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Characterization of a novel bullet-shaped lytic bacteriophage against extensively drug-resistant *Pseudomonas aeruginosa* isolated from human and domestic sources

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Abstract

Global spread and emergence of the extensively drug-resistant (XDR) strains of *P. aeruginosa* have become a concern, thus, searching for new alternative treatment approaches are required. This study was aimed to isolate and characterize a novel lytic phage against *P. aeruginosa*. Seventy XDR isolates of *P. aeruginosa* were collected from May to September 2018. Wastewater samples were used for isolation of lytic phage against XDR *P. aeruginosa* isolates. Host range, thermal and pH stability, adsorption rate, latent period, burst size and morphology of phage were determined following the standard protocols. Morphological characteristics of the phage revealed that it belonged to *Podoviridae* family and it was named vB-PaeP-007. Although the phage had a narrow host range, 47 out of 70 XDR isolates were susceptible to it. The adsorption rate, latent period and burst size of vB-PaeP-007 were approximately 89.80% in 8 min, 10 min and 93 phages per cell, respectively. Its lysis activity remained at a wide range of pH (4 up to 12) and temperature (– 20.00 up to 70.00 ˚C). Regarding the physiological features and host range of the vB-PaeP-007 phage, it could be a promising candidate for phage therapy and bio-controlling of infections from XDR isolates of *P. aeruginosa* in human and livestock storage centers.

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Introduction

Pseudomonas aeruginosa is a rod-shaped gram-negative bacterium that can cause serious opportunistic infections in humans. It is one of the major pathogens involved in nosocomial infections, and is reported as a critical global problem by the International Nosocomial Infection Control Consortium (INICC).[1] Moreover, the emergence of antibiotic resistance among bacterial pathogens has become a major public health concern, especially in health centers and hospitals, as it adversely affects the rate of complications caused by infection, the mortality rate and the and treatment cost.[2,3]

Bacterial isolates are classified into sensitive, multi-drug-resistant (MDR), extensively drug-resistant (XDR) and pan drug-resistant (PDR) based on their resistance to different antibiotics classes.[4] The MDR, XDR and PDR were defined as resistant to at least one agent in three or more antimicrobial categories, resistant to at least one agent in all but two or fewer antimicrobial categories and resistant to all agents in all antimicrobial categories, respectively.[5]

The World Health Organization (WHO) announced in 2017 that prevalence of carbapenem-resistant gram-negative bacteria, including *P. aeruginosa*, has dramatically increased over the last decade, so that the mean of carbapenem-resistant isolates was estimated to be 17.80% in Europe in 2015.[6] According to WHO's 2017 report, three bacterial species require urgent attention to find new antibiotics for treatment purposes, and carbapenem-resistant *Pseudomonas aeruginosa* (CRPsA) is the top priority of this list.[7]

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Overuse, improper and uncontrollable application of antibiotics over the past decades resulted in an increasing trend in the number of multiple resistances to antibiotics in microorganisms, consequently, finding and using alternative treatment methods, such as phage therapy, will be a promising approach for such purpose. Bacteriophages are a group of viruses that only infect bacteria and are host-specific, and do not cause damages to other bacteria except for the host. Theoretically, bacteriophages do not impose undesired and critical effects on the resident microflora of an organism such as human gut microbiome, and they have no severe side effects on the host organism as well. Studies conducted in this area have indicated the success of this method for eliminating antibiotic-resistant bacteria.[8,9] As a results, this study, isolates, identifies, and examines the morphological, physiological and bacteriological characteristics of new lytic bacteriophage against XDR isolates of *P. aeruginosa*.

Materials and Methods

***Pseudomonas aeruginosa* collection and anti-biogram.** From May to September 2018, the conventional biochemical tests were employed to identify the XDR isolates of *P. aeruginosa* obtained from human (100 samples) and domestic animals (22 cows, 29 goats and 21 sheep). Furthermore, the antibiotic resistance pattern of the isolates were determined using Kirby-Bauer disk diffusion method against recommended antibiotics by Clinical Laboratory Standards Institute,[10] including tobramycin (10.00 µg), amikacin (30.00 µg), ciprofloxacin (5.00 µg), levofloxacin (5.00 µg), ceftazidime (30.00 µg), cefepime (30.00 µg), piperacillin (100 µg), imipenem (10.00 µg), meropenem (10.00 µg), azteronam (30.00 µg) and polymyxin B (10.00 µg). Then the XDR isolates were identified according to the Centers for Disease Control and Prevention (CDC) protocol.[5,10]

Bacteriophage isolation, purification and propagation. Specific lytic phage was isolated using standard phage isolation protocol as previously described with some modifications.[11] Wastewater samples were collected from different regions of Arak (Iran) and were centrifuged at 5,000 *g* for 10 min. Then, 15.00 mL of each supernatant was mixed with 15.00 mL of 2X Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) containing the early-exponential phase of *P. aeruginosa* (Pae30) culture. After incubation overnight at 37.00 ˚C, each suspension was centrifuged at 8,000 *g* for 10 min and the supernatant was filtered with 0.45 µm syringe filter. Afterwards, 50.00 µL of an overnight culture of *P. aeruginosa* was mixed to 10.00 mL nutrient agar (0.70%) and poured onto the surface of solid nutrient agar (1.50%) layer. Subsequently, 10.00 µL of the phage filtrate was dropped on the surface of the double layer agar medium using the spotting method. After overnight incubation, the emergence of clear plaque was considered as the existence of lytic phage. Finally, a single plaque was selected by providing a dilution series of phage suspension and it was enriched. The purification step was repeated three times to ensure the purity of the phage.[12] The phage was propagated routinely using *P. aeruginosa* (Pae30) as host according to Sambrook and Russell.[13] Note that, *P. aeruginosa* (Pae30) was used in all further experiments as host.

Transmission electron microscopy. Phage suspension was concentrated using ultracentrifugation as described previously.[11] Then, 10.00 µL of the high titer phage suspension (10^{11} PFU mL^{-1}) was transferred to a carbon-coated 300 mesh copper grid surface and, after 45 sec, the grid was stained with uranyl acetate (2.00% w/v) for 30 sec. The remained color was removed and the grid was air-dried at room temperature. Morphological characteristics of the phage were analyzed using a transmission electron microscope (Philips, Amsterdam, The Netherlands) at an accelerating voltage of 100 kV.[8]

Host range determination. The host range was determined based on standard spot test as previously described.[12] A collection of *P. aeruginosa* (70 clinical XDR isolates and standard specie ATCC 27853), as well as standard and clinical isolates of other gram-positive and gram-negative pathogens (Table 1), was cultured individually on the upper layer agar using double layer method. After 30 min, 10.00 µL of the phage suspension (10^9 PFU mL^{-1}) was dropped onto the surface of the culture. The appearance of clear plaque after overnight incubation was considered positive.

pH and thermal stability. Phage suspension (approximately 10^9 PFU mL^{-1}) was incubated for one hour at different temperatures (– 20.00, 4.00, 25.00, 40.00, 50.00, 60.00, 70.00, 80.00 and 85.00 ˚C) to determine the thermal stability. To investigate the pH stability, a specific titer of phage (approximately 10^9 PFU mL^{-1}) was added to sodium chloride-magnesium sulphate SM buffer (100 mmol L^{-1} NaCl (Merck), 8.00 mmol L^{-1} $MgSO_4$ (Merck), 2.00% gelatin (Merck), 50.00 mmol L^{-1} Tris-HCl (Merck) at different pH values (2 to 12) and incubated for one hour at room temperature. A pH value of 7.00 and temperatures of 25.00 ˚C were considered as the control groups. After each test, the titer of phages was determined by double-layer agar plate method.[11] The rate of variation was expressed as a percentage in comparison with the control. This test was done in triplicate.

Phage adsorption assay. The kinetics of bacteriophage adsorption was determined based on the standard protocol. In short, bacterial/phage suspension was prepared at a MOI (multiplicity of infection) of 0.10 and incubated at 37.00 ˚C for 20 min with sampling at one minute intervals. The titer of free phages in the suspension was determined by double-agar layer method. Finally, the number of adsorbed phages was calculated by comparison

Table 1. Bacterial isolates used to determine phage host range.

Bacterial species	Description	Activity
Pseudomonas aeruginosa	ATCC 27853	L
Pseudomonas aeruginosa	Burn infection	L
***Pseudomonas aeruginosa* XDR (70 isolates)**	XDR isolates	L (47 isolates)
Shigella dysenteriae	PTCC 1188	N
Shigella sonnei	ATCC 9290)	N
Shigella sonnei	Wastewater	N
Shigella flexneri	ATCC 12022	N
Shigella flexneri	Wastewater	N
Escherichia coli	ATCC 25922	N
Escherichia coli	UTI	N
Klebsiella pneumonia	ATCC 13883	N
Streptococcus pneumonia	ATCC 49619	N
Enterococcus faecalis	ATCC 29212	N
Salmonella typhimurium	ATCC 14028	N
Proteus mirabilis	ATCC 43071	N
Staphylococcus saprophyticus	ATCC 15305	N
Staphylococcus epidermidis	ATCC 12228	N
Staphylococcus aureus	ATCC 35933	N
Staphylococcus aureus (MRSA)	ATCC 33591	N
Bacillus cereus	ATCC 11778	N
Bacillus subtilis	ATCC 12711	N
Enterobacter aerogene	ATCC 13048	N
Streptococcus pyogenes	ATCC 19615	N
Proteus mirabilis	ATCC 43071	N

L: lysis, N: no lysis

of the number of free phages at each time to the number of free phages at the initial time. All steps of the test were performed with three replications. This test was done in triplicate.[8,14]

One-step growth curve. One-step growth experiment was performed based on previous studies with a slight change in order to determine latency time and burst size of the phage. In short, the early-exponential phase culture of *P. aeruginosa* (OD600= 0.50) was mixed with the phage suspension (MOI = 0.01) and incubated at 37.00 ˚C for 10 min. The phage/host suspension was centrifuged (8,000 *g* for 5 min) and the pellet was subjected to fresh media and incubated again at 37.00 ˚C. Sampling was done at one minute intervals and the titer of phage was measured using double-layer method. Then, one-step growth curve was plotted and used to determine the latent time and the burst size of the phage.[15]

Statistical analysis. Data were analyzed with SPSS Software (version 19.0; IBM Corp., Armonk, USA) using the LSD test. The *p*-values less than 0.05 were considered statistically significant.

Results

Characteristics of *P. aeruginosa* isolates. The results showed that 70 isolates of *P. aeruginosa* had XDR phenotype according to the CDC. The antibiogram test revealed that all of the isolates were susceptible to polymyxin B (Fig. 1).

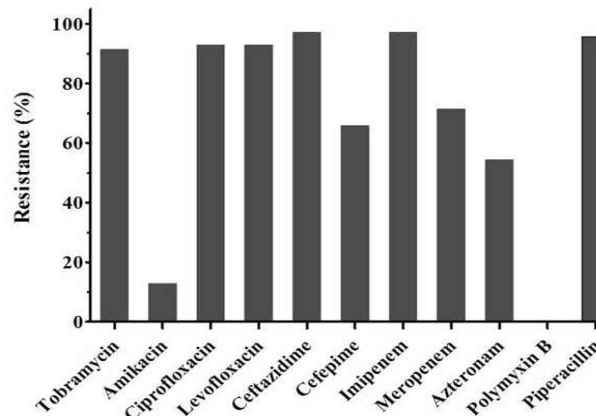

Fig. 1. Patterns of antimicrobial resistance of *P. aeruginosa* isolates.

Bacteriophage isolation and morphology. The formation of clear plaque on a culture of *P. aeruginosa* (Pae30) was the indicator of a lytic bacteriophage against the host bacterium. The size of transparent plaque formed in this research was about 2.00 mm. Transmission electron microscopy micrograph showed that this phage had a stretched appearance and an almost bullet-like shaped (155 ± 5.00 nm in length, 49.00 ± 3.00 nm in width at the thickest point) which was attached directly to a very short tail (17.00 ± 1.00 nm), (Fig. 2). Based on the morphological feature, this phage was classified in the *Podoviridae* family, and following the recommended nomenclature procedure by Kropinski *et al.* the name vB-PaeP-007 was assigned to the isolated phage.[16]

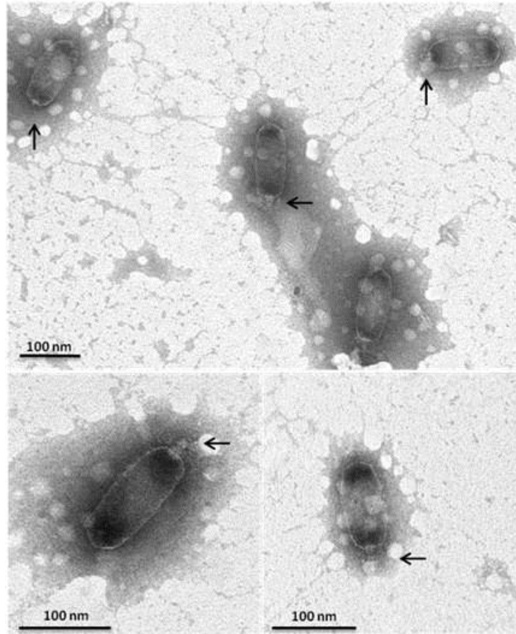

Fig. 2. Electron micrographs of vB-PaeP-007 with bullet-like shaped head.

Host range determination. Out of 24 groups of bacteria examined in the host range determination of the phage, only *P. aeruginosa* 27853 and some of the clinical isolates of *P. aeruginosa* were sensitive to vB-PaeP-007 (Table 1). These results showed that the host range of vB-PaeP-007 probably was limited to only *P. aeruginosa*. Out of 70 isolates of XDR *P. aeruginosa*, 47 isolates were lysed by vB-PaeP-007, and 23 isolates were not (Fig. 3).

pH and thermal stability. The vB-PaeP-007 tolerated the temperature range of – 20.00 to 50.00 ˚C well, while about 57.00% of the phages remained active at 60.00 ˚C. At 70.00 and 80.00 ˚C, the number of phages was reduced significantly, and finally it was plunged to zero at 85.00 ˚C. (Fig. 4A).

The result of the analysis of variance (LSD test) of different pH effects on the number of bacteriophages revealed a significant relationship between these pHs and reduction in the number of bacteriophages ($p < 0.05$). The number of phages was plunged to zero at pHs of 2.00, 3.00 and 13.00. There were no significant changes in the phage titer at pHs of 6.00, 8.00, and 9.00 compared to pH of 7.00. However, the number of phages was significantly reduced at pHs of 4.00, 5.00, 12.00 and 13.00. The lowest pH value of the phage, in which vB-PaeP-007 was able to be active was pH = 4.00 (2.10%) and pH = 12.00 (4.60%). Thus, with 95.00% confidence, it could be stated that the number of phages was depended on the medium pH (Fig. 4B).

Phage adsorption assay and one-step growth curve. As shown in (Fig. 4C), absorption of vB-PaeP-007 to the host cell surface was started immediately after 2 min and continued up to 8 min. In other words, the maximum level of phage attachment to the host, which was about 89.80%, occurred at 8 min after incubation (Fig. 4C). Analysis of the one-step growth curve revealed that vB-PaeP-007 had a latent period of 10 min, a rise phase of 20 min and a relatively large burst size of approximately 93 phages per cell (Fig. 4D).

Discussion

The *P. aeruginosa* is an opportunistic bacterium and causes serious nosocomial infections. Irregular and over-use of antibiotics has led to the emergence of antibiotic-resistant species.[2,17] A very high resistance rate of *P. aeruginosa* to most of the antimicrobial agents has been reported worldwide.[17,18] Among these MDR strains, the XDR and PDR ones are a serious threat to the public health, and require particular attention toward finding proper treatment procedures. Finding alternative treatment methods such as using lytic bacteriophages is a viable approach. The successful experiments on the isolation of the lytic bacteriophage against ESBL producing bacteria, MDR, and XDR strains of other important pathogens, confirm the promising role of bacteriophages as a replacement or a complement to antibiotic-therapy.[8,19,20]

In the case of *P. aeruginosa*, several lytic bacteriophages have been isolated from *Podoviridae*, *Myoviridae*, *Siphoviridae* families so far. To the best of our knowledge, the literature review demonstrated that the isolated bacteriophage in our study, the vB-PaeP-007 phage, was the first lytic bullet-shaped bacteriophage of the *Podoviridae* family against XDR *P. aeruginosa*. [21-23] The host range test revealed that vB-PaeP-007 was limited to *P. aeruginosa* only. In this regard, it was similar to φPA3 phage which only infected *P. aeruginosa*.[24] On the other hand; AZ1 phage reported by Jamal *et al.* was able to lyse *Achromobacter xylosoxidans* and *E.coli* in addition to *P. aeruginosa*.[25]

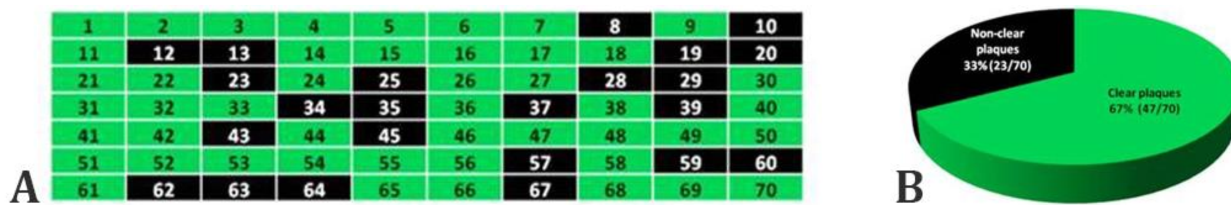

Fig. 3. Activity of vB-PaeP-007 against XDR *P. aeruginosa* isolates; **A**) lysed and non-lysed XDR *P. aeruginosa* isolates by vB-PaeP-007, **B**) Frequency of lysed and non-lysed XDR *P. aeruginosa* isolates by vB-PaeP-007.

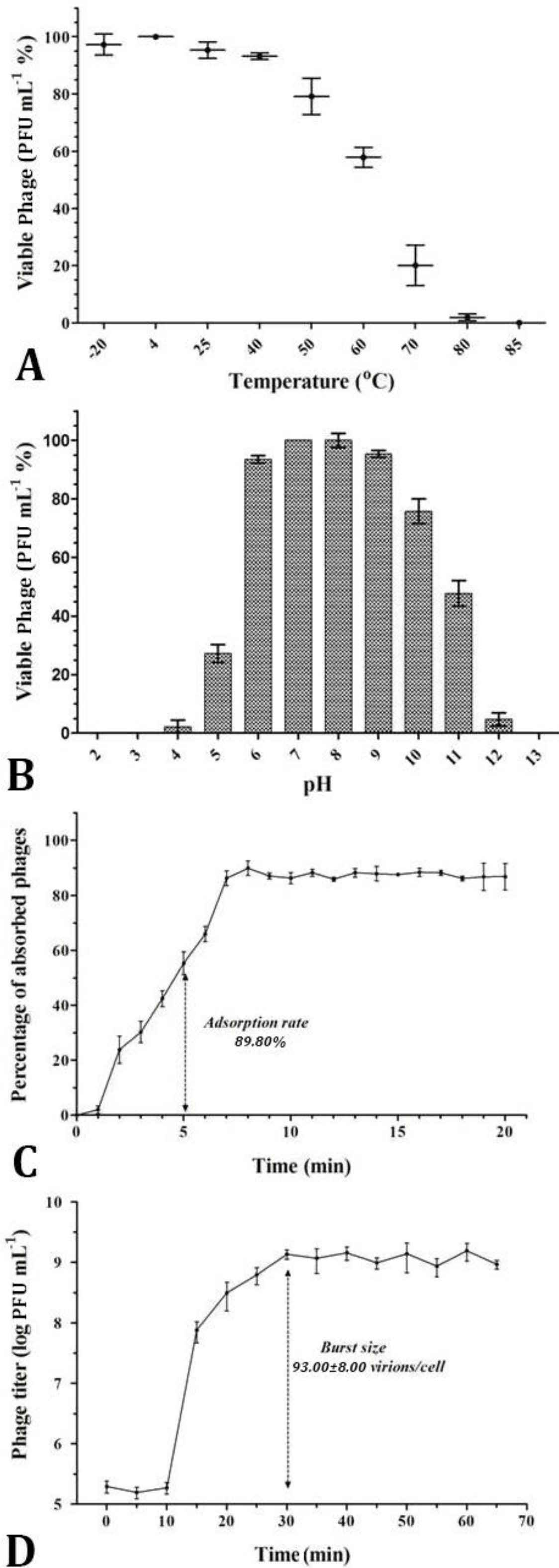

Fig. 4. The effect of different temperatures ranging from – 20.00 to 85.00 (A), different pH values (B) adsorption rate (C) and one step growth curve (D) of vB-PaeP-007. Values are expressed as the mean ± standard deviation taken from triplicate assays.

Although the narrow host range was considered as one of the main advantages of the phage (minimize the effect on natural normal flora), it should be mentioned that the ability of the phage to destroy different isolates of the host species was another advantage. In this regard, out of 70 isolates of *P. aeruginosa* XDR, vB-PaeP-007 was able to kill 47 isolates (67.00% of isolates). 23 isolates (less than 33.00% of the isolates) were not lysed. However, already reported phages LS1, LS2 and LS4 were able to lyse 41.20%, 54.20%, and 54.20% of the MDR isolates of *P. aeruginosa*, respectively.[26]

The pH stability range of vB-PaeP-007 was measured in a broad range from pH 4.00 to 12.00. The proper pH for maximum activity was from 6 and 10. Danis-Wlodarczyk *et al*. reported KTN6 and KT28 bacteriophages for *P. aeruginosa* with pH stability ranging from 3.00 to 12.00. Considering the fact that the reported pH of infectious wounds is mostly between 7.00 to 8.00, vB-PaeP-007 could be used in this range while maintaining its highest viability level.[27,28]

Moreover, vB-PaeP-007 remained active after an hour exposure to a range temperature from – 20.00 to 50.00 ˚C. At higher temperatures it decreased significantly the viable count, yet 57.80, 20.00 and 1.90% of the phages were still active at temperatures of 60.00, 70.00 and 80.00 ˚C, respectively. The thermal stability of AZ1 phage was reported from 37.00 to 65.00 ˚C. The other specific phages KTN6 and KT28 were tolerating temperatures from 40.00 to 70.00 ˚C, but they lost 90.00% of their activities after 15 min at 80.00 ˚C.[25,27] Therefore, vB-PaeP-007 phage could be easily used for bio-controlling of *P. aeruginosa* infections and maintaining very high viable counts at the body temperature.

Another important feature of a potential phage for phage therapy was having a short adsorption time, which represented a high attraction ratio of a phage to the host cells.[29] Binding of about 90.00% of the vB-PaeP-007 phage to the host cells within 8 min suggested that this phage could infect a high number of its bacterial hosts in a short time. The same short adsorption rate was similar to the previous report on φPA3 in which 95.00% of φPA3 phage particles were attached to *P. aeruginosa* surface in 5 min.[24] In contrast, only about Another important feature of a potential phage for phage therapy was having a short adsorption time, which represented a high attraction ratio of a phage to the host cells.[29] Binding of about 90.00% of the vB-PaeP-007 phage to the host cells within 8 min suggested that this phage could infect a high number of its bacterial hosts in a short time. The same short adsorption rate was similar to the previous report on φPA3 in which 95.00% of φPA3 phage particles were attached to *P. aeruginosa* surface in 5 min.[24] In contrast, only about 40.00% of the AZ1 phage particles were attached to *P. aeruginosa* after 30 min.[25] In the case of the latent period and the burst size various values were reported for

P. aeruginosa phages. For instance, the latent period and burst size of phage AZ1 and JG024 were approximately 33 min/326 phages per cell and 50 min/180 phages per cell, respectively.[30] The latent period of vB-PaeP-007 (10 min) was much shorter than that of the AZ1 and JG024, however, its burst size (approximately 100 phages per cell) was relatively lower.

In overall, due to the higher use of antibiotic and high-resistance-potential of the *Pseudomonas aeruginosa* as an opportunistic pathogen in humans and domestic animals to various antibiotics, it could be concluded that the phage therapy would be beneficial as a possible alternative method for localized infections in humans and domestic animals. On the other hand, the physiological results showed that the vB-PaeP-007 phage would be useful for the prevention of infections in stalls, fish breeding pools, hospitals, schools and homes for the aged and bio-controlling of infections as a new important strategy of therapeutic purposes.

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Conflict of interest

The authors declare that there is no conflict of interest.

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