

Efficacious antibacterial potency of novel bacteriophages against ESBL-producing *Klebsiella pneumoniae* isolated from burn wound infections

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ABSTRACT

Background and Objectives: Prevalence of extended spectrum β -lactamase (ESBL) leads to the development of antibiotic resistance and mortality in burn patients. One of the alternative strategies for controlling ESBL bacterial infections is clinical trials of bacteriophage therapy. The aim of this study was to isolate and characterize specific bacteriophages against ESBL-producing *Klebsiella pneumoniae* in patients with burn ulcers.

Materials and Methods: Clinical samples were isolated from the hospitalized patient in burn medical centers, Iran. Biochemical screenings and 16S rRNA gene sequencing were determined. The phages were isolated from municipal sewerage treatment plants, Isfahan, Iran. TEM and FESEM, adsorption velocity, growth curve, host range, and the viability of the phage particles as well as proteomics and enzyme digestion patterns were examined.

Results: The results showed that *Klebsiella pneumoniae* Iaufa_lad2 (GenBank accession number: MW836954) was confirmed as an ESBL-producing strain using combined disk method. This bacterium showed significant sensitivity to three phages including P ϕ Bw-Kp1, P ϕ Bw-Kp2, and P ϕ Bw-Kp3. Morphological characterization demonstrated that the phage P ϕ Bw-Kp3 to the *Siphoviridae* family (lambda-like phages) and both phages P ϕ Bw-Kp1 and ϕ Bw-Kp2 to the *Podoviridae* family (T1-like phages). The isolated bacteriophages had a large burst size, thermal and pH viability and efficient adsorption rate to the host cells.

Conclusion: In present study, the efficacy of bacteriophages against ESBL pathogenic bacterium promises a remarkable achievement for phage therapy. It seems that, these isolated bacteriophages, in the form of phage cocktails, had a strong antibacterial impacts and a broad-spectrum strategy against ESBL-producing *Klebsiella pneumoniae* isolated from burn ulcers.

Keywords: Bacteriophage therapy; Burn; *Klebsiella pneumoniae*; Extended spectrum beta-lactamase; Wound; Bacterial infections; Restriction endonuclease

INTRODUCTION

Despite all the advances in burns infection treatment and wound care techniques, infections caused by microorganisms including bacteria are significant causes of death in burn patients (1, 2). Control and

elimination of wound infection is one of the serious problems in burn hospital wards (3-5). The phenomenon of resistance to different antibiotics has been reported in a wide range of beta-lactamase-producing isolates in burn wounds (6-8). Phage therapy has been proposed as one of the ways to solve the

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serious problem caused by antibiotic resistant bacteria (9, 10). These viral particles, with a variety of about 10^{30} to 10^{32} types, include the most biologically diverse species on the earth and play a special role in controlling bacterial populations in the environment (11-13). Some bacteriophages have wide host ranges and attack multiple bacterial strains in a special species or even several related species (14, 15). Recently, with advances in genetic engineering, the design of synthetic and semi-synthetic phages with antibacterial properties has received much attention (16). *Klebsiella pneumoniae* is one of the most common pathogenic bacteria with high antibiotic resistant prevalence in patients with burn wounds (17). At present, clinical isolates of multi drug resistant (MDR) *K. pneumoniae* are evolved to extreme drug resistant (XDR) strains for which few treatment options are available (18). These bacteria have also become resistant to relatively strong antibiotics, which are a warning sign in the treatment of infections caused by them (19). Clinical MDR isolates of *K. pneumoniae* have widely become resistant to a broad range of antibiotics by accessing plasmids encoding broad-spectrum β -lactamase (ESBL) enzymes (20-23). Increased use of broad-spectrum β -lactam drugs and long-term hospitalization of patients has led to increasing prevalence of ESBL producing bacteria, which have achieved different resistant mechanisms such as achievement of efflux pumps, and reduction of membrane permeability (24-28). The progressive resistance of *K. pneumoniae* strains to various antibiotics has led to attempts for replacing conventional therapies with new therapeutic procedures. The aim of this study was to find effective bacteriophages against ESBL producing strains of *K. pneumoniae* isolated from burn wounds and to investigate their antibacterial activity so that they will be suggested for phage therapy of the infections caused by antibiotic resistant infections caused by *K. pneumoniae* strains. We identified the properties, stability and morphological characterization of bacteriophages infecting ESBL-producing *K. pneumoniae* isolated from burn ulcer infections and investigated the molecular identification of lytic phages. Evaluating the effectiveness of novel bacteriophages on ESBL-producing *K. pneumoniae* in burn ulcers is a high priority due to few research in this field. This study was accomplished based on the urgent need of the hospital community to treat drug resistance and the inhibiting of burn ulcer infections (bacterial infection)

that are not treated with common antibiotics.

MATERIALS AND METHODS

Screening and biochemical characterization of the isolates. A total of 50 burn wound samples were obtained from the patients, all of whom were hospitalized in various burn wards in Iran. Sampling was performed during three months for all isolates. Among the 50 Gram-negative bacteria, 5 *K. pneumoniae* isolates were identified. Biochemical tests including indole production, H_2S production, sugar fermentation in triple sugar iron agar (TSI) medium, urea hydrolysis, citrate utilization, and lysine decarboxylase were used for the identification of the isolates (29).

The bacterial DNA isolation and extraction. The nucleic acid of *K. pneumoniae* isolates was extracted by a DNA extraction kit (RIBO-prep, Russia) according to the manufacturer's instructions. For accurate identification of bacterial strains, all clinical isolates were molecularly identified by amplification and sequencing of 16S rRNA coding gene. The universal primers: 27F (5'AGAGTTTGATCCTGGCTCAG3') and 1492R (5'ACGGCTACCTTGTTACGACTT3') (Metabion, Germany) were used and the PCR reaction was accomplished in a thermal cycler (Bio Rad, Malaysia). The reaction was done under an initial denaturation at 95°C for 6 min, and 35 cycles including denaturation at 95°C for 45 s, primer annealing at 55°C for 40 s, and extension at 72°C for 45 s, followed by a final extension at 72°C for 5 min. All PCR products were sequenced (Applied Biosystems, USA) (30).

ESBL resistance patterns. Resistance of the isolates to common antibiotic was examined using Kirby-Bauer's standard disc diffusion method and then the antibiotic disks including 10 μ g ciprofloxacin (CPX), 30 μ g ceftazidime (CAZ), and 30 μ g cefotaxime (CTX) were used for ESBL resistant testing. After incubation at 37°C for 24 h, the diameter of the growth inhibition zones (mm) was measured and the results were recorded as resistant, susceptible, or intermediate, according to the clinical and laboratory standards institute (CLSI). For interpretation of the results, the bacterial growth inhibition zones ≥ 22 mm for ceftazidime, ≥ 27 mm for cefotaxime, and ≥ 25 mm for ciprofloxacin, were considered for detect-

ing broad-spectrum β -lactamase producing isolates. The results announced according to CLSI document M100-18 (31, 32).

Confirmation/phenotypic tests for detection of ESBL production. In order to phenotypically detect ESBL producing *K. pneumoniae* isolates, the combined three stages of agar disk diffusion method were used. In the first stage, cefexime and ceftazidime disks were examined simultaneously and if the bacterial strain was resistant to both disks, it would be initially considered as ESBL-producing strain. In the second stage, the combined ceftazidime disk and ceftazidime/clavulanic acid composite disk (30/10 μ g) was used; and in the final stage, cefotaxime disk and cefotaxime/clavulanic acid combined disk and ciprofloxacin/clavulanic acid (CONDA, Spain) were tested on the selected isolates. An increase of more than 5 mm in the growth inhibition zone created by the composite discs containing clavulanic acid compared to discs without it, indicated that the studied bacterial isolate belonged to the bacteria that produce β -lactamase enzymes (33, 34).

Isolation and concentration of bacteriophages. For screening the possible bacteriophages which were efficient on the *K. pneumoniae* isolates, samples were collected from inlet of Isfahan municipal wastewaters treatment plants in Iran, then were transferred to the Falavarjan research laboratory in sterile bottles and kept at 4°C. Samples were centrifuged at 8000 g (SIGMA.30 K3, Germany) for 15-20 min at 4°C. Then, 10 mL of each sample was filtered by a 0.22 μ m membrane filter (Cobetter, Japan) and the prepared filtrate was mixed with 100 μ L of each bacterial culture and 50 mL of 2 \times Brain Heart Infusion (BHI) broth medium (Ibresco, Iran), then the solution was completely mixed at 140 rpm at 37°C for 24 h (33). For concentration of the phages, 10% polyethylene glycol (PEG) and NaCl 1 M was added to each the mixture of bacteriophage and *K. pneumoniae* culture that gained from the previous stage. Every pellet was resuspended in SM buffer and added to a CsCl gradient tube. Afterwards the tubes were centrifuged at 30000 g for 60 min and were decomposed versus SM buffer (1 M Tris-HCl pH 7.5, 5.8 g NaCl, 2 g MgSO₄·6H₂O in 1 L distilled water) until obtain the concentrated phages. Finally, the concentrated bacteriophages were separated by using 0.45 μ m membrane syringe filters and stored at 4°C until used. (35, 36).

Detection of the effect of the bacteriophages on the bacteria by the spot test. For this purpose, 0.1 mL of overnight grown bacterial culture (1.5×10^8 CFU/ml) was prepared from each *K. pneumoniae* isolates and was added to 5 mL of melted soft BHI agar 0.7% (45°C) and overlaid on the surface of solid BHI (1.5% agar). Then, 10 μ L of the phage suspension was spotted on the bacterial culture. The drops allowed to completely absorb to the medium. After incubation at 37°C for 24 h, formation of clear plaques was confirmed the presence of phages on the bacterial lawn (37).

Detection of the effect of the bacteriophages on the bacteria by overlay (double layer agar) method. For this purpose, serial dilutions of each phage filtrate (10^8 PFU/mL) was prepared in 50 mL SM buffer and 0.1 mL of each dilution was distinctly mixed with 0.1 mL overnight grown bacterial culture (1.5×10^8 CFU/ml). Ultimately, prepared mixture was combined with 5 mL of molten soft BHI agar 0.7% (45°C) and was added to the surface of a BHI agar, then incubated for 24 h at 37°C. There upon, the created plaques were counted (37, 38).

Titration and phage plaques purification. For purification of bacteriophages using double layer method. Bacteriophage solutions with 10^{-4} to 10^{-8} dilutions were used. Single and clear plaques were cut and added to 1 mL SM buffer and thoroughly mixed for 1 min. The mixture was then centrifuged at 8000 g for 5 min, afterwards 0.1 mL of the supernatant was added to 0.9 mL of prepared SM buffer. At this stage, serial dilution was prepared from the supernatant and separately 5 mL of molten soft BHI agar 0.7% (45°C) was added to 0.1 mL overnight grown bacterial culture. The mixture was finally poured on the surface of the solid BHI agar that prepared with 1.5% agar. After incubation overnight at 37°C, the formed plaques were counted (39).

Detection of the phages host range. The host range of the studied phages was determined on different strains of *K. pneumoniae* including TM07, VRC10, ATCC 10031, Iaufa_lad1, Iaufa_lad2, and Iaufa_lad3. For this purpose, 0.1 mL of overnight grown bacterial culture (1.5×10^8 CFU/ml) was mixed with 5 mL of molten soft BHI agar 0.7% (45°C) and overlaid on the solid BHI (1.5% agar). After solidifying of the agar, 10 μ L of each dilution prepared from phage suspen-

sion (10^8 PFU/mL) was inoculated as spots on the bacterial lawns in separated areas. After 24 h incubation at 37°C, the phage lytic activity was announced as + for clear lysis and - for not clear lysis (37).

Determination of phages morphology by TEM. TEM imaging was used to observe phage particles and their nanometer scale size. The amount of 10 μ L of the high titer phage suspension ($\geq 10^8$ PFU/mL) was placed on 200 mesh copper grids with carbon-coat and stained with 2% (w/v) uranyl acetate for 1 min. Thereafter, the prepared sample was observed with a TEM (Philips EM 208S) at 100 KV. The latest changes in the International virus classification committee (ICTV) reports were used to further identify the possible family of the isolated phages (40, 41).

Observation of phage attack by FESEM. FESEM was used to study the interactions between phage and bacteria (bio-conjugation activity) as well as phage attack on host cells. The clear plaques obtained during the highest and most effective phage titer, were cut with a scalpel blade and mixed with 200 μ L SM buffer and then, transferred to the Arya Electron Optic laboratory. The samples were placed on a gold coated grids, then the prepared sample was observed with a FESEM (MIRA3 /TESCAN company) (42).

Determination of phage viability at different temperatures. In order to consider the antibacterial activity of phages against the *K. pneumoniae* strains at various temperature degrees, the plaque assay was used. For this purpose, 0.1 mL of each dilution prepared from phage suspension (10^4 - 10^8 PFU/mL) was applied on BHI agar medium comprising 5 mL overnight grown cultured bacteria (10^8 CFU/mL). The plates were exposed to different temperature degrees of 15, 20, 30, 37 and 42°C for 24 h. The most appropriate temperature for phage antibacterial activity was detected. The temperature at which the first clear phage plaques were observed was considered the optimum temperature. The experiment was repeated three times for each strain (36).

Determination of phage viability in different pH values. The viability of isolated bacteriophages was determined at several pH degrees. BHI broth medium with various pH degrees from 4-10 were prepared and equal amounts of the phage filtrate solution (10^8 PFU/mL) was inoculates to this medium. Afterward, the

suspension was placed at 37°C for 60 min and the degree of phage viability was determined by measuring the titer of active phages by overlay procedure (36).

Phage growth curve. At this stage, 1 mL of bacterial culture with (OD600 of 0.2) was mixed with the diluted phage suspension and incubated at 37°C. Afterwards, was centrifuged at 6000 g for 10 min to remove residual phage particles. Then, every 10 minutes, the virus titer was determined using overlay method in terms of PFU/mL. The burst size and latency period were measured from the growth curve one-step phage growth curve plotting (43).

Analysis of bacteriophage DNA digestion pattern. The prepared phage stock was first mixed with 1 μ g/mL *DNase I* and *RNase A* (Fermentas, America) for 20 min and incubated at 37°C, afterward passed through 0.22 μ m membrane filter. The mixtures were centrifuged at 28000 g for 60 min. Phage DNA extraction ($>10^8$ PFU/mL) was performed using an extraction kit (NORGEN, Canada) and DNA was obtained in the form of a precipitate. To remove protein residues, phenol/chloroform/isoamyl alcohol solution (25/24/1) was used. The precipitation of phage DNA was then done by using pure isopropanol (34, 44). Restriction endonuclease enzymes involving *ECOR I*, *KpnI*, *HaeI*, *HindIII*, *BamHI*, and *XhoI* (Fementas, USA) were used to digest phage genomic DNA. In this method, phage purified DNA was mixed with each of these enzymes according to the manufacturer's instructions and then placed at 37°C for 60 min. The results were obtained from enzymatic digestions was evaluated by 1% agarose gel electrophoresis at 75 V for 60 min (41).

Protein profile detection. For the analysis of the proteomics patterns, 20 μ L of each bacteriophage sample was mixed with 5 μ L loading buffer comprising (50 mM Tris-HCl [pH 6.8], 2% SDS, glycerol 10%, petothanol 5%, and aqueous bromophenol 0.001%). Afterwards, was heated in water for 10-15 min in order to denature the proteins. Then the proteomic pattern of the bacteriophages was analyzed based on the separate bands on SDS-PAGE 10% which were stained with Coomassie Brilliant Blue and compared to a protein ladder which included 10 fragments in the range of 10 to 180 KDa (34).

Statistical analysis of the data. One-way analy-

sis of variance (One-way ANOVA) was used for data analysis and all data were entered into Excel Microsoft. SPSS version 20 and Graph pad prism 8 was used for statistical analysis. Data output was presented as SEM \pm mean.

RESULTS

Identified *K. pneumoniae* strains. Bacterial strains isolated from burn wounds in different hospitals and confirmed using biochemical and molecular methods. The results of biochemical tests are given in Table 1. The results from molecular identification based on BLAST analysis of the amplified sequences in the 16S rRNA gene represented that the bacteria were strains of *K. pneumoniae* with 98-99% identity. Among the isolates, *K. pneumoniae* strain TA05 isolated from Tehran Motahari Hospital, that called *K. pneumoniae* strain Iaufa_lad2 (GenBank accession number: MW836954), was an extended-spectrum β -lactamase (ESBL) producer.

ESBL confirmation test. Fig. 1 shows the resistance pattern of the isolated ESBL *K. pneumoniae* strain Iaufa_lad2 causing nosocomial infection in

Table 1. Morphological and biochemical characteristics of the strains

Characteristics	Biochemical tests
-	Gram staining
Rods	Cell shape
Acid/Acid/H ₂ S -	TSI
+	Urease
Fermentative	O/F
Non-Motile	Motility
+	Voges Proskauer (VP)
-	Methyl Red (MR)
-	Oxidase
+	Catalase
+	Citrate utilization
-	Indole test
+	Nitrate reductase
+	ONPG test
-	Starch hydrolysis
-	Gelatin hydrolysis
+	Growth in KCN
<i>Klebsiella pneumoniae</i>	Identified species

+: Positive results; -: Negative results

the burn wound of a hospitalized patient based on agar disk diffusion method using combined disks including ceftazidime/clavulanic acid, cefotaxime/clavulanic acid, and ciprofloxacin/clavulanic acid.

The bacteriophages properties. Three lytic phages P ϕ Bw-Kp1, P ϕ Bw-Kp2, and P ϕ Bw-Kp3 were isolated from raw sewage inlet by double layered method. Observation of the phage's morphology by TEM showed that the phage P ϕ Bw-Kp3 which had a narrow long tail with a small head is similar to the family *Siphoviridae*. Both phages P ϕ Bw-Kp1 and P ϕ Bw-Kp2 had icosahedral shaped heads and non-contractile short tails, similar to the *Podoviridae* family (Fig. 2) (Table 2). According to the FESEM micrograph, the phages had bio-conjugated antibacterial complex and formed holes on the cell wall of pathogenic bacteria which led to the penetration of phage into the bacterial cell (Fig. 3).

The bacteriophages host ranges. The results from detection of the host range of the lytic bacteriophages P ϕ Bw-Kp1, P ϕ Bw-Kp2 and P ϕ Bw-Kp3 using spot method displayed that the plaques were formed on *K. pneumoniae* strain Iaufa_lad2 by all three bacteriophages. The strains *K. pneumoniae* Iaufa_lad3 and *K. pneumoniae* VRC10 were only lysed by the phage P ϕ Bw-Kp2 (Table 3).

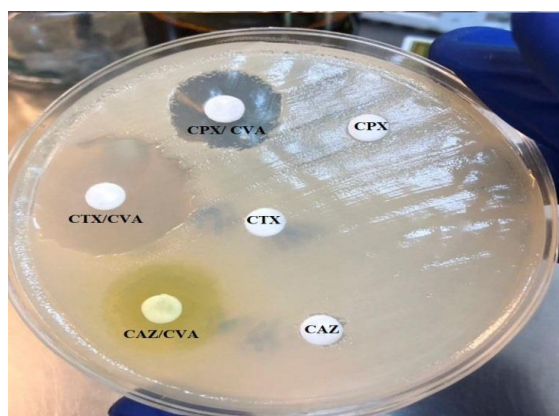


Fig. 1. Phenotypic confirmation test for ESBL-producing *K. pneumoniae* strain Iaufa_lad2. CPX: ciprofloxacin; CPX/CVA: ciprofloxacin/clavulanic acid; CTX: cefotaxime; CAZ: ceftazidime; CTX/CVA: cefotaxime/clavulanic acid; CAZ/CVA: ceftazidime/clavulanic acid; The bacterial growth inhibition zones ≥ 22 mm for ceftazidime, ≥ 27 mm for cefotaxime, and ≥ 25 mm for ciprofloxacin were indicated.

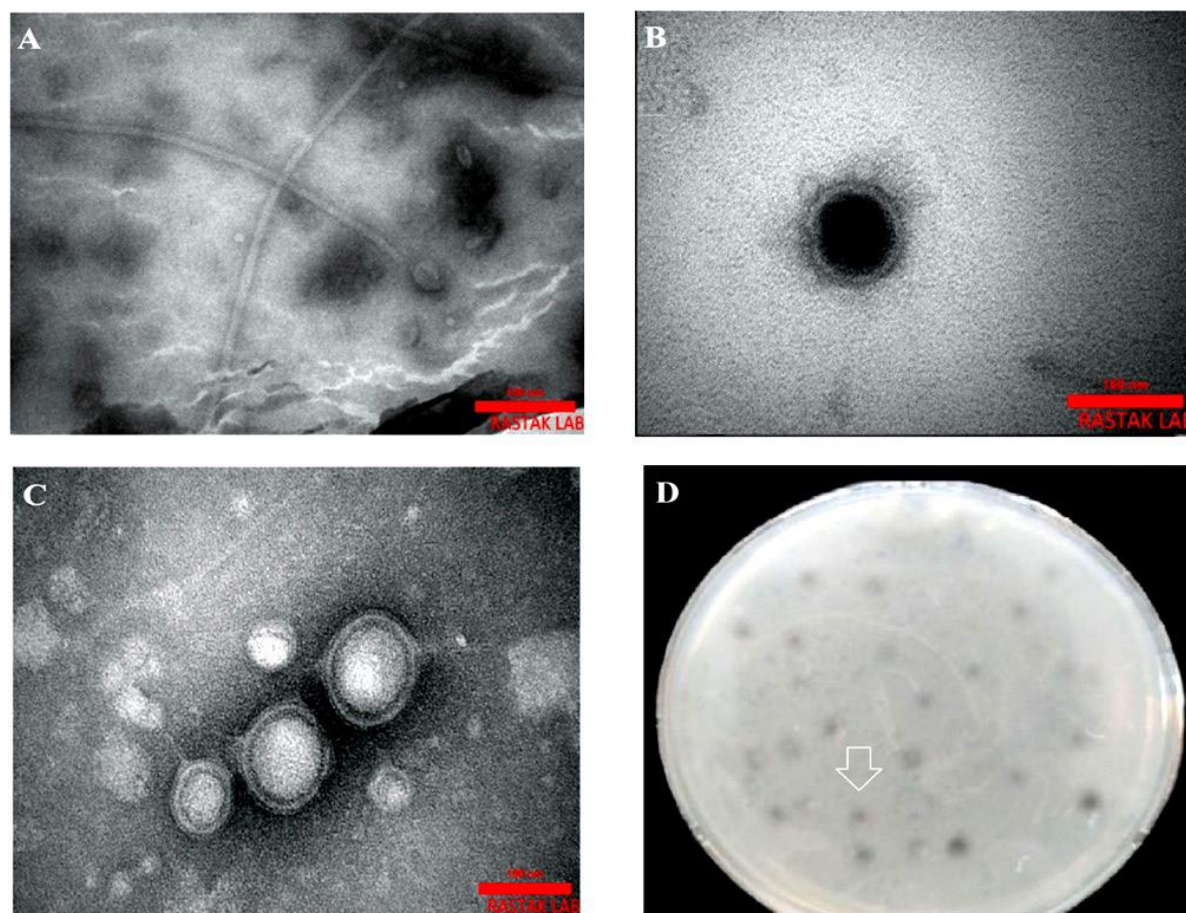


Fig. 2. Transmission electron microscopy (TEM) micrograph of the lytic phages of *K. pneumoniae* Iaufa_lad2 isolated from municipal sewage, Isfahan, Iran which were related to 3 families of bacteriophages. A: PφBw-Kp1 (*Siphoviridae*), B: PφBw-Kp2 (*Podoviridae*), C: PφBw-Kp3 (*Podoviridae*). A sample image of the clear plaque appearance (D); Scale bars showed 100-150 nm.

Table 2. Characteristics of the isolated bacteriophages

PφBw-Kp3	PφBw-Kp2	PφBw-Kp1	The isolated bacteriophage
<i>Caudovirales</i>	<i>Caudovirales</i>	<i>Caudovirales</i>	Order
<i>Podoviridae</i>	<i>Podoviridae</i>	<i>Siphoviridae</i>	Family Shape
A non-contractile short tail	A non-contractile short tail	A narrow long tail	(TEM) Plaque
Clear	Clear	Clear	shape Size
Head: 55 nm, Tail: 20 nm	Head: 60 nm, Tail: 10 nm	Head: 30 nm, Tail: 350 ± 10 nm	(nm)
T1-like phages	T1-like phages	lambda-like phages	Prototype
Wastewater treatment plant, south of Isfahan	Wastewater treatment plant, north of Isfahan	Wastewater treatment plant, south of Isfahan	Sampling area
33 × 10 ⁸	27 × 10 ⁸	35 × 10 ⁸	PFU/ml

The phages viability. The results from the impact of various pH degrees on the viability of specific bacteriophages PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3 demonstrated that all three bacteriophages had the highest stability of lytic activity at pH = 7, and

the lytic activity of all three phages was remarkably decreased at pH = 5 and pH=9, 10 (Fig. 4A). The results from the effect of various temperature degrees on the viability of the isolated bacteriophages at different time intervals (0, 5, 10, 15, 20, 25, and 30

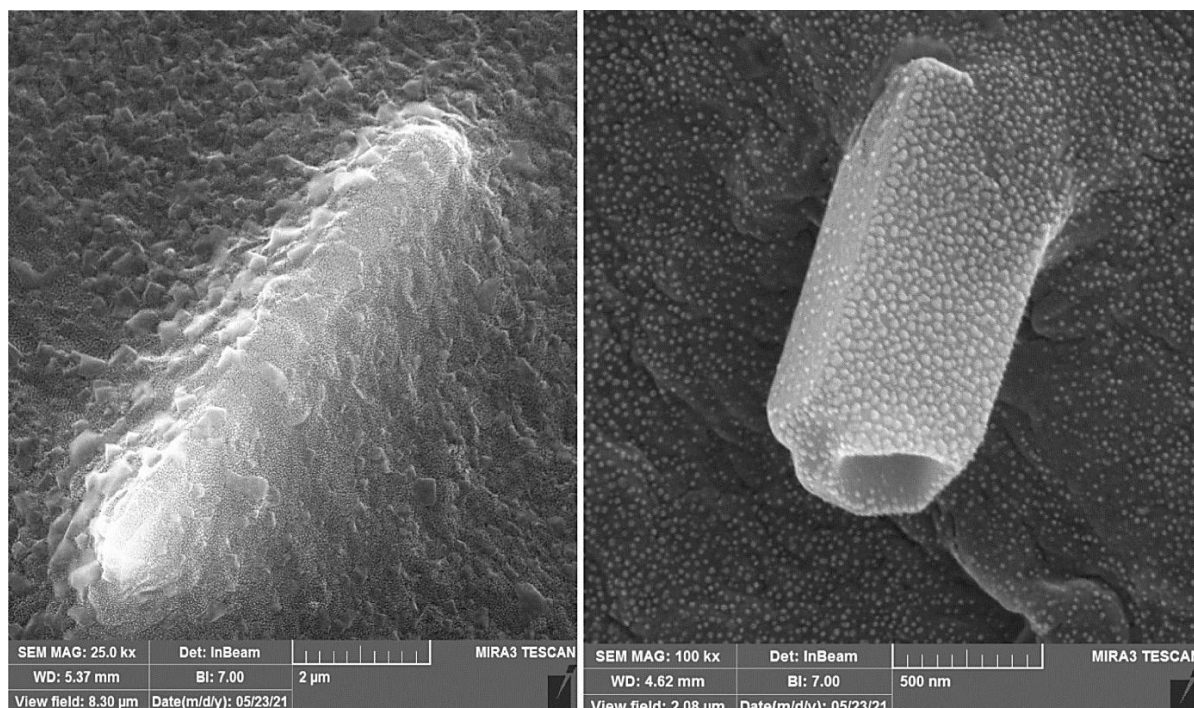


Fig. 3. Field Emission Scanning electron microscopy (FESEM) analysis of *K. pneumoniae* strain Iaufa_lad2 cells and phages on a gold coated grids. FESEM micrograph has taken 48 h after infection. The lytic activity of bacteriophage as antimicrobial agent against clinical *K. pneumoniae* strain Iaufa_lad2 was showed.

Table 3. Spot testing and investigation of phage sensitivity

Strain	Source	Spot test result		
		PφBw-Kp1	PφBw-Kp2	PφBw-Kp3
<i>K. pneumoniae</i> Iaufa_lad1	Clinical samples; Culture collection	-	-	-
<i>K. pneumoniae</i> subsp. <i>pneumoniae</i> Iaufa_lad2 ESBL (+)	Clinical samples; Culture collection	+	+	+
<i>K. pneumoniae</i> Iaufa_lad3	Clinical samples; Culture collection	-	+	-
<i>K. pneumoniae</i> TM07	Clinical samples; Culture collection	-	-	-
<i>K. pneumoniae</i> VRC10	Clinical samples; Culture collection	-	+	-
<i>K. pneumoniae</i> ATCC 10031	Institute Pasteur of Iran	-	-	-

*ESBL – extended-spectrum β-lactamase producing strain. (+): Clear plaques; (-): no plaques formation

min) showed that the absorption level was reached to about 90% in 10 to 15 min. On the other hand, approximately 20 to 30% of the remaining phages were not absorbed by the host cell (Fig. 4B). The results from the impact of various temperatures on the viability of isolated bacteriophages at several temperatures (15, 20, 30, 37, 42, and 45°C) demonstrated that all three bacteriophages had the highest stability at temperatures between 15 and 20°C, with an approximate value of 90 to 95% (Fig. 4C).

Latent period and burst size. In the one-step growth curve that was plotted based on the growth pattern and lytic activity of the bacteriophages PφBw-Kp1, PφBw-Kp2 and PφBw-Kp3, on ESBL producing *K. pneumoniae* Iaufa_lad2 showed that the growth of the phages reached to a constant rate (burst size) at the times between 80-90 min (Fig. 5).

DNA digestion pattern. Analysis of the restriction enzymes digestion pattern showed that the DNA of

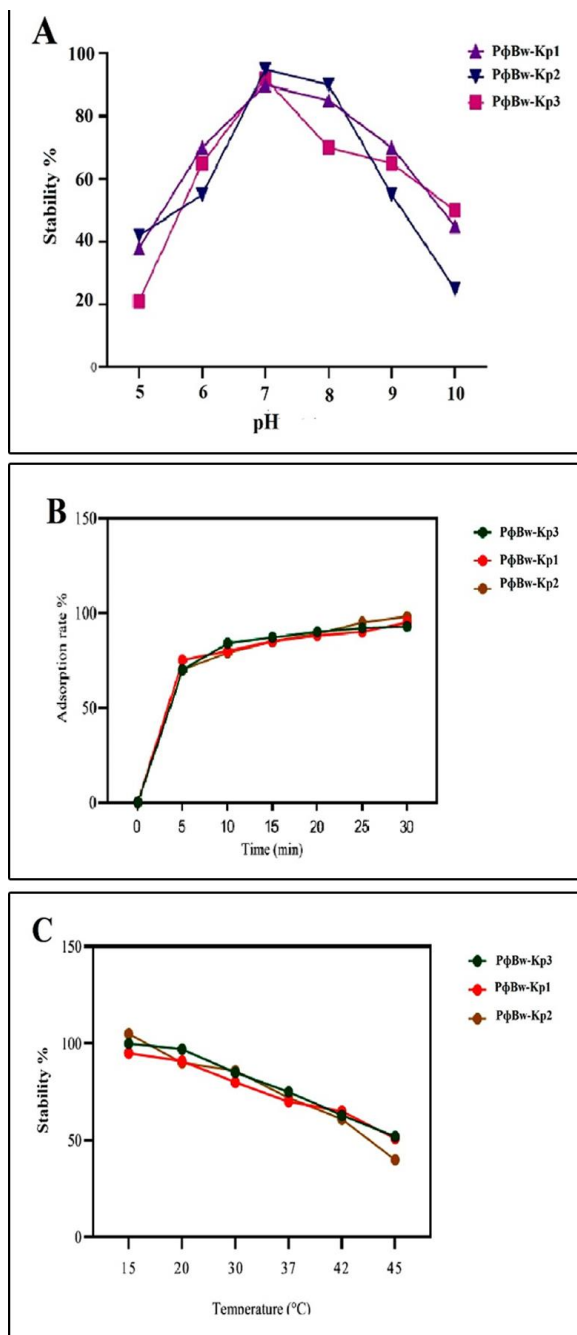


Fig. 4. Stability of the bacteriophages PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3 in various pH values for 1 h at 37°C (One-way ANOVA $P \geq 0.05$; $F = 0.120$) (A). Adsorption rate of the bacteriophages PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3 at various times for 1 h ($P \geq 0.05$; $F = 0.0003$) (B). Temperature stability of bacteriophages PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3 at several temperatures for 1 h ($P \geq 0.05$; $F = 0.051$) (C). There were no significant differences between the three phage groups. Identical results were obtained in 3 repetitions.

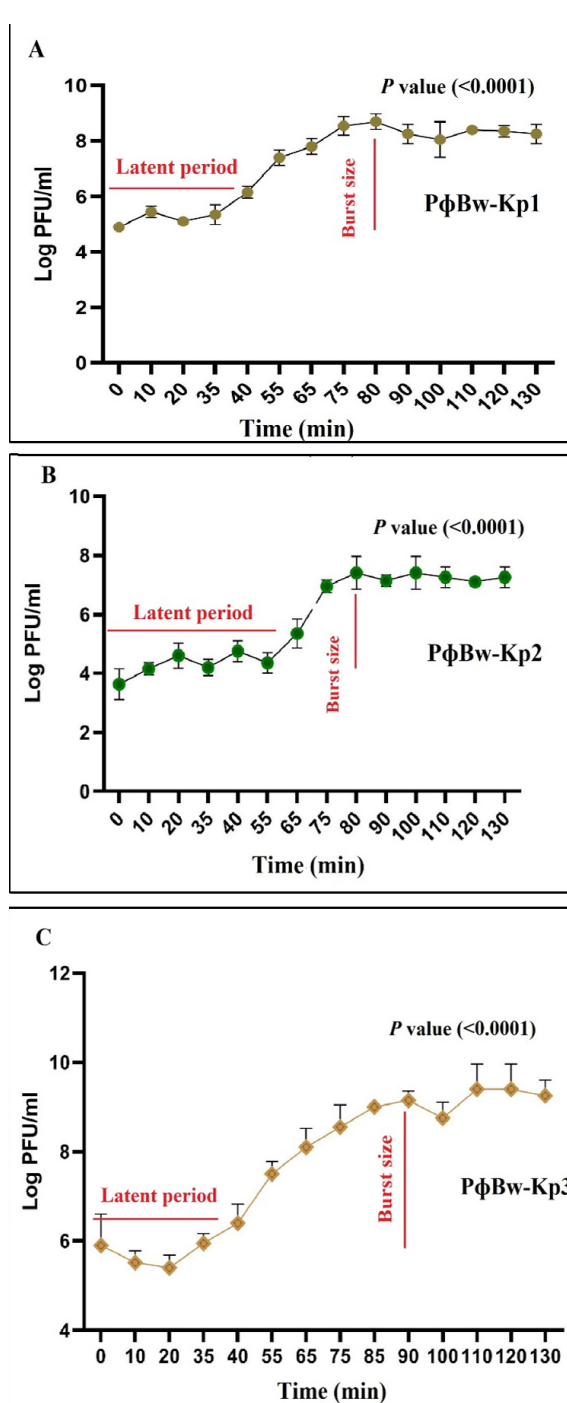


Fig. 5. One-step growth curve of the isolated bacteriophages on ESBL producing *K. pneumoniae* laufa_lad2. One-way ANOVA displayed that a significant difference was observed in each chart ($P < 0.0001$). The latency period of PφBw-Kp1 was 20 min and the burst time was 80 min. The latency period of PφBw-Kp2 was 55 min and the burst size was 80 min. The latency period of PφBw-Kp3 was 40 min and the burst time was 90 min. The data show the mean \pm SD (P value < 0.0001 for all phage groups). Identical results were obtained in 3 repetitions.

the phage P ϕ Bw-Kp1 was digested by *Hind III*, *Bam HI*, and *Kpn I* enzymes, the DNA of the phage P ϕ Bw-Kp2 was digested by all used restriction enzymes, and the phage P ϕ Bw-Kp3 was digested by *Hind III* and *EcoRI* enzymes (Fig. 6).

Protein profiles. The results are shown in Fig. 7. The size of bacteriophages P ϕ Bw-Kp1, P ϕ Bw-Kp2, and P ϕ Bw-Kp3 according to protein profile in SDS-PAGE was detected as 60 to 75 KDa, 55 to 65 KDa and 25 to 63KDa, respectively.

DISCUSSION

The present study attempted to evaluate the effectiveness of phages to inhibit burn wound infecting *K. pneumoniae* including an ESBL producing strain, Iaufa_lad2, which showed significant sensitivity to three phages including P ϕ Bw-Kp1, P ϕ Bw-Kp2, and P ϕ Bw-Kp3. TEM results showed that P ϕ Bw-Kp3 to the *Siphoviridae* (lambda-like phages), and that both phages P ϕ Bw-Kp1 and P ϕ Bw-Kp2 to the *Podoviridae* family (T1-like phages). The isolated bacteriophage had large burst sizes, thermal and pH stability and high adsorption rate to the host cells in the first few hours. Keşik-Szeloch et al. (2013) isolated and identified 32 phages, eight from *Myoviridae*, eight from *Siphoviridae*, and 16 from *Podoviridae*. They observed ESBL-producing *K. pneumoniae* among the isolates. The isolated *Siphoviridae* genomes (KP16 and KP36) contained low restriction sites similar to the strategy

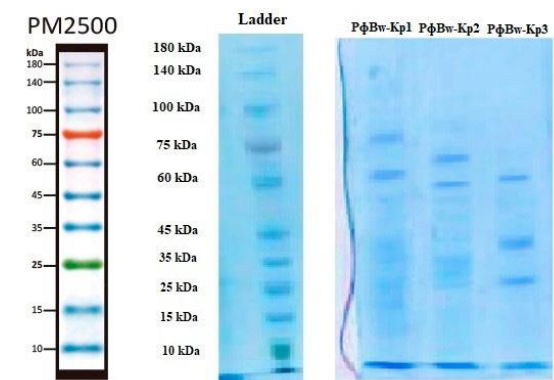


Fig. 7. The proteins profile of 3 specific phages. SDS-PAGE results showed major bands with molecular weight ranging from 60 to 75 KDa, 55 to 65 KDa and 25 to 63KDa, respectively, for phages P ϕ Bw-Kp1, P ϕ Bw-Kp2 and P ϕ Bw-Kp3.

found in T7-like phage (KP32). KP34 genome was digested by all used endonucleases in their research (*EcoRV*, *EcoRI*, *HindIII*, *NsiI*, *NcoI*, *PaeI*, and *SnaBI*) (24). Karumidze et al. (2013) isolated six lytic bacteriophages against *Klebsiella* from sewage-contaminated river water and proposed them as phage therapy candidates. Two of the phages, vB_Klp_5 and vB_Klox_2, were considered in more detail. Biological attributes including phage structure, nucleic acid composition, host range, growth phenotype, and thermal and pH viability were determined for all six phages. Limited sample sequencing was performed to define the phylogeny of the *K. pneumoniae*- and *K. oxytoca* specific bacteriophages, vB_Klp_5 and vB_Klox_2, respectively. Both of the studied phages

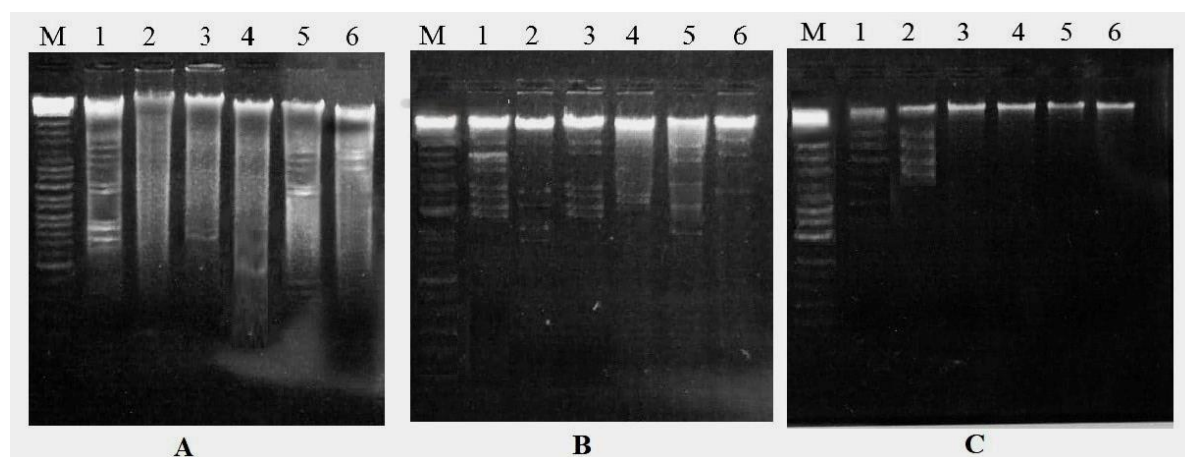


Fig. 6. Restriction map of the bacteriophages DNA, digested by different restriction endonuclease enzymes including *Hind III* (lan 1), *Eco RI* (lan 2), *Xho I* (lan 3), *Hae III* (lan 4), *Bam HI* (lan 5), and *Kpn I* (lan 6). M: 25 Kb DNA ladder (Fermentas/Thermo Fisher Scientific, USA). A: P ϕ Bw-Kp1; B: P ϕ Bw-Kp2; C: P ϕ Bw-Kp3.

had large burst sizes, efficient rates of adsorption and were stable under various adverse conditions. Phages reported in their study were double-stranded DNA bacteriophages belonged to the families *Podoviridae* and *Siphoviridae*. Their results demonstrated that one or more of the six phages were able of effectively lyse ~63 % of *Klebsiella* strains (45). The results of these researchers were consistent with our results. Wintachai et al. (2021) identified phage KP1801 as an alternative for antibiotics and biocontrol agents against extended spectrum β lactamase-producing *K. pneumoniae* (ESBL-KP) infection. The phage KP1801 was classified as being in the order *Caudovirales*, belonging to the *Siphoviridae* family using TEM. The effect of various temperatures and pH degrees of phage KP1801 stability were examined. Also, adsorption rate and one-step growth curve were determined. The results demonstrated that, it was stable at 25°C and pH = 7. Phage adsorption rate increased in the first 20 to 30 min. This uptrend lasted up to 80 min and was almost constant from 80-120 minutes. The growth curve indicated that in the 1 h at OD = 600, the absorption was about 0.2 nm and from this time to 8 h, it was close to zero (46). While in our research, bacteriophages which were identified as *Siphoviridae* and *Podoviridae* families, order *Caudovirales*, had a great burst size and viability. Our phages had highest percentage of viability and antibacterial activity at pH = 7 and at the temperature range between 15 to 20°C. Park et al. (2017) investigated on two phages, PKO111 specific for *Klebsiella oxytoca* and PKP126 specific for *K. pneumoniae*. Host specificity was differed in the phages. The phages were stable at 4°C to 60 and pH 3 to pH = 11, but in our study, the lytic activity of all three phages was remarkably decreased at pH=5 and pH=10, and the bacteriophages in our report showed the highest viability at temperatures between 15°C and 20°C (47). Maciejewska et al. investigated the effects of myovirus isolates KP15 and KP27 on multi-drug-resistant strains of *K. pneumoniae* and *K. oxytoca*. Molecular analysis showed that KP27 phages were insensitive to endonuclease digestion (48). Pallavali et al. isolated and identified the bacteriophages that were effective against MDR bacterial isolates from septic ulcer infections, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *K. pneumoniae*. The antibacterial effect of phage was detected using two-layer agar (overlay) method. Phage PA DP4 was effective on *Pseudomonas aeru-*

ginosa, phage KP DP1 was effective on *K. pneumoniae*, phage SA DP1 was effective on *Staphylococcus aureus* and phage EC DP3 was effective on *Escherichia coli*. The Gram-positive isolates were 100% MDR, while the Gram-negative isolates were 86% drug resistant. The results of that study also demonstrated that studied phages can be a good choice for prophylaxis against septic wound. The results of this study were almost consistent with our study (49). Cao et al. showed an effect of the phage KP 1513 on multidrug resistant *K. pneumoniae* (MRKP). Phage 1513, had strong stability under different pH and temperature. It produced a clear plaque with a halo and was classified as *Siphoviridae*. It had a short latent period of 30 min, a burst size of 264 and could inhibit *K. pneumoniae* 1513 growth *in vitro* with a dose-dependent pattern. The type of the phage was consistent with the phage isolated in our report in terms of family and bacterial host (50). Komijani et al. (2017) detected a new lytic bacteriophage named VB- KpneM-Isf48 against *K. pneumoniae* carrying antibiotic-resistant genes (TEM, SHV and CTX M) which isolated from infected wounds. The host range, morphology, thermal, pH, saline stress viability, and molecular identification of the bacteriophage were evaluated. In that study, 41 strains of *K. pneumoniae* were isolated from 193 infected wounds over a two-year period in three hospitals in Isfahan. Phenotypic and genotypic methods were used to recognize ESBL strains. A total of 36 from 41 isolates had one or more antibiotic resistant coding genes. The results demonstrated that the difference between phenotypic and genotypic identification methods was significant. Morphological features of phage placed it in the *Myoviridae* family and 38 of 41 clinical isolates were susceptible to the bacteriophage (51). Furthermore, Horváth et al. (2020) isolated and characterized a lytic bacteriophage, vB_KpnS_Kp13, which was effective against metallo- β -lactamase producing *K. pneumoniae* isolates originating from hospital samples (urine, blood, sputum and feces). Morphological characterization of vB_KpnS_Kp13 showed that the newly identified phage belonged to the *Siphoviridae* family, and phylogenetic analysis showed that it is a part of a distinct clade of the *Tunavirinae* subfamily. Functional analysis revealed that vB_KpnS_Kp13 had relatively short latent period time (18 minutes) compared to the other bacteriophages *K. pneumoniae*. In our study, the isolated phages belonged to the *Siphoviridae* and *podoviridae* families, although had

high latent period times (52). Zurabov and Zhilenkov (2021) identified the morphology of four phages by TEM electron microscopy and investigated; their host range, lytic activity, adsorption rate, burst size, latency period, and temperature and pH of the activity. The genomes of all isolated bacteriophages were studied through digestion by restriction endonucleases including *HindIII*, *HinfI*, *HaeIII*, *SspI*, *BamHI*, *EcoRV*, *NotI*, *EcoRI*, *KpnI*, *MspI*, *VspI*, *NdeI*, *BglII*, *BglIII*, *PvuI*, and *SmaI*. The 4 lytic bacteriophages belonged to *Siphoviridae*, *Myoviridae* and *Podoviridae* families. Studied phages showed wide host range and great viability at various temperature and pH degrees (53), that consistent with our study. In our study, analysis of the restriction enzymes digestion pattern demonstrated that PφBw-Kp1 phage was susceptible to *Hind III*, *Bam HI* and *KpnI* enzymes and the partial digest obtained from enzymatic digestion. PφBw-Kp2 was sensitive to all of the restriction enzymes and phage PφBw-Kp3 was sensitive to *HindIII* and *EcoRI* enzymes.

CONCLUSION

In this study, we proposed an effective antibacterial agent for burn wounds infections using novel phages against ESBL-producing *K. pneumoniae*. The effect of bacteriophages against ESBL promises a dramatic success for phage therapy. It is believed that these three isolated bacteriophages, in the form of phage cocktails, can also have a significant effect on other closed ESBL strains. Each of the isolated phages alone had a good antibacterial effect, but if used as a cocktail, they would create a broad-spectrum strategy. It is hoped that by conducting different complementary studies and evaluating the effectiveness of phages, they can be used clinically as effective therapeutic agent against ESBL-producing *K. pneumoniae* in burn patients.

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REFERENCES

- Galeiras R, Lorente JA, Pértega S, Vallejo A, Tomicic V, de la Cal MA, et al. A model for predicting mortality among critically ill burn victims. *Burns* 2009; 35: 201-209.
- Bloemsma GC, Dokter J, Boxma H, Oen IM. Mortality and causes of death in a burn centre. *Burns* 2008; 34: 1103-1107.
- Panjeshahin MR, Lari AR, Tali A, Shamsnia J, Alaghehbandan R. Epidemiology and mortality of burns in the south west of Iran. *Burns* 2001; 27: 219-226.
- D'Avignon LC, Hogan BK, Murray CK, Loo FL, Hospenthal DR, Cancio LC, et al. Contribution of bacterial and viral infections to attributable mortality in patients with severe burns: an autopsy series. *Burns* 2010; 36: 773-779.
- Mayhall CG. The epidemiology of burn wound infections: then and now. *Clin Infect Dis* 2003; 37: 543-550.
- Bennett JW, Robertson JL, Hospenthal DR, Wolf SE, Chung KK, Mende K, et al. Impact of extended spectrum beta-lactamase producing *Klebsiella pneumoniae* infections in severely burned patients. *J Am Coll Surg* 2010; 211: 391-399.
- Hubab M, Maab H, Hayat A, Ur Rehman M. Burn wound microbiology and the antibiotic susceptibility patterns of bacterial isolates in three burn units of Abbottabad, Pakistan. *J Burn Care Res* 2020; 41: 1207-1211.
- Gupta M, Naik AK, Singh SK. Bacteriological profile and antimicrobial resistance patterns of burn wound infections in a tertiary care hospital. *Heliyon* 2019; 5(12): e02956.
- Vahedi A, Soltan Dallal MM, Douraghi M, Nikkhahi F, Rajabi Z, Yousefi M, et al. Isolation and identification of specific bacteriophage against enteropathogenic *Escherichia coli* (EPEC) and *in vitro* and *in vivo* characterization of bacteriophage. *FEMS Microbiol Lett* 2018; 365(16): fny136.
- Herridge WP, Shibu P, O'Shea J, Brook TC, Hoyles L. Bacteriophages of *Klebsiella* spp., their diversity and potential therapeutic uses. *J Med Microbiol* 2020; 69: 176-194.
- Abedon ST (2008). Bacteriophage ecology: population growth, evolution, and impact of bacterial viruses. *Advances in Molecular and Cellular Microbiology*. 1st ed. Cambridge University Press. Cambridge and New York.
- Hemminga MA, Vos WL, Nazarov PV, Koehorst RB, Wolfs CJ, Spruijt RB, et al. Viruses: incredible nano-

- machines. new advances with filamentous phages. *Eur Biophys J* 2010; 39: 541-550.
13. Fernández L, Gutiérrez D, García P, Rodríguez A. The perfect bacteriophage for therapeutic applications—a quick guide. *Antibiotics (Basel)* 2019; 8: 126.
 14. Donlan RM. Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends Microbiol* 2009; 17: 66-72.
 15. Kropinski AM. Measurement of the bacteriophage inactivation kinetics with purified receptors. *Methods Mol Biol* 2009; 501: 157-160.
 16. Barbu EM, Cady KC, Hubby B. Phage therapy in the era of synthetic biology. *Cold Spring Harb Perspect Biol* 2016; 8(10): a023879.
 17. Church D, Elsayed S, Reid O, Winston B, Lindsay R. Burn wound infections. *Clin Microbiol Rev* 2006; 19: 403-434.
 18. Palmeiro JK, de Souza RF, Schörner MA, Passarelli-Araujo H, Grazziotin AL, Vidal NM, et al. Molecular epidemiology of multidrug-resistant *Klebsiella pneumoniae* isolates in a Brazilian tertiary hospital. *Front Microbiol* 2019; 10: 1669.
 19. Gootz TD. The global problem of antibiotic resistance. *Crit Rev Immunol* 2010; 30: 79-93.
 20. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* 2005; 18: 657-686.
 21. Amirmozafari N, Tehrani HF, Tavaf Langeroodi Z, Abdullahi A. Survey of drug resistance due to extended spectrum β -lactamases in *Klebsiella pneumoniae* strains isolated from hospitalized patients. *Res Med* 2007; 31: 241-245.
 22. Feizabadi MM, Mahamadi-Yeganeh S, Mirsalehian A, Mirafshar SM, Mahboobi M, Nili F, et al. Genetic characterization of ESBL producing strains of *Klebsiella pneumoniae* from Tehran hospitals. *J Infect Dev Ctries* 2010; 4: 609-615.
 23. Lee CH, Su LH, Tang YF, Liu JW. Treatment of ESBL-producing *Klebsiella pneumoniae* bacteraemia with carbapenems or flomoxef: a retrospective study and laboratory analysis of the isolates. *J Antimicrob Chemother* 2006; 58: 1074-1077.
 24. Keşik-Szeloch A, Drulis-Kawa Z, Weber-Dąbrowska B, Kassner J, Majkowska-Skrobek G, Augustyniak D, et al. Characterising the biology of novel lytic bacteriophages infecting multidrug resistant *Klebsiella pneumoniae*. *Virology* 2013; 10: 100.
 25. Agnihotri N, Gupta V, Joshi RM. Aerobic bacterial isolates from burn wound infections and their antibiograms—a five-year study. *Burns* 2004; 30: 241-243.
 26. Turner PJ. Extended-spectrum beta-lactamases. *Clin Infect Dis* 2005; 41 Suppl 4: S273-275.
 27. Bandekar N, Vinodkumar CS, Basavarajappa KG, Prabhakar PJ, Nagaraj P. Beta lactamases mediated resistance amongst gram-negative bacilli in burn infection. *Int J Biol Med Res* 2011; 2: 766-770.
 28. Shrestha S, Amatya R, Dutta R. Prevalence of extended spectrum beta lactamase (ESBL) production in gram-negative isolates from pyogenic infection in tertiary care hospital of eastern Nepal. *Nepal Med Coll J* 2011; 13: 186-189.
 29. Naghavi NS, Golgoljam M, Akbari M. Effect of three sewage isolated bacteriophages on the multi drug resistant pathogenic bacteria. *J Biol Sci* 2013; 13: 422-426.
 30. Cheng F, Li Z, Lan S, Liu W, Li X, Zhou Z, et al. Characterization of *Klebsiella pneumoniae* associated with cattle infections in southwest China using multi-locus sequence typing (MLST), antibiotic resistance and virulence-associated gene profile analysis. *Braz J Microbiol* 2018; 49 Suppl 1(Suppl 1): 93-100.
 31. Gharavi MJ, Zarei J, Roshani-Asl P, Yazdanyar Z, Sharif M, Rashidi N. Comprehensive study of antimicrobial susceptibility pattern and extended spectrum beta-lactamase (ESBL) prevalence in bacteria isolated from urine samples. *Sci Rep* 2021; 11: 578.
 32. Clinical and Laboratory Standards (CLSI). Development of in vitro susceptibility testing criteria and quality control parameters for veterinary antimicrobial agents; approved guideline- 3rd Ed. CLSI document M37-A3. Clinical and Laboratory Standards Institute. Wayne, Pennsylvania. USA. 2008.
 33. Linscott AJ, Brown WJ. Evaluation of four commercially available extended-spectrum beta-lactamase phenotypic confirmation tests. *J Clin Microbiol* 2005; 43: 1081-1085.
 34. Rudresh SM, Nagarathnamma T. Extended spectrum β -lactamase producing Enterobacteriaceae & antibiotic co-resistance. *Indian J Med Res* 2011; 133: 116-118.
 35. Mendes JJ, Leandro C, Mottola C, Barbosa R, Silva FA, Oliveira M, et al. *In vitro* design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections. *J Med Microbiol* 2014; 63: 1055-1065.
 36. Ghasemi SM, Bouzari M, Emtiazi G. Preliminary characterization of *Lactococcus garvieae* bacteriophage isolated from wastewater as a potential agent for biological control of lactococcosis in aquaculture. *Aquacult Int* 2014; 22: 1469-1480.
 37. Beheshti Maal K, Soleimani Delfan A, Salmanizadeh S. Isolation and identification of two novel *Escherichia coli* bacteriophages and their application in wastewater treatment and coliform's phage therapy. *Jundishapur J Microbiol* 2015; 8(3): e14945.
 38. Ács N, Gambino M, Brøndsted L. Bacteriophage enumeration and detection methods. *Front Microbiol* 2020; 11: 594868.
 39. Carroll-Portillo A, Coffman CN, Varga MG, Alcock J, Singh SB, Lin HC. Standard bacteriophage purification

- procedures cause loss in numbers and activity. *Viruses* 2021; 13: 328.
40. Krupovic M, Dutilh BE, Adriaenssens EM, Wittmann J, Vogensen FK, Sullivan MB, et al. Taxonomy of prokaryotic viruses: update from the ICTV bacterial and archaeal viruses subcommittee. *Arch Virol* 2016; 161: 1095-1099.
 41. Rahimzadeh Torabi L, Doudi M, Naghavi NS, Monajemi R. Isolation, characterization, and effectiveness of bacteriophage P ϕ -Bw-Ab against XDR *Acinetobacter baumannii* isolated from nosocomial burn wound infection. *Iran J Basic Med Sci* 2021; 24: 1254-1263.
 42. Sillankorva S, Neubauer P, Azeredo J. *Pseudomonas fluorescens* biofilms subjected to phage phiIBB-PF7A. *BMC Biotechnol* 2008; 8: 79.
 43. O'Flaherty S, Coffey A, Edwards R, Meaney W, Fitzgerald GF, Ross RP. Genome of staphylococcal phage K: a new lineage of *Myoviridae* infecting gram-positive bacteria with a low G+C content. *J Bacteriol* 2004; 186: 2862-2871.
 44. Shang Y, Sun Q, Chen H, Wu Q, Chen M, Yang S, et al. Isolation and characterization of a novel *Salmonella* phage vB_SalP_TR2. *Front Microbiol* 2021; 12: 664810.
 45. Karumidze N, Kusradze Ia, Rigvava S, Goderdzishvili M, Rajakumar K, Alavidze Z. Isolation and characterisation of lytic bacteriophages of *Klebsiella pneumoniae* and *Klebsiella oxytoca*. *Curr Microbiol* 2013; 66: 251-258.
 46. Wintachai P, Naknaen A, Thammaphet J, Pomwiset R, Phaonakrop N, Roytrakul S, et al. Characterization of extended-spectrum- β -lactamase producing *Klebsiella pneumoniae* phage KP1801 and evaluation of therapeutic efficacy *in vitro* and *in vivo*. *Sci Rep* 2020; 10: 11803.
 47. Park EA, Kim YT, Cho JH, Ryu S, Lee JH. Characterization and genome analysis of novel bacteriophages infecting the opportunistic human pathogens *Klebsiella oxytoca* and *K. pneumoniae*. *Arch Virol* 2017; 162: 1129-1139.
 48. Maciejewska B, Roszniowski B, Espaillet A, Keşik-Szeloch A, Majkowska-Skrobek G, Kropinski AM, et al. *Klebsiella* phages representing a novel clade of viruses with an unknown DNA modification and biotechnologically interesting enzymes. *Appl Microbiol Biotechnol* 2017; 101: 673-684.
 49. 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.
 50. Cao F, Wang X, Wang L, Li Z, Che J, Wang L, et al. Evaluation of the efficacy of a bacteriophage in the treatment of pneumonia induced by multidrug resistance *Klebsiella pneumoniae* in mice. *Biomed Res Int* 2015; 2015: 752930.
 51. Komijani M, Bouzari M, Rahimi F. Detection and characterization of a novel lytic bacteriophage (vBK-pneM-Isf48) against *Klebsiella pneumoniae* isolates from infected wounds carrying antibiotic resistance genes (TEM, SHV, and CTX-M). *Iran Red Crescent Med J* 2017; 19.
 52. Horváth M, Kovács T, Koderivalappil S, Abraham H, Rakhely G, Schneider G. Identification of a newly isolated lytic bacteriophage against K24 capsular type, carbapenem resistant *Klebsiella pneumoniae* isolates. *Sci Rep* 2020; 10: 5891.
 53. Zurabov F, Zhilenkov E. Characterization of four virulent *Klebsiella pneumoniae* bacteriophages, and evaluation of their potential use in complex phage preparation. *Virol J* 2021; 18: 9.