

Isolating anti *Pseudomonas aeruginosa* phages from wastewater and sewage samples: characterization of vB_PaeM_kmuV and vB_PaeM_kmuB

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Abstract

In this study, 20 samples were searched for phages specific to *Pseudomonas aeruginosa* and 11 phages were isolated. Two phages were more effective on clinical antibiotic-resistant isolates. Therefore, further investigations were performed to characterize them. The electron micrographs showed that both phages belong to Myoviridae family with large heads and long tails. SDS-PAGE of the structural proteins also showed that both are large phages. Their genomes were sensitive to digestion by restriction endonucleases (*HindIII*, *EcoRI* and *AluI*). Both phages could tolerate a pH range of 3 to 9.5 and temperatures from 4 to 50°C.

Phage vB_PaeM_kmuB was adsorbed after 2 minutes and vB_PaeM_kmuV after 8 minutes of exposure to the host. Both phages could prevent the growth of the antibiotic resistant biofilm producing *P. aeruginosa*

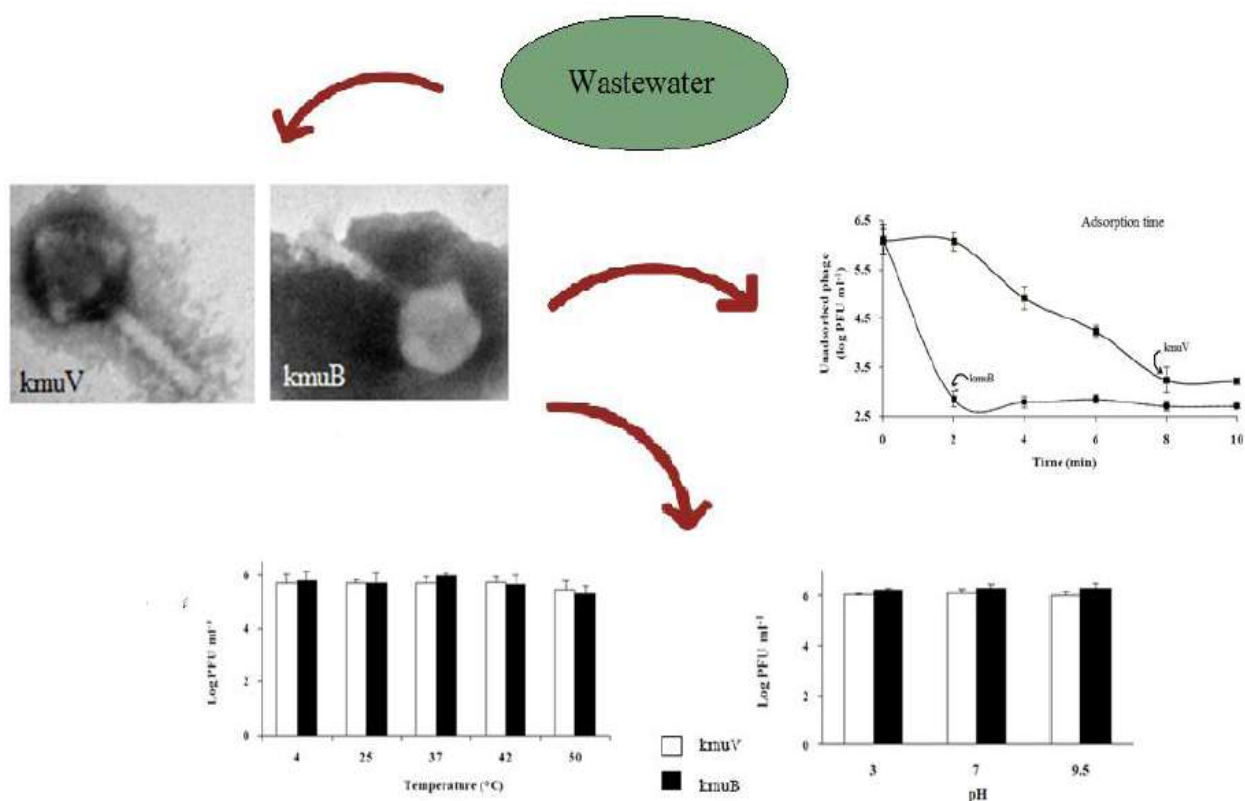
strain in liquid culture even at low multiplicity of infections. However, kmuB showed higher efficacy in this test. Phages kmuV and kmuB may be suitable candidates for phage therapy.

Keywords: *Pseudomonas*, Lytic phages, Phage therapy, Antimicrobials, Myoviridae.

Introduction

Pseudomonas aeruginosa, a gram-negative bacterium, abundant in different natural habitats, is one of the most clinically important bacterial strains. Its colonization in human tissues especially in hospitalized, immunodeficient and burn patients causes serious health problems and even death¹⁷. It is also a dominant pathogen in cystic fibrosis that causes patients' death¹⁸. Treatment of *P. aeruginosa* infections is a challenge, since it forms biofilm and shows resistance to antibiotics and disinfectants^{4,13,16,23}.

Graphical abstract



Because of the above reasons, researchers are searching for new therapeutics; novel antibiotics, vaccines and bacteriophages^{1,3,7,8,10,19}. Phage therapy, an old method that attracted attentions recently, uses lytic bacteriophages (phages) in a fight against bacteria. Phages are the natural weapons to control bacterial populations. A lytic phage attaches and penetrates to its host, hijacks the host cell metabolic systems to multiply and bursts the host to get out of it⁵. Several studies applied phages for *P. aeruginosa* infections in animals and human cases^{1,12,17,24}.

In comparison to antibiotics, phages are advantageous in some ways. Phages are host-specific, so do not affect body normal flora and mammalian cells; are self-restricting; proliferate in the presence of the host and disappear after eliminating the host cells and are suitable candidates for removal of biofilms (matrixes that are impenetrable to antibiotics)^{20,29}. Additionally, isolating and purifying phages is economical than producing new antibiotics²².

Phage therapy has offered new hopes for treatment of antibiotic-resistant infections. Therefore, in this study, we report isolation of 11 lytic phages against *P. aeruginosa* and partial characterization of two of these phages.

Material and Methods

Bacterial strains: Three *P. aeruginosa* strains were purchased from microbial collections. Two other strains were clinical antibiotic-resistant isolates from hospitalized patients. One of these isolates (*P. aeruginosa* DS1) could also form strong biofilm³⁰. Table 1 presents the list of used bacterial strains.

Table 1
List and sources of bacterial strains

Bacterial strain	Source
<i>P. aeruginosa</i> ATCC 9027	American Type Culture Collection, Manassas, VA
<i>P. aeruginosa</i> ATCC 1074	American Type Culture Collection
<i>P. aeruginosa</i> PTCC 1430	Persian Type Culture Collection, Tehran, Iran
<i>P. aeruginosa</i> DS1	Clinical isolate, Afzalipour Hospital, Kerman, Iran
<i>P. aeruginosa</i> PI11	Clinical isolate, Imam Khomeini Hospital, Tehran, Iran

Phage isolation: Twenty lake, pond and raw urban and hospital wastewater samples were collected from some parts of Iran (12 samples from Tehran, four from Kerman, two from Gilan, one from Hormozgan and one from North Khorasan provinces). Purification and enrichment of the samples were done based on the protocol presented by Salem and colleagues²⁷ with some modifications. Briefly, the collected samples were mixed by vortexing and left for 20 min, then centrifuged at 10000 g for 5 min to isolate particulate material. A portion of 10 ml of the cleared

supernatants was added separately to 100 ml LB broth culture of *P. aeruginosa* (OD600 of 0.3).

The suspensions were incubated at 37°C overnight (2 h without shaking and then shaking at 50 rpm). Chloroform (1/10) was added to and mixed with the suspensions to lyse bacteria and the lysate settled for 1 h at 4°C. The upper part was collected in a new tube and centrifuged at 10000 g for 5 min to separate bacteria and bacterial debris. The residual chloroform was evaporated by leaving open-capped tubes in a laminar-flow cabinet for 30 min.

Detection of the presence of phage particles at enriched samples was done by spot assay method described previously and a single plaque for each sample was purified three times²⁹. *P. aeruginosa* ATCC 9027 was used as the host strain in this step, but *P. aeruginosa* DS1 was used after determining the phage host range (because of the stronger effect of the phages on it).

Determination of phage host range: 5 µl spot of isolated phages were tested on plates seeded with bacterial strains (OD600 of 0.3) presented in table 1. The clear zones were checked after overnight incubation. For positive samples (with clear zones of bacterial lysis), spot assay was repeated using serially diluted samples to overrule the lysis from without⁶. In this step, two phages that were more effective were selected for further study.

Transmission Electron Microscopy (TEM): High-titre (10^9 PFU ml⁻¹) lysates were sedimented for 90 min at 20000 g and washed twice under the same conditions in neutral ammonium acetate. Phage particles were then deposited on carbon-coated Formvar films on copper grids stained with 2% uranyl acetate and examined in a Zeiss EM900 transmission electron microscope.

Structural proteins: Sedimented phage particles (10^9 PFU ml⁻¹) were suspended in SDS-PAGE loading buffer (2% SDS, 10% glycerol, 2.5% β-mercaptoethanol, 0.0025% bromophenol blue and 6.25 mM Tris/HCl, pH 6.8). The suspension was boiled for 10 min and loaded onto 12% polyacrylamide gel. After electrophoresis, the gel was stained with coomassie brilliant blue R250 (Merck) and destained with destaining solution (30% methanol and 10% acetic acid in distilled water).

Phage DNA isolation and digestion: Phage DNA was extracted by the method described by Salem et al²⁷. Briefly, DNase I and RNase A were added to remove bacterial sources of contamination. Then, EDTA and Proteinase K and SDS were added. Finally, phenol: chloroform extraction and ethanol precipitation were done and the purified DNA was dissolved in distilled water. The extracted genome was then digested with restriction endonucleases (HindIII, EcoRI, AluI and SmaI; Fermentas) according to the supplier's recommendation and run on 0.7% agarose gel.

Adsorption time: Phage particles were added to bacterial suspension at mid-log phase at a multiplicity of infection (MOI) of 10^{-1} . Sampling was done at desired intervals and the samples were centrifuged to sediment the phage particles adsorbed to bacterial cells. The supernatants were diluted. Each dilution was mixed with the host cells and plated on LBA (Luria-Bertani agar). The next day, the plaques, which showed the number of unadsorbed phages, were enumerated.

Effect of temperature on phages: Phage particles (10^7 PFU ml^{-1}) were incubated at different temperatures (4, 25, 37, 42 and 50°C) for 30 minutes. One hundred μl of each sample was serially diluted and plated on LBA after mixing with host cells. After overnight incubation, the plaques were counted.

Effect of pH on phages: Phage particles (10^7 PFU ml^{-1}) were incubated at three pH values (3, 7 and 9.5) for 30 minutes at room temperature. Glycine-HCl (pH 3) and glycine NaOH (pH 9.5) were added to the culture medium to adjust pH²⁵. One hundred μl of each sample was serially diluted and plated on LBA after mixing with host cells. After overnight incubation, the number of plaques was counted.

The optimal MOI for infection: *P. aeruginosa* DS1 was cultured to mid-log phase. The suspension was diluted to obtain 1×10^5 CFU ml^{-1} . The suspension was mixed with phage particles at different MOIs (10^{-1} , 10^0 , 10^1 and 10^2) and incubated at 37°C without shaking. Sampling was done at desired intervals and the absorbance at 600 nm was read and compared to bacterial control. The experiment was done 3 times.

Statistical analysis: The results are expressed as the mean $\pm$ SD of 3 experiments. One-way ANOVA was used to compare obtained results of pH and temperature effects.

$p < 0.05$ was considered as statistically significant. Analysis was performed in SPSS software version 18 (IBM Corp., Armonk, NY, USA).

Results and Discussion

Phage isolation and host range: Eleven phages were isolated from 20 samples. All phages were tested to determine host range (Table 2). Two phages were more effective on *P. aeruginosa* DS1 (the antibiotic-resistant biofilm producing strain). These two phages were also the only isolated particles that could lyse *P. aeruginosa* PI11 (the antibiotic-resistant strain). Therefore, these two phages (named kmuV and kmuB) were selected for further analyses.

Transmission Electron Microscopy (TEM) and structural proteins: Both phages showed icosahedra heads and contractile tails, the characteristics of Myoviridae family of phages. Generally, it is estimated that 96% of isolated phages are tailed. This fact applies also for *Pseudomonas* phages and about 30% of known tailed *Pseudomonas* phages belongs to Myoviridae family¹¹. The head and tail sizes of vB-PaeM-kmuV were calculated as 100 nm and 118×20 nm respectively (Fig. 1A). For vB-PaeM-kmuB, the head was 93 nm and the tail was 131×21 nm (Fig. 1B).

Six protein bands were obviously observed in a coomassie-stained SDS polyacrylamide gel of phage kmuV and 8 bands for phage kmuB (Fig. 1C). The most abundant peptide is a 75 kDa band which is present in both phages and may be the major protein of capsid. The structural proteins of the two phages are very similar, which is not surprising, since they belong to the same family and have similar behaviors. The small differences seen on the gel may be related to the minor differences between the structure of the two phages or even the fewer amounts of those capsid or tail subunits in kmuV.

Table 2
Host range and sources of isolated phages

Sampling place	<i>P. ae</i> Phage	9027	1074	1430	DS1	PI11
Untreated sewage, Kerman	kmuV	++	-	-	+++	++
Baqiatallah hospital, Tehran	BH	+	-	-	++	-
Environmental, Mashhad	EM	+	-	-	++	-
Almahdi park lake, Tehran	AP	+	-	-	++	-
Processing and disposal complex Aradkou, Tehran	A	+	-	-	++	-
Ghaem hospital, Rasht	GH	+	-	-	++	-
Imam Khomeini hospital, Tehran	IK	+	-	-	++	-
Urban sewage, Kerman	OR	+	-	-	++	-
Imam Hasan hospital, Fuman	IH	+	-	-	++	-
Zoo lake, Tehran	BL	+	-	-	++	-
Zoo wastewater, Tehran	kmuB	++	-	-	+++	++

-: no lysis

Lysis at concentrations +: 10^6 and above, ++: 10^4 to 10^5 , +++: 10^3 PFU ml^{-1} and below

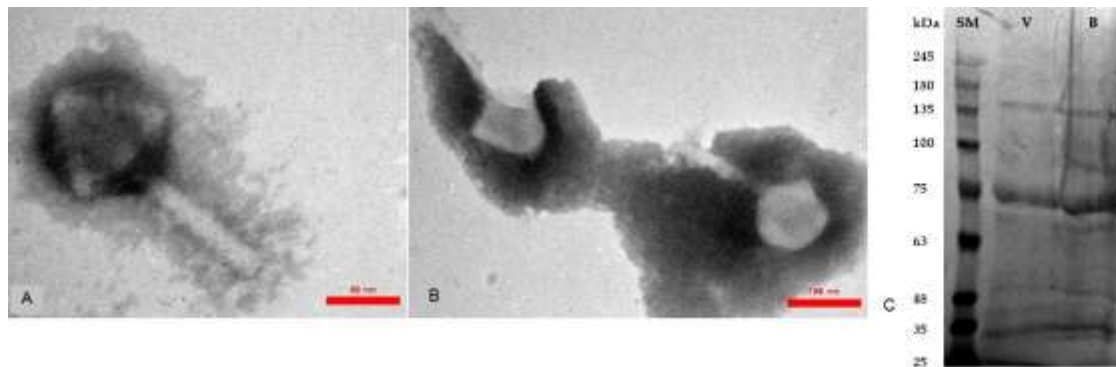


Fig. 1: The electron micrographs of (A) phages kmuV and (B) kmuB stained with uranyl acetate and (C) SDS-PAGE of Phages kmuV (line V) and kmuB (line B) separated by 12% SDS polyacrylamide gel and stained with Coomassie blue. The sizes of protein markers (line SM) are indicated on the left. A pre-stained protein size marker from Sinaclon was used.

Phage DNA isolation and digestion: The genomes of both phages were digested by HindIII, EcoRI and AluI restriction enzymes into many pieces, but not with SmaI (Fig. 2). It also seems that the genomes of the two phages are similar. Both genomes are resistant to SmaI restriction enzyme, which is a common phenomenon in phages²⁹. Another hypothesis is that they have no cut site or have just one cut site for this enzyme that converts the circular DNA into a linear molecule. Albeit, the second hypothesis seems unlikely as kmuV and kmuB are Myoviruses by large genome sizes and SmaI is a restriction enzyme that recognizes a six-base-pair sequence.

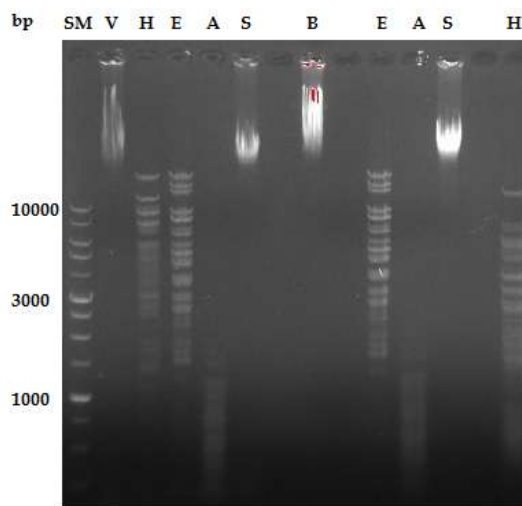


Fig. 2: Digestion of genomes of phages kmuV and kmuB. From left SM: size marker, V: undigested genome of kmuV, kmuV genome digested with H: HindIII, E: EcoRI, A: AluI, S: SmaI; B: undigested genome of kmuB, kmuB genome digested with E: EcoRI, A: AluI, S: SmaI and H: HindIII.

Adsorption time, effect of temperature and pH on phages and the optimal MOI: As shown in fig. 3, phage kmuV started to adsorb to the host cells after 3 minutes and this adsorption continues until 8 minutes, but phage kmuB was adsorbed efficiently on bacteria after 2 minutes. Unlike kmuV, kmuB was rapidly adsorbed into the host. The

difference may be related to the difference of phage receptors on bacterial cells or their need to some cations for adsorption (such as Mg^{2+} or Ca^{2+}). This shows that in spite of high similarity, kmuV and kmuB behave differently.

Both phages were stable at tested temperatures (Fig. 4A). There were no statistically significant differences between the temperatures (p -value>0.05). Also, both phages were stable at tested pH values at room temperature (Fig. 4B). There were no statistically significant differences between the pH values (p -value>0.05). The stability of phages to pH values is different. Some phages prefer alkaline conditions whereas others are sensitive to high pH values, a third group is sensitive to both acidic and alkaline conditions and a fourth group is resistant to both conditions¹⁴. The temperature is also important for storage and using phages in phage therapy. It seems that phages are more resistant to thermal inactivation compared to bacteria. It may be related to disulfide bonds of capsid proteins¹⁴. Tailed phages are highly resistant to low temperatures².

Also, Myophages may survive better in poorer environmental conditions¹⁵. Scarascia et al²⁸ isolated 7 phages against *P. aeruginosa* that were able to infect the host cells within a range of temperatures from 25 to 45°C and pH range from 5 to 8. PA5oct, a jumbo phage of *P. aeruginosa*, was stable even at 60°C and a pH range of 5-11⁹. Yazdi et al³¹ also isolated a phage which was effective on a biofilm producing *Proteus mirabilis* that was resistant to high temperatures and pH from 3 to 11. Phage ASP-1 (against *Aeromonas salmonicida*) was stable at 4 to 50°C and pH 4 to 11. The highest stability reported by the authors was at pH 11²¹.

Different MOIs were tested to evaluate the effect of phages kmuV and kmuB on *P. aeruginosa* DS1. All MOIs could efficiently prevent from the growth of the host, but phage B was more effective (Fig. 5). Although the two phages were very similar, kmuB infected the host cells more efficiently in LB broth medium. This may be due to the shorter adsorption time of kmuB. The host growth in LB which is seen especially for phage kmuV may be related to the

incidence of resistance in some host cells. In fact, a problem in phage therapy is occurrence of resistance to applied phage in the host bacteria. To overrule this phenomenon, scientists

recommend using more than one type of phage for therapy, especially if phages use different receptors to enter into the bacterial cells²⁶.

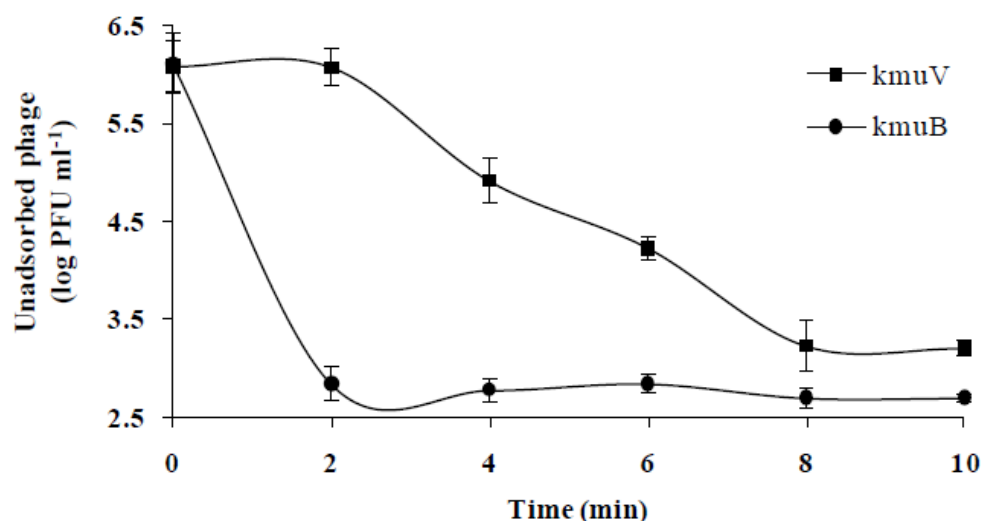


Fig. 3: Adsorption times of phages kmuV and kmuB on *P. aeruginosa* DS1. N=3

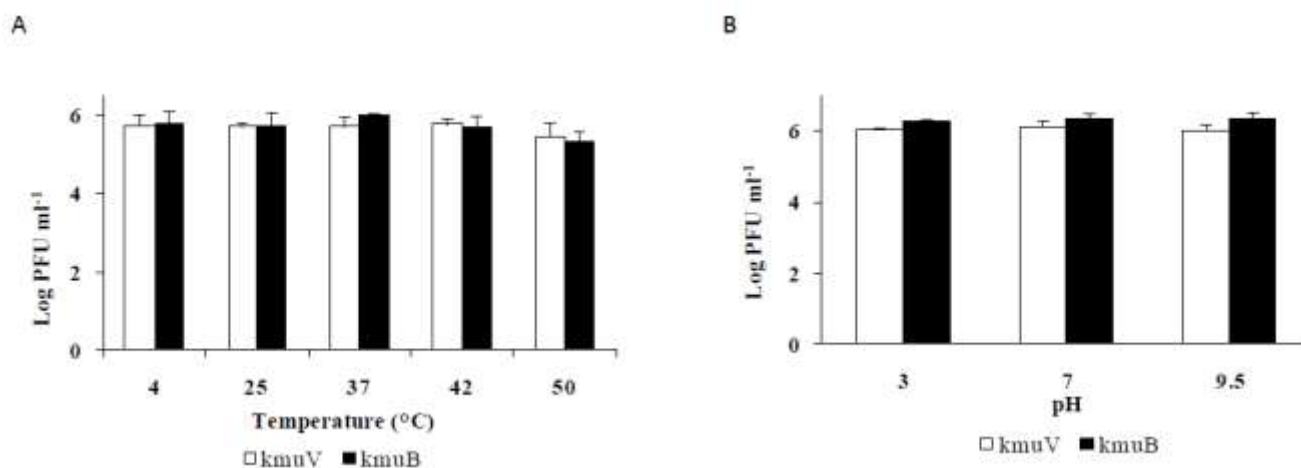


Fig. 4: Effect of (A) temperature and (B) pH on phages kmuV and kmuB. N=3

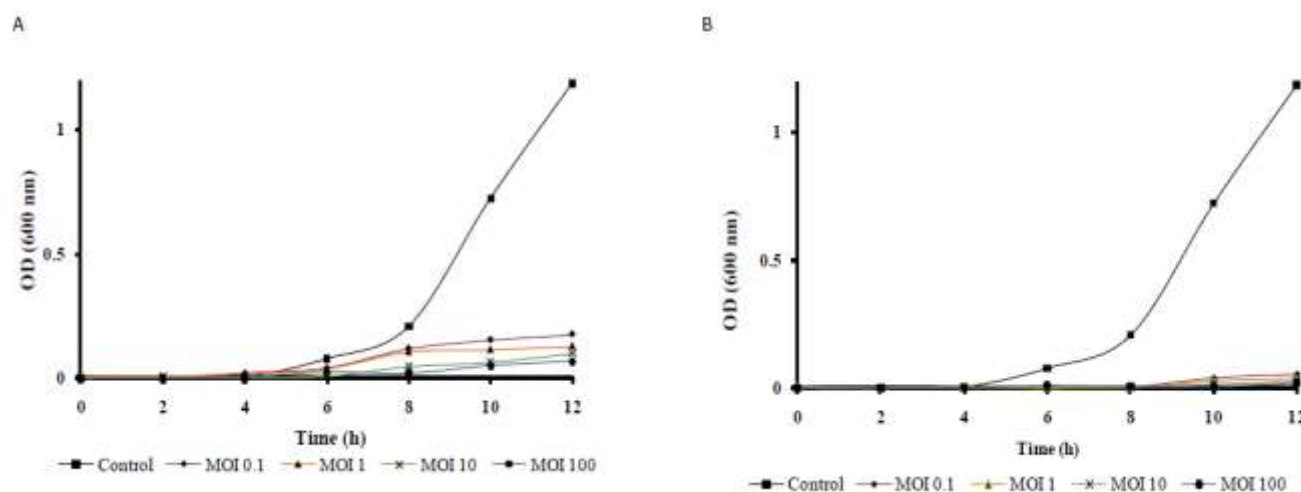


Fig. 5: Effect of (A) phages kmuV and (B) kmuB on *P. aeruginosa* DS1 in LB broth at different MOIs. No phage particles were added to the control. N=3. Error bars are not shown

Conclusion

Based on the sizes of the heads, the number of DNA fragments after digestion with restriction endonucleases and the sizes of the capsid and tail proteins in SDS-PAGE, it is estimated that kmuV and kmuB are large phages. Although kmuV and kmuB are very similar and may be related as their adsorption time into the same host in the same conditions is different, the hypothesis is that their receptors may be different. These two phages may be suitable candidates for phage therapy and can be used as a cocktail.

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