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Isolation and Identification of a Sewage *Pseudomonas aeruginosa*-infecting Bacteriophage

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ABSTRACT

Introduction: *Pseudomonas aeruginosa*, a nosocomial pathogen, causes respiratory infections, particularly in individuals with cystic fibrosis (CF); it poses a significant challenge due to escalating antibiotic resistance. Phage therapy is emerging as a potential alternative approach to treating antibiotic-resistant bacteria. This study aimed to isolate and identify a highly efficacious bacteriophage targeting *P. aeruginosa*. **Methods:** Sewage samples from Mofid Children's Hospital were collected and titrated using a double-layer plaque assay. After plaque detection, the phage was isolated, and then the host range, adsorption rate, and stability were evaluated through spot tests and double-layer plaque assays. Phage morphology was determined using transmission electron microscopy (TEM). **Results:** Of the three sewage samples collected, one contained phage. We isolated a lytic bacteriophage that exhibited clear plaque formation and induced lysis in planktonic *P. aeruginosa*. TEM analysis revealed that the phage belonged to the *Myoviridae* family. The phage had a broad host range and demonstrated optimal stability at 37 °C and pH 7. **Conclusion:** The isolated phage can potentially be used for treating *P. aeruginosa* lung infections, offering an alternative approach to address drug-resistant isolates.

INTRODUCTION

P. aeruginosa, a Gram-negative bacterium, exhibits colonization and persistence in various environments, encompassing natural sources like soil and water, as well as clinical and hospital settings [1, 2]. It poses a significant risk as an opportunistic pathogen, causing severe infections in humans and animals. The emergence of multidrug-resistant (MDR) strains has severely impeded treatment efforts, presenting a grave concern for hospitals and individuals with immunodeficiency, including patients with cystic fibrosis (CF) [3].

P. aeruginosa is a prevalent pathogen in CF patients, often implicated in pulmonary complications and disease progression. It employs diverse strategies to colonize the CF lung, with numerous virulence factors leading to lung inflammation and tissue damage, potentially exacerbating the severity of pulmonary exacerbations [1, 2]. The escalating antibiotic resistance poses significant challenges for the CF community, given the frequent reliance on antibiotic treatment to manage pulmonary exacerbations [2].

To tackle these challenges, phage therapy, which utilizes bacteriophage viruses to combat bacterial infections, has emerged as a promising alternative for treating MDR infections [4]. Compared to antimicrobial treatment, phage therapy offers numerous advantages, including high specificity and effectiveness, particularly against multidrug-resistant bacteria. It exhibits self-propagation and protects against mutation-mediated antibiotic resistance development [1, 4]. However, phage therapy encounters several challenges, including (1) phage selection, (2) limitations in host range, and (3) lack of familiarity with phages [5].

Extensive research has been conducted on applying phage therapy in human medicine to combat antibiotic-resistant *P. aeruginosa* infections, particularly in the context of pulmonary diseases. Most of these studies have reported successful outcomes in phage therapy against *P. aeruginosa* [1, 4].

This study aimed to identify and isolate a potent bacteriophage targeting *P. aeruginosa* strains obtained from cystic fibrosis patients. The objective was to address and mitigate antibiotic-resistant infections in this patient population.

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MATERIAL AND METHODS

***P. aeruginosa* isolation from cystic fibrosis patients.** Pharyngeal swab samples were obtained from 121 cystic fibrosis patients referred to the Iranian CF Center at the Children's Medical Center in Tehran, Iran, between November 2018 and January 2019. *P. aeruginosa* isolates were obtained from these samples and then stored at -70 °C using Tryptic Soy Broth (TSB) supplemented with 15% glycerol without antibiotics.

Bacteriophage isolation and enrichment. Sewage samples from Mofid Children's Hospital in Tehran, Iran, were used to isolate the bacteriophage. Sampling was conducted thrice, with approximately 2 L of sewage collected using a sterile bottle. The samples were transported to the Medical Microbiology Laboratory at Alzahra University in a cool box at 4 °C and stored at the same temperature. To eliminate or reduce bacteria and debris, 40 mL of the sample was centrifuged at 10 000 × g for 15 min using a ROTINA 380 centrifuge (Hettich, Germany) [6]. The transparent supernatant was combined with 5 mL of nutrient broth (Liofilchem, Italy) and incubated overnight at 37 °C. Five mL from an overnight broth culture of *P. aeruginosa* ATCC 27853, obtained from the Iranian Research Organization for Science and Technology (IROST), was inoculated into the mixture and incubated at 37 °C in a shaking incubator (Kuhner SHAKER X, Lab-Therm) at 110 RPM. After 24 h, the flask contents were centrifugated and filtered through 0.45 µm pore size filters, which allowed the phages to pass through but not the bacteria [6].

Evaluation of lytic activity of the isolated phage. The lytic activity and titers of the phage lysates were determined using a double-layer plaque assay (DLA). The recovered supernatant was serially diluted in a saline phosphate buffer (PBS). Amounts of 0.1 mL of the diluted phage and 0.5 mL of *P. aeruginosa* ATCC 27853 (adjusted to a 0.5 McFarland standard) were added to 3 mL of soft nutrient agar (0.75% agar at 45 °C). The mixture was layered onto a nutrient agar plate and allowed to solidify before overnight incubation at 37 °C. The plaque-forming units (PFU) per milliliter were calculated by multiplying the number of plaques by 10 and the inverse of the dilution factor [4, 7].

Transmission electron microscopy (TEM). TEM visualized phage morphology; the images were prepared at Rastak Lab. A high-concentration phage solution was applied onto a Cu Mesh 300 grid coated with formvar-carbon. The grid was fixed with 1% glutaraldehyde, stained using the standard negative staining method with 2% uranyl acetate, and examined under an EM 208S transmission electron microscope (Zeiss, Germany) operating at 100 kV [4].

Host range determination. The host range for the isolated bacteriophage was determined against 23 bacteria isolates, including 20 *P. aeruginosa* isolates from cystic fibrosis patients, *P. aeruginosa* ATCC 27853, *Klebsiella pneumoniae*, and *Staphylococcus aureus*. The host range was determined using a spot test; a 0.5 mL aliquot of bacterial culture was mixed with 5 mL of nutrient-soft agar (0.75% agar at 45 °C) and poured into an 8 cm petri dish (Biotest) containing 15 mL of 1.5% nutrient bottom agar. After brief drying, 10 µL of the filtered supernatant was added to the solidified soft agar. The plate was then incubated overnight at 37 °C and the next day was examined for clearing zones [8].

Phage stability tests. The thermal stability of the isolated phage was assessed by incubating the phage suspension at temperatures of -20, 4, 37, 50, 60, and 70 °C for an hour. Following the incubation, the phage count was determined using the double-layer plaque assay. For pH stability tests, 0.1 mL of the phage suspension was mixed with 0.99 mL of nutrient broth within the pH range of 4-10. The mixture was then incubated at 37 °C for 1 h before phage titration [4, 9].

Phage adsorption assay. The adsorption rate of the phage to the host bacterium was assessed by incubating 1 mL of phage with 9 mL of host bacterium (adjusted to a 0.5 McFarland standard). The phage-host mixture was maintained at 37 °C, and aliquots were collected at 5 and 10-minute intervals. Each aliquot underwent centrifugation at 8,000 g for 10 minutes to precipitate the phages bound to the bacteria. The concentration of unabsorbed phages in the supernatant was subsequently determined by applying the double-layer plaque assay [4].

Ethics statement. The study adhered to the Declaration of Helsinki guidelines and obtained approval from the ethics committee of Alzahra University in Tehran, Iran (Code: IR.AlZAHRA.REC.1401.015).

RESULTS

Bacteriophage isolation and enrichment. Of the three sewage samples collected, one contained phage. In the DLA assay, clear plaques (Fig. 1) indicated the lytic activity of isolated phage against *P. aeruginosa*. The titer of the purified phage was 10^9 PFU/ml.

TEM. The phage had an icosahedral head with a diameter of ~148.29 nm and a tail length of ~ 204.40 nm, displaying a member of the order *Caudovirales.* Based on the morphology, the phage was classified into the *Myoviridae* family (Fig. 2).

Host range. The newly isolated phage successfully infected 100% of *P. aeruginosa* isolated obtained from CF patients and the standard strain (ATCC 27853). However, the recovered phage did not affect *K. pneumoniae* or *S. aureus*.

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Fig. 1. DLA assay displaying plaques caused by phages in P. *aeruginosa* culture.

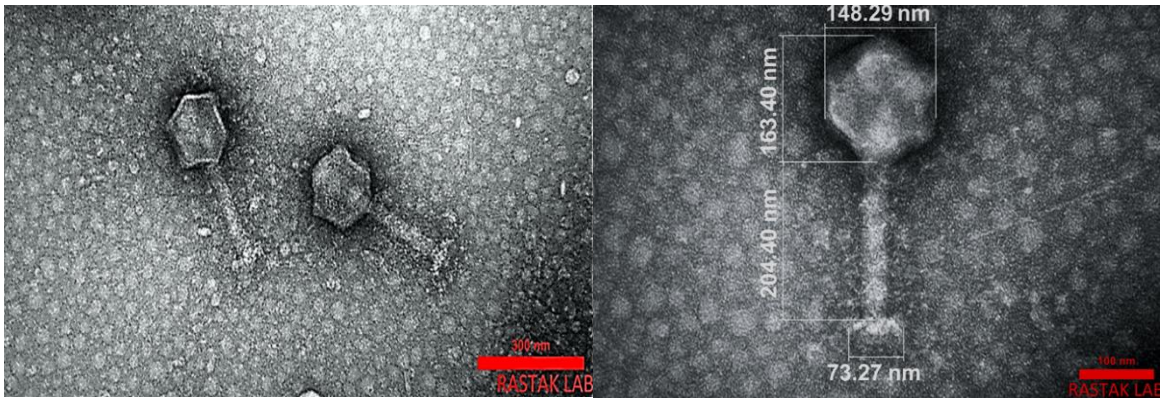

Fig. 2. TEM image of negatively-stained phage. The scale bar represents 100 nm.

Phage stability tests. The isolated phage exhibited lytic activity within the temperature range of -20 to 50 °C. However, a decline in lytic activity was observed at higher temperatures of 60 and 70 °C. Notably, the optimal temperature for lytic activity was 37 °C (Fig. 3A). Lytic activity was observed within the pH range of 5-9, with pH 7 identified as the optimal pH for lethal activity. However, a significant decrease in lytic activity was observed beyond the pH range of 5-9, and complete inactivation was observed at pH 3 (Fig. 3B).

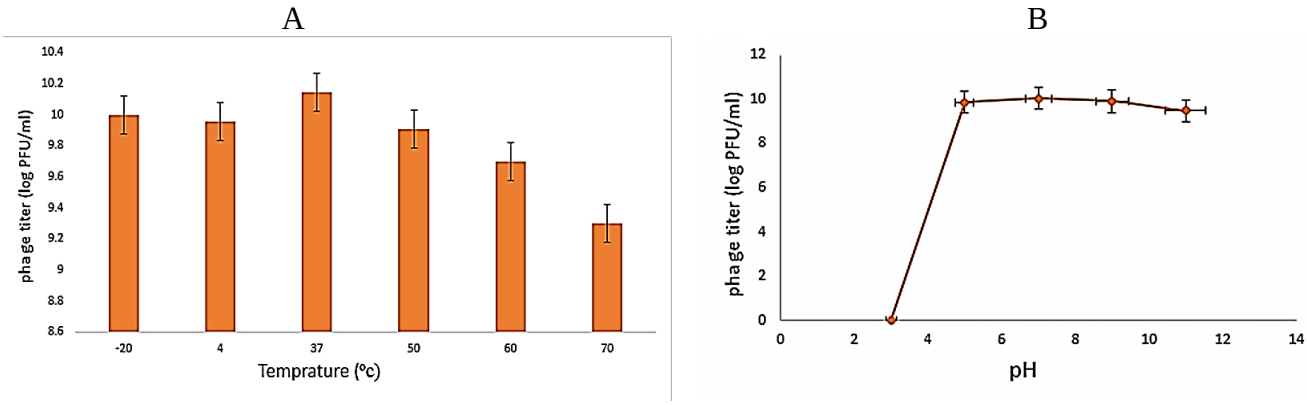

Fig. 3. The phage stability at various temperatures (A) and pH levels (B).

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Phage adsorption assay. The bacterial host demonstrated adsorption of 90% of phage particles within 10 min.

DISCUSSION

P. aeruginosa, a prominent opportunistic pathogen, exhibits remarkable adaptability, allowing it to thrive in diverse environments [10]. Its high antibiotic resistance poses a significant threat in hospital settings, particularly for individuals with compromised immune systems, leading to various diseases. Consequently, treating this bacterium has become increasingly challenging [11]. In response to the escalating antibiotic resistance crisis, the World Health Organization (WHO) has designated *P. aeruginosa* as a Priority 1 (Urgent) pathogen, highlighting the pressing need for the development of new antimicrobial alternatives [11]. Phage therapy presents a promising option for managing *P. aeruginosa* infections [4]. Over the past decade, numerous researchers have dedicated their efforts to isolating and characterizing lytic phages specific to *P. aeruginosa* [1]. Our study identified a novel bacteriophage with lytic activity against *P. aeruginosa* isolated from hospital sewage. TEM analysis revealed the presence of an icosahedral head and a short tail, classifying this phage as a member of the order *Caudovirales*, which encompasses tailed viral particles exhibiting *myovirus* morphology. Notably, most *P. aeruginosa* phages also belong to the order *Caudovirales* [1, 12, 13]. For instance, Yang *et al*. (2021) demonstrated the robust lytic activity of a KPP10 phage, classified under the *Myoviridae* family, against *P. aeruginosa* [10].

The isolated phage exhibited a broad spectrum of lytic activity against *P. aeruginosa*, effectively lysing all clinical strains obtained from cystic fibrosis patients. These findings were further supported by Pallavali *et al*. (2017), who successfully isolated and identified multiple bacteriophages targeting *P. aeruginosa*, demonstrating exceptional antimicrobial efficacy against this extensively drug-resistant pathogen [14]. Conversely, the isolated phage exhibited remarkable specificity, displaying no activity against samples of *K. pneumoniae* or *S. aureus*. This exceptional specificity and ability to infect a wide range of target bacterial species renders this phage highly suitable for potential therapeutic applications.

The stability of the isolated phage was assessed under various conditions, including temperature and pH. Our findings revealed that the phage remained stable within a temperature range of -20 °C to 70 °C, indicating its suitability for storage at low temperatures. Preservation under frozen conditions reduces molecular mobility and subsequent chemical reactivity, offering enhanced protection for the phage capsid and other proteins [14]. Moreover, the isolated phage exhibited sustained activity within a temperature range of 37 °C to 70 °C. The impact of pH on phage activity was also assessed, revealing robust lytic activity within the pH range of 5 to 9 while exhibiting a notable decrease in activity outside this range. Consistent with the findings of Lerdsittikul *et al*. (2022), our study demonstrated that the titer of phage VL1 remained relatively stable when incubated within a pH range of 7 to 10. However, complete inactivation of the phage occurred upon exposure to strongly acidic pH values of 2 and 1. Moreover, phage VL1 demonstrated remarkable stability at 25 °C and 37 °C, in contrast to the storage conditions at 4 °C. However, exposure to temperatures ranging from 40 °C to 70 °C resulted in a significant decline in the activity of phage VL1 [1]. The exceptional stability of phages over a broad range of pH and temperature conditions presents significant advantages for their potential utilization in phage therapy and treating bacterial lung infections [15].

The phage particles were adsorbed on the bacterial host within 10 min. Specifically, phages belonging to the *Myoviridae* family exhibited absorption by the target bacteria within the same time frame [16].

In this study, we isolated a lytic bacteriophage from hospital sewage that shares similarities with the *Myoviridae* family. This phage exhibited a broad host range and demonstrated remarkable stability under diverse environmental conditions, including variations in temperature and pH. In this study, we evaluated the efficacy of isolated bacteriophages against *P. aeruginosa* strains obtained from cystic fibrosis patients, comparing our findings to previous studies. Additional investigations are required to provide a comprehensive molecular characterization of bacteriophages, encompassing genome-based taxonomic analysis and assessments of the absence of lysogeny-related genes, antibiotic resistance genes, and virulence genes.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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