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Isolation and Molecular Characterization of *Pseudomonas aeruginosa* Lytic Bacteriophages as a Potential Therapeutic Alternative to Traditional Antibiotics

Aya M. Elgawish[1], Tahany K. Alkhoudher[2], Abdelghafar M. Abu-Elsaoud[1, 3], Waleed M. El Kazzaz[1*]

[1]Botany and Microbiology Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.

[2]Botany Department, Faculty of Science, Port Said University, Port Said, Egypt.

[3]Department of Biology, College of Science, Imam Muhammad bin Saud Islamic University (IMSIU), Riyadh 11623, Saudi Arabia.

***Correspondence**
Corresponding author: Waleed M. El Kazzaz
E-mail address: Walid_elkazaz@science.suez.edu.eg

Abstract

The emergence of antibiotic-resistant bacteria has led to increased bacterial infections that are difficult to treat. Bacteriophage therapy presents a potential solution to this problem. In this study, three phages isolated from different water sources in Ismailia were identified as potential candidates for bacteriophage therapy to control *Pseudomonas aeruginosa* bacteria. The phages were characterized according to morphology, host range, physical characteristics, and genetic differences. Based on their head and tail features, TEM images were utilized to identify two phages from the *Siphoviridae* family and one from the Myoviridae family. The phages were also tested for their tolerance to different physical and chemical factors such as temperature, pH, salinity, chloroform, and exposure to the laser, blood plasma, and essential oils. The three phages exhibited different preferences and tolerances to these factors, suggesting that they may be effective against different bacterial strains of *P. aeruginosa* in different environments. The study demonstrates the potential of phage therapy as an alternative to antibiotic therapy and highlights the importance of understanding phage characteristics in developing effective phage formulations.

KEYWORDS
Pseudomonas aeruginosa, MDR, Phage therapy, Bacteriophage

INTRODUCTION

Antimicrobial resistance is a growing public health concern worldwide as bacteria evolve and resist multiple antibiotics (Aslam *et al.*, 2021). One such bacterium that has been associated with multidrug resistance is *Pseudomonas aeruginosa*. *P. aeruginosa* is a Gram-negative, rod-shaped, opportunistic pathogen commonly associated with various human infections (Pang *et al.*, 2019). This bacterium is known to cause many diseases, including respiratory tract infections, urinary tract infections, wound infections, sepsis, and even death in immunocompromised individuals. *P. aeruginosa* infections are particularly challenging to treat due to their resistance to a wide range of antibiotics and their ability to form biofilms, often leading to chronic infections (Cappelletty, 1998; Wu *et al.*, 2015). The emergence of antibiotic-resistant strains of *P. aeruginosa* has increased interest in alternative therapies such as bacteriophage therapy. Understanding the pathogenesis and virulence factors of *P. aeruginosa* is crucial for developing effective treatments and preventing infections caused by this bacterium (Langendonk *et al.*, 2021; Pachori *et al.*, 2019).

Bacteriophage therapy has emerged as a promising alternative to traditional antibiotics for treating infections caused by multidrug-resistant bacteria (Broncano-Lavado *et al.*, 2021; Kakasis and Panitsa, 2019). Bacteriophages are viruses that can infect and kill bacteria and are highly specific to their target bacteria, making them a potentially powerful tool for combating antibiotic-resistant infections. *P. aeruginosa* is a particularly challenging pathogen, causing many infections and often exhibiting resistance to multiple antibiotics (Duckworth and Gulig, 2002; El-Shibiny and El-Sahhar, 2017; Principi *et al.*, 2019). Bacteriophages specific to *P. aeruginosa* have been identified and studied and show promise for effectively targeting and eliminating this troublesome pathogen. In this paper, we will explore the potential of bacteriophage therapy for combating antibiotic resistance, focusing on *P. aeruginosa* and the use of bacteriophages to treat infections caused by this pathogen. The use of individual phage isolates has been improved by combining them into a cocktail, leading to increased applicability for particular preparations (Chegini *et al.*, 2020; Duan *et al.*, 2022).

In this study, three phages were isolated from water/wastewater samples and characterized based on their physiological, host range, and molecular characteristics, with significant genetic differences, identified. Bacteriophages resist adverse physical and chemical factors, allowing them to thrive in harsh environments. However, their diverse characteristics can differ between and within families, and high resistance to external factors is critical for phage preparation stability.

MATERIALS AND METHODS

Bacterial cultures, storage, and antimicrobial susceptibility testing

In this study, the bacterial host *P. aeruginosa* ATCC 9027 was

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used to isolate bacteriophages and carry out experiments. A laboratory collection of bacterial strains was used for host range determination. Different *P. aeruginosa* isolates from (pus, blood, urine, septum, and ear), *E. coli* from (urine), *S. aureus* from (pus), *K. pneumonia*e from (pus), *A. baumannii* from (urine), and *P. mirabilis* from (pus and urine) were obtained. All bacteria were grown aerobically at 37°C. The VITEK 2 (bioMérieux, France) system was used for isolate identification and antibiotic susceptibility determination. For medium-term bacterial culture storage, agar slants were made. Reserving Eppendorf's was made by mixing 100 µl of glycerol (20%–30%) with 400 µl of broth medium and keeping it at -20 °C for up to six months.

Bacteriophage isolation, purification, and high-titer preparation

Water samples from the Suez Canal University Hospital Wastewater, El Temsah Lake, and Serapeum wastewater treatment facility influent were collected in pre-sterilized 1L glass bottles and transported to the laboratory in an icebox. The samples were spun at 4000 rpm for 10 minutes to get rid of large particulates, subsequently filtered for bacterial contamination using a 0.22-um syringe filter (Millipore GP, Millipore), and the filtrates were kept at 4°C until they were used.

To enrich phages, an exponentially growing *P. aeruginosa* culture (5 mL) was mixed with 85 mL of filtered samples, and 10 mL of 10x nutrient broth, and incubated for 24 hours at 37°C in a shaking water bath (120 rpm). Cultures were centrifuged at 4000 rpm and the supernatant was filtered through a 0.22 syringe filter to obtain enriched phages (Twest and Kropinski, 2009).

Phage selection was performed according to the modified Double-Layer Agar protocol described by Kauffman and Polz (2018). Briefly, 10 ul of phage dilution was mixed with 100 ul of overnight bacterial culture on the top of a Nutrient Agar plate, followed by the addition of 3 ml of melted soft agar (50°C), mixed vigorously, let solidify (20 min), and incubated at 37°C. Plates were observed after 24 hours of incubation at 37°C, and positive samples were recorded as plaque-forming units (pfu/ml).

The plaque-streak technique is used to isolate phage from samples. Morphologically distinct plaques were selected and purified after three rounds of streaking onto top agar seeded with bacteria. The spot-test method was used for phage enumeration using the 10-fold serial dilution method according to Champagne and Gardner (1995). High-titer phage stock was prepared by agar overlay procedures according to Patterson *et al.* (1987).

DNA extraction

Phage gDNA was extracted from high-titer filtered lysate (approximately 1011) as previously described (Gautam, 2022). In this procedure, 1 mL of high titer filtered lysate was digested for 1.5 hours at 37oC with 10 uL/mL DNaseI and 10 uL/mL RNaseA. EDTA was added at a concentration of 20 mM to inactivate DNaseI and RNaseA. NaCl (0.5 mol/L) and polyethylene glycol (PEG) 8000 (1 g/10 mL) were gently mixed into the filtrate and incubated for 15 minutes at room temperature before centrifugation at 10,000 rpm for 10 minutes at 4 oC. The pellets were dissolved in 100 uL of pH 8.0 TE buffer, followed by 100 uL of phenol: chloroform: isoamyl alcohol (25:24:1), vortexed, and centrifuged at 12,000 rpm for 10 minutes. The upper layer was collected, and the DNA was precipitated by the standard ethanol precipitation protocol. Nanodrop (Thermo Fischer. USA) determined the purity and quantity of the isolated DNA.

Restriction Enzyme Digest and Gel Electrophoresis of DNA

The study uses EcoRI, HindIII, Dra, and BSU restriction enzymes (NEB, England) to cut DNA at specific sequences for analysis. Each enzyme has a specific recognition site, which generates a unique pattern of fragments when digesting the DNA sample. Following the manufacturer's instructions, restriction digestion reactions were done with 0.5 ug of phage DNA. Digested DNA patterns were visualized using the standard 1% Agarose gel electrophoresis and photographed with a UV transilluminator supplied by a digital camera.

Transmission Electron Microscopy

The morphology of isolated phages is examined using a transmission electron microscope. A high-titer phage lysate was dropped onto a carbon-coated copper grid for adsorption, then stained for 1 minute with 2% phosphotungstate, allowed to dry, and examined with a transmission electron microscope at 100 kV (H-7650, Hitachi, Japan). For phage classification based on morphological characteristics, the International Committee on Taxonomy of Viruses (ICTV) guidelines were followed.

Temperature, pH, salt, essential oils, and plasma stability tests

A potential phage for use as a bio-control agent must be stable in a wide range of physicochemical conditions, such as temperature, pH, and salinity (Phothaworn *et al.*, 2020). The temperature tolerance of the bacteriophage was assessed by incubating one milliliter of phage at various temperatures ranging from -18 to 60 degrees Celsius for one hour. A titer of about 10^6 PFU/ml was achieved by adding phages to Eppendorf's holding sterile SM buffer with a salt content of 1 to 30%. The samples were then left to marinate at 25 degrees Celsius for an hour. Phage solutions were kept at pH values between 2 and 12 for 1 hour to ascertain the phages' tolerance at these ranges. Furthermore, 100 ul of phage (10^9 PFU/mL) was mixed with 900 uL of castor, argan, jojoba, coconut, and almond oils as well as plasma for 1 h at 25°C to examine the effect of essential oils and plasma on phage stability. The double-layer agar method was used to measure phage titers in each of the methods (Twest and Kropinski, 2009). There were three separate replicas for each of these evaluations.

One-step growth curve

This scientific experiment involves the culture of bacteria and the addition of phage to study their efficiency in infecting and replicating within the host bacteria. Analysis of a one-step growth curve was used to ascertain the latent period of the phages that were isolated as well as their burst size (Kutter *et al.*, 2004). One-step growth curve was conducted according to Kropinski (2018) In a microfuge tube, the phage-bacteria suspension that had a multiplicity of infection of 0.01 was kept in an incubator at 37 degrees Celsius for five minutes without being shaken for adsorption. Following centrifugation at 13,000g for one minute, the pellets containing partially infected cells were reconstituted in 10 mL of pre-warmed LB broth at a concentration of $1x10^7$ CFU/mL, and the mixture was then re-incubated at 37 degrees Celsius without being shaken. Up to two hours' worth of samples were collected at 10-minute intervals, after which the phage titers were immediately measured using the double-layer agar technique (Twest and Kropinski, 2009). The size of the burst was determined by determining the ratio of the final titer of liberated phages to the original count of bacterial cells that had been infected.

Host Range Identification

the host range of the isolated phages was tested against 22 non-repetitive clinical *P. aeruginosa*, *E. coli*, *K. pneumonia*, *S. aureus*, *A. baumannii*, and *P. mirabilis* strains, Using the standard spot assays (Tan *et al.*, 2020). Briefly, 5 uL of diluted phage lysate (~10^6 PFU/mL) were spotted on dried 4 mL soft agar seeded with 200 uL of exponentially growing bacterial strains and incubate at 37°C. the plates were checked for plaques after 24 hours and the appearance of a clear zone in bacterial culture was recorded as a positive result (Shahin *et al.*, 2022). Host range experiments were repeated twice.

Random Amplification of Polymorphic DNA (RAPD) PCR Fingerprinting

Random Amplified Polymorphic DNA (RAPD) is used to study genetic diversity among different *P. aeruginosa* strains to ensure non-duplicate strains. Twenty-five strains of *P. aeruginosa* were tested using RAPD primer OPM-09 with sequence (5′→3′), (GTCTTGCGGA) obtained from RAPD kit (Eurofin). The amplified DNA fragments were analyzed using gel electrophoresis to identify genetic differences and similarities between the strains.

Statistical analyses

The data was collected, analyzed, and presented using tables and figures. Statistical tests were conducted to determine whether the data were parametric or nonparametric, and descriptive statistics were used to evaluate the data. Inferential statistics, such as two-way ANOVA, repeated measures ANOVA, and one-way ANOVA, were used to compare treatment groups, time points, and dilutions at significance levels of 0.05. Duncan multiple range tests were used to compare treatment groups, and the statistical package for social science SPSS (IBM-SPSS ver. 28.0 for Mac OS) was used to analyze the data.

RESULTS

Random Amplification of Polymorphic DNA (RAPD) PCR Fingerprinting

Random Amplified Polymorphic DNA (RAPD) was employed to create unique fingerprints for each bacterial isolate by amplifying random DNA regions. The OPM-09 RAPD primer (5′→3′ sequence: GTCTTGCGGA) obtained from the Eurofins Bio diagnostics kit was used to study 22 *P. aeruginosa* isolates. Analysis of the results confirmed the primer's ability to differentiate between the 22 isolates, indicating the genetic diversity of the samples (Fig. 1).

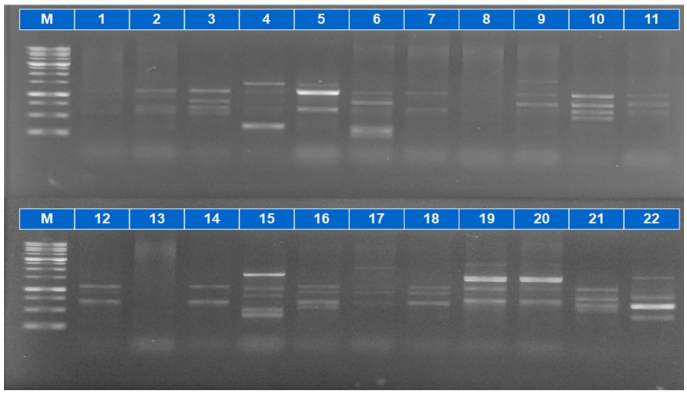

Fig. 1. RAPD genotyping of *P. aeruginosa* strains from clinical sources (1-25), using OPM-09 primers; M) 1 Kbp DNA Ladder (Genedirex).

Antimicrobial susceptibility testing and host range identification.

The antibiotic susceptibility of 22 *P. aeruginosa* strains was evaluated, revealing that most strains were resistant to the antibiotics used against them. This highlights the need for alternative methods, such as phage therapy, to treat infections caused by antibiotic-resistant bacteria.

The percentage of resistant *P. aeruginosa* strains to antibiotic agents is presented in Fig. 2. The highest resistance rates were observed for ampicillin (89%), cefepime (65.38%), and ciprofloxacin (61.54%), while the lowest rate of resistance was noticed for colistin (15.38%). At the same time, the resistance rates for tobramycin, gentamycin, ticarcillin/clavulanic acid, levofloxacin, amikacin, aztreonam, ceftazidime, piperacillin, imipenem, meropenem, and polymyxin B were 57.69%, 57.69%, 53.85%, 53,85%, 50%, 50%, 46.15%, 46.15%, 46.15%, 34.62%, and 30.77%, respectively.

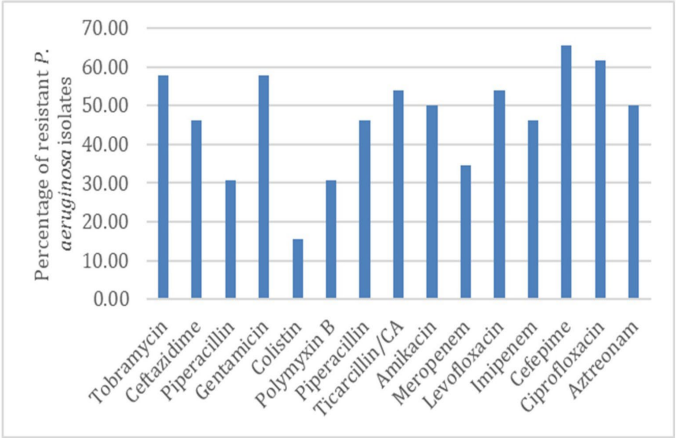

Fig. 2. shows the percentage of resistance among 22 *P. aeruginosa* strains using different antibiotics (Tobramycin, Ceftazidime, Ampicillin, Gentamicin, Colistin, Polymyxin B, Piperacillin, Ticarcillin/CA, Amikacin, Meropenem, Levofloxacin, Imipenem, Cefepime, Ciprofloxacin, and Aztreonam).

Isolation and plaque morphology of bacteriophages from different water samples.

Three phages were isolated using bacterial host strain *P. aeruginosa* serotype ATCC 9027, and their ability to create clear zones of lysis on bacterial lawns indicated their lytic properties against bacteria, which is desirable for biocontrol applications. The morphological, host range, and physical characteristics of three phages isolated from different water sources, including Serabioum wastewater treatment plant effluent, El-Temsah Lake, and Suez Canal University Hospital Wastewater, were determined in this study. The isolation rate of phages was found to be high, likely due to the diverse source material containing a wide range of bacteria and viruses and the selected bacteriophages. This diversity may have provided a favorable environment for the growth and proliferation of bacteriophages. The three relevant phages were cultured on *P. aeruginosa* culture plates and formed plaques with varied morphologies. Three phage plaques are depicted in Fig. 3, each with a different physical structure and different plaque sizes (small, medium, and large). Fig. 3a shows a phage (VB_PaeS-AW01) with 2-4 mm plaque sizes and a bright semi-circular plaque. Fig. 3b shows phage (VB_PaeM-AW02) with 3-5 mm plaque sizes and semi-circular layers of plaque: bright center layer, clear middle layer, and turbid outer layer. Fig. 3c shows phage (VB_PaeS-AW03) with distinct 6-8 mm plaque sizes and three semicircular layers of plaque: a large bright center layer, a small slightly turbid middle layer, and a small turbid outer layer.

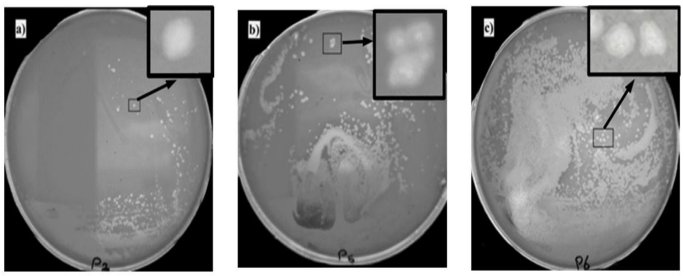

Fig. 3. The three relevant phages' plaque morphology. Plated on the host *P. aeruginosa* were a), d) VB_PaeS-AW01 phage; b), e) VB_PaeM-AW02 phage; c), f) VB_PaeS-AW03 phage.

Transmission electron microscope

The three bacteriophages of interest were photographed by TEM to evaluate their morphology. Figure 4 shows the difference in phage structure as determined by TEM. Figure 4-a depicts phage VB_PaeS-AW01 of the *Siphoviridae* family, which has an icosahedral head, a capsid diameter (nm) of 49±15, and a long, non-contractile tail with a length (nm) of 99±14. Figure 4b, depicts phage VB_PaeM-AW02 of the Myoviridae family with an icosahedral head, capsid diameter (nm) 51±13, and contractile tail length (nm) 99±14. Fig. 4c depicts phage VB_PaeS-AW03 of the *Siphoviridae* family, which has an icosahedral head, a capsid diameter (nm) of 55±9, and a long, non-contractile tail with a length (nm) of 100±6. The phage capsid diameters did not differ noticeably. Table 1 shows the capsid diameter (nm) and tail length (nm) of three phages as described.

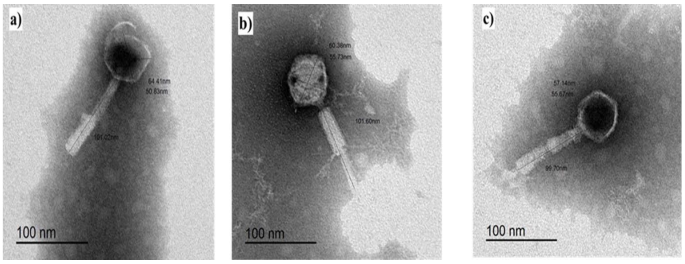

Fig. 4. Interested phages' transmission electron micrographs. a) VB_PaeS-AW01; b) VB_PaeM-AW02; c) VB_PaeS-AW03.

Molecular characterization and restriction enzyme digest

To obtain more detailed information on the three isolated phages, genomic characterization was carried out using restriction enzymes (EcoRI, HindIII, DraI, BSU) to digest the phage DNA and analyze their diversity. The results showed successful digestion of the phage genome by all the tested restriction enzymes, indicating that the genome was composed of double-stranded DNA. Additionally, the restriction enzyme patterns obtained were able to distinguish between the three phages, suggesting differences in their genomes. The diversity of the restriction patterns clearly showed that the three phages were distinctly different.

Phage's host range

The study also investigated the host range of three phage isolates targeting *P. aeruginosa* against 22 clinical strains and 3 type culture strains of the same species. Results showed that (Figs. 6 and 7), the isolated phages were polyvalent and capable of infecting multiple strains of *P. aeruginosa*. VB_PaeS-AW01 showed the highest spectrum of lytic activity against 96% (24), followed by VB_PaeS-AW03 and VB_PaeM-AW03 with lytic spectrum activities of 88% (22) and 76% (19) of the tested strains, respectively. On the other hand, the phage cocktail containing the three phages showed lytic activity against all the tested strains (Fig. 7). However, the results were negative when tested against other bacterial species, such as *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. mirabilis*, indicating that the phage isolates were specific only to *P. aeruginosa* strains. The data indicate that the three phages have a very wide host range of *P. aeruginosa* strains which supports that these phages may bind to multiple receptors hence they are capable of infecting more diverse host strains.

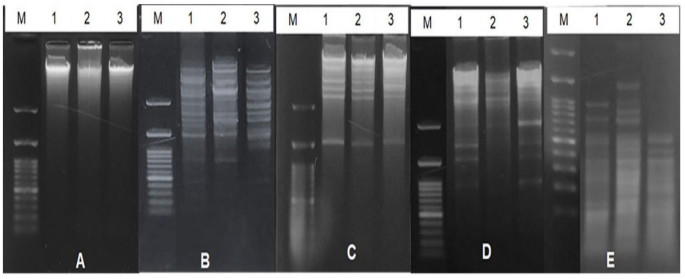

Fig. 5. Agarose gel electrophoresis for phages DNA restriction digestion using A) Non, B) EcoRI, C) DraI, D) HindIII, and E) BsuI restriction enzymes. M) 100 bp DNA Ladder (Genedirex)1); VB_PaeS-AW01; 2) VB_PaeM-AW02; 3) VB_PaeS-AW03.

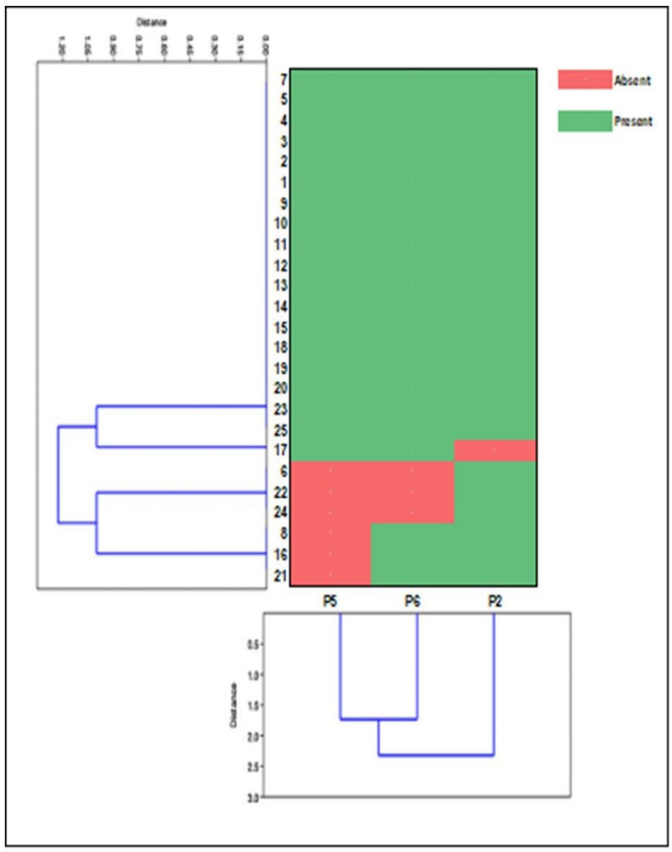

Fig. 6. Heatmap showing the absence and presence of the three phage isolates among 25 (22 clinical and 3 type culture strains) different bacterial strains, where red color represents the absence of lytic activity, and green color represents the presence of lytic activity.

One step growth curve Experiment

The one-step growth curve characteristics of three bacteriophages, VB_PaeS-AW01, VB_PaeS-AW02, and VB_PaeS-AW03, were examined, and their latent phase and burst size were determined (Fig. 8). The latent phase for VB_PaeS-AW01 was 30 minutes with a burst size of 95; for isolate VB_PaeS-AW02, the latent phase was 20 minutes, and the burst size was 73; and for phage VB_PaeS-AW03, the latent phase was 40 minutes, with a burst size of 175 (Table 2). These findings indicate that the burst size

and latent phase can significantly vary among different bacteriophages.

Table 1. The capsid diameter (nm) and tail length (nm) of three phages of interest (nm).

Phage	Diameter of the cap (nm)	Length of the tail (nm)
VB_PaeS-AW01	49±15	99±14
VB_PaeM-AW02	51±13	107±2
VB_PaeS-AW03	55±9	100±6
ANOVA (p-value)	p<0.001***	p<0.001***

*, **, *** significant at p<0.05, <0.01, <0.001, ns, non-significant at p>0.05

Table 2. Latent period and burst size of the isolated phages.

Phage	Latent period (Minutes)	Burst size (PFU/cell)
VB_PaeS-AW01	30	95±06
VB_PaeM-AW02	20	73±04
VB_PaeS-AW03	40	175±13

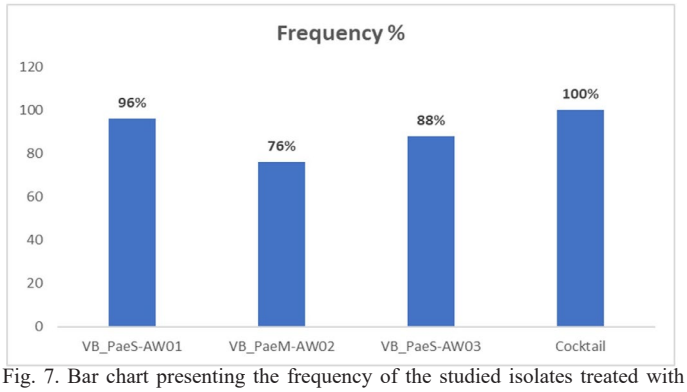

Fig. 7. Bar chart presenting the frequency of the studied isolates treated with different bacterial strains.

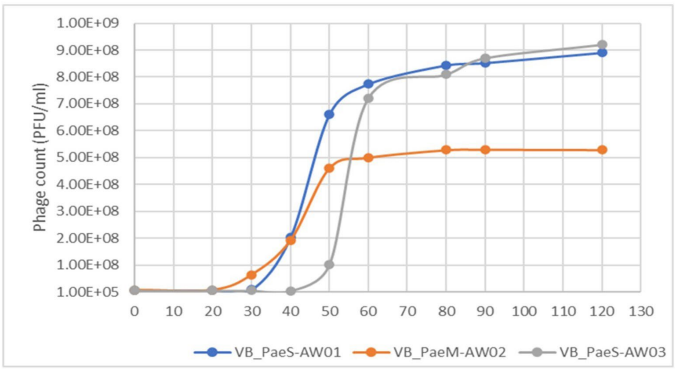

Fig. 8. One-step growth curve of bacteriophages VB_PaeS-AW01, VB_PaeM-AW02, and VB_PaeS-AW03 measured against *P. aeruginosa* at an MOI of 0.1. The phage titer as PFU per ml is shown at different time points. Each data point is a mean from three experiments.

The effects of variable incubation temperatures on phage stability

The behavior and stability of the phages were investigated at various incubation temperatures. The plaque titer of each phage was measured at different temperatures to determine the optimal and limiting conditions for infection. All the isolated phages showed stability after one-hour incubation at the temperature range from -18 to 40°C. VB_on the other hand, plaque counts started to drop sharply at 50 and 60°C. None of the phage isolates showed detectable titers at temperatures above 70°C (Fig. 9).

The effect of variable pH levels on phage stability

Figure 10 illustrates the impact of different pH levels on phage stability and the three *P. aeruginosa* phages' infectivity was evaluated. It was observed that extremely acidic (pH 2-3) and highly alkaline (pH 12-13) conditions had a negative impact on phage infectivity. At pH 2, no plaques were formed in any of the tested phages, indicating a loss of phage viability. However, the phages remained stable at pH levels ranging from 4 to 10, suggesting that this pH range is suitable for maintaining phage viability. These results highlight the importance of pH in phage stability.

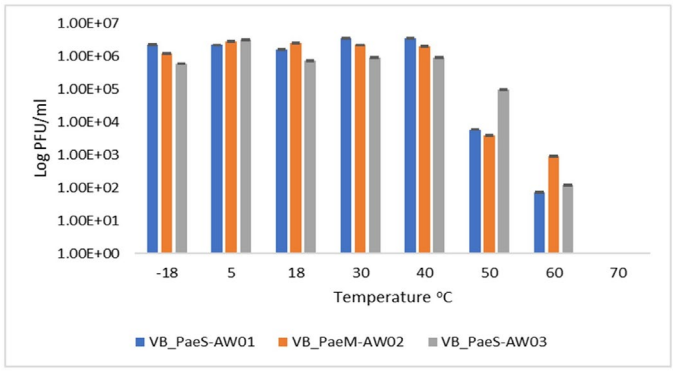

Fig. 9. Bar chart presenting the mean and standard deviation of *P. aeruginosa* phage stability at different temperatures.

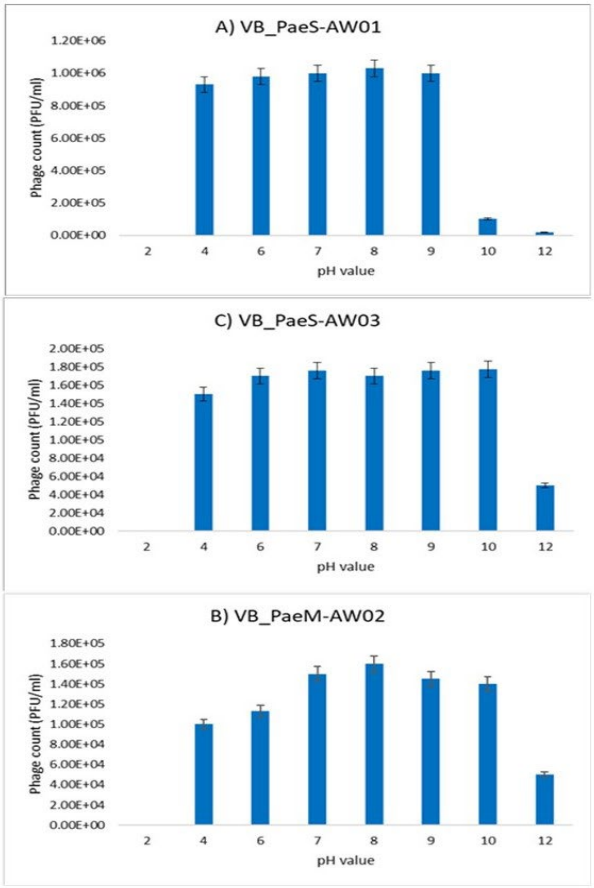

Fig. 10. Bar chart presenting the mean and standard deviation of *P. aeruginosa* phage stability at different pH levels.

The effect of salinity on phage stability

To evaluate the impact of NaCl concentration on phage stability and plaque formation, the three isolated phages were exposed to varying salt concentrations ranging from 0.5% to 40%. Results indicated that isolate 2 was tolerant to a maximum salt concentration of 1% and a minimum of 25%. Isolate 5 demonstrated the highest salt tolerance at 20%, with a minimum of 25%. Isolate 6 exhibited a maximum salt tolerance of 15% and a

minimum of 5%. These findings reveal that the phages' tolerance to NaCl concentrations is highly variable and dependent on the individual phage isolate (Fig. 11).

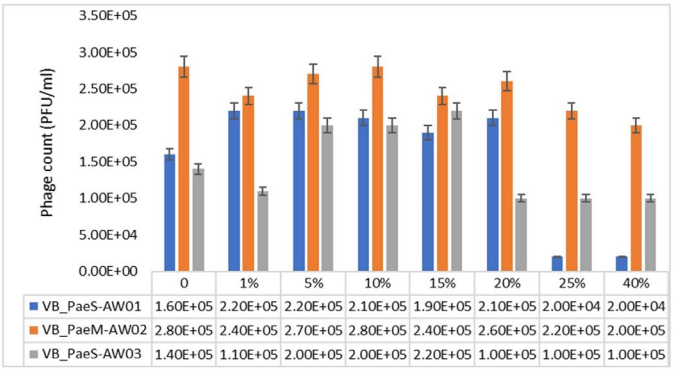

Fig. 11. Bar chart presenting the mean and standard deviation of *P. aeruginosa* phage stability at different salinity levels.

The effect of blood plasma and essential oils on phage stability

The impact of blood plasma and essential oils on the stability of three phages was investigated. The results showed that blood plasma has a strong negative impact on plaque count for all the tested phages (Fig. 12). Regarding the essential oils, only argan oil positively affects phage stability for isolate 2. In contrast, all other oils including jojoba oil, castor oil, coconut oil, and sweet almond oil had a negative impact on plaque count for all three phage isolates.

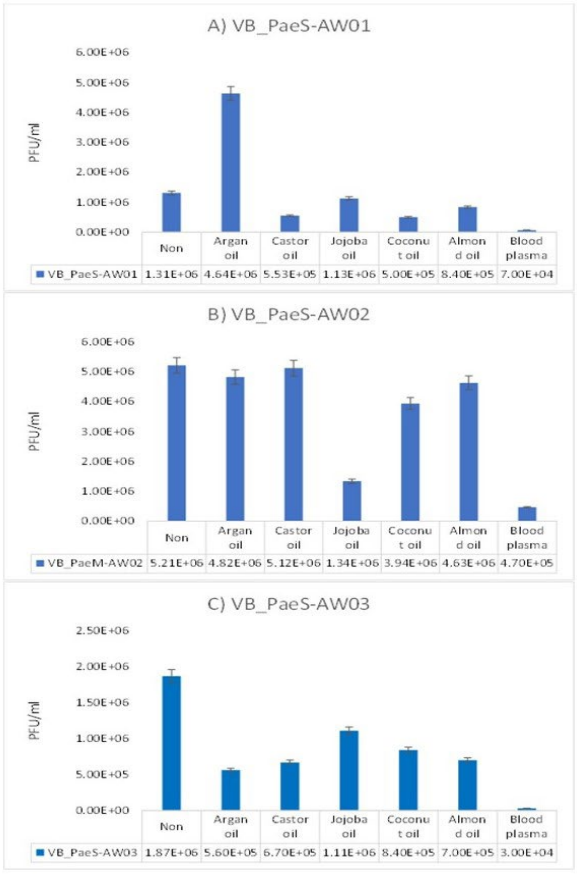

Fig. 12. Bar chart presenting the mean and standard deviation of *P. aeruginosa* phage stability in blood plasma and different oils.

DISCUSSION

The world is looking into bacteriophages (phages) again because more reports of bacteria are resistant to antibiotics and because it is harder to find and make new antibiotics 10.1042/ETLS20170005, 10.1128/CMR.00066-18).In phage therapy, obligately lytic phages are primarily used to destroy the bacterial hosts intended for them while leaving human cells unharmed and minimizing the broader impact on commensal bacteria frequently caused by antibiotic use (10.1042/ETLS20170005). Rapid advancements in phage therapy have led to several clinical trials and instances of its life-saving therapeutic application (Maddocks *et al.*, 2019).

Phage therapy is resurfacing as a promising treatment option for serious bacterial infections. Possible candidates must be powerful, stable, and strictly lytic against the intended pathogens to be effective in phage therapy. Therefore, to ensure that newly isolated phages match these requirements, they are screened and put through various phenotypic and genotypic characterization (Maddocks *et al.*, 2019; Aslam *et al.*, 2020; Petrovic Fabijan *et al.*, 2020).

The primary goal of this study was to isolate and characterize virulent phages against *P. aeruginosa*. To that purpose, samples of untreated water from various locations in Ismailia, including Serabioum Wastewater, El Temsah Lake, and University Hospital Wastewater, were collected and utilized as a source for the isolation of phages unique to *P. aeruginosa*.

In the current work, three lytic phages were successfully isolated from prior samples using *P. aeruginosa* NCTC 12924 / ATCC 9027. The ability of phages to create a clear zone of lysis (plaques) on bacterial lawns revealed that they were naturally virulent phages with lytic properties against bacteria, which is preferred in biocontrol applications (Clokie *et al.*, 2011).

In this study, it was found that the rate of isolating phages was surprisingly high. This can be traced back to the source material that was used. The source material used in this study was diverse and contained a wide range of bacteria and viruses, in addition to our selected bacteriophages. This diversity in the source material may have provided a favorable environment for the growth and proliferation of bacteriophages. The lytic spectrum of a bacteriophage is considered one of the most important biological features that determine its potential applications. The lytic spectrum of a phage is defined by the range of bacteria genera, species, and strains it can infect. This biological feature is critical in phage biocontrol applications, as it determines the phage's ability to target specific bacterial strains (Kutter, 2009).

In the context of phage biocontrol, selecting virulent phages with broad lytic spectra over temperate phages with narrow lytic activity is crucial. The reason behind this selection is due to the potential ability of temperate phages to transfer virulence or antibiotic resistance genes. Furthermore, the inability of narrowly lytic phages to target a wide range of bacterial strains also emphasizes the importance of selecting virulent phages with broad lytic spectra in phage biocontrol applications (Kutter *et al.*, 2004; Hagens and Loessner, 2010).

The lytic spectra of the phages in this study were investigated when different host strains of clinical *P. aeruginosa* were used, demonstrating phage diversity. The phages' lytic spectra would not be considered narrow because the three phages consistently lysed most of the 25 clinical host strains of *P. aeruginosa* that were evaluated. The variations in susceptibility of the bacterial strains could be due to diverse host factors/mechanisms that bacteria have developed to combat phage infection and death; one example is the loss of phage receptors from the bacterial surface. Phage attachment to a specific receptor on the bacterial surface is the first important step in phage infection, and any change in the surface profile, whether by controlled expression or receptor mutation, results in phage insensitivity. The presence of restriction endonucleases in some bacterial cells is another host factor that contributes to bacterial resistance. These restriction systems work by digesting foreign viral DNA, thereby preventing phage replication (Shin *et al.*, 2012).

In this study, it didn't appear that bacterial strains could have possessed any resistance mechanisms and didn't have resistance to most of our phages. Also, using a phage cocktail could increase

the possibility of bacterial destruction and reduce any chances of resistance. Our three phages demonstrated high capability to lyse most of the 25 clinical strains of *P. aeruginosa* and have a broad lytic profile and were selected for further physiological and molecular characterization.

In this study, phages were examined by TEM and found to be diverse in structure. All three phages had icosahedral heads ranging from 49 to 64 nm. Two of these, VB_PaeS-AW01 and VB_PaeS-AW03, have long, non-contractile tails ranging from 99 to 113 nm, and these characteristics indicate that these two phage isolates are members of the *Siphoviridae* family. The third phage, VB_PaeM-AW02 has a long, contractile tail; based on its tail structure, which measures 107–109 nm in length, it is a member of the Myoviridae family (Ackermann, 2007; 2011; Tolstoy *et al.*, 2018).

To further characterize the three phages, genomic characterization was performed on agarose gel electrophoresis using restriction enzymes (EcoRI, HindIII, Dra, and Bsu) to digest the phage DNA to analyze the diversity among the phages. The successful restriction enzyme digestion observed in this investigation revealed that the phage genome was indeed double-stranded DNA, and the restriction enzymes used for this analysis were able to identify the three phages due to differences in their patterns. Similarly, morphological identification using TEM revealed differences between these phages (Addy *et al.*, 2018).

In addition to biological features, newly isolated phages should be evaluated for their stability and persistence when exposed to various external conditions that they may face in the environment to confirm their biocontrol potential. As a result, another study goal was to look at how the three phages reacted to changes in physicochemical stress factors that could occur during phage manufacture or biocontrol application of formulated phages (Hagens and Loessner, 2010).

During the experiment, the behavior and stability of three phages were investigated at various incubation temperatures. All phage isolates showed no titers at temperatures above 70 °C, likely due to the effect of high temperatures on phage proteins. There could be several potential reasons for these results. One possibility is that the optimal temperature for phage infectivity varies between different phage isolates. Each phage may have a unique set of proteins and enzymes that function optimally at different temperatures. Additionally, the temperature range in which the phages remained stable may be related to the temperature tolerance of their bacterial hosts. Another possibility is that higher temperatures cause the denaturation of phage proteins, leading to a loss of phage viability. This denaturation could be irreversible, leading to a sharp drop in plaque titers. Additionally, the temperature may affect the mobility of the phage particles as well as their diffusion rate toward their host cells. Therefore, temperature changes may have a direct impact on the likelihood of phage particles encountering their host cells, leading to changes in plaque titer. Other studies (Litt and Jaroni, 2017) have reported the ability of some phages to resist high temperatures, where they remain stable for up to 24 hours at a temperature range of 40–60 °C and for 90 days in cold storage. The results of this study indicated that the phages were thermally stable at high temperatures (45 °C–55 °C), suggesting their suitability for use on phage carcasses/meat in combination with other food processing technologies that involve thermal treatments to enhance food microbial safety.

During the experiment examining the effect of different pH levels on phage stability, it was observed that extremely acidic (pH 2) and highly alkaline (pH 12–13) conditions negatively impacted the infectivity of the three *P. aeruginosa* phages.

Because they have phosphate and carboxyl groups on their surfaces, viruses like phages have a negatively charged surface when they are in the water. When the environment is acidic, there are a lot of hydrogen ions (protons) in the solution. These can interact with the negatively charged groups on the surface of the phage, making them protonated. This protonation can change the charge on the phage surface from negative to neutral or even positive, which can lead to repulsion between the phage and the negatively charged bacterial surface. This can make it hard for the phage to attach to the bacterial cell and infect it, which makes the phage less infectious. So, the potential reason for this study result could be the effect of pH on the structure and stability of the phage particles. On the other hand, highly alkaline conditions may have caused the surface of the phage to lose its proton, which caused the phage particles to stick together and lose their ability to spread disease. The phages were stable at pH levels from 4 to 10, which may be because the phage particles were neutrally charged. This may have made it easier for the phages to stick to the surface of the bacteria and spread the infection. Also, the pH tolerance of phages may depend on the type of phage and the type of bacteria used in the experiment. This is because different types of phages and bacteria may react differently to changes in pH. Previous research has shown that almost all phages are resistant to pH levels of 7-8 (Hazem, 2002). Other studies have indicated that a large proportion of tailed phages exhibit stability within a pH range of 5.0 to 9.0. Our research has yielded similar results, further supporting the notion that the majority of tailed phages can withstand acidic or alkaline conditions within this pH range. This information can be of great significance in the development of phage-based therapeutic and biocontrol applications, as it provides insight into the potential stability of these phages within different environments. (Fan *et al.*, 2017).

The different levels of salt tolerance seen in the three phage isolates in this study could be caused by several factors. Phages can be affected by osmotic stress from high salt concentrations, which could affect their stability and plaque formation. Based on these results, it seems that phages' ability to handle salt is a complicated, multi-step process that is likely affected by both their genes and their environment. More research needs to be done to fully understand how phage salt tolerance works and what it might mean for phage therapy. In a study by Islam *et al.* (2020), it was found that the lytic activity of JSF4 bacteriophages steadily decreased when treated with less than or more than 0.9 N NaCl solution, indicating the impact of bacteriophage osmotic stress (Islam *et al.*, 2020). Similarly, Chandrarathna *et al.* (2020) demonstrated that AHP-1 phage infectivity was maintained regardless of the concentration of NaCl studied (0.1%–3.5%) (Chandrarathna *et al.*, 2020). In contrast, Jończyk, *et al.* (2011) reported that bacteriophages could be inactivated by osmotic shock. Furthermore, Weinbauer (2004) observed that psychrophilic *Pseudomonas* phages (wy and ps1) showed decreased persistence in highly concentrated NaCl or sucrose solutions .

This study investigated the effects of blood plasma and essential oils on phage stability for three phage isolates, and the results showed that blood plasma had a negative impact on plaque count for all phage isolates. This may be because parts of blood plasma, such as proteins and lipids, get in the way of phage infection. These parts might have made it harder for phages to attach to bacterial host cells or get through the walls of bacterial cells, making plaques less likely to form. The study also found that essential oils had a negative effect on the number of plaques for all three phage isolates. Most essential oils, like jojoba oil, castor oil, coconut oil, and sweet almond oil, could be cytotoxic or antibacterial, which would make them bad for plaque counts for all three phage isolates. These properties may have hurt the bacterial host cells, making them less likely to be infected by phages. Argan oil may have helped the VB_PaeS-AW01 phage stay stable because it has a unique mix of fatty acids and antioxidants, which may have protected the phage from environmental stresses and kept its ability to spread. On the other hand, argan oil might have helped the bacterial host cells grow, making more of them available for the phages to infect. Overall, the effects of blood plasma and essential oils on phage stability will likely be specific to the phage isolate and the type of oil or plasma used. The outcome of these interactions may depend on the makeup of the oil or plasma, as well as the properties of the phage and the bacterial host cells. The reference study by Shinde Stamatos *et al.* (2022) investigated the impact of human plasma (HP) on the infectivity of staphylococcal bacteriophages (Shinde *et al.*, 2022). The results revealed

a significant reduction in infectivity in the presence of HP. The degree of inhibition varied depending on the bacterial strain and the bacteriophage used, ranging from 48% to 81% for two methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates infected with the same bacteriophage and 98% for a third MRSA clinical isolate infected with a different bacteriophage. Interestingly, the addition of HP did not affect the infectivity of an Enterococcus faecalis clinical isolate. It is hypothesized that plasma proteins bind to staphylococcal surface proteins, masking the receptors that are involved in bacteriophage attachment and thereby reducing infectivity.

The growth curve of bacteriophages is an important part of phage biology that has been studied a lot to figure out how phages interact with bacteria. The size of a bacteriophage's burst and how long it stays dormant are two important parameters that can be used to describe its growth curve. The growth curve of bacteriophages is based on how different factors, like MOI and burst size, work together. The MOI shows how often the bacterium is exposed to the phages, while the burst size shows how well the phages infect and reproduce inside the bacterium. The results show that when phages are exposed to more bacteria, their replication is stronger, and their burst sizes are bigger. But the size of the burst can also be affected by other things, such as how the phages and the host bacteria work. The reference study by Pajunen *et al.* (2000) investigated the one-step growth curve of φYeO3-12 on Y. enterocolitica serotype O:3 strain 6471/76-c. The study showed that the phage exhibited an eclipse period of 15 minutes, followed by a latent period of 25 minutes and a growth period of 10 minutes, with a burst size of 100 to 140 PFU per infected cell. Phage was added at an MOI of 0.1. These findings were consistent with the values observed for T7 group phages, suggesting that φYeO3-12 belongs to this group (Pajunen *et al.*, 2000).

The goal of the study was to find out what kinds of hosts the three phage isolates that attack *P. aeruginosa* can attack. The isolates were tested against 25 clinical strains of *P. aeruginosa* from diverse sources. They were found to be positive, indicating that they were polyvalent phages capable of infecting many strains of the same species. However, when tested against different bacterial species such as *E. coli*, *K. pneumonia*e, *A. baumannii*, and *P. mirabilis*, the results were negative, showing that the phage isolates were specific only for *P. aeruginosa* strains. These results highlight the importance of evaluating the host range of phages to determine their potential use in treating specific bacterial infections (Greene and Goldberg, 1985).

CONCLUSION

The emergence of antimicrobial resistance and the limited discovery and development of new antibiotics has led to a resurgence of interest in bacteriophage (phage) investigations worldwide. Phage therapy utilizing obligately lytic phages has emerged as a promising treatment option for serious bacterial infections. This therapy selectively destroys bacterial hosts while minimizing the impact on commensal bacteria, which is a frequent adverse effect of antibiotics. The present study isolated and cultivated virulent phages against *P. aeruginosa* strains from untreated water sources in Ismailia, including Serabioum Wastewater, El Temsah Lake, and University Hospital Wastewater, and described their biological and physical qualities for use in biocontrol and restricting antibiotic resistance. The isolated phages demonstrated broad lytic activity and have the potential to be used as biocontrol agents against *P. aeruginosa* infections. The selection of virulent phages with broad lytic spectra can minimize the impact on commensal bacteria and human cells while effectively controlling bacterial infections. The study also revealed that the phage isolates are polyvalent and specific to *P. aeruginosa* strains, highlighting the need for alternative treatments such as bacteriophage therapy due to the resistance of *P. aeruginosa* strains to conventional antibiotics. Overall, the findings emphasize the importance of carefully selecting virulent phages with broad lytic spectra to maximize their potential as biocontrol agents against bacterial infections.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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