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Review

The History and Applications of Phage Therapy in *Pseudomonas aeruginosa*

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Abstract: The *Pseudomonas aeruginosa* is one of the bacteria that cause serious infections due to resistance to many antibiotics can be fatal in severe cases. Antimicrobial resistance is a global public health concern. To solve this problem, interest in phage therapy has revived; some studies are being developed to try to prove the effectiveness of this therapy. Thus, in this opinion article, several historical aspects are addressed as well some applications of phage therapy against *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*; bacteriophages; therapy; resistance; antibiotic

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1. Introduction

Bacterial resistance to antibiotics is a public health problem that raises concern worldwide, with both clinical and economic implications, and is associated with inappropriate use of antibiotics. The United Nations Organization (UNO) estimates about 10 million deaths per year in the 2050s. For this reason, new therapeutic strategies, beside traditional antibiotics, must be urgently developed. One of these strategies can be the use of bacteriophages (phages).

Phages are viruses that infect bacteria. These are the most ubiquitous and diversified biological group residing on Earth. They have high specificity for the host and high permanency in natural systems [1]. Due to their obligate requirement of a bacterial host, phages are abundantly found distributed essentially anywhere their host exists in the biosphere. Their abundance in nature was estimated as ten to hundreds of millions of phages in every gram of soil, water, and billions on and inside the human body at any moment [2].

Phages can also be used as therapeutic agents [3]. In contrast to antibiotics, phage therapy specifically lyses the host bacteria and does not affect non-host bacteria [4]. After the inactivation of the host pathogenic bacteria, the phage abundance is in proportion to that of the host pathogenic bacteria and thus, when the host bacteria diminish, the phage count also decreases, which maintains the microbial stability and diversity [3].

Due to the high rates of resistance of *Pseudomonas aeruginosa* to antibiotics, there is a growing concern about finding alternatives to antibiotics, such as phages. As of March 2019, there were about 137 phages sequenced and published in a public bank, targeting *P. aeruginosa*. Most of these phages belong to the Caudovirales family [5].

The specific phages for *Pseudomonas* been described in the mid-20th century [6].

2. Pseudomonas Aeruginosa

P. aeruginosa is a Gram-negative bacillus, belong *Pseudomonadaceae* family, included in the gammaproteobacteria class, mono-flagellated, straight or slightly curved [7]. It has basic nutritional needs, is non-fermenting carbohydrates, and has a high tolerance for stress, physical and chemical factors [7].

High number of virulence factors, such as enzymes that degrade effector molecules of the immune system and elements essential to the host's cellular and tissue structure, such as proteases and elastases, are present in *P. aeruginosa*. It also includes phenazine and its derivatives, such as pyocyanin, which are associated with the alteration of states of cellular oxidation, inter-cellular communication, and regulation of gene expression, assuming a crucial role in cell survival, especially in the context of infection. Pyoverdin, a greenish pigment, is a siderophore that mediates the absorption of iron ions, an essential element for the survival and growth of the organism [7]. It is also important to highlight the presence of membrane transport systems, namely type III secretion systems, which, despite not being virulence factors themselves, mediate the efflux of proteins responsible for the destruction of epithelial barriers and host cells, and interfere with the activity of macrophages and neutrophils.

This bacterium is able to alternate between planktonic lifestyle and growth in biofilm, which helps to survive in specific niches, such as catheters and several hospital surfaces, giving it a greater tolerance for disinfectants and antibiotics. It is also associated with chronic colonization of internal devices and prosthetic material, as well as the lungs of patients with cystic fibrosis or bronchiectasis [8]. The polar flagellum and pili type IV are involved in the formation of biofilm, and they mediate the capacity for mobility and are relevant in adherence to host tissues [9].

The genome of *P. aeruginosa* is relatively large (5.5–7 Mbp) compared to other sequenced bacteria such as *Bacillus subtilis* (4.2 Mbp), *Escherichia coli* (4.6 Mbp) and *Mycobacterium tuberculosis* (4.4 Mbp) and encodes a large proportion of regulatory enzymes important for the metabolism, transport and efflux of organic compounds. This coding capability of *P. aeruginosa* to the genome allows great metabolic versatility and high adaptability to environmental changes [10]. *P. aeruginosa* has been recognized as an opportunistic pathogen that is the most common bacterium associated with healthcare-associated infections (HAI) such ventilated-associated pneumonia (VAP), one of the most common HAI [10] It rarely affects healthy individuals, but causes high morbidity and mortality in patients with cystic fibrosis (CF) and immunocompromised individuals [10].

With the increase of antimicrobial resistance and the tendency to evolve to multiple resistance when in the presence of antibiotics for long time, it is necessary to understand the resistance mechanisms at their disposal, the means of evolution and dissemination, and to find alternative methods [11].

When subjected to selective antibiotic pressure, the induced response facilitates bacterial survival and develops antimicrobial resistance [12].

The Problem of Bacterial Resistance in P. aeruginosa

Portugal is a country with a high consumption of antibiotics, and antimicrobial resistance has grown sharply. The development of resistance is a natural process due to the selective pressure exerted by the use of antibiotics, and it has undergone a progression due to the inappropriate use of antibiotics [12].

P. aeruginosa developed the ability to resist to several antibiotics and it became a challenge to treat this type of infection [10]. The WHO recently revealed its antimicrobial

resistance to carbapenems, emerging the development of new therapies in the treatment of this type of infections [10].

P. aeruginosa has shown high resistance to a range of antibiotics, including aminoglycosides, quinolones, and β-lactams. The excessive use of antibiotics culminates in the increase of resistance of this strain and consequent inefficiency of the result of the empirical antibiotherapy [13].

The main resistance mechanisms are intrinsic, acquired, and adaptive resistance. Intrinsic resistance is done through the low permeability of the outer membrane, expression of efflux pumps inactivating enzymes and production of antibiotics. Acquired resistance may occur for horizontal transfer of resistance genes or gene mutational changes. Adaptive resistance is the ability of bacteria to adapt to the antibiotic creating mechanisms to protect against it [13].

The most important mechanisms of resistance to β-lactams is the production of β-lactamase enzymes, however, the resistance can also be caused by overexpression of efflux systems by changing the membrane permeability and the synthesis of binding protein to penicillin (PBPs) with low affinity for β-lactams. The chromosomal β-lactamase AmpC is intrinsic to this species and confers resistance to some β-lactamase [14].

B-lactamases can inactivate β-lactam antimicrobials by breaking the β-lactam ring, by destroying the amide bond. The hydrolysis of β-lactam antibiotics occurs by the formation of an ester bond between the active serine site (or with zinc ions, in the case of metallo-β-lactamases) of the β-lactamase enzyme and the β-lactamic ring of the antimicrobial. In *P. aeruginosa* these enzymes are present in the periplasmic space and inactivate the β-lactamic after they cross the outer membrane, before binding to PBPs [15].

A large part of the genes encoding the β-lactamases enzymes are in mobile regions of bacterial DNA, such as plasmids and class 1 integrons, which contribute to the increase in bacterial resistance. The most frequently acquired β-lactamases are pseudomonas-specific enzymes (PSEs) PSE-1 and PSE-4.

Transferases are important in resistance to aminoglycosides, since catalyse acetylation, adenylation or phosphorylation of antibiotic inactivating it. Methylases are also relevant in resistance to these pharmacological agents because transform the 30S ribosomal subunit and prevent the binding of clinically relevant compounds such as gentamicin, tobramycin and amikacin [14].

Chromosomal gene mutations such as depression of the *amp*C gene, loss of the OrpD porin and horizontal gene transfer are extremely important in conferring resistance to antibiotics. The genes responsible for the expression of β-lactamases or aminoglycoside-modifying enzymes are acquired by natural transformation processes. Generally, these genes are inserted in integrations, genetic elements that capture exogenous genes and guarantee their expression, inside plasmids or transposons, which allow their transfer between cells. At *P. aeruginosa*, these integrations contribute to the development of multidrug resistance, since they rarely include only one element of resistance to antibiotics [16].

3. Bacteriophages

3.1. History of Phage Therapy

Several visionary scientists contribute over the time to the development of what is now referred of phage therapy (Figure 1). This was a very long history that benefits from the contribution of many.

In 1896, Ernest Hanbury Hankin, a British bacteriologist working as the Chemical Examiner and Bacteriologist to the Government of the United Provinces and of the Central Provinces of India, demonstrated the presence in two rivers in India, the Ganga River and the Yamuna River, of an unidentified substance with high activity against *Vibrio cholerae*, limiting the spread of the cholera epidemic [17–19].

Figure 1. Phage therapy timeline. Adapted from Green et al. [20].

In 1915, Frederick Twort, a British microbiologist, observed and described transparent plaques in *Staphylococcus* cultures that appeared successively in different strains after appropriate filtration [21]. Twort published an article describing a filterable agent responsible for bacterial lysis, raising the hypothesis that it is a virus [21].

Two years after this report, Felix d'Herelle, a microbiologist at the Pasteur Institute in Paris, was commissioned to investigate a serious outbreak of haemorrhagic dysentery of bacterial origin in French troops. From faecal patient samples, he obtained a bacteria-free filtrate which was later incubated with *Shigella* spp. He observed a result similar to that described by Twork, which he named lysis plates [18].

In 1917, Felix d'Herelle, proposed that the phenomenon was caused by an obligate intracellular parasite able of parasitizing bacteria, for the first-time naming bacteriophages [22]. During this year, were isolated phages able to lysepathogenic bacteria as

Salmonella typhi, *Escherichia coli*, *Pasteurella multocida*, *Vibrio cholerae*, *Yersinia pestis*, *Streptococcus species*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* [23].

In 1923 was raised in Georgia the Eliava Institute (EIBMV), by d'Herelle e Georgi Elliava.

During World War II, certain regions of the Soviet Union and Eastern Europe had limited access to antibiotics and, consequently, developed phage therapy. The practice of phage therapy in the Soviet Union has been well advised and is still widely used in Russia and Eastern European countries for more than 80 years, especially at the Eliava Institute in Tbilisi, Georgia [2].

In 1952, at the Hirszfed Institute, a program was developed to treat phage patients with suppurative infections [24]. In the 1980s, controlled studies with animals were published in the English scientific literature. In recent years, some Western European countries have started approved therapeutic use [25].

In 2016, Paul Turner and colleagues reported isolating a phage that could restore antibiotic sensitivity in multidrug-resistant P. aeruginosa. This phage was later used to treat a patient with a longstanding aortic graft infection that did not respond to multiple surgical interventions and aggressive antibiotic therapy with a single application of phage [26].

3.2. Phage Structure and Taxonomy

Phage vary in their complexity, structure, genetic material, shape (tail filaments and icosahedral), and size [27]. The genome is composed of single or double stranded DNA or RNA of very variable length, arranged in a linear or circular shape. Its size varies from a few thousand base pairs to 498 kilobase (Kb) pairs in G phage, the largest phage sequenced up to the moment [2,28].

Most bacteriophages are composed of an icosahedral head associated with a helical symmetry protein tail [28]. The head is formed by the capsid, constituted by repeated structural protein subunits that surrounds the nucleic acid. The capsid protects the nucleic acid and possesses proteins which confer specificity for certain bacterial cells [29]. The neck connects the head to the tail, a heteroligomer composed of various proteins that ensure genome release when the virion is bound to the host cell.

The distal portion of the tail has a basal plate to which tail fibers and spicules are attached which have proteins capable of binding to membrane receptors of certain bacteria [28]. The tail may have different contractility and dimensions, may exhibit accessory structures such as spicules, collar, lipid envelope or absence of envelope [28].

Virus taxonomy is currently the responsibility of the International Committee on the Taxonomy of Viruses (ICTV), and the Bacterial and Archaeal Subcommittee (BAVS) within the ICTV that focuses on phages [30] which published its first report in 1971 [29]. The system is based on the evaluation of a variety of phage properties including the molecular composition of the virus genome (ss/ds, DNA, or RNA), the structure of the virus capsid and whether it is enveloped or not, the host range, pathogenicity, and sequence similarity [30].

Phages are classified through a structural and sequence-based taxonomic system (Table 1); initially into families, and each family is further categorized in accordance to the capsid structure, the structural and chemical composition of the genes and the mechanism of their mRNA production [2].

Phages can be classified in to Siphoviridae, Podoviridae, Myoviridae, and Filamentous phages (Table 1), according to morphology and nucleic acid (International Committee on Taxonomy of Viruses, ICTV) [3]. Virulent tailed phages of the Caudovirales order have been the best described for phage therapeutic applications. Within this group, the Myoviridae have a large capsid head and contractile tail, the Siphoviridae have a relatively small capsid and a long flexible non-contractile tail, and the Podoviridae have a small capsid head and short tail [1].

Table 1. Phage classification according to ICTV [3].

Form	Nucleic Acid	Order	Family	Details	Examples
With tail	dsDNA (L)	Caudovi-rales	Myoviridae	Contractile tail	T4, Um, P1, P2
			Siphoviri-dae	Long tail, not contractile	Λ, T5, HK97, N15
			Podoviri-dae	Short tail	T7, T3, φ29, P22
Polyhedral	ssDNA (C)	Petitvira-les	Microviri-dae	Capsomers conspicuous	PM2
	dsDNA (C,S)	Linavira-les	Corticovi-ridae	Complex capsid, lipids	PRD1
	dsDNA (L)	Kalamavi-rales	Tectiviri-dae	Double capsid, lipids, pseudo-tail	PRD1
	dsDNA (L)		SH1 *	Double capsid, lipids	SH1
	dsDNA (C)		STIV *	Tower-shaped protrusions	STIV
	ssRNA (L)	Levivira-les	Leviviridae	Like poliovirus	MS2
	dsRNA (L, M)	Mindivi-rales	Cystoviri-dae	Envelope, lipids	Φ6
Filamentous	ssDNA (C)	Tubulavi-rales	Inoviridae	Long filamentous, short stem	M13
	dsDNA (L)	Ligamen-virales	Lipothixvi-ridae	Envelope, lipids	TTV1
	dsDNA (L)		Rudiviro-dae	Rigid rods type, TMV	SIRV-1
Pleomorphic	dsDNA (C,S)		Plasmavi-ridae	Envelope, without lipid capsid	L2
	dsDNA (C, S)		Fusellovi-ridae	Lemon shape, envelope	SSV1
	dsDNA (L,S)		**	Lemon shape, envelope	His1
	dsDNA (C, S)		Guttaviri-dae	Drop shape	SNDV
	dsDNA (L)		Ampulla-viridae *	Bottle shape, NC helical	ABV

Legend: * unnamed; ** unclassified; dsDNA—double strand DNA; ssDNA—single strand DNA; C, circular; L, linear; M, multiparty; NC, nucleocapsid, S, supercoiled.

3.3. Phage Infection Process

Phages are mandatory intracellular parasites do not have their own metabolism, for this reason, requiring the metabolism, energy resources and material resources of the hosts to replicate. They need to bind to specific locations on the host's surface. This connection is always necessary regardless of its propagation cycle, which can be classified as lytic, tempered and chronic phages [2].

The life cycle of the phage may differ only lytic or lysogenic, depending on the type of phage and the physiological state of the bacterial cell.

3.3.1. Nucleic Acid Recognition, Adsorption, and Injection

This stage is common to both life cycles of bacteriophages.

In the first stage of the infection process, the phage recognizes the receptors present on the bacterial surface. A variety of components present in the bacterium, such as flagella, pill, capsule, LPS and proteins, are potential receptors for phages [31,32]. The initial contact with the bacteria occurs through Brownian movements of the viral particle [31].

Subsequently, a reversible bond occurs, mediated by the tail fibres [33]. Then the specific and irreversible binding of the Bio Protein Receptor (RBPs) of the phage with the target receptors of the bacterium, promotes a change in the conformation of the viral particle allowing the release of its genome through the capsid [34], thus transferring the nucleic acid into the bacterial cytoplasm.

Phages that have contractile tails can penetrate the cell wall and inject the nucleic acid directly into the cytoplasm to bacterium [32]. After the Phage introduces its nucleic acid

into the bacterial cell, the life cycle may diverge to lithic cycle or lysogenic cycle, depending on the type of phage and the physiological state of the bacterium. If the phage is virulent, it initiates a lytic cycle, which will promote cell lysis. If the phage is temperate, these have genes that regulate the two types of cycles; the type of cycle that will occur may be influenced by various factors [32].

3.3.2. Lytic Cycle

In the lytic cycle (Figure 2), phages bind to specific receptors on the surface of the host cell by injecting its genome. After the entry of the nucleic acid, viral transcription begins, through RNA polymerase [32].

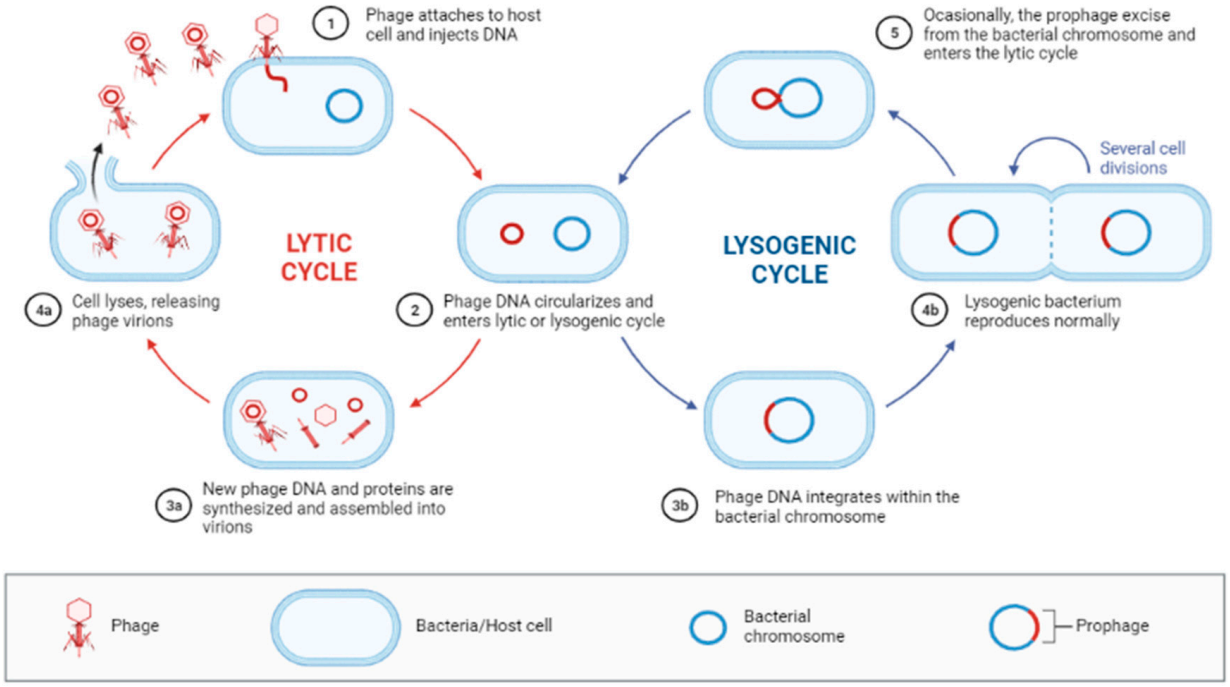

Figure 2. Lytic cycle and Lysogenic cycle. Lytic cycle: Immediately following injection into the host cell, the phage genome synthesizes early proteins that break down the host DNA, allowing the phage to take control of the cellular machinery. The phage then uses the host cell to synthesize the remaining proteins required to build new phage particles. The heads and sheaths are assembled separately, the new genetic material packed into the head and new daughter phage particles constructed. During this process, the host cells gradually become weakened by phage enzymes and eventually burst, releasing on average 100–200 new phage progeny into the surrounding environment. Lysogenic cycle: Following the injection of the phage DNA into the host cell, it integrates itself into the host genome, with the help of phage-encoded integrases, where it is then termed a prophage. The prophage genome is then replicated passively along with the host genome as the host cell divides for as long as it remains there and does not form the proteins required to produce progeny.

Subsequently, the synthesis of structural and catalytic proteins occurs with the capacity to form the structures of new phages. Genes are expressed to encode the synthesis of holin and lysine, enzymes that have the capacity to change the cytoplasmic membrane and the bacterial cell wall, that is responsible for promoting cell lysis, and consequent release the newly formed viral particles, which has the capacity to infect other bacteria [35].

3.3.3. Lysogenic Cycle

Tempered viruses integrate their genome into the host chromosome or plasmid (Figure 2), which is replicated whenever the cell reproduces. The incorporated genome,

also called a prophages, can activate the lytic cycle in the cell. The phage remains in a latent state and replicates its DNA together with that of the bacteria [32] and this state of latency is not irreversible, occasionally these bacteria can enter the lytic cycle.

The factors that lead to the activation of the temperate phage lytic cycle are not well known, but it is known that factors causing stress in the host cell or causing DNA damage, have been shown to induce temperate phage lytic cycle.

In phage infection, the host cell is helpless against the invading virus. However, bacteria have defense mechanisms against phages. In response, phages developed a series of methods to circumvent these mechanisms [36].

3.4. Phage-Host Interactions

Phages attach to the bacterial surface through the adsorption process. Bacteria have resistance mechanisms that impede the phage adsorption process; however, phages are able to adapt their binding proteins to the receptor through a mutation [36].

Pseudomonas spp. has a great capacity for biofilm formation, due to a range of polysaccharides extracellular and other biofilm-forming components. After infection, phages are able to prevent superinfection with other phages, modulating the expression of cell receptors. After adsorption, phages inject their DNA into the host cell. However, many bacteria encode restriction endonucleases to cut foreign DNA and prevent phage infection. In turn, the phages developed anti-restriction strategies to defend their DNA against cleavage by restriction endonucleases [37].

A different antiviral mechanism that protects the host through the degradation of foreign DNA is the CRISPR-Cas System which acts as an adaptive bacterial immune system. Us CRISPR-Cas systems, the system is activated when the virus enters the bacterium: this recognizes the exogenous DNA and enzymes (Cas) cut pieces of this material and introduce them into a specific genomic region of the bacterium, called the CRISPR locus. In the next viral infections, the bacteria that contain these pieces of virus DNA inserted into the CRISPR locus generate an RNA from this sequence. This RNA will associate with the Cas enzyme and then make its way to the viral DNA, which is then cleaved and thus inactivated [38].

Phages have conceived ways to disable CRISPR-Cas systems (Figure 3). In total, 10 different families of anti-CRISPR proteins (Acr) were identified in *Pseudomonas* temperate phages. Phages appear able to escape CRISPR interference through specific mutations [39].

3.5. Advantages and Disadvantages of Phage Therapy

Phage therapy has several advantages over antibiotics treatment for bacterial infections [40], as shown in Table 2:

Despite the numerous advantages of phage therapy, challenges and limitations exist and must be considered.

Some of the concerns of using this therapy are: (i) Most infections are polymicrobial, and it is necessary to use an effective and efficient phage cocktail, with a greater spectrum of action [2]; (ii) Lack of approval by the Food and Drug Administration (FDA) or the European Medicines Agency (EMA), due to concerns about phage resistance and the role of phage in the evolution of the bacterial genome; (iii) Phages as evolution drivers: unlike antibiotics, they are biological agents based on proteins that contain DNA/RNA that have the potential to interact with the body's immune system and other microbial cells in the body and can actively replicate and evolve inside the body. This evolution can, in turn, result in the evolution of the bacterial communities of the commensal host and possibly even affect the composition of the niche microbiome; (iv) Phage selection criteria: factors such as the phage's ability to infect bacteria in stationary phase, phage enzymes, stability to serum inactivation and mutation rate have proved to be important and deserve further investigation; (v) Lack of well-organized public phage libraries [2,41].

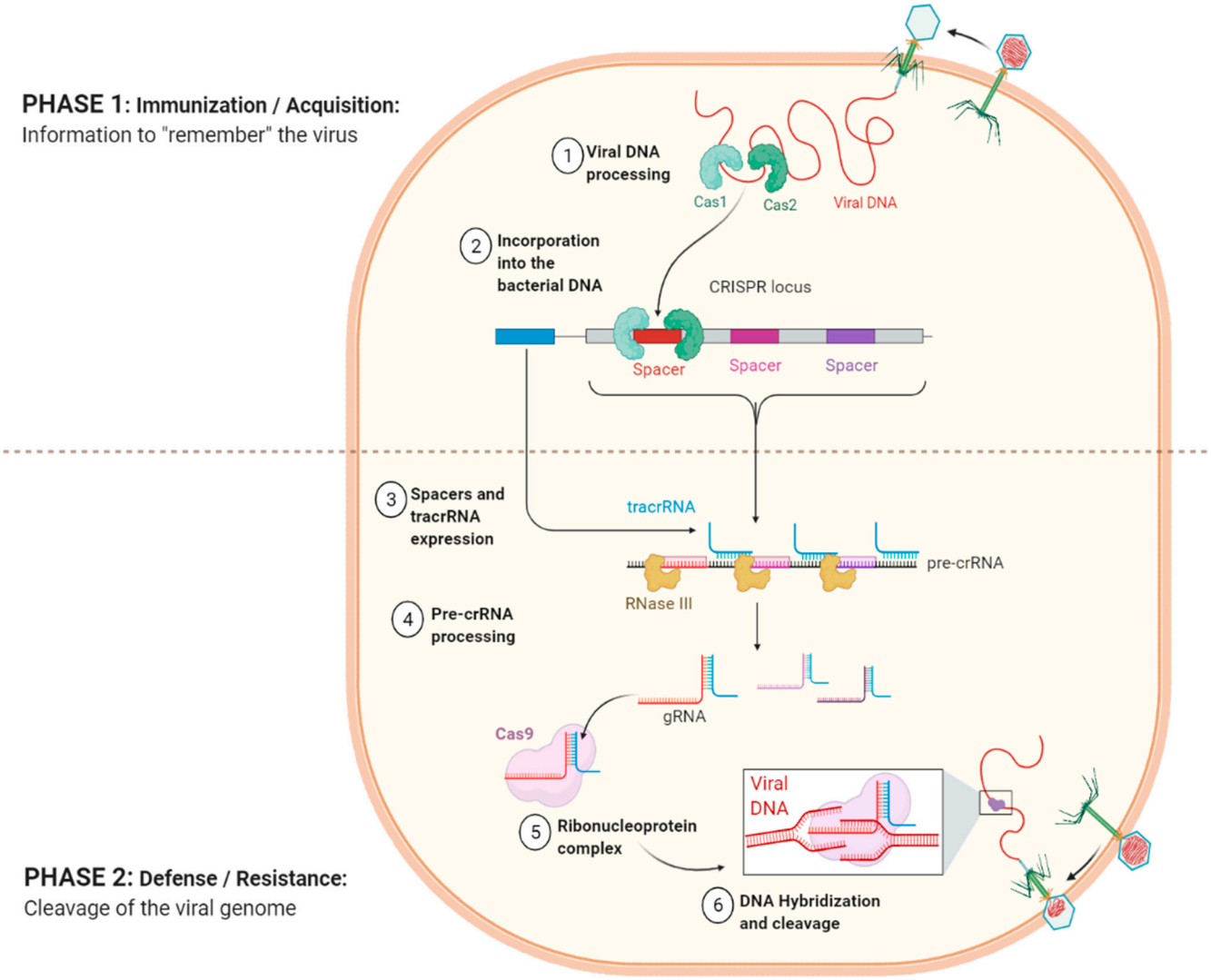

Figure 3. CRISPR-Cas9 Adapative Immune System of *Pseudomonas aeruginosa* against bacteriophage. After infection, the Cas1-Cas2 complex recognizes the invading DNA and integrates a portion of it into the CRISPR array, giving rise to a new spacer. The bacterium incorporates a fragment of the invading phage DNA into its genome as a spacer into the CRISPR array, this spacer will serve as memory allowing the bacterium to recognize the same threat upon reinfection. The CRISPR array is transcribed as a long precursor CRISPR RNA (pre-crRNA). The host RNase III recognizes the tracrRNA:crRNA-Cas9 complex and cleaves both tracrRNA and crRNA. The Cas9 protein and the gRNA form a ribonucleoprotein complex through interactions between the gRNA scaffold and surface-exposed positively-charged grooves on Cas9. Cas9 undergoes a conformational change upon gRNA binding that shifts the molecule from an inactive, non-DNA binding conformation into an active DNA-binding conformation.

Table 2. Phages therapy advantages.

Advantages	Description
Specificity	Very specific to the host. infections with more than one host, it is necessary to use a phage cocktail
Bactericidal versus bacteriostatic	Lytic phages infect target host bacteria and cause cell death, compared to certain bacteriostatic antibiotics
Active on-site propagation	Phages increase the concentration in the host as they replicate, theoretically requiring only one therapeutic dose
Low inherent toxicity	Phages exist in large amounts in the biosphere, it is possible to isolate and purify the phage required to achieve certain bacteria.
Formulation and application versatility	Various phages can be converted in a cocktail to target several bacteria simultaneously. The type of administration can also vary, liquid, powder, ointment, tablets.
Narrow potential for antibiotic cross-resistance	Since the phage and bacterial resistance mechanisms are different, bacteria resistant to certain antibiotics can be treated with the use of phage therapy.
Biofilm clearance	Phages can penetrate through biofilms. Part of this capacity is due to the presence of depolymerases and lysins
Relatively low discovery and production cost	The costs associated with discovering phage isolation and purification are relatively low
Low environmental impact	Phages are natural components of the environment

Font: Adapted from [40].

3.6. Phage Therapy Applications

For phage therapy to be a viable alternative to antibiotics, clear efficacy data from randomized controlled clinical trials are needed. In recent years, several clinical trials have been carried out, but only a few are concluded [6].

In 2009, the first controlled clinical trial of a therapeutic bacteriophage preparation was carried out, which showed efficacy and safety in antibiotic-resistant *P. aeruginosa* chronic otitis [42]. Also in 2009, another randomized, double-blind controlled trial addressed the safety of a phage cocktail targeting *P. aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* for the treatment of venous leg ulcers [6].

Between 2013 and 2017 PhagoBurn, funded by the European Commission, was the first clinical trial of phage therapy using good production practices (GMP) [2].

Sextaphage is one such commercial pharmaceutical phage composition from the Russian company Microgen. This phage therapeutic contains phages against six specific pathogens, with the intent of treating urinary tract infections in pregnant women [2].

In 2016, Paul Turner and colleagues reported isolating a phage that could restore antibiotic sensitivity in multidrug-resistant *P. aeruginosa*. This phage was later used to treat a patient with a longstanding aortic graft infection that did not respond to multiple surgical interventions and aggressive antibiotic therapy with a single application of phage. The same team recently reported the treatment of two cystic fibrosis patients with antibiotic-resistant infections. Aside from the wild type phage isolated from natural sources, genetically engineered phages have also been reported for phage therapy. A young patient with cystic fibrosis and bilateral lung transplantation who had developed a *Mycobacterium abscessus*

infection was reportedly treated with a phage cocktail of genetically engineered lytic phages that were administered intravenously [43].

In the United States, they successfully utilized intravenous phage therapy to treat a patient with a severe systemic infection caused by multidrug-resistant organisms. The phages used in that case were isolated from various environmental samples [43].

In France, the national health regulator has authorized the first treatment of patients with extremely drug-resistant and difficult to treat infections using phage therapy. Since then, six cases with various bacterial infections have been successfully treated [44].

In January 2018, the Federal Government of Belgium, in cooperation with researchers and specialists from the Federal Agency for Medicines and Health Products, took an important step in phage therapy, developing a regulation for the production and clinical application of phages. The procedure is based on the legal authorization of a pharmacist to prepare a medical product with phages individually. The active ingredients used in its preparation must meet the requirements of the European, Belgian or other official Pharmacopoeia [45].

According to several reviews, there are many advantages to using phages as antibacterial therapy [25].

3.7. Phage Therapy and P. aeruginosa, Studies In Vivo and In Vitro

Several in vitro studies have been carried out in recent years to assess the efficacy of phages on *P. aeruginosa*, including multiresistant strains [5].

Pseudomonas-specific phages were first described in the mid-20th century [5]. There are about 137 sequenced phages targeting *P. aeruginosa* in public databases, most of them from the *Caudovirales* order [46].

In 2010, a study was published on the effects of lytic phages in preventing *P. aeruginosa* biofilm formation in hydrogel-coated catheters [47]. Another study used a combination of phage *Podoviridae* LUZ7 and streptomycin on *P. aeruginosa PAO1* [48].

At the beginning of the year 2019, the result of the first clinical study on phage therapy directed at *P. aeruginosa* was reported. This study consisted of using an anti-*P.aeruginosa* phage cocktail for burn patients. The test result showed that pre-determined stutterers might not be the solution. Some strains of P. aeruginosa will not be susceptible or will quickly become resistant to the phages administered. In order to make phage therapy effective, it is necessary to formulate phage cocktail in a personalized way [49].

Some clinical trials were carried out using phages for *P. aeruginosa*. A study evaluated the efficacy and safety of a therapeutic phage preparation (Biophage-PA) [5].

Another study used a topical application of phage in a burn patient colonized with *P. aeruginosa* [5].

One study used a BFC-1 phage cocktail to treat nine patients with acute burns colonized by multidrug-resistant *P. aeruginosa* and *Staphylococcus aureus* [50].

Tables 3–5 describe some in vivo and in vitro and clinical studies carried out in recent years to test the effectiveness of phage therapy against *Pseudomonas aeruginosa* [46,49,51–93].

Regarding in vitro studies, to date, four have been used as models of cystic fibrosis, chronic rhinosinusitis cancer and burn wound infection. The main bacterial strains used were PAO1 with one without pilin (PAO1-NP). Various phages were used, namely Pa193, Pa204, Pa222, Pa223, PEV1, PEV2, PEV20, PEV61, DMS3, vB_PaeM_GUMS6, vB_PaeM_GUMS32, and vB_PaeM_GUMS45 with application ranging from 4 to 48 h and the main result is significant reduction of multidrug resistant (MDR) P. aeruginosa by phages PEV1, PEV20, and PEV61 in cystic fibrosis. DMS3 and PEV2 phages were able to inhibit bacterial growth in chronic rhinosinusitis. It is also known that a single dose of phage is able to significantly reduce biofilms formed in vitro. In burn wound infections, the combination of two phage-antibiotics (vB_PaeM_GUMS6, vB_PaeM_GUMS32, vB_PaeM_GUMS45) had the highest inhibition efficiency against the *P. aeruginosa* strain.

Table 3. In vitro studies about phage therapy for *Pseudomonas aeruginosa*.

Study	Subject	Pathology	Bact Strain	Phage	Duration	Outcome
Chang, R.Y.K. [93]	Lung alveolar macrophage and epithelial cells	Cystic fibrosis	PAV237	PEV1, PEV20 and PEV61	24 h	Significantly reduces the multidrug-resistant (MDR) *P. aeruginosa* burden in mouse lungs.
Fong, S.A. [73]	44 isolates *Pseudomonas aeruginosa* from humans	Chronic rhinosinusi-tis	PAO1	Pa 193, Pa 204, Pa 222, Pa 223	48 h	A single dose of phages is able to significantly reduce biofilms formed in vitro.
Shiley, J.R. [74]	Lung cell A549 and U937 monocyte	Cancer	PAO1-WT, pilin lacking PAO1-NP	DMS_3, PEV_2	24 h	DMS_3 and PEV_2 were able to inhibit bacterial growth in a PAO1-WT and PAO1-NP infection model, respectively.
Aghaee, B.L. [88]	Human	Burn wound infection	*P. aeruginosa*	vB_PaeM_GUMS6, vB_PaeM_GUMS32, vB_PaeM_GUMS45	4, 8, 12, 24 and 48 h	The combination of two phage-antibiotics had the highest inhibition efficiency against the *P. aeruginosa* strain. The phages tested showed low stability at high temperatures, acid pH values, and in both lotions.

Legend: PAO1—*Pseudomonas aeruginosa* serotype O1; WT—wild type; NP—pilin-lacking; MDR—multidrug-resistant.

Table 4. In vivo studies about phage therapy for *Pseudomonas aeruginosa*.

Study	Subject	Participants	Age	Pathology	Bacterial Strain	Phage	Duration	Outcome
Antoine, C. [82]	*Galleria mellonella*	450 Larvae	N.D.	Canine otitis	PAV237	PEV2	24, 48 and 72 h	Despite the ineffectiveness as life-saving antimicrobials in this model, PEV2 is active against *P. aeruginosa* in *G. mellonella*, even if this did not result in the complete elimination of bacteria at the tested MOIs.
Olszak, T. [60]	*Galleria mellonella*	Larvae	N.D.	N.D.	PAO1	PA5oct Jumbo phage	8, 18, 24, 48 and 72 h	The use of a phage mixture targeting different extracellular structures as receptors causes a huge impact on bacterial fitness, virulence, and pathogenicity.
Cafora, M. [94]	*Danio rerio*	Embryos	N.D.	Cystic fibrosis (CF)	PAO1	vB_PaeP_PYO2, vB_PaeP_DEV, vB_PaeM_E215, vB_PaeM_E217	20, 24, 26, 48 hpf (to block the embryo pigmentation from 24 h post fertilization)	The lethality and bacterial burden were both decreased following phage therapy application.
Jang, H.J. [95]	*Drosophila melanogaster*	15–30 M/F	5–7 days	PA infection	PAO1	MPK1,MPK6,D3112, and PP7	48 h	N.D.
Alvi, I.A. [90]	Mice	81 F	6–8 weeks	CF	PAO1	vB PaeP- SaPL	1, 6, 12, 24, 48 and 96 h	The majority of tested MDR *P. aeruginosa* isolates are highly susceptible to SaPL, which inhibits their growth for longer time. SaPL is a potential candidate that can be used in phage therapy against MDR *P. aeruginosa* infections.

Table 4. *Cont.*

Study	Subject	Participants	Age	Pathology	Bacterial Strain	Phage	Duration	Outcome
Lin, Y. [92]	Mice	F	8–10 weeks	CF, bronchiectasis and chronic obstructive pulmonary disease (COPD)	FADD1-PA001	PEV20	N.D.	Co-spray dried phage PEV20 and ciprofloxacin combination powder showed synergistic bacterial killing effect compared with single PEV20 or ciprofloxacin powders in an acute mouse lung infection model caused by *P. aeruginosa*.
Chow, M.Y.T. [93]	Mice	F	6–8 weeks	Acute respiratory infection	FADDI-PA001	PEV31	26 h	Pulmonary delivery of phage PEV31 in mice can reduce the MDR bacterial burden.
Lin, Y.W. [55]	Rats	17 (14F; 3M)	8 weeks	CF	*Pandrug-resistant (PDR) isolate P. aeruginosa 112* and *P. aeruginosa* ADDG.	øPEV20	48–72 h	This studied showed positive outcomes in the utilization of PEV20 phage, in Rats, with simullation results predicting an infection erradication 12 h after the treatment, but some inconsistencie in in vivo models.
Abd El-Aziz, A.M. [65]	Mice	Three groups of F mice (15 each)	10-week-old	Lung infection	*P. aeruginosa* clinical strains	MMI-Ps1	72 h post infection	In vitro and animal model studies showed that MMI-Ps1 effectively killed different serotypes of *P. aeruginosa*, including mucoid and nonmucoid strains. In addition, phage therapy significantly protected mice from lung and bloodstream infection caused by mucoid strains of *P. aeruginosa*.

Table 4. *Cont.*

Study	Subject	Participants	Age	Pathology	Bacterial Strain	Phage	Duration	Outcome
Międzybrodzki, R. [70]	Mice	N.D.	8–16 weeks at the begin-ning	Murine collagen-induced arthritis	*P. aeruginosa* 119x	119x phage	56 days	Preliminary results do not suggest that phages may aggravate the symptoms of rheumatoid arthritis
Chang, R.Y.K. [95]	Mice	4	8 to 10 weeks	Lung infection	FADDI-PA001	Phage PEV20	24 h	The study showed that intratracheally delivered aerosolized phage powder can significantly reduce MDR *P. aeruginosa* load in mouse lungs.
Roach, D.R. [76]	Mice	6	N.D.	CF, acute pneumonia	*P. aeruginosa* PAKlumi	PAK_P1	14 days	The study shows that successful phage curative and pro-phylactic therapies of acute respiratory infections depend on an essential synergy between phage lysis and the host's own immune defenses.
Waters, E.M. [77]	Mice	60	6–8 weeks	CF, COPD	*P. aeruginosa* LESB65 and NP22_2	Phage PELP20	48–72 h and 7 days depending on the treatment.	The study shows that phage administered intranasally up to 6 days after establishment of chronic lung infection were efficient in reducing bacterial numbers in the lungs of mice infected with P. aeruginosa, demonstrating the potential for phage therapy in the treatment of established and recalcitrant chronic respiratory tract infections.

Table 4. *Cont.*

Study	Subject	Participants	Age	Pathology	Bacterial Strain	Phage	Duration	Outcome
Stratton, C.W. [79]	Mice	Rodents	N.D.	Catheter-induced rat aortic valve endocarditis	*P. aeruginosa* strain CHA	Cocktail of 12 phages agaisnt *P. aeruginosa*	6 h	This study provide a strong proof of concept for the use of phage cocktails combined with antimicrobial agents for therapy of deep-seated and systemic infections.
Oechslin, F. [51]	Mice	Rodents	N.D.	Induced rat aortic valve endocarditis	33 strains of *P. aeruginosa* reference strains and clinical isolates	12 phages contained in the PP1131 cocktail	18 h	Single-dose phage therapy was active against *P. aeruginosa* EE and highly synergistic with ciprofloxacin. Phage-resistant mutants had impaired infectivity.
Furusawa, T. [83]	Mice, horse	Rodents	N.D.	Bacterial keratitis	Pseudomonas sp. (Pa12, Pa18, Pa26, and Pa50) and *P. aeruginosa* strain NE-126	ΦR18 and ΦS12-1	3 h	The phages used in this study rapidly adsorbed to *P. aeruginosa* and widely killed these isolates from horse lesions. One of the phages was shown to completely prevent keratitis in a keratitis mouse model.
Danis-Wlodarczyk, K. [84]	Wax moth	Larvae	N.D.	Infection	Pseudomonas aeruginosa (PAO1)	KTN4 phage	36 h	Considering all these characteristics, KTN4 phage is a suitable and promising for applications in treatment and prophylaxis in lung infections.

Table 4. *Cont.*

Study	Subject	Participants	Age	Pathology	Bacterial Strain	Phage	Duration	Outcome
Forti, F. [94]	Mice and wax moth	F	8–10 weeks	Acute respiratory infection (mice) and bacteremia (wax moth)	PAO1; PA14; PAK-lumi; LESB58; AG5; AG3; AA10; GJY9; CL1; CL2; VR8; AG6; DV4; CH7; AA2; AA43; AA44; TR1;TR66; TR67; E1 to E9 and PaPh1 a PaPh33.	Cocktail composed of four novel phages (PYO2, DEV, E215 and E217) and two previously characterized phages (PAK_P1 and PAK_P4)	After two hours post-infection (P.I.) (mice); 1 h P.I. (wax moth)	Our findings indicate that in mice, lethal acute respiratory infection can be cured by treatment with the cocktail. Compared to our previous data obtained with a single phage, the cocktail showed the advantage of having more rapid efficacy in reducing the bacterial load.

Legend: F—female; M—male; N.D.—Not determined; PA—*Pseudomonas aeruginosa*; PAO1—*Pseudomonas aeruginosa* serotype O1; CF—Cystic fibrosis; COPD—chronic obstructive pulmonary disease; MDR—multidrug-resistant; PDR—pandrug-resistant.

Table 5. Clinical studies about phage therapy for *Pseudomonas aeruginosa*.

Study	Subject	Participants	Age	Pathology	*P. aeruginosa* Strain	Phage	Duration	Outcome
Tkhila-ishvili [71]	Human	1F	83 years old	Periprosthetic joint infection	Clinical isolate of *P. aeruginosa*	N.D.	5 days	The wound healed and the patient was discharged after 14 days with oral antibiotics for six weeks. The patient died four months after bacteriophage treatment due to left ventricular assist device pump thrombosis.
Madd-ocks, S. [61]	Human	1F	77 years old	Adenocarcinoma	Clinical isolate of *P. aeruginosa*	Lytic anti-*P.aeruginosa* phages	7 days	Intravenous antibiotics, bacteriophage therapy was well tolerated, with no adverse events detected either during therapy or subsequently. In combination with antimicrobials, bacteriophage therapy was associated with resolution of infection and with apparent eradication of *Pseudomonas* colonization. The successful decolonization would have occurred in the absence of phage treatment is unlikely, given the extent of disease and the sequential development of antimicrobial resistance.
Aslam, S. [62]	Human	3 (2F;1M)	51 years old	Lung transplant	Clinical isolate of *P. aeruginosa* among other strains	PA193; Pa204;Pa222;Pa223; Pa176; Paϕ1; PaSKWϕ17,PaSKWϕ22; PaATFϕ1; PaATFϕ3; BdPF16phi428.	60 days	Bacteriophage therapy was well tolerated and associated with clinical improvement when used as an adjunct to antibiotics in lung transplant recipients with against multidrug-resistant respiratory infections otherwise not responsive to antibiotics alone.

Table 5. *Cont.*

Study	Subject	Participants	Age	Pathology	*P. aeruginosa* Strain	Phage	Duration	Outcome
Law, N. [69]	Human	1M	26 years old	Cystic fibrosis	Clinical isolate of *P. aeruginosa*	Cocktail AB-PAo1	9 months	Given the concern for multidrug-resistant *P. aeruginosa* infections in cystic fibrosis patients, bacteriophage therapy may offer a viable anti-infective adjunct to traditional antibiotic therapy.
Gupta, P. [63]	Human	20	36 years old	Nonhealing wound	Clinical isolate of *P. aeruginosa* among other strains	Cocktail from water sources	21 days	A significant improvement was observed in the wound healing, and there were no signs of infection clinically and microbiologically after 3 to 5 doses of topical bacteriophage therapy. Seven patients achieved complete healing on day 21 during follow up while in others healthy margins and healthy granulation tissue were observed.
Jault, P. [66]	Human	27	18 years or older	Burn wound infection	Clinical isolate of *P. aeruginosa*	PP1131	8 days	Clinically relevant reduction in bacterial burden was observed in the phage group, with numerically fewer serious adverse events seen in those treated with the phage cocktail than the standard of care, indicating a favourable potential of phage therapy.

Legend: F—female; M—male; N.D.—Not determined; PA—*Pseudomonas aeruginosa*.

Regarding in vivo studies, to date the mice has been the most used animal model followed by *Galleria mellonella* and wax moth. Different pathologies were studied, being CF the most used model followed by COPD and bacterial induced endocarditis. All phages showed positive results, in some cases even after one dose, reducing the bacterial burden and in some cases curing the bacterial infection, all the phages' cocktails studied showed were capable of curing the infections, in some cases this were complemented with antibiotic therapy. The studies duration ranged from 1 h to 56 days.

The clinical trials showed positive results in every trial. In the trials that phage therapy was complemented with antibiotics all patients healed completely and the bacterial infection were eradicated, when the phage therapy was used alone, not all patients got cured but all showed a reduction in bacterial burden and improvement in the healing process. The age of the patient doesn't seem to be an important variant in the therapy results, the results were also identical in all pathologies. All studies were done using Pseudomonas clinical isolates and the duration varied from 5 days to 9 months.

4. Conclusions

The significant increase in antibiotic resistant strains and the scarcity of new antibiotics require, in the immediate future, the discovery of new effective therapies, such as phage therapy.

Their diversity, low production cost, low capacity for adverse effects, and their specificity for target cells make phages very attractive.

P. aeruginosa is responsible for serious infections, and over the last few years it has shown high levels of resistance to antibiotics on the market, being one of the emerging bacteria in terms of discovering new effective therapies.

Although there are some studies that demonstrate the clinical success of phage therapy, more studies are needed on the safety, efficacy, and even development of phage resistance as an alternative therapy in *P. aeruginosa* infections, to solve the serious problem of resistance and lack of alternatives to antibiotics on the market today. Additionally, phages can be used in combination with antibiotics or other antimicrobials for improved performance.

The growing interest in phage therapy by patients and physicians and the consequent increase in phage orders from around the world highlights a growing need for the establishment of phage banks with well-characterized phages that can facilitate access by the international community.

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