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Experimental Phage Therapy on Multiple Drug Resistant *Pseudomonas aeruginosa* Infection in Mice

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Abstract

With the rising prevalence of multiple-antibiotic resistant-bacteria (MDRs) and the lack of development of new antibiotics by the pharmaceutical industries, there is an urgent need to develop novel approaches to combat MDRs, especially *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus*. Bacteriophage therapy has been applied for decades as a means of treating bacterial infections in some parts of the world and numerous encouraging results have been documented. Here, we present evidence in murine models that animals infected with MDRs *P. aeruginosa* can be successfully treated with specific bacteriophages that target these MDRs microbes. We utilized three different forms of bacterial infections on Stage II and III wound on deep lower back of animals; deep wound infection and chronic infection treated the each of the infections by respective dermal application of phages. Furthermore, we successfully tested phage therapy for both acute and chronic infections. We evaluate the potential use of lytic phage on wound contraction; we observed drastic changes on the wounds after 24-hours of phage application. Pros and cons of phage therapy to treat human MDRs are discussed.

Keywords: Phage therapy; Lytic phage; Multiple drug resistant; Infectious diseases; Phage; *E. coil*; *Pseudomonas aeruginosa*; Mice; Antibiotics; Infection

Introduction

The emergence of pathogenic multiple drug resistant (MDRs) microorganisms have become a critical problem in modern medicine, particularly because of the concomitant increase in immunocompromised patients due to transplantation, autoimmune disorders and various viral infections, particularly HIV-1 [1].

Bacterial predators, i.e. bacteriophages (phages)–viruses that infect and rapidly destroy bacteria, were discovered almost a century ago and there have been many attempts to apply phages to treat bacterial infections [2]. While phage treatment has been successfully used in Russia, Georgia and Poland, it has been largely ignored in the West. The emerging crisis of antibiotic resistance and the uncertain outlook for development in new antibiotics have dramatically altered landscape of MDRs, generating renewed interest in phages as a means of eradicating drug-resistant microorganisms.

Lytic phages are very specific and only attack and lyse specific bacterial species, disrupt bacterial metabolism and cause the bacterium to lyse. In addition, phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacteria. The therapeutic phages have some theoretical advantages over antibiotics due to their rapid bacteriolytic activity and phages have been reported to be more effective than antibiotics in treating certain infections in humans [3-5], and experimentally infected animals [6]. However, the target species of bacteria can develop resistant to a particular type of phage and some patient may experience diarrhea.

From a clinical standpoint, phages appear to be innocuous. During the long history of phages utility as therapeutic agents in Eastern Europe and the former Soviet Union (and, before the antibiotic era, in the United States), phages have been administered to humans (i) orally, in tablet or liquid formulations (10^5 to 10^{11} PFU/dose), (ii) rectally, (iii) locally (skin, eye, ear, nasal mucosa, etc.), in tampons, saline rinses, and creams, (iv) as nasal aerosols or intrapleural injections and (v) intravenously, [7-11], albeit to a lesser extent than the first four methods, and there have been virtually no reports of serious complications associated with phage-therapy [12,13]. In the United States, phi X174, apparently a harmless phage, has been used to monitor humoral immune function in adenosine deaminase-deficient patients [8], and to determine the importance of cell surface-associated molecules in modulating the human immune response [14,15]. In this study, we evaluate the potential use of lytic phage on wound contraction. Extensive study need to be done to determine if there is any effect of enhancement of epithelization by phage therapy.

Materials and Methods

Isolation and purification of MDR microbes

Bacterial cultures of the putative MDRs *Pseudomonas aeruginosa* were isolated from a clinical specimen from a local hospital (clinical isolates and Lab collection). The specimens were streaked on Luria-Bertan (LB) agar (Sigma Co.-USA), and after 48 hours, individual colonies were picked and their purity were determined by Gram-staining, morphological characteristics and specific biochemical analyses [16].

Determination of Minimal Inhibitory Concentrations (MIC)

The MDRs microbe's antibiotic plates were prepared by adding the following antibiotics at final concentrations of 5-200 µg/ml from the

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stock solutions of Ciproflaxin, Nalidixic Acid, Ampicilin, Gentamycin, Methicillin, Vancomycin and Minomycin in sterilized LB agar. The purified *P. aeruginosa* were re-plated to verify the purity of culture. Minimum inhibitory concentration (MIC) of various colonies of this MDRs strain was done by standard MIC method [16].

Isolation and purification of bacteriophage

Lytic Bacteriophages were isolated from clinical specimen (Urine sample from patients) with urinary tract infections (UTI) by plaque assay and spot assay techniques [16].

Isolation of phage from clinical specimen

Urine sample from a patient (24 years old female athlete) suffering from severe UTI, was centrifuged at 6000 rpm to remove solid matters and the supernatant was then passed through a sterile 0.45 µm pore size nitrocellulose filter (EMD Millipore Co.). 50 µl of filtrate and 100 µl of early exponential phase, *P. aeruginosa* were mixed in 3 ml of melted L.B soft agar and plated at 37°C overnight.

Phage isolation, purification and quantifications

Phages were purified by successive single plaque and propagation method, as described previously [16,17]. Briefly, a single plaque was picked from a plate using a sterile capillary tube and added to a mid-log-phase *P. aeruginosa* culture (10^8 CFU/ml) supplemented with 0.1M $CaCl_2$. 10 µl of culture mixture and phage mixture were incubated at 37°C overnight. The lysate was filtered through a 0.45 µm-pore-size sterile filter (EMD Millipore Co.). For quantification and titration, serial dilutions of the phage containing filtrates were made, and plaques were allowed to develop on a lawn of the same host bacterial culture. Single plaques were purified through 3 successive rounds of plaquing and repeated three additional times, after which purified phages were obtained. All lysates were stored at 4°C.

Determination of phage interaction with bacteria

A. **Plaque assay by double layer method:** 100 µl of early exponential phase bacterial culture and 50 µl of respective lysates were mixed with $CaCl_2$ and $MgSO_4$ (0.1 M final concentrations) into 3 ml of melted LB soft agar tube. It was then poured on LB agar plate and incubated at 37°C overnight. Negative control contained no lysates [13,18].

B. **Spot assay by double agar layer method:** 100 µl of the early exponential phase culture of bacterial culture was mixed into 3 ml of melted LB soft agar and plated on a LB agar plate. After solidification, 10 µl of phage lysate were applied on the bacterial lawn and incubated at 37°C, overnight.

Transmission electron microscopy

Phage morphology was studied by precipitating of 500 µl lysate with PEG 6000 (Promega Co. USA) and NaCl to a final concentration of 8% and 4%, respectively, and incubated at 4°C, overnight. The pellet was re-suspended in 100 µl of double deionized distilled water. Four hundred mesh carbon coated grids were negatively stained with 2% uranyl acetate for 30 seconds and examined by transmission electron microscope (GOEL-JEM-1200 EX II).

Phage DNA extraction

DNA from the phage was extracted from phage PS5 lysate using DNA extraction kit (Promega Co.-USA).

Polymerase Chain Reaction (PCR) amplification

The phage DNA was used as PCR templates. PCR products were generated by using F: 5′-CCC GGG ATC CGA T-3′ and R: 5′-ATG CCA TCC CGG G-3′ primers. The amplification was carried by denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing temperature 60°C for 30 seconds and extension at 72°C for 2 min. Amplified products were sized by 2% agarose gel electrophoresis and documented by photography.

DNA sequencing and computer analysis

Sequencing reaction was performed by using ABI 3130 (Applied Biosystems, Foster City, CA, USA). For alignment and comparison of similar new sequences, ClustalW.2 was used. The similarity between our data sequence and the sequence database was assessed by the use of BLAST-NCBI.

Establishing of MDRs *P. aeruginosa* infection in mouse model

Pathogen free 7 week's old BALB/c male mice (in groups of two) were used for infection experiments. MDRs *P. aeruginosa* culture was grown in LB medium at 37°C. Log–phase culture of MDR *P. aeruginosa* to optical density at 600 nm of 1.1 (5×10^8 CFU/ml), followed by centrifugation at 3000 rpm was used. Pellet was washed and re-suspended in PBS and was stored at 4°C. This suspension was used as infectious agent on Stage II and III wound on deep lower back of the animals. Wound model were either Stage II or Stage III. The Stage II was caused by surficial scratches, mainly to epidermis layer and perhaps and dermis layer at some places, whereas the stage III involved deep wound penetrating the dermis and subcutaneous tissue [19]. The Stage III models were treated with low concentration of phage application Animals were maintained in strict sterile condition, according to guidelines for the housing of rodent in scientific institutions [19]. Bedding (single type), cage, cage shelter, bars, cage lid, water container were autoclaved and changed every day. Food was stored in a clean, dry, vermin-free, well-ventilated sterile area to reduce any possible contamination. Water delivery system was monitored during study to ensure proper function.

Phage application as therapeutic agent for treatment of mice infected with MDRs *P. aeruginosa*

The efficacy of phage therapy was evaluated in five groups of mice, using MDRs *P. aeruginosa* as pathogen. Three groups were evaluated for the effect of phage dose on established infected wound, for this purpose, first group of the animals were challenged by local infection of MDR *P. aeruginosa*; 3×10^8, second group; 3×10^6, third group; 3×10^4 CFU. Whereas, the forth group served as positive control without phage therapy, but infected *P. aeruginosa* and the The fifth group served as the negative control, which only received 3×10^4 CFU of phage without bacterial infection.Each animal in these groups were treated with a two injections of lytic phage PS5 of the appropriate dose of phage (~9×10^8 PFU), administered i.p 30 min after bacterial challenge. 24 hours after the first injection, the animals were administered a second injection of phage and then given a daily dose of phage orally (~3×10^8). The positive control group was not treated with phage. Infected animals and controls were observed under sterile condition for one week and the status of the wounds were monitored and recorded by photography.

Phage therapy on chronic MDRs infection

One week after infections with MDRs *P. aeruginosa* (positive

control group). The chronically infected mice were treated with two doses of lytic phage PS5, first *via* intraperitoneal (i.p) injection (~9×10^8 PFU) and a second dose of phage, 24 hours after the first infection, and were given a daily dose of phage orally (~3×10^8). Infected mice were observed for one week and photographed.

Results and Discussion

In this study, *Pseudomonas* lytic phage was isolated from a clinical specimen. The phage was further characterized by utilizing the local isolated MDRs strain of *P. aeruginosa* as a bacterial lawn for detection of the host-phage interaction. Plaque assay of filtered sterilized phage lysates on the lawn of *P. aeruginosa* (Figure 1A) exhibited clear plaques suggesting a highly lytic activity of both types of phages. These phages were propagated as virulent phages in MDRs *P. aeruginosa* (PS5) (Figure 1B). The electron microscopic examination indicated that these phages belong to *Myoviridea* phage based on morphology (Figure 2).

BLAST analysis of PCR products highlighted by designed primers [20] for phage PS5 showed homology with SAM domain (accession number: KC351753) (Figure 3). Sterile alpha motif (SAM) domains are known to exhibit diverse protein-protein interaction modes. SAM domain is a putative protein interaction motif present in a wide variety of proteins involved in diverse biological processes. The SAM domain that is evolutionarily conserved from lower to higher eukaryotes is a ~ 70-amino acid protein sequence that participates in protein-protein, protein-lipid and protein-RNA interactions. They can form multiple self-association folds and also bind to various non-SAM domain-containing proteins [21]. The SAM domain can potentially function as a protein interaction module through the ability to homo- and hetero-oligome with other SAM domains [21]. The SAM domain is involved in the regulation of multiple-processes among prokaryotes and diverse eukaryotes. Another interesting feature was presence of radical SAM domain proteins phage PS5 [21].

Our analyses of the SAM domain of *P. aeruginosa LESB58* (GeneID: XM_001276480) and phage PS5 PCR product by BLAST-NCBI, exhibited 99% similarity with *P. aeruginosa LESB58*.

In addition to demonstrating that the radical SAM domain contains essential motifs to coordinate the [4Fe-4S] cluster and cofactor SAM, is essential for the antiviral activity of viperin [20], which mediated by interferon gamma (IFNγ), Viperin has been shown to be induced by other viruses, such as vesicular stomatitis virus, dengue virus, yellow fever virus, human polyomavirus JC and hepatitis C virus (HCV), in cultured cells and *in vivo* [22].

It has been known that *P. aeruginosa* is a common agent of serious infections in severe burn patients. Acute burn wounds cause a breach in the protective skin barrier and suppress the immune system, rendering the patients highly susceptible to bacterial infections. *P. aeruginosa* colonization in the severe burn wounds and its rapid replication within the damaged tissues often leads to disseminated infections, resulting in bacteremia and septic shock and high rates of mortality and morbidity [23,24]. Treatment of such infections is confounded by the MDRs *P. aeruginosa* [25]. Multiple studies have demonstrated the benefits of phage therapy for a variety of bacterial infections in animal model systems [26].

In our investigation, the efficacy of phage treatment in murine model, after superficial infection with MDRs *P. aeruginosa*, showed that administration of lytic phage PS5 in the infected animal animal resolved the infection (Figures 4-6). Concerning the timing of phage treatment, our results showed that administration of phage 30-45 minutes after the bacterial challenge was very effective. The scientific literature shows, simultaneous injection is the easiest method for examination of the antibacterial effects of phage or drug *in vivo* [27]. Furthermore, the bacteria and the phage immediately transferred into blood, as this is assumed to be the most suitable *in vivo* situation for the phage–bacterium interaction [27].

We also tested the efficacy of phage PS5 on chronic bacterial skin infection (6 days old infection) (Figures 7A, 8A and 9A). In these animals, the lesion was not cleared by the immune system of the animals, but healing was apparent by the lytic phages. We demonstrated complete recovery in chronically MDRs-infected animals post phage-treatment (Figures 7-9).

Recovery of the animals was achieved by administering two doses of phage daily for one week and maintaining a continuous infusion of phage *via* the drinking water. The effectiveness of lytic phage PS5

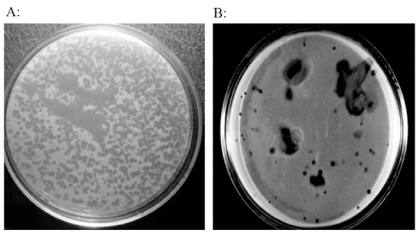

Figure 1: Plaque assay of lytic phage.

Plaque assay of lytic phage on the lawn of different strains of multiple drug resistance *P. aeruginosa*. (A) Plaque assay of phage PS5 on the lawn of multi drug resistant *Ps. aeruginosa*. (B) Spot assay of phage PS5 on the lawn of multi drug resistant *P. aeruginosa*.

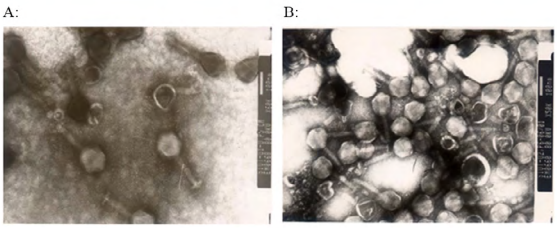

Figure 2: Electron Micrograph of Phage PS5.

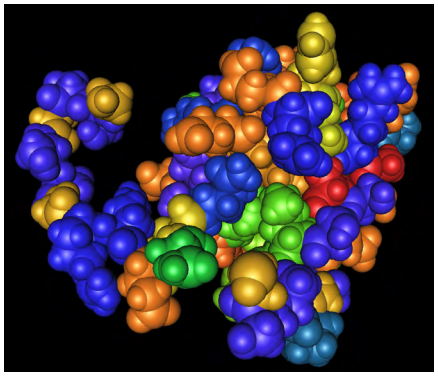

Figure 3: 3D structure of phage PS5 "Sterile alpha motif" (SAM) protein (accession number: KC351753).

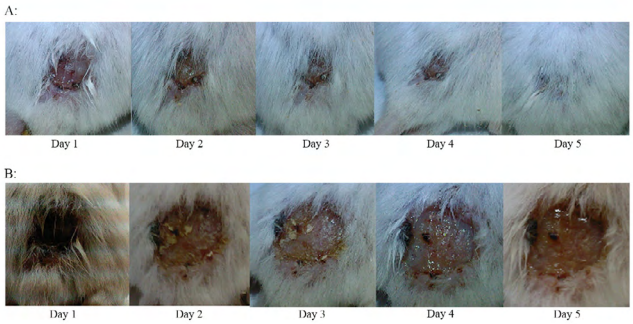

Figure 4: Phage therapy of MDR *P. aeruginosa* acute infection.

Pseudomonas Phage PS5, application (3×10^8) after 30 minutes on Lesion of mice infected with MDR *P. aeruginosa*. (A) Phage application and time needed to heal the lesion on the animal back.
(B) Control animals infected with MDR *Pseudomonas aeruginosa*

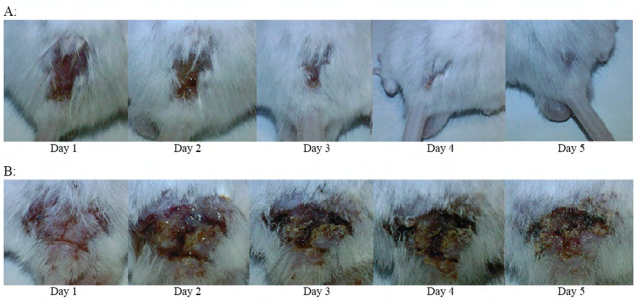

Figure 5: Phage therapy of MDR *P. aeruginosa* acute infection.

Pseudomonas Phage PS5, application (3×10^6) after 30 minutes on Lesion of mice infected with MDR *P. aeruginosa*. (A) Phage application and time needed to heal the lesion on the animal back.
(B) Control animals infected with MDR *Pseudomonas aeruginosa*

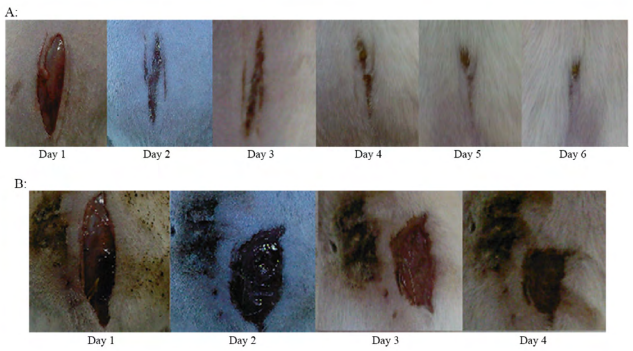

Figure 6: Phage therapy of MDR *P. aeruginosa* acute infection.

Pseudomonas Phage PS5, application (3×10^4) after 30 minutes on Lesion of mice infected with MDR *P. aeruginosa*. (A) Phage application and time needed to heal the lesion on the animal back.
(B) Control animals infected with MDR *Pseudomonas aeruginosa*

in treatment of experimental infected murine model exhibited by MDRs *P. aeruginosa* in these animals strongly suggested that MDRs *P. aeruginosa* is susceptible for both oral and i.p applications of the lytic phage PS5. Effectiveness of phage treatment was apparent in the chronic lesions (within 6 days) after treatment as determined by complete recovery of the infected rodents. Of note, the chronic infection model would closely mimic the real like human condition, when an infected patient who was diagnosed with MDRs infection would be a candidate for phage therapy.

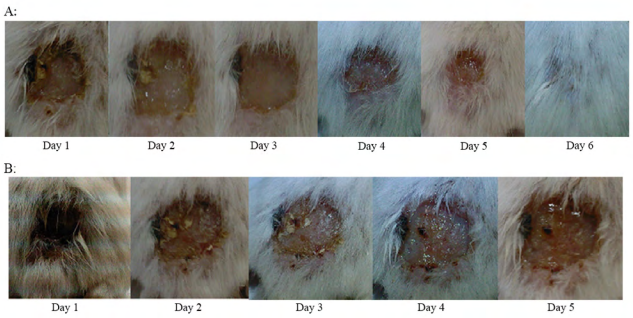

Figure 7: Phage therapy of MDR *P. aeruginosa* chronic Infection.

(A) *Pseudomonas* Phage PS5 (3×10^8), application after 30 minutes on infected lesion of mice infected with MDR *Pseudomonas.*
(B) Control: Lesion on mice skin infected with MDR *Pseudomonas* without Phage therapy.

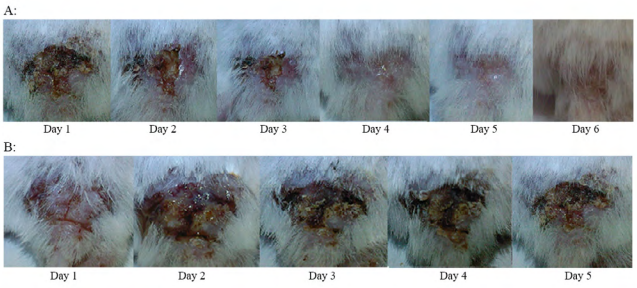

Figure 8: Phage therapy of MDR *P. aeruginosa* chronic Infection.

(A) *Pseudomonas* Phage PS5 (3×10^6), application after 30 minutes on infected lesion of mice infected with MDR *Pseudomonas*
(B) Control: Lesion on mice skin infected with MDR *Pseudomonas* without Phage therapy

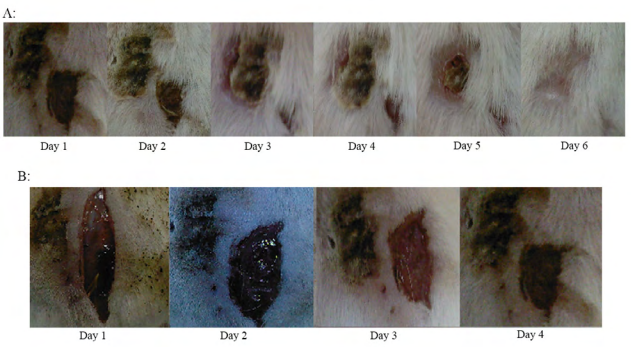

Figure 9: Phage therapy of MDR chronic infection.

(A) Healing of chronically infected burned lesion of *Pseudomonas* by Phage PS5 Therapy (3×10^4).
(B) Control: Experimentally infected burned mice with MDR *P. aeruginosa*.

The possibility of bacterial resistance to phage may be an obstacle in the development of an effective phage therapy system [28]. Data from the literature help to understand that even if the bacteria develop phage resistance new phage that may have lytic activity against that particular bacteria can be utilized. It is also possible to prepare a mixture of different strains of phages that can attach to different receptors and may prevent the emergence of a bacterial-phage resistant during phage treatment. This will be similar to current treatment of HIV-1 with multiple drug cocktails, known as highly active antiretroviral therapy (HAART) or three drug cocktail for tuberculosis (i.e. isoniazid, rifampin and ethmbutol) [28,29].

We believe that some of the reasons that phage therapy has not been globally recognized and applied may be due to three major concerns described in the literature [9]. First, the rapid lyses of a large numbers of microbes, especially Gram negative that may release endotoxin (i.e. LPS). In our study, we did not observe any so called the Jarisch-Herxheimer reaction, in any of our experimental animals. Such results are also reported by others when mass bacteriolyses occurred after antibiotic treatment [30]. Therefore, in all experiments, the animals recovered due to rapid bacterial lysis do not appear to be a serious situation. Phage-treated mice, in fact, remained healthy weeks after treatment. The above results become more convincing in light of numerous reports documenting phage efficacy *in vivo* against several bacterial species [31], including *S. aureus* [18], *E. coli* [32], *P. aeruginosa* [33] methicillin-resistant *Staphylococcus aureus* (also known as meat-eating bacteria) and vancomycin-resistant *Enterococcus faecium* [33].

The second important concern is the "by-standard effect", where phages may destroy other non-target microbes and disturb the normal flora. This is not real concern since phages are highly receptor-specific and no such data has been reported elsewhere. And the retrospective history of using phage administration by different routes in several countries has reported no such outcome. There have been almost no report of serious complication related to their use [34], as phages are common entities in the environment and regularly consumed in foods, the development of neutralizing antibodies should not be a significant obstacle during the initial treatment of acute infection, because the kinetics of phage action or lytic enzymes is much faster than immune recognition and antigen processing system by the adoptive immunity. However, even if antibodies are generated by a host against a particular host's immune system, it is unlikely that the same host will be receiving the exact same phage therapy twice [35-39].

Thirdly, there is a possibility that phage preparations may contain residual bacterial antigens or endotoxins [32]. To address this, bacteriophage production for clinical trials have to follow specific Good Manufacturing Practice (GMP) guidelines with appropriate quality controls [40], and to meet specific standards for purity and sterility.

In summary, our study provides clear evidence that not only for superficial infection lytic phage can be locally applied, but also it may be used in systematic infections. Our studied on MDRs *Ps aeruginosa* phages, to control bacterial infection shows therapeutic promise. Since the treatment of ever increasing worldwide incidence of the MDRs bacteria is a challenge, use of the specific well-characterized phages can be an alternative strategy to combat this problem. However, it is quite clear that the safe use of these phages as therapeutic modality will require complete target characterizations to avoid any potential side effects.

Finally, our experimental phage therapy and bioinformatics analysis suggested that phage treatment is a useful tool for the treatment of *Pseudomonas* infections. Given the encouraging results of this study, it may be useful to develop more precise experiments that may allow the use of these phages, in case of serious human clinical infections with MDRs, where all known antibiotics have failed to treat the infection.

Acknowledgments

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Authors' Contributions

Zhabiz Golkar carried out complete bench work, participate in sequence analysis by applying bioinformatics tools, inference of computational analysis, sequence submission to NCBI and writing the manuscript. Omar Bagasra coordinated and assisted in writing and editing the manuscript. Nusrat Jamil conceived the study and designed the experiments.

Animals in Research

The research has complied with our relevant institutional policies. It was approved by PCMD (Panjwani Center for Molecular Medicine and Drug) Research Institute animal care policy and guideline.

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