacteria thrive, their main habitat being the ocean as well as topsoil. There are an estimated 10^{31} bacteriophages on Earth (Keen, 2015; Letarov, 2020). In *The Bacteriophages: Biology and Application* monograph, the authors vividly compare these viruses to spaceships considering their intricate structure and the fact that each phage particle (virion) contains a nucleic acid genome (DNA or RNA) enclosed in a protein or lipoprotein coat, or capsid. The combined nucleic acid and capsid form the nucleocapsid (Salmond and Fineran, 2015).

Phage infection begins with a multi-step process of adsorption, when the virion eventually attaches itself to a host cell via a specific receptor present on the surface of the bacterium. It must be noted that every bacteriophage only infects the type of bacteria to which it can bind. Then, it proceeds to inject or eject its nucleic acid into the cell in a process called translocation. In addition, bacteriophage replication is divided into a lysogenic cycle and lytic cycle. Lysogeny refers to a process when viruses integrate their nucleic acid into the host's genome or exist within the host's cells as plasmids, without directly killing it (temperate phages). The lytic cycle, on the other hand, leads to the rapid destruction of the infected cell (virulent phages).

The International Committee on Taxonomy of Viruses effectuated a classification of bacteriophages based on a concept that a species is defined by a combination of characteristics which

may or may not all be present in a given representative. Phages are classified by large differences in their structure and nucleic acid components. Nonetheless, it must be mentioned that bacteriophage classification is still open and regularly updated since the process of discovering new phages continues.

The largest, most widespread, and probably oldest group of phages are Caudovirales—tailed phages with double-stranded DNA and complex genetic maps which account for over 96% of all phages. They infect eubacteria as well as single-celled organisms named Archaea. These viruses are also further divided into three main families: Myoviridae, Podoviridae, and Siphoviridae. Contrastingly, tailless phages, which are sorted into 10 families with narrow host ranges, only incorporate 190 viruses, less than 4% of all bacterial viruses (23). There are three types of them: polyhedral (e.g., Microviridae, Leviviridae), filamentous (e.g., Inoviridae, Rudiviridae), and pleomorphic (e.g., Plasmaviridae) (Kasman and Porter, 2021).

A brief history of phage therapy

Since their discovery in the preantibiotic era, bacteriophages have been closely inspected as possible therapeutic agents in bacterial infections, though highly controversial from the very beginning. Félix d'Hérelle himself entertained this concept and attempted to put this idea into practice—he first tried to treat *Shigella*-induced dysentery in rabbits as well as avian typhoid in chickens. Then, after initial success, he moved on to humans suffering from bacillary dysentery. In the latter part of his scientific work, d'Hérelle played a pivotal role, together with a fellow researcher from the Pasteur Institute, George Eliava, in creating an establishment specializing in bacteriophages and their therapeutic capabilities. The center was founded in 1923 in Tbilisi, Georgia, and bears the name of the Eliava Institute—today, it is one of the most significant bacteriophage-centered research organizations in the world. Interestingly, at the outset of phage-mediated therapy, bacteriophages were studied and used as antibacterial agents in Poland, France, and the United States. Unfortunately, the early studies were criticized not only for inappropriate controlling measures but also for inconsistent results. Commercially available phage medications were oftentimes ineffective due to applied preservatives, such as organomercury, which led to decrease in phage activity in the same way as lack of refrigeration and proper purification did. In spite of the commencing scientific skepticism centered around the idea that phage therapy's positive anti-infection effect was a result of stimulation of specific antibacterial immunity or nonspecific phagocytic activity rather than default physiological functions of phages, further research *in vivo* conducted on mice infected with *Bacterium typhosum* demonstrated that phages, being efficacious against a particular bacterial strain, could be indeed used as therapeutic agents in animals (Kasman and Porter, 2021). The aforesaid anti-infection outcome was eventually deemed to derive from the physiological functions of phages themselves. In addition, scientists from Yale University determined that factors in blood, tissue, and bile had only minimal, if any, effect on phage potency. Unfortunately, in those days, statistically controlled trials, standardized methods, and double-blind studies were not a gold standard in science,

leading to raging debates over the biology and application of phages as well as the *in vitro* and *in vivo* impact of these viruses on bacteria. At the moment when phage therapy was in its infancy and needed further development, the era of antibiotics came upon the Western world, diverging the attention from bacteriophages. Regardless, phage therapy continued to be used in parts of Asia as well as in Eastern Europe, particularly in Poland (Hirszfild Institute in Wrocław) and the Soviet Republic. Notwithstanding, the studies done in Eastern European countries were also not up to standards—nonrandomized and uncontrolled—according to the authors of the book *The Bacteriophages*; therefore, they still required further authorization (Kasman and Porter, 2021).

The Western scientific community rediscovered, so to speak, phage therapy in animals sometime during the 1980s. Human experiments started in the 2000s, and the first randomized trial was published in 2009 in the United States (Wittebole *et al.*, 2014). It evaluated the safety of the so-called phage cocktail against *P. aeruginosa*, *S. aureus*, and *E. coli* in 42 patients suffering from chronic venous leg ulcers. In 2004, the Phage Summit was held in Florida being the first international conference in decades, dedicated to bacteriophages and their potential to be an asset to modern-day medicine. The year of 2016 marked the time when the first person in the United States was treated with intravenous phage therapy—the patient felt sick after contracting an infection with dangerous *A. baumannii*. He eventually woke up from a coma and made a full recovery.

Phages, as natural enemies of bacteria, could play a vital role in the treatment of multidrug-resistant bacterial infections. However, since they tend to have quite narrow host ranges, antibiotics, being much cheaper and effective against a wider range of bacteria, overshadowed the development of phage therapy and, so far, have remained the basic way of treating infectious diseases. Nonetheless, due to the ever-increasing antibiotic resistance discussed in the earlier chapter of this work, phage therapy progressively is gaining more worldwide attention and continues to be reexamined all around the globe. Of course, due to specific features of phages being bacterial parasites, unique and even atypical issues arise according to their potential exploitation. Figure 1 presents some advantages as well as potential risks involved in phage therapy.

Phage preparations: administration and storage

To begin with, one of the most important steps is to select the right phages and isolate them. There are two ways of doing so, either by choosing multiple phages which have a wide spectrum of activity and assembling them in a so-called phage cocktail or by firstly isolating the pathogenic bacterium and testing it against a collection of phages—in this case, the bacterium responsible for the infection must be properly identified and phages, to which it is vulnerable, should be screened and confirmed. The standard method for phage selection is a two-layer agar plate (Cui *et al.*, 2019). The general aim is to identify phages exhibiting good antibacterial properties, low potential to harm the patient, and the capability to reach target bacteria *in situ*. Phages, as exceptionally abundant viruses, can be taken from any environmental sources accommodating the target pathogen. It must

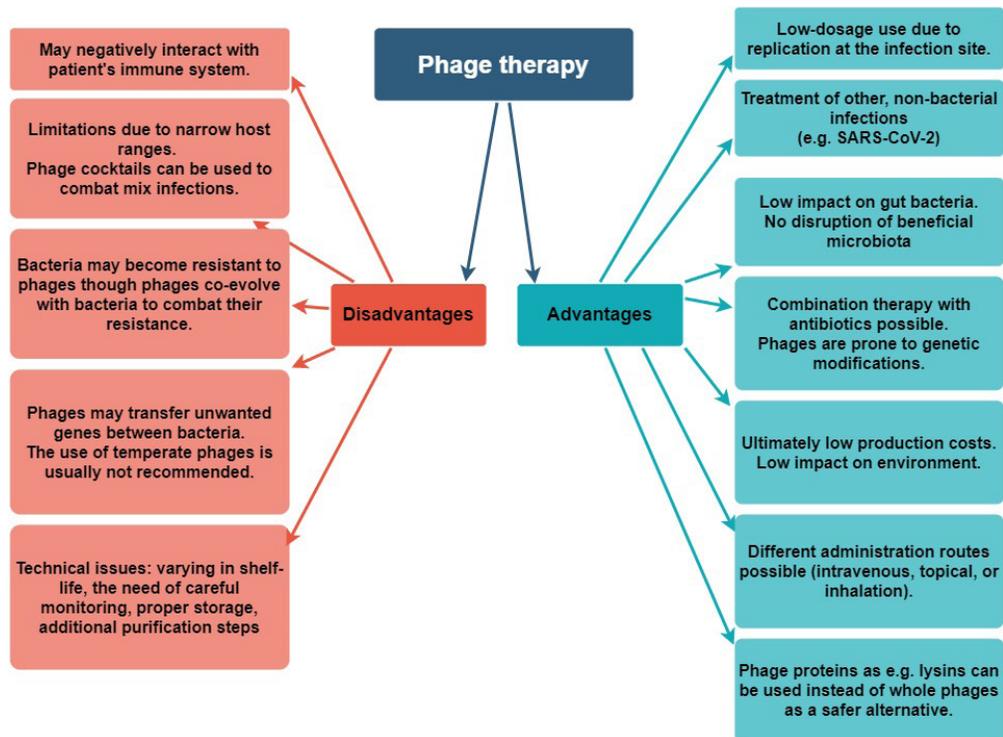


Figure 1. Possible disadvantages and advantages of phage therapy.

be remembered that this form of treatment should be chosen only if traditional antibacterial agents prove ineffective. Fungal, viral, or parasite-related infection cases should not be treated with phage therapy—at least it is not recommended at this point—however, the newest research may suggest otherwise, as it will be discussed in the upcoming part of this work. Additionally, the procedure of phage isolation usually entails a form of phage enrichment as well as a detailed process of purification to remove any unwanted microorganisms, for instance, by clarification of lysed cultures through centrifugation or filtration. Following isolation, phages need to be genetically sequenced and characterized. As in the case of other antibacterial agents, the most important criteria for selecting a suitable bacteriophage are the specificity, efficacy, and possible lack of adverse effects. The selected bacteriophage should efficiently absorb and be lytic for the target bacterial host (Kakasis and Panitsa, 2019).

It is ill-advised to use temperate phages in the treatment of infections since lysogeny can eventually lead to phage resistance in bacteria by amplifying the virulence of the target pathogen. Nevertheless, temperate phages may be useful for other reasons—they may act as modulators of mammalian immune responses (Ciešlik *et al.*, 2021). In general, it is crucial to use long-circulating bacteriophages or change their immunogenicity to avoid clearance by the reticuloendothelial system. Reactions of the immune system and synthesis of antibodies may lead to phage neutralization, therefore reducing their antibacterial activity—but it does not have to indicate a treatment failure and emergence of antibodies takes time to happen.

Phage administration can be topical or internal or can be done by injection or inhalation. It is necessary to avoid any parallel use of other medications that would lead to phage inactivation.

Also, phages may not successfully diffuse in the human body. As a consequence, it must be kept in mind that the finest way of phage administration is delivering them as close to the pathogen as possible. For instance, capsules protecting phages from stomach acid are preferred in gastrointestinal bacterial diseases, for respiratory tract infections aerosolized phage preparations are suggested to be used, phage powder or lotions are recommended for skin infections, and for bloodstream infections intravenous administration should be considered (Cui *et al.*, 2019). Selecting a suitable route of administration will ensure success in therapy. Furthermore, there must be a minimum of 10 virions for every bacterial cell to decrease the target bacterial population (10^8 bacteriophages/ml) (Kakasis and Panitsa, 2019).

Formulations containing phages must be stored properly in view of the fact that these viruses are vulnerable to several factors such as pH value, high temperature, and mechanical stress—therefore, any extreme environmental conditions should be avoided. Certain techniques have been developed to ensure appropriate storage (Loh *et al.*, 2020). Phage preparations must be free of endotoxins, kept with high titers, and stored at a low temperature (cannot be placed above 37°C) with a pH value between 7.35 and 7.45. The spray-drying and freeze-drying (lyophilization) techniques remain a method of choice in phage formulation. Moreover, preparations should not be refrozen, and their shelf-life must be regularly monitored (Leung *et al.*, 2018; Malik *et al.*, 2017).

Safety issues

In general, bacteriophage therapy is considered quite safe, given the everyday human exposure to these viruses and their presence in the body. Overall, no major life-threatening immune

reactions have been reported (Furfaro *et al.*, 2018). As a further matter, because of their narrow host ranges and high specificity, phages affect gut microflora in a marginal way. However, there are some concerns about phage-induced immunological reactions and their impact on body tissues. After all, they are biological agents, and this fact should not be forgotten. The ability of phages to modify bacterial targets by transduction of DNA between bacteria or by genetic expression of virulence factors can also bring about unfavorable side effects, making bacteria more moribund—this is the reason why scientists do not recommend utilizing temperate phages for treatment purposes (in favor of lytic ones) (Nilsson, 2019). Their ability to display lysogeny, which makes them incorporate their genomes into bacteria, may have a detrimental outcome on the patient's health. Unfortunately, it must be emphasized that not all phages facilitate a good therapeutic result. Clearly, nontemperate phages may also carry bacterial virulence factor genes. They too exhibit transduction but tend to destroy the host or if they possess predominately or only bacterial genes, they do not reproduce. It must be mentioned that some concerns also arise from phage-induced bacterial lysis, which results in the release of bacterial endotoxins, as well as from production-based endotoxins present in phage preparations (Szermer-Olearnik and Boratyński, 2015). The last problem may be resolved by using a sufficient purification process; the first one may place a limit on the treatment of Gram-negative systemic infections. For the most part, bacteriophages are composed mostly of proteins and nucleic acid molecules; therefore, they themselves are relatively nontoxic, and because of their capability of self-replication, smaller preparation doses may be needed, which directly transfers to lesser expenses. It must be recalled that phages and bacteria are subjects to an everlasting process of coevolution. In theory, it is possible for bacteria to develop resistance to phages by either chromosomal mutations or antiviral mechanisms, such as via changing the structure of their surface phage receptors (analogous as in the development of antibiotic resistance). In order to tackle this issue, combining both phages and antibiotics might be a good solution. It has been observed that they could act together to prevent resistance, and surprisingly, phage resistance may also result in antibiotic resusceptibility and bacterial virulence reduction, although further research is needed, especially considering the antibiotic dosage (Oechslin, 2018). Also, multiple phages introduced in phage cocktails could combine efforts in targeting various receptors on the surface of bacteria, which may contribute to a lower chance of resistance in empirical therapy. The strategy of the so-called personalized phage treatment which involves using single phages or targeted phage cocktails, effective against isolation from the patient bacterium, is generally more expensive, though it may prove to be a better way to counteract bacterial resistance.

In conclusion, the emergence of resistance must be carefully monitored, and phage therapy should be used with a great dose of supervision. Fortunately, new phages will always be present in nature since, as mentioned earlier, they are also a subject of evolution, and bacteria, even when resistant to present-

day phages, have shown weakness toward past and future ones (Oechslin, 2018).

As established before, antibiotic resistance poses a significant threat to modern-day medicine and in agriculture, mainly in livestock and food production, among others (Wernicki *et al.*, 2017) (Fig. 2).

PHAGE THERAPY: APPLICATIONS IN MEDICINE

In medical sciences, the growing number of infections has become harder to treat. Since its early days, phage therapy has been used by physicians to treat various health conditions. Today, many types of phage applications are reexamined as potential therapeutic options.

Dermatology

Skin is the largest external sensory organ in the human body being susceptible to various types of damage. Hard-to-treat wound infections and dermatological problems affect thousands of people around the world each year. Pathogens such as *Klebsiella pneumoniae*, *P. aeruginosa*, and *S. aureus*, often resistant to antibiotics, frequently colonize wounds especially in hospital environments. In cases of severe infections, bacteriophage therapy can be used experimentally. Usually, delivering phages to the infection site of the wound requires liquid formulations or powders, possibly creams too. Medicines can be applied by dripping the solution onto the wound or applying soaked gauze, although it may lead to losing phages that may get stuck in the gauze or drip down from the wound (Chang *et al.*, 2020). Preparations in the form of gels and creams are meant for topical application and mucous membranes. A successful safety trial of phage therapy against skin ulcerations and other wounds was completed in the year of 2008 with positive results. Phage therapy has been used in the treatment of such conditions as postoperative infections, infected venous stasis ulcers as well as infected wounds, and diabetic foot complications (Duplessis and Biswas, 2020). However, it must be noticed that in many trials a fluctuant level of efficacy of the applied phage preparations was observed, possibly due to, for instance, insufficient concentrations. A few side effects have been observed, mostly revolving around increased localized pain and eczematous changes. Following the Eliava Institute research, bacteriophage therapy can aid physicians in treating skin conditions such as *hidradenitis suppurativa*, complications of acne, staphylococcal- and streptococcal-associated skin diseases, and folliculitis (Kutateladze and Adamia, 2008).

General medicine

In internal medicine, respiratory tract infections are one of the most common problems. Phage-based treatment could be used as an alternative or supplementary option for patients suffering from RT ailments, such as laryngitis, pneumonia, chronic bacterial bronchitis, and sinusitis. The accepted method for delivering these viruses into the respiratory tract is nebulization with suitable devices, properly adapted to phage size. In general, inhaled phage therapy is considered safe, and although it needs more detailed and rigorous *in vivo* studies, it has already been successfully used in

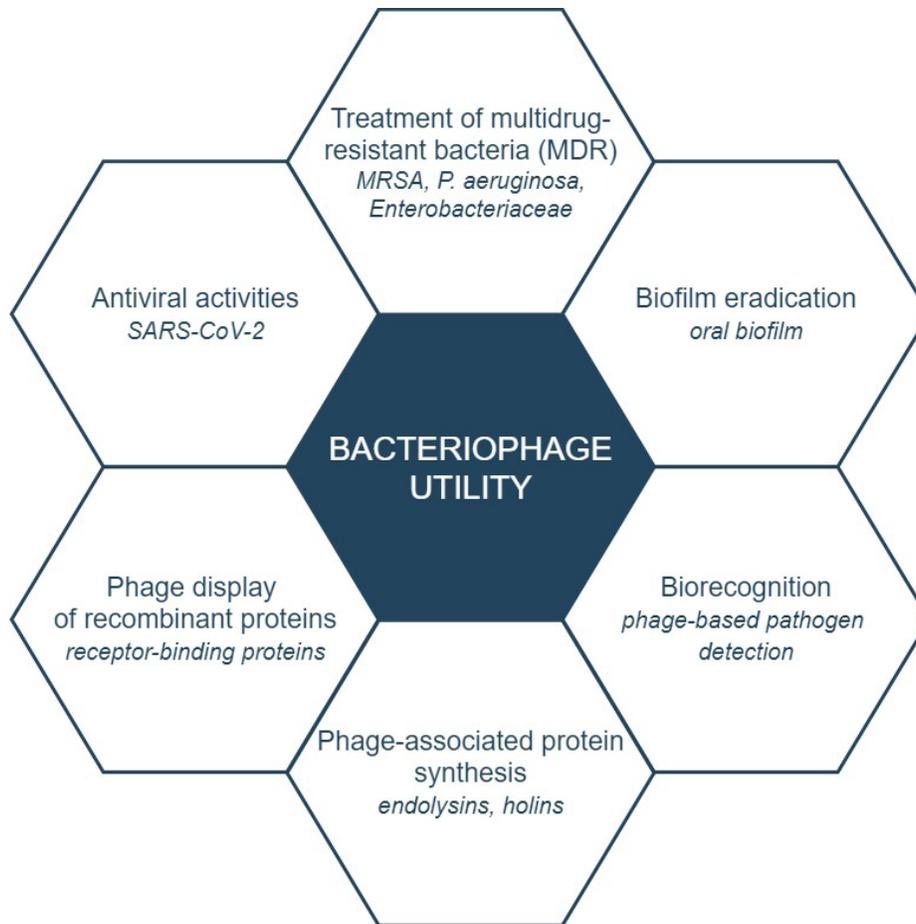


Figure 2. Examples of the most promised directions in bacteriophage application.

the treatment of bacterial infections of the lungs, both in animals and in humans (Li *et al.*, 2021). Additionally, gastrointestinal diseases, such as gastritis, irritable bowel syndrome, enteritis, colitis, diarrhea, and constipation, can also be treated with phage therapy (Kutateladze and Adamia, 2008; Maronek *et al.*, 2020; Vale *et al.*, 2008).

It should be recalled that gut microbiota plays a surprisingly important role in the human body—in metabolism, health, and physiology. Gut bacteria may be involved in the development of many chronic diseases and disorders, such as obesity, alcoholic liver disease, diabetes mellitus, and autism symptoms, and can even impact the brain itself through their metabolites (e.g., butyrate). Bacteriophages may regulate microbiota quality as well as quantity and therefore could be used as a method of modulation and manipulation of pathogenic bacteria present in the gut, when a controlled diet does not deliver the expected benefits for the patient (Paule *et al.*, 2018).

Clostridioides difficile-related contagious infections are a frequent ailment in hospitalized patients, especially those who undergo an antibiotic treatment, leading to diarrhea, inflammation of the colon, and even death in severe cases. Bacteriophages that target *C. difficile* have been identified, albeit temperate phages, since no lytic ones have been found. Nevertheless, research suggests that even these phages, usually not recommended for healthcare-related use, could eventually be employed against

this type of infection (Sangster *et al.*, 2014). Phage therapy may also prove to be a useful therapeutic tool against *E. coli*, a major pathogen, as well as *H. pylori* which colonizes the stomach in about half of the human population and is associated with peptic ulcers, gastritis, and stomach cancers. However, further advances in research are needed. Sepsis, an uncontrolled inflammatory response, is a life-threatening condition that remains a demanding clinical challenge and a leading cause of the death of critically ill patients in the intensive care unit. Bacteriophages, which have been shown to possess immunomodulating abilities (e.g., potent anti-inflammatory properties), may become a positive treatment option in the future, as animal-based and experimental studies on humans suggest (Górski *et al.*, 2020a). These viruses stimulate an increase in phagocytosis which constitutes a significant antisepsis strategy (Górski *et al.*, 2017).

SARS-CoV-2/COVID-19

The newest, continuous research has pointed scientists toward evaluating beneficial attributes of phage therapy in the treatment of COVID-19 patients. Phages, surprisingly, revealed an ability to interfere with eukaryotic viruses, both *in vivo* and *in vitro*. Polish scientists from the Institute of Immunology & Experimental Therapy, the Polish Academy of Sciences in Wrocław, and from the Medical University of Warsaw suggest a possible explanation of this phenomenon. Antiviral activities of phages could depend

on phage-induced interferon production or on competition of phages and eukaryotic viruses for the same receptors on the cellular surface. Ultimately, phages may stimulate the production of antiviral antibodies cross-reacting with pathogenic viruses (phage vaccines) (Górski *et al.*, 2020b).

Their scientific research also gives an indication that the T4 phage may serve as a protection for epithelial cells, coating human lungs and kidneys, when they are infected with *adenovirus* (adsorption and reproduction inhibition) and that bacteriophages present in the body may transfer themselves from other organs and infiltrate into epithelium layers without a damaging aftereffect (Górski *et al.*, 2020b). A sort of biological shield against eukaryotic virus particles could be set up in the organism, following a rise in phage transcytosis by epithelial cells, driven by bacteriophage therapy—it could have a protective effect against coronavirus-mediated invasion of the lungs. Likewise, the aforementioned T4 phage may be able to reduce coronavirus's attachment to target cells via its KGD sequence. Anti-inflammatory activity of phages (inhibition of NF- κ B) as well as induction of probable antiviral immunity through the expression of antiviral cytokines (for instance, IFN- α and IL-12) could also have a potentially good therapeutic effect on patients infected with the infamous SARS-CoV-2. Scientists noticed that bacteriophages are always present in a healthy respiratory virome and incidences of viral microorganisms causing infections in the body are linked to a lower percentage of phages in the RT. Apart from that, phage therapy could be utilized in the treatment of COVID-19 bacterial complications, occurring in many cases. In general, even though it is not recommended to use bacteriophages against nonbacterial infections, as it was mentioned before, the newest promising research suggests that they can, in fact, prove exceptionally useful against viral (Epstein-Barr virus, SARS-CoV-2, adenovirus) as well as fungi-induced diseases (by, e.g., *Candida albicans*, *Aspergillus fumigatus*) (Górski *et al.*, 2020b).

More effective treatment of biofilm using bacteriophages

The biofilm phenomenon and its detrimental function in the hospital environment and humans' health have been well known for decades (Costerton *et al.*, 1999). Biofilm-forming microorganisms include, e.g., *S. aureus*, *P. aeruginosa*, and *E. coli* as well as *Actinomyces* spp., *Lactobacillus* spp., *Streptococcus* spp., and *Enterococcus* spp. while the last four play the main role in oral biofilm. A functional role of biofilms is to defend the living bacterial community against (negative) influences from the environment. In this context, biofilm-forming cells are hardly accessible for treatment options, such as disinfectants or antibiotics, when compared to those living beyond this structure (Doub, 2020). Not surprisingly, MRSA and *P. aeruginosa* are generally responsible for the most serious hospital-acquired infections. On the other hand, an implant and oral biofilms may lead to common complications in dental treatment and are associated with a chronic inflammation state which, in turn, may lead to teeth or implant loss (Lafaurie *et al.*, 2017).

Phage therapy is now considered to be the most useful strategy in the treatment of oral biofilm since the traditional antibiotic-based approach has been ineffective due to the fact that an outer coat of cells hinders the interaction of antibiotics with the bacteria. Bacteriophages can break up biofilm consistency

through the action of depolymerase—a phage enzyme that allows degrading polysaccharides mostly contained in biofilms (Azeredo *et al.*, 2021).

Other applications of phage therapy in medicine include ear infections, such as otitis media and otitis externa, and urological and gynecological disorders, such as chronic bacterial prostatitis, acute and chronic vaginitis, cystitis, and cervicitis (Kono *et al.*, 2021; Landlinger *et al.*, 2021; Leitner *et al.*, 2021).

Bacteriophage-derived vaccines and treatment with endolysins

Bacteriophages, since their development, have attracted researchers' attention as promising, genetically modifiable delivery vehicles and platforms for vaccines which can be mass-produced, being cost-efficient as well. The use of phage-associated proteins allows omitting the hurdles arising when working with fully functional bacteriophages while at the same time enabling scientists to induce modifications by means of genetic engineering techniques. Nowadays, a variety of bacteriophage proteins have been taken into consideration. These encompass, e.g., receptor binding proteins (RBPs), virion-associated peptidoglycan hydrolases, endolysins, and holins. Nanoparticles- (NPs-) based vaccines tend to arouse both humoral and cellular immune responses against specific pathogens, thus providing host protection against infection. Although produced by a bacteriophage, the NPs can protect the organism against a broad range of pathogens including those of viral, bacterial, and parasitic origin depending on the genetic recombinant construct that was utilized (Kingston *et al.*, 2019; Purwar *et al.*, 2018; Roth *et al.*, 2021; Tao *et al.*, 2019).

Phage-based detection of emerged microorganisms

One of the primary advantages of using bacteriophages is their specificity toward the host and the fact that they can infect only live bacterial cells. This, in contrast with the other biorecognition elements such as enzymes, antibodies, and nucleic acids, makes phages an ideal tool for the identification of different emergent pathogens. RBPs of tailed phages composed of tail tips, fibers, and spikes can selectively interact with receptors present on cellular surfaces of bacteria (Arnaud *et al.*, 2017).

Phage display of recombinant antibodies

In medicine, there are two types of phage-virus vaccines: phage-displayed vaccines (more popular) and phage DNA vaccines. Displayed vaccines consist of bacteriophages which demonstrate, on their surface, immunogenic proteins/peptides through transcriptional fusion or by utilizing predisposed peptides which capture antigens via their binding receptors (Khan *et al.*, 2015). In phage DNA vaccination, in order to immunize the organism, a eukaryotic expression cassette is replicated into the genome of a phage and then the whole phage particles are applied (Kuhl *et al.*, 2012). These vaccines have been examined as a possible tool of prevention for certain diseases caused by other viruses (HIV, hepatitis B), bacteria (*Yersinia pestis*, *Chlamydia abortus*) as well as fungi (*C. albicans*), parasites (*Taenia solium*), and even cancer (Lewis lung carcinoma, melanoma) (Bao *et al.*, 2019).

As an alternative to using entire phages in traditional therapy, making use of single phage-encoded enzymes, in particular endolysins, in the fight against bacteria-induced infections has also been proposed. Endolysins come about as highly specified, powerful, quickly effective bacteriolytic agents. They are particularly helpful with Gram-positive bacterial infections. Sadly, in the case of Gram-negative bacteria, the outer membrane defends their peptidoglycan from exogenous endolysins. Altogether, endolysins lead to a quick and efficient release of phage progeny from the host bacterium. Phage lytic enzymes, which have been correctly purified, could be used as a method of control directed at moribund bacteria present on the mucous membranes—it may potentially prevent diseases that begin with mucosal membranes infections. However, it is possible that gut microbiota will also be affected by endolysins because of their broad lytic activity. Nonetheless, further research is required, though at this moment endolysins seem to bear no risk of virulence transduction and bacterial resistance (Viertel *et al.*, 2014).

CONCLUSION

In the past two decades, scientific understanding of bacteriophages' nature has changed enormously, gaining a new dimension, so to speak. These viruses displayed a tremendous potential to become highly beneficial for humankind. The research seems to support phage therapy and phage-derived enzymes as a promising, future alternative to antibiotics in the treatment of multidrug-resistant bacterial infections. So far, several scientific reports have documented the current achievements in the development of antibacterial, bacteriophage-based resources. Although the historical background and the discoveries of milestones in phage research remain usually constant, the authors' point of view often relies on different factors such as journal topic or even geographical location. Consequently, the discovery or rise of new infectious agents such as SARS-CoV-2 will focus researchers toward developing new fighting strategies (Golkar *et al.*, 2014; Lin *et al.*, 2017).

It is worth mentioning that Poland has an over-100-year tradition of phage therapy with the establishment of the Hirsfeld Institute as a milestone. Its scientists conduct pioneer, world-leading research, publish numerous articles, submit international patent applications, and systematically gain new data about bacteriophage therapy, even in terms of its implementation in the treatment of nonbacterial diseases. It constitutes a great opportunity for the country to advance on phage therapy and soon utilize it as a legally accepted, therapeutic method.

Future perspectives and recommendations

It must be remembered that the use of phages in the European Union, Australia, and America is still experimental, though human studies are progressing and the number of documented cases, where phage therapy was successfully applied as a therapeutic method, is steadily increasing. In the food industry, instead, there are several commercial phage preparations available that are used for the suppression of bacterial pathogens. These encompass, e.g., *Salmonella* spp., *Listeria monocytogenes*, MRSA, *E. coli* O157:H7, *M. tuberculosis*, *Campylobacter* spp., and *Pseudomonas syringae* (Lin *et al.*, 2017). However, it should also be highlighted that phage therapy is definitely not a panacea for modern-day medicine. There is still much to be learnt in the field of phage immunomodulatory

effects, the possibility of horizontal gene transfer, phage host range, and bacterial resistance to phages (even so, it should not be thought of as the same as antibiotic resistance).

The decline of the antibiotic era is coming, and healthcare providers will soon be met with a difficult challenge—progressively, more people will die due to severe, hard-to-treat, drug-resistant infections. Therefore, the advantages and disadvantages of phage-centered therapy must be carefully analyzed, with a reminder that we are dealing with biological organisms subjected to ever-lasting evolution and there is always a chance that their use will bring about detrimental effects, or perhaps, with further research, bacteriophages will indeed become our superheroes in the fight against superbugs.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

Izabela Durbas (ID) was principally responsible for the overall concept and design of the study. ID was also responsible for searching and receiving publications from databases. ID and Grzegorz Machnik (GM) performed analysis, selection, and interpretation of data. ID and GM prepared the manuscript and figures. Bogusław Okopień (BO) participated in the processing and contributed to the critical revision of the manuscript, proofreading, and supervision. All authors read and approved the final version of the manuscript to be published.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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