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Characterization of Bacteriophage vB_PaeS_TUMS_P6 Infecting *Pseudomonas aeruginosa*

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ABSTRACT

Background: *Pseudomonas aeruginosa* is an important pathogen in healthcare settings that poses significant challenges due to its ability to rapidly develop antibiotic resistance. Its propensity to form biofilms and adapt to host defenses makes it even more difficult to treat, leading to prolonged and debilitating illnesses. So, it is vital to prioritize efforts to develop new strategies for treating infections caused by this pathogen. In the present work, morphological and biological characteristics of vB_PaeS_TUMS_P6 (P6), a lytic phage against *P. aeruginosa*, belonging to the genus Luzseptimavirus were fully described.

Methods: *P. aeruginosa* ATCC 27853 was used for propagation and biological characterization of P6. Its morphology was assessed using transmission electron microscopy (TEM). Adsorption rate assay, one-step growth curve analysis and time-kill experiment were analyzed. Host Range of P6, as well as pH and thermal stability were also determined.

Results: The results showed that it was of classic podovirus morphology and had a short latent period. It could kill bacteria at multiplicity of infection as low as 0.01 and also infect some multidrug-resistant clinical isolates. Stability data suggested that P6 remained stable in various temperatures and pH levels, which is a beneficial characteristic for phage therapy in different situations.

Conclusion: This study presents promising data supporting the future use of P6 as a candidate for phage therapy.

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Introduction

Pseudomonas aeruginosa is a common cause of opportunistic nosocomial infections that can afflict a wide range of patients, especially those with compromised immune systems. Several challenges already exist when it comes to managing *P. aeruginosa* infections including a wide range of virulence factors, biofilm formation, and adaptation to the host immune system, leading to persistent infections and making it a significant public health concern (1). Moreover, overexposure to antibiotics as well as remarkable capacity of *P. aeruginosa* to confer resistance via multiple mechanisms have resulted in increased emergence of multidrug resistant traits that have spread extensively and rendered traditional therapies often ineffective (2). Therefore, *P. aeruginosa* has been designated as a priority 1 or critical pathogen by WHO, for which innovative approaches are required to help curtail this growing global crisis (3).

The urgent need for alternative antibacterial strategies has renewed attention to phage therapy, the bacteriophage-based treatment of bacterial infections, which has shown promising therapeutic potential in many studies (4-6). Phages enjoy some advantages over antibiotics including minimal effect on natural flora, high diversity, ease of isolation, anti-biofilm properties, and ability to destroy drug resistant bacteria. However, it has also been accompanied by some possible drawbacks that, indeed, tend to be relatively insignificant and can be overcome (7). Besides, for this approach to be fully utilized, these natural enemies of bacteria must be carefully chosen and fully characterized to meet existing requirements while ensuring the eradication of the target pathogens (8).

In this study, morphologic, biologic and antibacterial characteristics of vB_PaeS_TUMS_P6 (P6), previously isolated from wastewater and sequenced (Genbank accession number OL519842.1), were investigated.

Materials and Methods

Bacterial Strains and Growth Condition

The bacterial strains used in this study are listed in Table 1. *P. aeruginosa* ATCC 27853 was used for propagation and biological characterization of P6. Clinical isolates were collected from medical labs and PCR-based assay using specific primers was employed for rapid and precise differentiation of *Pseudomonas aeruginosa* from other species (9). Single colonies were grown aerobically in Luria-Bertani (LB) broth except for lactobacilli that Man Rogosa Sharpe (MRS) medium was used. All strains were preserved using 20% glycerol at −80 °C until further use.

Bacteriophage isolation and propagation

Untreated wastewater samples were gathered and then processed by being centrifuged at 10,000×g for 10 minutes to eliminate particles and further sterilized using 0.22 µm low protein binding PES membrane filters. After filtration, the samples were combined with a broth culture of P. aeruginosa (with an OD600 of 0.4) and supplemented with 2 mM $CaCl_2$. The mixture was then kept at 37 °C with gentle shaking overnight. Following this, the mixtures were once again centrifuged at 10,000×g for 10 minutes and filtered. The presence of phages was confirmed by spot testing, and samples that produced a clear zone were cultured to obtain separate plaques. One well-isolated plaque was then subjected to multiple rounds of plaque purification, and the purified sample was subsequently propagated for further utilization.

Antibiotic Susceptibility Profile

To test the susceptibility of all clinical *P. aeruginosa* isolates to antimicrobial agents, the Kirby-Bauer Disk Diffusion Method was used and the results were evaluated by the standards set by the Clinical and Laboratory Standards Institute (CLSI). Each isolate was tested using four categories of antimicrobial agents, used to define multidrug resistance (MDR) in *P. aeruginosa* (10), including antipseudomonal carbapenems (imipenem or meropenem), aminoglycosides (gentamicin or amikacin), antipseudomonal fluoroquinolones (ciprofloxacin), and penicillins along with beta-lactamase inhibitors (piperacillin-tazobactam).

Host Range Determination and EOP Analysis

A total of 40 standard strains and 83 clinical isolates were assessed to determine the host range using spot testing, and sensitive isolates identified in the spot test were selected to determine relative efficiency of plating (EOP). To do this, 100 µL of the host in a mid-exponential phase was added to 4 mL of molten agar (48 °C), poured onto the agar plates, and left to solidify at room temperature. Then, 10 µL of the purified phage suspension (~10^{10} PFU/mL) was transferred onto freshly seeded lawns and allowed to absorb. The plates were then incubated overnight at 37 °C and checked for clearing. The next day, a more detailed analysis was carried out by spotting diluted phage stocks on agar overlays. EOP was then calculated by dividing the average PFU on each bacterium by the average PFU on *P. aeruginosa* ATCC 27853 (15).

Electron Microscopy

The phage lysate was purified using 50 kDa MWCO Amicon Ultra-0.5 Centrifugal Filter Unit and washed twice with 0.1 M neutral ammonium acetate solution. A small amount of the sample was applied onto a carbon-coated Formvar film on 300 mesh copper grid (EMS) followed by negatively staining with 2% uranyl acetate. The grid was allowed to air dry and examined on a Philips EM208S transmission electron microscope at an accelerating voltage of 100 kV (11).

Adsorption Rate Assay

The phage's attachment ability was evaluated as previously described (12). The bacteria were grown until mid-log phase, diluted to an OD600 of 0.1-0.2, and counted using a serial dilution method. A 9 ml suspension of the cells was combined with 1 ml of phage lysate (multiplicity of infection (MOI) =0.0001) and gently agitated whilst incubated at 37 °C. Every minute, 50 µl of the mixture was transferred into a 950 µl LB solution, which was chilled on ice and saturated with chloroform before being vigorously mixed for 10 seconds. Bacteriophages that were not absorbed were enumerated with agar overlay plates. LB medium was employed as negative control and the adsorption rate constant was calculated by the following equation:

$$k = \frac{2.3}{Bt} \log \frac{Po}{P}$$

where k is the adsorption rate constant, in mL/min; B is the initial bacterial concentration in colony forming unit (CFU)/ml; and t is the time span in which the titer decreases from P0 (the initial concentration of the bacteriophage) to P (the final concentration of the bacteriophage). The experiment was performed independently three times.

One-Step Growth Curve Analysis

This experiment was performed according to Kropinski's method (13). Briefly, 100 µL of the phage preparation (~10^{7} plaque forming unit (PFU)/mL) was added to 9.9 mL of a log phase

bacterial culture containing 2 mM $CaCl_2$ and incubated at 37 °C (MOI=0.001). After 5 min, a 100 µL sample was taken and added to 9.9 mL of fresh pre-warmed medium, which was then diluted 10 times twice. At different time intervals, a 100 µL sample was mixed with the host culture and poured onto underlay plates. The plates were incubated overnight and the number of plaques, and then the latent period and average burst size were determined.

Time-kill experiment

P. aeruginosa was grown until it reached an OD600 of 0.5, and subsequently diluted to a concentration of 10^7 CFU/ml. Afterwards, phage stock solution at different MOI values including 0.01, 0.1, 1, 10, and 100 were used to infect host cells. After incubation at 37 °C with gentle shaking, bacterial concentration was measured by quantifying the bacterium titer at different time points. The study was done in triplicate and the LB medium was used as negative control (14).

Thermal and pH stability

To determine pH stability, 100 µL phage suspension (~3.0×10^{10} PFU/mL) was added to 900 µL LB broth medium adjusted to pH values of 3, 5 and 9 with NaOH or HCl and incubated at 25 °C. For thermal stability tests, 100 µL phage suspension was used to inoculate 900 µL LB broth medium and incubated at 40, 50, and 60 °C. Aliquots were collected every 15 min for 90 min and the phage titer was determined using the double-layer agar method. LB medium at pH=7 and constant temperature of 25 °C was used as control. All tests were performed in triplicate.

Statistical analysis

Statistical analysis was carried out using SPSS statistics version 20.0. The presence of significant differences was determined using one-way ANOVA model followed by Tukey's HSD Post hoc test. A P value of ≤ 0.01 was considered significant.

Result

Antibiotic susceptibility and host range determination

Antibiotic susceptibility test of the clinical isolates suggested that 55.5% were resistant to at least one antimicrobial category while the prevalence of MDR was more than 24%, and P6 could lyse about 33.7% of them(of which 20% were MDR) with high EOP but did not display any lytic activity against non-*Pseudomonas* Gram-negative as well as Gram-positive bacteria.

Electron Microscopy

Plaque analysis showed that P6 produced large round plaques with clear centers and turbid edges, ranging in size from 1 to 3 mm in diameter after incubation for 18 h at 37 °C on 0.5% LB agar medium (Figure 1a) that could be a result of decreasing lytic activity caused by the lysis inhibition phenomenon (16). TEM images of the phage revealed a capsid of about 70-nm-diameter and a short tail which is typical for podovirus morphology (Fig. 1b).

Adsorption rate assay

Measuring adsorption kinetics showed that the phages quickly attached to the host bacteria, with more than 93% of them attaching within the first 5 minutes. Furthermore, the value for the adsorption rate constant of P6 to its host was calculated to be 7.4×10^{-10} mL/min at the 10 min interval (Figure 2).

One-step growth curve analysis

One-step growth curve of P6 is shown in Figure 3. According to the obtained data, the latent period for P6 was approximately 36 min and the average burst size was calculated to be 30 PFU/infected cell.

Time-kill experiment

To study the killing activity of P6 against *P. aeruginosa* planktonic cells the Time-kill kinetics assay was performed using colony count which is generally seen as the gold standard for bacterial enumeration. The data on the lysis effect of P6 on *P. aeruginosa* is displayed in Figure 4. The results suggested that the number of viable bacteria was significantly dropped after 2 h of incubation even at MOI: 0.01 ($p \leq 0.001$). In addition, there were no significant differences between various MOIs although bacterial count was lower at higher MOI.

Thermal and pH Stability

The results of stability tests were shown in Figure 5 (a and b). Phage titer showed no obvious change after 90 min incubation at 40 and 50 °C as well as pH values of 5 and 9 but the activity of the phage decreased by either increasing temperature or decreasing pH. This data suggests that P6 remains stable in various temperatures and pH levels, which is a beneficial characteristic for phage therapy in different situations.

Discussion

Currently, *P. aeruginosa* and other nosocomial pathogens are posing a severe public health hazard worldwide due to their resistance to various antimicrobial agents for which novel antibacterials are necessary. For this reason, researchers and medical professionals throughout the world have been working hard to identify promising alternate antimicrobial methods, such as phage therapy that, over the past few years, has made significant advances in combating difficult-to treat infections. However, there is yet limited information on this therapeutic option (17).

In this study, biological characteristics of P6, a lytic phage against *P. aeruginosa*, that was previously isolated from wastewater and sequenced, were investigated. Wastewater is generally known to be a good source of phages against multiple antibiotic-resistant bacterial strains because, due to contamination from fecal and hospital wastes, there is a wide range of microorganisms present in it (18).

Adsorption assay showed that P6 particles efficiently attached to *P. aeruginosa* cells, resulting in a 10-fold reduction in non-adsorbed phage particles only 4 min after infection. Different factors such as cell growth phase, presence of certain cations, temperature, cell size and density, and the nature of phage receptor can affect the binding rate constant and high absorption rates in some conditions where the phage reproduction is as important as host killing, such as biofilm eradication, where bacterial density is high, can be advantageous (19). The growth characteristics of P6 demonstrated relatively short burst time and low burst size. The lysis time and burst size are typically positively correlated, and research has shown that phages that have short lytic cycles and high burst sizes can improve the effectiveness of phage therapy. However, the relationship between these characteristics and the success of phage therapy is not yet fully comprehended (20).

The ability of a phage to suppress the growth of a host strain over time is very important for therapeutic applications; thus, a growth suppression analysis of P6 was completed. The results of this study have shown that bacterial count rapidly decreased to the minimum and bacterial growth inhibition in the presence of the phage remained significant at least for 16 h despite the number of phage-resistant bacteria began to rise. While the emergence of resistant trait seems unavoidable and is natural in the process of bacteria-phage

Table 1. List of bacterial strains used in this study.

Bacterial strain	Source
Acinetobacter baumannii	ATCC BAA-747
Acinetobacter baumannii	laboratory strain
Bacillus cereus	laboratory strain
Bacillus subtilis	laboratory strain
Citrobacter freundii	laboratory strain
Enterobacter aerogenes	laboratory strain
Enterococcus faecalis	laboratory strain
Escherichia coli	laboratory strain
Escherichia coli	ATCC 25922
Escherichia coli	ATCC 8739
Klebsiella pneumoniae	laboratory strain
Klebsiella pneumoniae	laboratory strain
Lactobacillus jensenii	laboratory strain
Lactobacillus plantarum	laboratory strain
Lactobacillus salivarius	laboratory strain
Lactobacillus reuteri	laboratory strain
Methicillin-resistant	laboratory strain
Staphylococcus aureus(MRSA)	
Methicillin-resistant	laboratory strain
Staphylococcus aureus (MRSA)	
Proteus mirabilis	laboratory strain
Pseudomonas aeruginosa	ATCC 27853
Pseudomonas aeruginosa	ATCC 9027
Pseudomonas putida	laboratory strain
Pseudomonas syringae	ATCC 10205
Salmonella enterica	laboratory strain
Salmonella enteritidis	ATCC 13076
Salmonella enteritidis	laboratory strain
Salmonella paratyphi	laboratory strain
Salmonella paratyphi C	laboratory strain
Salmonella typhi	laboratory strain
Salmonella typhimurium	ATCC 14028
Serratia marcescens	laboratory strain
Shigella flexneri	laboratory strain
Staphylococcus aureus	ATCC 23591
Staphylococcus aureus	ATCC 25923
Staphylococcus aureus	ATCC 33591
Staphylococcus aureus	ATCC 6538
Staphylococcus aureus	ATCC 6538p
Staphylococcus epidermidis	laboratory strain
Staphylococcus epidermidis	ATCC 12228
Staphylococcus saprophyticus	laboratory strain
ATCC: American Type Culture Collection	

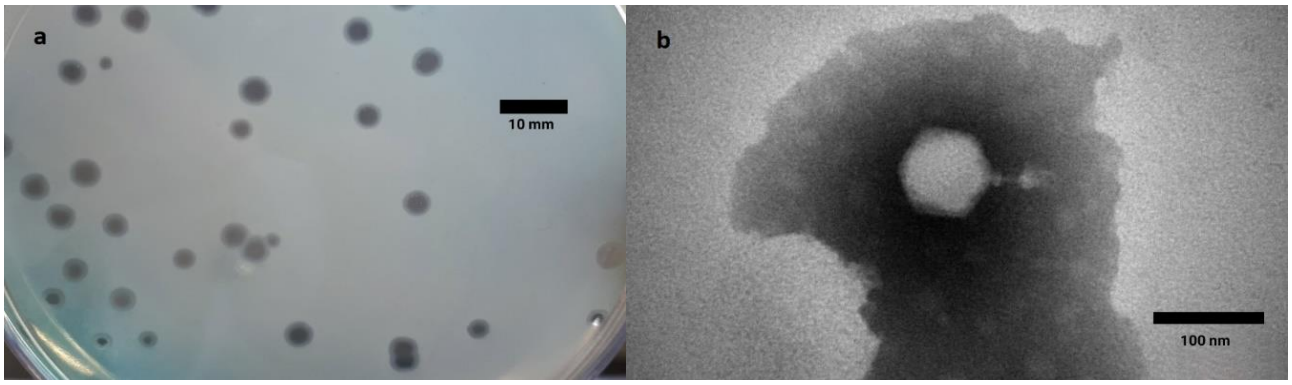

Figure 1. a) Plaque morphology of P6 on the lawn of *Pseudomonas aeruginosa* ATCC 27853; b) Transmission electron micrograph of P6.

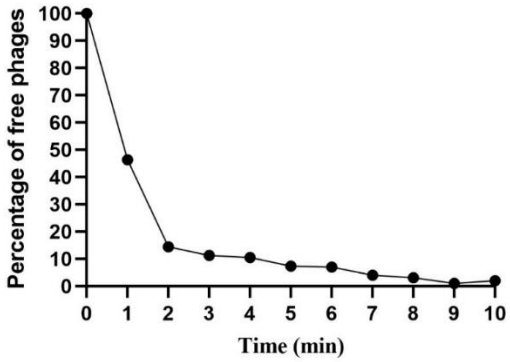

Figure 2. Adsorption kinetics of P6 to *Pseudomonas aeruginosa* ATCC 27853.

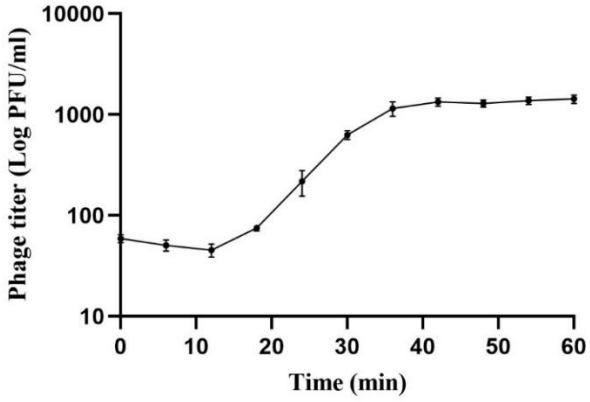

Figure 3. One-step growth curve of P6.

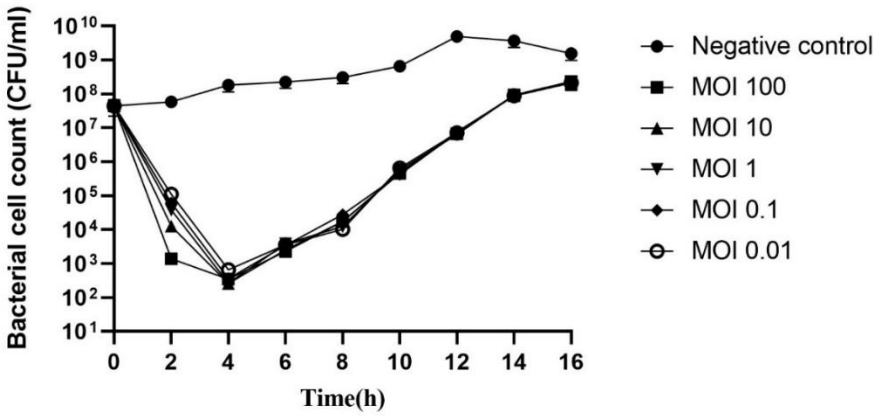

Figure 4. Kinetics of lytic activity of P6 against *Pseudomonas aeruginosa* ATCC 27853 at different MOIs.

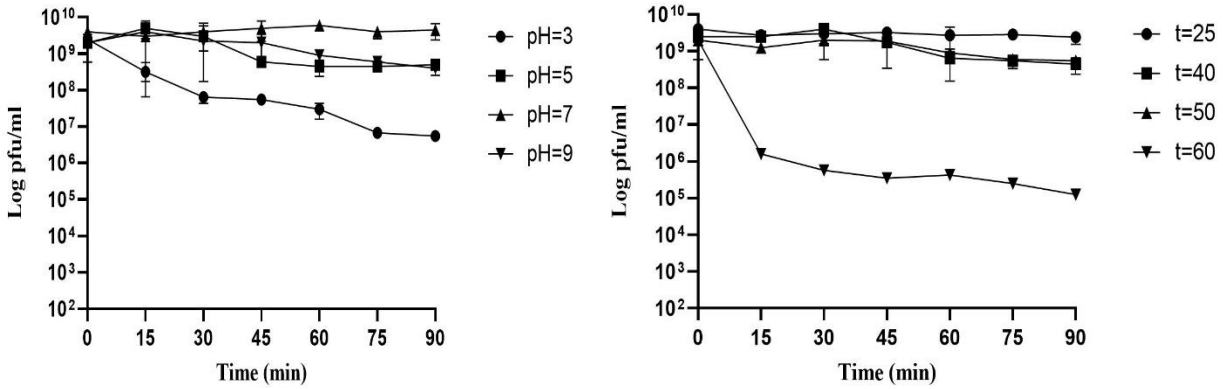

Figure 5. a) pH stability and b) Thermal stability of P6; All assays were performed in triplicate .

coevolution, it has been reported that it does not affect the effectiveness of therapy in vivo, where the immune system may have a significant role (21). Moreover, to reduce the possibility of the development of resistant mutants, use of a combination of different phages or a combination of phages with antibiotics using different resistance mechanisms might be helpful (7).

Conclusion

Pseudomonas aeruginosa is a common cause of infections that occur in medical settings and is known to have a high degree of inherent resistance to most antibiotics. While options for combatting this bacteria are limited, phage therapy has revived as a promising approach. This research examined the characterization of a recently isolated phage, vB_PaeS_TUMS_P6 and results showed that it can be a potential antibacterial agent against *P. aeruginosa*. However, additional efforts to isolate

and evaluate new phage candidates are necessary to expand the host range for resistant isolates.

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Ethics approval and consent to participate

This study was approved by Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.TIPS.REC.1399.034).

Conflict of interest

The authors declare no conflict of interest.

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