Iranian Journal of Microbiology
Volume 14 Number 2 (April 2022) 203-213

ORIGINAL ARTICLE

Isolation and characterization of lytic bacteriophages against *Pseudomonas aeruginosa* isolates from human infections in the north-west of Iran

Raheleh Majdani*, Elham Shams Ghahfarokhi

Department of Biology, Faculty of Basic Sciences, University of Maragheh, Maragheh, Iran

Received: July 2021**, Accepted:** February 2022

ABSTRACT

Background and Objectives: With the emergence of *Pseudomonas aeruginosa* antibiotic-resistant strains, using the antibacterial potential of bacteriophages could be an effective approach to combat bacterial infections.

Materials and Methods: In this study, after evaluation of antibiotic sensitivity of 20 clinical bacterial isolates of *Pseudomonas aeruginosa*, isolation of lytic phages was performed against 15 isolates using double-layer agar overlay technique. Molecular analysis of isolated phages was carried out using *EcoR*V and *Hind*III endonucleases. Then, the host range of the phages was evaluated and the phage with the broadest host range (PPaMa1/18) was selected to morphological characteristics by TEM. Also, its one-step growth curve was determined and the stability of the phage to environmental parameters (temperature and pH) was evaluated.

Results: All isolates of *Pseudomonas aeruginosa* were resistant to five antibiotics. In total, 15 phages were successfully isolated from the sewage sources against each of 15 used bacterial isolates. Molecular analysis of the phages showed a high rate of genomic variation. In the morphological analysis of the selected phage (PPaMa1/18) using TEM, an icosahedral head with a size of 90 nm × 75 nm and a long contractile tail (215 nm) were observed which indicated its similarity to the *Myoviridae* family. The latent period of the PPaMa1/18 was about 20 minutes and its burst size was estimated to be 58 PFU/cell. Also, the PPaMa1/18 phage antibacterial activity was not significantly affected at pH 4-10 and temperature 4-40C°.

Conclusion: Findings demonstrated that isolated bacteriophage (PPaMa1/18) with wide host range, strong lytic effect and high stability can be used as a promising candidate for the treatment of antibiotic-resistant infections caused by *Pseudomonas aeruginosa.*

Keywords: Phage; Antibiotic resistance; *Pseudomonas aeruginosa*; Clinical isolates; Bacterial infections

INTRODUCTION

Pseudomonas aeruginosa is one of the major causes of community-acquired infections, considered in 9-10% of nosocomial opportunistic infections (1, 2). *P. aeruginosa* is found in various environments including water and soil and also it can infect humans, animals, and plants (3, 4). This bacterium is one of the most important causes of malignant external otitis, endocarditis, meningitis, pneumonia, septicemia, bacteremia, urinary tract infections and surgical site infection, especially in people with immunodeficiency (4, 5).

Various strains of *P. aeruginosa* have biofilm formation capability. Under special circumstances, this bacterium can form tightly-packed structures called

*Corresponding author: Raheleh Majdani, Ph.D, Department of Biology, Faculty of Basic Sciences, University of Maragheh, Maragheh, Iran. Telefax: +98-4137276060 Email: rahelehmajdani@yahoo.com

biofilms and prevent the entrance of antimicrobial agents into the bacterial cell (6). They have a wide range of virulence factors and a set of resistance mechanisms to antibiotics with highly adaptive features in different environments (7). *P. aeruginosa* can attack a host cell through its wide range of virulence factors and protect itself from the host's defense (4).

Moreover, the innate and adaptive resistance mechanisms of *P. aeruginosa* provide high resistance to several antibiotics. These mechanisms include: reducing the permeability of cell coatings, efflux pumps, obtaining resistance genes through plasmids and transposons, and changing the expression and function of chromosomally encoded mechanisms through mutation (8, 9). Infections caused by *P. aeruginosa* are often severe and fatal due to low sensitivity to different types of antibiotics and the emergence of antibiotic resistance during therapeutic stages (10). The excessive use of antibiotics and also increasing of antibiotic-resistant strains has made critical challenges to treat infections caused by this bacterium (9).

Nowadays, phage therapy or the use of phages to deal with multi-drug resistant (MDR) bacteria is a new therapeutic strategy (11). Phages are viruses that contain a genome (DNA or RNA) surrounded by a protein coating (capsid) and can infect bacteria (12-14). These bacterial viruses are found remarkably in a variety of environments (15).

The major advantages of phages, in comparison with antibiotics, can be their high specificity to bacterial targets, cost-effectiveness, easy isolation, abundance in the environment, ability to remove biofilms, proliferation at the site of infection, minimal effect on natural flora and non-serious side effects in eukaryotic cells (12, 14, 16, 17). Various studies have shown the successful use and antimicrobial function of *P. aeruginosa* phages to cope with antibiotic-resistant infections in *in vitro* and *in vivo* studies (18-20).

In this study, 15 lytic phages were isolated separately against antibiotic-resistant isolates of *P. aeruginosa* from Maragheh sewage. After determining the range of phages hosting, one of the phages was chosen with the widest spectrum of hosts and named "selected Phage". Then the morphology, burst size and other physiological characteristics of this phage were investigated.

MATERIALS AND METHODS

Identification of bacterial isolates of *Pseudomonas aeruginosa*. Clinical samples were taken from hospitals of Tabriz, East-Azarbaijan, Iran. The collected samples were cultured on selective media (MacConkey agar, Blood agar, and EMB) and after incubation, bacterial cultures were evaluated for morphological and biochemical characteristics. Biochemical tests were performed including catalase, oxidase, IMVIC, SIM, TSI, and carbohydrate metabolism.

Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates. Antibiotic sensitivity of 20 isolates of *P. aeruginosa* from patients was evaluated to 12 different antibiotics. Antibiotic susceptibility patterns of isolates were determined by Kirby-Bauer disk diffusion method on the Muller-Hinton agar medium (Hi-Media, India) (21).

Different antibiotic discs (Padtan Teb, Iran) including penicillin (10 µg), amoxicillin (25 µg), gentamicin (10 µg), nalidixic acid (30 µg), tetracycline (30 µg), imipenem (10 µg), amikacin (30 µg), kanamycin (30 µg), tobramycin (10 µg), cefepime (30 µg), cefixime (5 µg), rifampicin (5 µg) were used in this study. After incubation time, the diameter of the non-growth holes was measured by the ruler and the isolates were classified into sensitive, semi-sensitive and resistant groups, according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (22).

Isolation and purification of phages. To isolate the phages against MDR *P. aeruginosa* isolates, samples were collected from the sewage of Maragheh city, East-Azarbaijan, Iran. The collected sewage samples were centrifuged for 5 minutes at 2500 rpm and the supernatant solution was then filtered through 0.45 µm syringe driven filters (Jet Biofil, Canada). Then, 5 ml of cultured *P. aeruginosa* (OD = 0.4 / 0.5) and 5 ml of 2× LB broth (Luria Bertani Broth) were added to 45 ml of the filtered solution and incubated for 24 hours at 37°C at 180 rpm (23). After incubation, 3 ml of chloroform was added to the suspension and centrifuged at 3500 rpm for 30 min at 4°C (The chloroform addition process was repeated to ensure purification of the phage) and finally, the supernatant was filtered through 0.45 µm syringe driven filters. Chloroform does not affect the performance of phages and results in the bacterial killing in a phage solution (23) with

some minor modifications.

Evaluation of forming plaques with bacteriophages was carried out using Adams double layer agar method with minor modifications (23). 200 µl of the bacterial culture of *P. aeruginosa* and 100 µl of purified phage solution were mixed and the solution was incubated for 5 minutes to absorb the phage into the bacteria and then the phage-bacterial solution was added to a sterile tube containing 5 ml soft agar (0.7% agar). The mixture was transferred to the base agar surface and incubated for 24 hours at 37°C. The phages of formed plaques on the plate surface were collected and stored in SM buffer (NaCl 5.8 g/L MgSO4·7H2O 2 g/L, 1M Tris HCl (pH7.5) 50 ml/L and 2% gelatin 5 ml/L) (24).

Extraction and digestion of the genomic DNA of phages. The genomic DNA of each isolated phage was extracted according to the kit (Phage DNA Isolation, Norgen, Canada) manufacturer's instructions. *Hind*III and *EcoR*V restriction enzymes were used to digest the extracted DNA of the phages based on the manufacturer's protocols (Thermo Fisher Scientific, USA). In summary, the DNA of the phages was digested for 5 hours at 37°C and electrophoresed on 0.8% agarose gel. After staining the gel with Safe Red (iNtRON Biotechnology, South Korea), the bands were visible when exposed to UV radiation.

Determining the host range of phages. Spot test was used to determine the host range of each of the isolated phages. The host range of each of the phages was determined by adding 5 µl of the desired phage (10^{12} PFU/ml) to the surface of the cultivated plates with each of antibiotic-resistant *P. aeruginosa* isolates. The plates were incubated for 12 hours at 37°C and the formation of plaques on the surfaces of the plates was evaluated (24). Phage with the broadest host range was selected for more detailed assays for determining its characteristics.

Transmission Electron Microscopy (TEM) of selected phage. The purified suspension of selected phage was added to the surface of copper grades and negative staining with 2% uranyl acetate was performed. The extra color was adsorbed by paper filters and the collected phage samples were dried on copper grades. The morphology of the phage was observed by the Zeiss LEO 906 TEM at the voltage of 100 kV (Zeiss, Germany). The image was taken in Drug Applied Research Center, Tabriz University of Medical Sciences , Tabriz, Iran.

Determination of one-step growth of the selected phage. A one-step growth test was performed to determine the latent period and burst size of the selected phage using the method of Pajunen et al. (25). Selected phage at MOI = 0.01 was added to the culture of *P. aeruginosa*.

In order to absorb the phage to bacteria, the mixture was incubated for 15 minutes at 37°C. After incubation, the mixture was centrifuged for one hour at 4°C and the supernatant was discarded. Pellets containing bacterial contaminant phages were transferred to the fresh LB medium and incubated at a temperature of 37°C at 180 rpm. Then, the mixture was sampled at ten-minute intervals and the phage titer was evaluated using the double-layer agar method. Burst size and latent period were estimated from triplicate experiments ± SEM.

Temperature and pH stability of the selected phage. The selected phage stability to different temperatures and pHs was assessed according to the method described by Bao et al. and Verma et al. with minor modifications. The tube containing 1 ml of the selected phage solution was incubated at 4, 10, 20, 30, 40, 50, 60, 70°C for one hour. After incubation at selected temperatures, the selected phage's survival rate was evaluated using "soft agar overly" method (26). To determine the stability of selected phage to different pHs, nutrient broth culture media (Hi-Media, India) with pH 2, 4, 6, 8, 10, and 12 prepared using 1M HCl and 1M NaOH solutions and then, 1 ml of the selected phage solution was added to 9 ml of nutrient broth medium with adjusted pH and incubated for one hour at 37°C. After the completion of incubation, the selected phage's survival rate was evaluated at each pH using the "soft agar overly" method (27). These experiments were performed three times, and the results are reported as the mean of three observation ± SEM.

RESULTS

Isolation and identification of bacterial isolates of *Pseudomonas aeruginosa.* In this study, based on standard biochemical tests, 20 collected bacterial isolates from hospital samples were confirmed as *P. aeruginosa.*

Antibiotic susceptibility of *Pseudomonas aeruginosa* isolates. Antibiotic susceptibility of 20 *P. aeruginosa* isolates obtained from clinical specimens was determined according to the CLSI 2017 guidelines, the results are shown in the Table 1. All isolates of *P. aeruginosa* were resistant to penicillin (10 µg), amoxicillin (25 µg), nalidixic acid (30 µg), kanamycin (30 µg) and cefixime (5 µg) antibiotics. And the resistance rate of *P. aeruginosa* isolates to cefepime (30 µg), imipenem (10 µg), rifampicin (5 µg), tetracycline (30 µg), tobramycin (10 µg), gentamicin (10 µg) and amikacin (30 µg) were 95%, 90%, 55%, 35%, 30%, and 15% respectively. Among these bacteria, 15 of the most resistant isolates to antibiotics were used for the isolation of lytic phages against. The susceptibility pattern of these isolates to various antibiotics was shown in Table 2.

Table 1. Antibiotic susceptibility of *P. aeruginosa* clinical isolates from patients

Antibiotics	N=20		
	R%	I%	S%
Penicillin (P)	100	0	0
Amoxicillin (AMX)	100	0	0
Gentamicin (GM)	30	0	70
Nalidixic acid (NA)	100	0	0
Tetracycline (TE)	40	0	60
Imipenem (IMP)	90	10	0
Amikacin (AK)	15	5	80
Kanamycin (K)	100	0	0
Tobramycin (TOB)	35	10	55
Cefepime (FEP)	95	5	0
Cefixime (CFM)	100	0	0
Rifampicin (RA)	55	0	45

R: Resistant, I: Intermediate, S: Sensitive

Isolation and purification of phages. In this study, one phage was isolated against each of the 15 MDR *P. aeruginosa* isolates from the sewage of Maragheh city, East-Azarbaijan, Iran. Each of these phages formed clear plaques on the surface of plates inoculated with their host bacterial isolate, which two of the plates as shown in Fig. 1 (A and B).

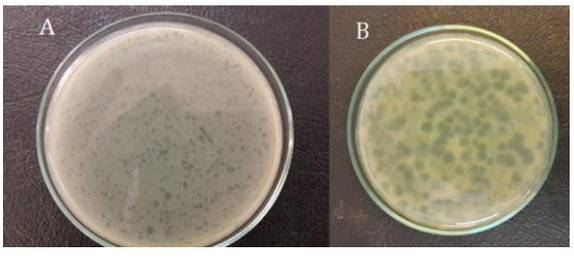

Fig. 1. (A and B). Plaque formation of lytic phages on double layer agar plates. Plaque assay of lytic phages on a lawn of MDR-*P. aeruginosa* isolates. A: Plaque assay of phage 6 on the lawn of MDR-*P. aeruginosa* isolate 6. B: Plaque assay of phage 7 (PPaMa1/18) on the lawn of MDR-*P. aeruginosa* isolate7.

Table 2. Sensitivity pattern of antibiotic-resistant *P. aeroginosa* isolates

P. aeruginosa isolates	Resistance
Isolate 1	P, NA, IMP, FEP, CFM, RA, AMX, K
Isolate 2	IMP, AMX, NA, P, TE, K, FEP, GM, CFM, TOB
Isolate 3	P, CFM, GM, NA, AMX, TE, IMP, AK, K, TOB, FEP, RA
Isolate 4	AMX, TE, IMP, NA, K, FEP, CFM, P
Isolate 5	NA, P, IMP, AMX, K, FEP, CFM, RA
Isolate 6	K, P, AMX, CFM, NA, IMP, FEP, RA
Isolate 7	P, AMX, GM, NA, TE, IMP, AK, K, TOB, FEP, CFM, RA
Isolate 8	NA, TE, IMP, P, K, FEP, CFM, RA, AMX
Isolate 9	P, NA, AMX, IMP, K, FEP, CFM
Isolate 10	P, RA, IMP, FEP, K, CFM, AMX, NA
Isolate 11	FEP, AMX, K, P, NA
Isolate 12	AK, NA, P, CFM, K, IMP, FEP, RA
Isolate 13	P, IMP, NA, AK, CFM, RA, K
Isolate 14	P, FEP, AMX, K, NA, CFM, RA, IMP
Isolate 15	IMP, AMX, NA, RA, K, P, FEP, CFM

P: Penicillin, AMX: Amoxicillin, GM: Gentamicin, NA: Nalidixic acid, TE: Tetracycline, IMP: Imipenem, AK: Amikacin, K: Kanamycin, TOB: Tobramycin, FEP: Cefepime, CFM: Cefixime, RA: Rifampicin

Extraction and restriction endonuclease digestion of phage DNA. Extraction of genomes of the 15 isolated phages was observed by electrophoresis on agarose gel (Fig. 2). The results showed that the genome of all phages was double-stranded DNA, but differences in the size of extracted DNAs were observed. Then, for genome analysis, the pure DNA of each phage was digested by two endonuclease enzymes *Hind*III and *Eco*RV. As shown in Fig. 3 (A and B) different restriction patterns obtained by using both of the enzymes, indicated a wide variation in the phage genomes sequences.

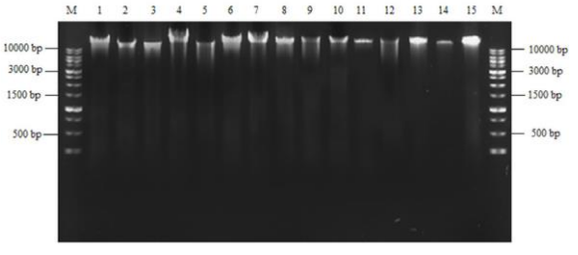

Fig. 2. Genome of phages: 0.8% agarose gel. M shows 1 kb DNA Ladder (Cinagene, Iran) and Lane (1-15), show bands of phage DNA. Lane 7 shows band of phage PPaMa1/18 DNA.

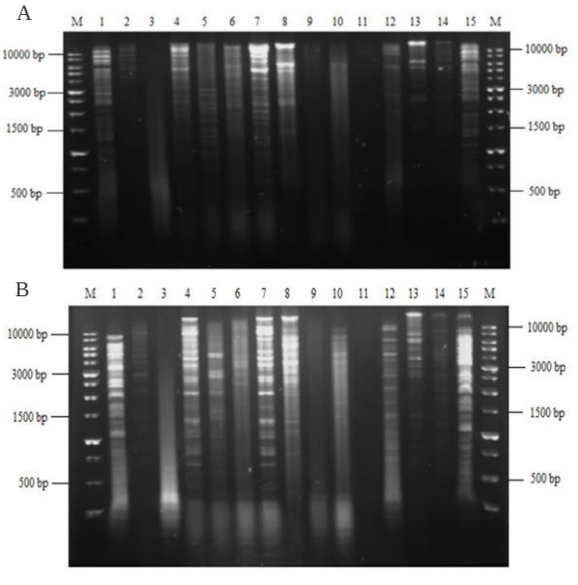

Fig. 3. A. Enzyme digestion analysis of *P. aeruginosa* phages DNA with *Hind*III. M:1 kb DNA Ladder (Cinagene, Iran), Lanes (1-15) contained: digested genome of *P. aeruginosa* phages with *Hind*III. Lane7: digested genome of phage PPaMa1/18 with *Hind*III. B. Enzyme digestion analysis of *P. aeruginosa* phages DNA with *Eco*RV. M:1 kb DNA Ladder (Cinagene, Iran), Lanes (1-15) contained: digested genome of *P. aeruginosa* phages with *Eco*RV. Lane7: digested genome of phage PPaMa1/18 with *Eco*RV.

Determination of the host range of isolated phages. The host range of each phage against the *P. aeruginosa* isolates was determined by using spot test. The results of the evaluation of the host range of 15 phages were shown in Table 3. It indicated that Phage 7 (phage against isolate 7) has had the broadest spectrum of the host with an effect on 85.7% of *P. aeruginosa* isolates that was known as selected phage and was named PPaMa1/18.

phage PPaMa1/18 as a candidate for phage therapy, were used in subsequent analyses such as Transmission Electron Microscopy (TEM), evaluation of pH and temperature stability and, determination of one-step growth curve.

Transmission electron microscopy (TEM) of phage PPaMa1/18. The image obtained from the TEM showed that phage PPaMa1/18 had a large, icosahedral head (dimensions were 90 nm × 75 nm) and a long contractile tail of 215 nm in length (Fig.4A and B). According to the International Committee on Taxonomy of Viruses (ICTV), this phage belonged to the *Myoviridae* family of the *Caudovirales* order.

One-step growth curve of the phage PPaMa1/18. The One-step growth curve in this study consisted latent phase, log phase, and stationary phase. Based on this curve, the latent period was estimated about 20 minutes and the burst size was calculated to be approximately 58 PFU/cell (Fig. 5).

Stability of phage PPaMa1/18 to pH and temperature. In this study, survival rate of the phage PPaMa1/18 was evaluated at different temperatures. The results of thermal stability showed that the phage PPaMa1/18 was stable at the temperature range from 4°C to 40°C. In addition, at temperatures above 40°C, the phage's titer dropped sharply so that at 70°C the phage completely deactivated (Fig. 6).

The phage PPaMa1/18 stability to different pH values also investigated. The phage PPaMa1/18 had an appropriate lytic activity in pH ranges from 4 to 10. However, after an hour of incubation of the phage at pH 12, there was a significant reduction in the lytic activity of the phage. Another noteworthy point is that in pH 2, the phage was completely inactivated (Fig. 7).

Table 3. The lytic spectrum of isolated phages on 15 *P. aeruginosa* isolates collected from hospitals of Tabriz, East-Azarbaijan, Iran

P.aeruginosa isolates	Phages														
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
Isolate 1	+	+	+	-	-	+	+	+	-	+	+	-	+	+	+
Isolate 2	-	+	-	+	-	-	+	+	-	+	+	+	-	-	+
Isolate 3	-	-	+	+	+	+	-	+	+	+	-	+	-	+	+
Isolate 4	+	-	+	+	+	-	+	+	+	-	-	+	+	+	-
Isolate 5	+	-	+	-	+	-	+	+	-	+	+	-	+	+	+
Isolate 6	-	+	-	-	-	+	+	-	+	+	-	-	-	-	+
Isolate 7	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+
Isolate 8	+	+	+	+	+	-	+	+	-	+	+	+	+	+	-
Isolate 9	-	+	+	-	-	+	+	+	+	-	+	+	-	-	+
Isolate 10	+	+	-	+	+	+	-	-	+	+	-	-	+	+	+
Isolate 11	+	-	-	+	-	+	+	+	+	+	+	+	-	+	-
Isolate 12	-	-	+	-	+	-	+	+	-	+	-	+	-	-	-
Isolate 13	+	-	+	-	+	+	+	+	+	-	+	+	+	+	+
Isolate 14	+	+	+	+	-	+	+	-	-	-	-	-	-	+	-
Isolate 15	+	-	+	+	+	+	-	+	+	-	+	+	+	+	+

+: lysis; -: no lysis
P: Phage
P7: phage PPaMa1/18

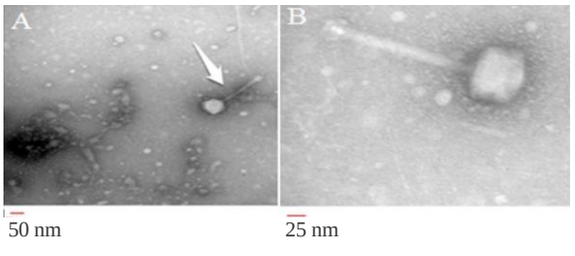

Fig. 4. Transmission electron micrographs of phage PPaMa1/18 negatively stained with uranyl acetate. The Scale bars represent (A) 50 nm and (B) 25 nm.

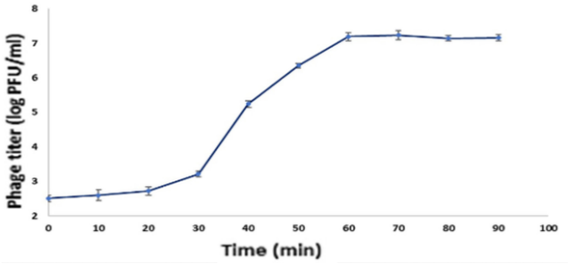

Fig. 5. One-step growth curve of phage PPaMa1/18 on *P. aeruginosa*. Data represent mean ± SEM from three triplicate experiments

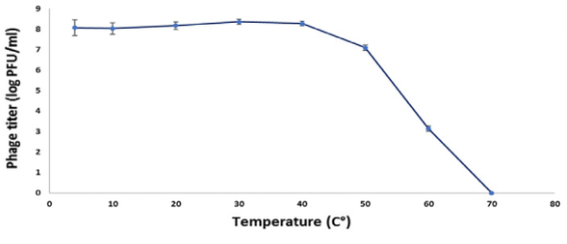

Fig. 6. Thermal stability of phage PPaMa1/18 treated with different temperature for 1 h. Data represent mean ± SEM from three triplicate experiment.

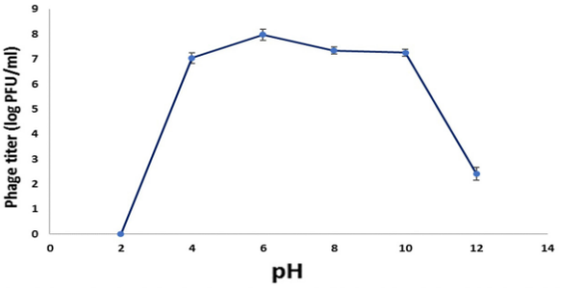

Fig. 7. pH stability of phage PPaMa1/18 treated with different pH for 1 h. Data represent mean ± SEM from three triplicate experiment.

DISCUSSION

Many of the infections caused by *Pseudomonas aeruginosa* strains are highly resistant to antibiotic treatments. One of the reasons refers to the innate resistance of the bacteria but another important cause of this resistance is related to some other factors such as inappropriate use of various antibiotics (28). In recent years, with the rapid expansion of antibiotic-resistant bacteria and the decline of the pharmaceutical industries desire to invest in the production of new antibiotics, especially against Gram-negative bacteria due to increased research costs, finding effective and inexpensive therapy approaches for controlling bacterial infections is taken into consideration (29). In this regard, the isolation and characterization of lytic bacteriophages against clinical antibiotic-resistant isolates of *Pseudomonas aeruginosa* in different geographical area could be very helpful to combat these infections that threaten human healthcare recently. Then, we isolated and characterized lytic bacteriophage with antibacterial potential against *P. aeruginosa* isolates originated from north west of Iran as a candidate for phage therapy.

In our study, all of the isolates were resistant to at least five used antibiotics, also the rate of resistance to five antibiotics including cefixime, penicillin, amoxicillin, nalidixic acid, kanamycin was 100%. Antibiotic resistance to amoxicillin, kanamycin, tobramycin, gentamicin and, tetracycline was significantly higher than those reported by Pallavali et al. (23). Comparing to some studies in Iran, bacterial isolates in our study showed more resistance to imipenem, lower resistance to amikacin and gentamicin and similar resistance to cefepime (30). In another study, isolates of *P. aeruginosa* revealed 50%, 63.9%, 16.7%, 8.3%, and 16.7% resistance to imipenem, cefepime, amikacin, tobramycin, gentamicin, respectively which is lower than the current study (31). Brzozowski et al. reported lower rate of resistance to imipenem and cefepime and higher rate of resistance to tobramycin, amikacin, and gentamicin (32). Recently, phage therapy was studied as one of the renewed biologic approaches to combat multi-drug resistant bacteria in therapeutic fields. Wastewater is known to be a rich source of various microorganisms like bacteriophages (33) that can be exploited to control pathogenic bacterial infections. Lytic phages are preferable to phage therapeutics due to their rapid proliferation in host bacteria than lysogenic phages (34). *P. aeruginosa* lytic phages have been used in several studies to treat infections caused by antibiotic-resistant strains of *Pseudomonas aeruginosa* effectively. The lytic phage PAXYB1 isolated from wastewater was used to infect *P. aeruginosa* strain PAO1 and other clinical isolates of MDR *P. aeruginosa* from different sources. The PAXYB1 was able to efficiently infect and kill clinical isolates of *P. aeruginosa* (35). The lytic phage AZ1 was isolated against the MDR clinical strain of *P. aeruginosa*-2995 and the activity of this phage was determined against *P. aeruginosa*-2995 in both planktonic cells and the biofilm. The phage showed desirable activity in the destruction of MDR *P. aeruginosa*-2995 of both planktonic cells and the biofilm. However, combination of phages was needed to complete elimination (36). In another work, bacteriophages isolated from sewage and evaluated against MDR-bacterial isolates (*P. aeruginosa, S. aureus, K. pneumoniae* and *E. coli*) from patient with septic wound infections. These phages demonstrated perfect lytic activity against the MDR bacteria causing septic wounds. Therefore, the authors suggested the phages as therapeutic options for treating septic wounds (23). In addition, *P. aeruginosa* phage isolated from sewage of Ilam had tremendous effects against a variety of clinical and general laboratory strains (ATCC15693) of *P. aeruginosa* (37). In our study, according to molecular analysis, highly genomic variation was observed using endonuclease enzymes among genomes of 15 isolated bacteriophages and just three and two genomes were not digested with *Hind*III and *Eco*RV endonuleases, respectively. In other studies, the digestion of isolated genomes of bacteriophage against *Pseudomonas aeruginosa* was reported by using some endonucleases (*Eco*RI and *Dra*I) and also the insensitivity of the genomes to other enzymes (*Pst*I and *Bam*HI) was shown (38). Molecular analysis of the genomes of isolated *P. aeruginosa* phages in Kwiatek showed digestion of the genomes with *Eco*RI, *Hin*dIII, and *Sma*I enzymes but no digestion was observed by using *Bam*HI (39). Genomic variety could be a good sign of possibility of isolating different effective bacteriophages against bacterial infections which could be helpful to introduce more effective packages against infections of *P. aeruginosa*. Rate of host range is one of the important factors to introduce appropriate candidates for phage therapy. Phage PPaMa1/18 showed the highest host range against 87% of

used isolates that was in accordance to the study of Garb et al. which showed high rate of lytic effects of the phage JG02 (84%) on P. *aeruginosa* clinical isolates (40). In another study, broad spectrum of isolated bacteriophages against *P. aeruginosa* isolates was confirmed (41). Also, the determination of host range analysis in a study showed that 51.4% of the *P. aeruginosa* bacterial strains investigated were susceptible to phage BrSP1 (42). Large burst size and short latency period are two significant features for bacteriophages in phage therapy and phage propagation has a closely relationship with the burst size (43). Then, large burst size of a bacteriophage is a clear advantage to select a phage as an antibacterial agent. Based on one-step growth characteristics, the latent period of the phage PPaMa1/18 was short, that is desirable for phage therapy but its burst size was approximately low. In spite of lower rate of burst size of PPaMa1/18, strong lytic efficiency and also broad spectrum of its lytic effect on clinical isolates can select it as an appropriate candidate to phage therapy. Selecting a potentiated bacteriophage for phage therapy needs to be regarded to different factors not just one characteristic. There are reports of significant changes in the latent period and the burst size of *Pseudomonas aeruginosa* phages. In some researches, *Pseudomonas aeruginosa* phages with longer latent periods and larger burst sizes have been introduced such as phage PAXYB1 (latent period of about 30 minutes and burst size approximately 141 PFU/cell) and phage PPA-ABTNL (latent period of about 35 minutes and burst size approximately 110 PFU/cell) (35, 24). In other reports, some phages with shorter latent period and larger burst sizes have been introduced such as phage ø/PSZ1 (latent period of about 12 minutes and burst size approximately 100 PFU/cell) and phage ø/PSZ2 (latent period of about 10 minutes and burst size approximately 100 PFU/cell) (44). Differences in the latent period and the burst size of phages can also be due to differences in the medium, environmental factors (pH and temperature), and host cell (45). In TEM morphological analysis, PPaMa1/18 was belonged to *Myoviridea* family of the *Caudoviridals* order (head size of 90 nm × 75 nm and a long contractile tail of 215 nm in length), which was similar to several other *Pseudomonas aeruginosa* phages such as MPK1, JG024 with the head diameter of 80 nm × 75 nm and tail length of 130 nm, SL1 (head diameter: 70 nm and length tail: 130 nm), SL2 (head diameter: 120 nm and length tail: 170 nm), C11 (head diameter: 65 nm and length tail: 122 nm), vB_paeM-10 (head diameter: 90 nm × 91 nm and length tail: 175 nm) in previous studies (46-48, 40, 49). The phages as candidates for phage therapy should be more stable in different environmental conditions (50). PPaMa1/18 showed high survivability in different temperatures and different pH values. High rate of lytic effects between temperature 4-40°C and pH range of 4 to 10 was determined. The phage was completely deactivated at 70°C and pH 2. Inactivation of phage at pH 2 may be due to denaturation of proteins in acidic conditions (51). Denaturation of virus particles and other irreversible damages are shown due to high temperatures. Also, aggregation process can cause the decrease of phage concentration in acidic solutions (52). Different stability of phages in different temperature and pH conditions have been shown in several studies. Didamony et al. showed that *Pseudomonas aeruginosa* phages ø/PSZ1 and ø/PSZ2 were stable in a temperature range of 30-50°C and were inactivated after incubation for an hour at 60°C. This indicates that the phage PPaMa1/18 is more stable than phages ø/PSZ1 and ø/PSZ2 at higher temperatures (44). In another report, the phage AZ1 was stable at temperature range of 37-65°C and its activity decreased with increasing temperature and it was completely deactivated at 80°C, indicating that AZ1 phage is more stable at higher temperatures comparing to our results (36). Two specific phages against *Pseudomonas aeruginosa* P2S2 and *P. aeruginosa* P5U5 exhibited significant stability within the pH range of 4 to 11 (53).

CONCLUSION

According to our results, using lytic phages can be potentiated for combatting infections due to *Pseudomonas aeruginosa* bacteria. The high rate of genomic variation of isolated phages refers to the abundance of lytic phages even in the same area. Because of the wide host range and effective lytic activity and likewise high stability of the phage PPaMa1/18 in various environmental conditions (temperature and pH), it can be used as a promising candidate for the treatment of antibiotic-resistant infections caused by *Pseudomonas aeruginosa*.

ACKNOWLEDGEMENTS

We are deeply grateful to the Microbiology Laboratory of the University of Maragheh for our access to their lab equipment and for their precious assistance in conducting this research work. Also, the authors declare that they have no conflicts of interest.

REFERENCES

1. Driscoll JA, Brody SL, Kollef MH. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs* 2007; 67: 351-368.
2. Hancock RE, Speert DP. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. *Drug Resist Updat* 2000; 3: 247-255.
3. Battle SE, Rello J, Hauser AR. Genomic islands of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 2009; 290: 70-78.
4. Pires DP, Boas DV, Sillankorva S, Azeredo J. Phage therapy: a step forward in the treatment of *Pseudomonas aeruginosa* infections. *J Virol* 2015; 89: 7449-7456.
5. Snyder LA, Loman NJ, Faraj LA, Levi K, Weinstock G, Boswell TC, et al. Epidemiological investigation of *Pseudomonas aeruginosa* isolates from a six-year-long hospital outbreak using high-throughput whole genome sequencing. *Euro Surveill* 2013; 18: 20611.
6. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 2002; 15: 167-193.
7. Essoh C, Blouin Y, Loukou G, Cablanmian A, Lathro S, Kutter E, et al. The susceptibility of *Pseudomonas aeruginosa* strains from cystic fibrosis patients to bacteriophages. *PLoS One* 2013; 8(4): e60575.
8. Lister PD, Wolter DJ, Hanson ND. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev* 2009; 22: 582-610.
9. Lambert PA. Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J R Soc Med* 2002; 95 Suppl 41(Suppl 41): 22-26.
10. Aloush V, Navon-Venezia S, Seigman-Igra Y, Cabili S, Carmeli Y. Multidrug-resistant *Pseudomonas aeruginosa:* risk factors and clinical impact. *Antimicrob Agents Chemother* 2006; 50: 43-48.
11. Hall AR, De Vos D, Friman VP, Pirnay JP, Buckling A. Effects of sequential and simultaneous applications of bacteriophages on populations of *Pseudomonas aeruginosa in vitro* and in wax moth larvae. *Appl Environ Microbiol* 2012; 78: 5646-5652.
12. Clark JR, March JB. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends Biotechnol* 2006; 24: 212-218.
13. Ackermann HW. 5500 Phages examined in the electron microscope. *Arch Virol* 2007; 152: 227-243.
14. Haq IU, Chaudhry WN, Akhtar MN, Andleeb S, Qadri I. Bacteriophages and their implications on future biotechnology: a review. *Virol J* 2012; 9: 9.
15. Henry M, Debarbieux L. Tools from viruses: bacteriophage successes and beyond. *Virology* 2012; 434: 151-161.
16. Endersen L, O'Mahony J, Hill C, Ross RP, McAuliffe O, Coffey A. Phage therapy in the food industry. *Annu Rev Food Sci Technol* 2014; 5: 327-349.
17. Pires D, Sillankorva S, Faustino A, Azeredo J. Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC 10145 biofilms. *Res Microbiol* 2011; 162: 798-806.
18. Fu W, Forster T, Mayer O, Curtin JJ, Lehman SM, Donlan RM. Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an *in vitro* model system. *Antimicrob Agents Chemother* 2010; 54: 397-404.
19. Wright A, Hawkins CH, Änggård EE, Harper DR. A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa;* a preliminary report of efficacy. *Clin Otolaryngol* 2009; 34: 349-357.
20. Watanabe R, Matsumoto T, Sano G, Ishii Y, Tateda K, Sumiyama Y, et al. Efficacy of bacteriophage therapy against gut-derived sepsis caused by *Pseudomonas aeruginosa* in mice. *Antimicrob Agents Chemother* 2007; 51: 446-452.
21. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1996; 45: 493-496.
22. Wayne PA. Performance Standards for Antimicrobial Susceptibility Testing. Clinical and Laboratory Standards Institute. CLSI M100-S27. 2017.
23. Pallavali RR, Degati VL, Lomada D, Reddy MC, Durbaka VRP. Isolation and *in vitro* evaluation of bacteriophages against MDR-bacterial isolates from septic wound infections. *PLoS One* 2017; 12(7): e0179245.
24. Cao Z, Zhang J, Niu YD, Cui N, Ma Y, Cao F, et al. Isolation and characterization of a "phiKMV-like" bacteriophage and its therapeutic effect on mink hemorrhagic pneumonia. *PLoS One* 2015; 10(1): e0116571.
25. Pajunen M, Kiljunen S, Skurnik M. Bacteriophage phiYeO3-12, specific for *Yersinia enterocolitica* serotype O: 3, is related to coliphages T3 and T7. *J Bacteriol* 2000; 182: 5114-5120.
26. Bao H, Zhang H, Wang R. Isolation and characterization of bacteriophages of *Salmonella enterica* serovar Pullorum. *Poult Sci* 2011; 90: 2370-2377.

27. Verma V, Harjai K, Chhibber S. Characterization of a T7-like lytic bacteriophage of *Klebsiella pneumoniae* B5055: a potential therapeutic agent. *Curr Microbiol* 2009; 59: 274-281.
28. Breidenstein EB, de la Fuente-Núñez C, Hancock RE. *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* 2011; 19: 419-426.
29. Vieira A, Silva YJ, Cunha A, Gomes NC, Ackermann HW, Almeida A. Phage therapy to control multidrug-resistant *Pseudomonas aeruginosa* skin infections: *in vitro* and *ex vivo* experiments. *Eur J Clin Microbiol Infect Dis* 2012; 31: 3241-3249.
30. kouchaksaraei FM, Shahandashti EF, Molana Z, kouchaksaraei MM, Asgharpour F, Mojtahedi A, et al. Molecular detection of Integron genes and pattern of antibiotic resistance in *Pseudomonas aeruginosa* strains isolated from intensive care unit, Shahid Beheshti Hospital, North of Iran. *Int J Mol Cell Med* 2012; 1: 209-217.
31. Hosu MC, Vasaikar S, Okuthe GE, Apalata T. Molecular detection of antibiotic-resistant genes in *Pseudomonas aeruginosa* from nonclinical environment: public health implications in Mthatha, Eastern Cape province, South Africa. *Int J Microbiol* 2021; 2021: 8861074.
32. Brzozowski M, Krukowska Ż, Galant K, Jursa-Kulesza J, Kosik-Bogacka D. Genotypic characterisation and antimicrobial resistance of *Pseudomonas aeruginosa* strains isolated from patients of different hospitals and medical centres in Poland. *BMC Infect Dis* 2020; 20: 693.
33. Chan BK, Abedon ST, Loc-Carrillo C. Phage cocktails and the future of phage therapy. *Future Microbiol* 2013; 8: 769-783.
34. Goodridge LW (2008). Phages, bacteria and food. In: *bacteriophage ecology: population growth, evolution and impact of bacterial viruses*. Ed, ST Abedon. Cambridge University Press, 1st ed, Cambridge, UK, pp. 302-305.
35. Yu X, Xu Y, Gu Y, Zhu Y, Liu X. Characterization and genomic study of "phiKMV-Like" phage PAXYB1 infecting *Pseudomonas aeruginosa*. *Sci Rep* 2017; 7: 13068.
36. Jamal M, Andleeb S, Jalil F, Imran M, Nawaz MA, Hussain T, et al. Isolation and characterization of a bacteriophage and its utilization against multi-drug resistant *Pseudomonas aeruginosa*-2995. *Life Sci* 2017; 190: 21-28.
37. Azizian R, Nasser A, Askari H, Kalani MT, Sadeghifard N, Pakzad I, et al. Sewage as a rich source of phage study against *Pseudomonas aeruginosa* PAO. *Biologicals* 2015; 43: 238-241.
38. Akhwale JK, Rohde M, Rohde C, Bunk B, Spröer C, Boga HI, et al. Isolation, characterization and analysis of bacteriophages from the haloalkaline lake Elmenteita, Kenya. *PLoS One* 2019; 14(4): e0215734.
39. Kwiatek M, Mizak L, Parasion S, Gryko R, Olender A, Niemcewicz M. Characterization of five newly isolated bacteriophages active against *Pseudomonas aeruginosa* clinical strains. *Folia Microbiol (Praha)* 2015; 60: 7-14.
40. Garbe J, Wesche A, Bunk B, Kazmierczak M, Selezska K, Rohde C, et al. Characterization of JG024, a *Pseudomonas aeruginosa* PB1-like broad host range phage under simulated infection conditions. *BMC Microbiol* 2010; 10: 301.
41. Essoh C, Latino L, Midoux C, Blouin Y, Loukou G, Nguetta SP, et al. Investigation of a large collection of *Pseudomonas aeruginosa* bacteriophages collected from a single environmental source in Abidjan, Côte d'Ivoire. *PLoS One* 2015 ;10(6): e0130548.
42. de Melo ACC, da Mata Gomes A, Melo FL, Ardisson-Araújo DMP, de Vargas AP, Ely VL, et al. Characterization of a bacteriophage with broad host range against strains of *Pseudomonas aeruginosa* isolated from domestic animals. *BMC Microbiol* 2019; 19: 134.
43. Amarillas L, Rubí-Rangel L, Chaidez C, González-Robles A, Lightbourn-Rojas L, León-Félix J. Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrug-resistant *Escherichia coli*. *Front Microbiol* 2017; 8: 1355.
44. El Didamony G, Askora A, Shehata AA. Isolation and characterization of T7-like lytic bacteriophages infecting multidrug resistant *Pseudomonas aeruginosa* isolated from Egypt. *Curr Microbiol* 2015; 70: 786-791.
45. Müller-Merbach M, Kohler K, Hinrichs J. Environmental factors for phage-induced fermentation problems: replication and adsorption of the *Lactococcus lactis* phage P008 as influenced by temperature and pH. *Food Microbiol* 2007; 24: 695-702.
46. Jurczak-Kurek A, Gąsior T, Nejman-Faleńczyk B, Bloch S, Dydecka A, Topka G, et al. Biodiversity of bacteriophages: morphological and biological properties of a large group of phages isolated from urban sewage. *Sci Rep* 2016; 6: 34338.
47. Cui X, You J, Sun L, Yang X, Zhang T, Huang K, et al. Characterization of *Pseudomonas aeruginosa* phage C11 and identification of host genes required for virion maturation. *Sci Rep* 2016; 6: 39130.
48. Latz S, Krüttgen A, Häfner H, Buhl EM, Ritter K, Horz HP. Differential effect of newly isolated phages belonging to PB1-Like, phiKZ-Like and LUZ24-Like Viruses against Multi-Drug Resistant *Pseudomonas aeruginosa* under varying growth conditions. *Viruses* 2017; 9: 315.
49. Heo YJ, Lee YR, Jung HH, Lee J, Ko G, Cho YH. Antibacterial efficacy of phages against *Pseudomonas aeruginosa* infections in mice and Drosophila melanogaster. *Antimicrob Agents Chemother* 2009; 53: 2469-

2474.
50. Ahmadi M, Karimi Torshizi MA, Rahimi S, Dennehy JJ. Prophylactic bacteriophage administration more effective than post-infection administration in reducing *Salmonella enterica* serovar Enteritidis shedding in Quail. *Front Microbiol* 2016; 7: 1253.
51. Hazem A. Effects of temperatures, pH-values, ultra-violet light, ethanol and chloroform on the growth of isolated thermophilic *Bacillus* phages. *New Microbiol* 2002; 25: 469-476.
52. Tan CW, Rukayadi Y, Hasan H, Abdul-Mutalib NA, Jambari NN, Hara H, et al. Isolation and characterization of six Vibrio parahaemolyticus lytic bacteriophages from seafood samples. *Front Microbiol* 2021; 12: 616548.
53. Santos TM, Ledbetter EC, Caixeta LS, Bicalho ML, Bicalho RC. Isolation and characterization of two bacteriophages with strong *in vitro* antimicrobial activity against *Pseudomonas aeruginosa* isolated from dogs with ocular infections. *Am J Vet Res* 2011; 72: 1079-1086.