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

Ladan Rahimzadeh Torabi¹, Nafiseh Sadat Naghavi², Monir Doudi¹, Ramesh Monajemi²

¹Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
²Department of Biology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran

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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* laufa_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 PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3. Morphological characterization demonstrated that the phage PφBw-Kp3 to the *Siphoviridae* family (lambda-like phages) and both phages PφBw-Kp1 and φBw-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

¹Corresponding author: Nafiseh Sadat Naghavi, Ph.D, Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran. Tel: +98-3137420134 Fax: +98-3137420136 Email: nafiseh_naghavy@yahoo.com

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serious problem caused by antibiotic resistant bacteria (9, 10). These viral particles, with a variety of about $10^{10}$ to $10^{12}$ 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* have 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$_2$S 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’AGAGTTTGTATCCTGGCTCAG3’) and 1492R (5’ACGGCTACCTTGTACGACTTT3’) (Metabione, 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 μg ciprofloxacin (CPX), 30 μg ceftazidime (CAZ), and 30 μg 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, cefotaxime and cefazolin 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 μg) 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-A.30 K3, Germany) for 15-20 min at 4°C. Then, 10 mL of each sample was filtered by a 0.22 μm membrane filter (Cobetter, Japan) and the prepared filtrate was mixed with 100 μL of each bacterial culture and 50 mL of 2x 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. 7H₂O in 1 L distilled water) until obtain the concentrated phages. Finally, the concentrated bacteriophages were separated by using 0.45 μm 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 × 10⁶ 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 μL 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 (10⁴ 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 × 10⁶ 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 10⁸ 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, Iaefa_lad1, Iaefa_lad2, and Iaefa_lad3. For this purpose, 0.1 mL of overnight grown bacterial culture (1.5 × 10⁶ 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 μL of each dilution prepared from phage suspen-
sion (10^6 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 μL of the high titer phage suspension (≥10^6 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 μL 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 (10^1-10^6 PFU/mL) was applied on BHI agar medium comprising 5 mL overnight grown cultured bacteria (10^5 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 (10^6 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 μg/mL DNase I and RNase A (Fermentas, America) for 20 min and incubated at 37°C, afterward passed through 0.22 μm membrane filter. The mixtures were centrifuged at 28000 g for 60 min. Phage DNA extraction (≥10^6 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, Kpnl, Hael, HindIII, BamHI*, and *Xhol* (Fermentas, 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 μL of each bacteriophage sample was mixed with 5 μL loading buffer comprising (50 mM Tris-HCl [pH 6.8], 2% SDS, glycerol 10%, petethanol 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 ± 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 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 PφBw-Kp1, PφBw-Kp2, and PφBw-Kp3 were isolated from raw sewage inlet by double layered method. Observation of the phage's morphology by TEM showed that the phage PφBw-Kp3 which had a narrow long tail with a small head is similar to the family Siphoviridae. Both phages PφBw-Kp1 and PφBw-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 leaded 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 PφBw-Kp1, PφBw-Kp2 and PφBw-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 PφBw-Kp2 (Table 3).

| Characteristics            | Biochemical tests                        |
|-----------------------------|------------------------------------------|
| -                           | Gram staining                            |
| Rods                        | Cell shape                               |
| Acid/Acid/H2S -             | 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                            |

Klebsiella pneumoniae Identified species

*: Positive results; <: Negative results

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* Isfahan 2 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 |
|----------|----------|----------|---------------------------|
| **Podoviridae** | **Podoviridae** | **Siphoviridae** | Order |
| A non-contractile short tail | A non-contractile short tail | A narrow long tail | Family Shape |
| Head: 55 nm, Tail: 20 nm | Head: 60 nm, Tail: 10 nm | Clear | (TEM) Plaque |
| T1-like phages | T1-like phages | Head: 30 nm, Tail: 350 ± 10 nm | shape Size |
| Wastewater treatment plant, north of Isfahan | Wastewater treatment plant, south of Isfahan | lambda-like phages | (nm) |
| 33 × 10\(^6\) | 27 × 10\(^6\) | 35 × 10\(^6\) | Prototype |
| | | | Sampling area |
| | | | 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 re- 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 laufa_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 laufa_lad2 was showed.

Table 3. Spot testing and investigation of phage sensitivity

| Strain                                      | Source                      | Spot test result |
|---------------------------------------------|-----------------------------|------------------|
| *K. pneumoniae* laufa_lad1                  | Clinical samples; Culture collection | -                |
| *K. pneumoniae* subsp. pneumoniae laufa_lad2 ESBL (+) | Clinical samples; Culture collection | +                |
| *K. pneumoniae* laufa_lad3                  | Clinical samples; Culture collection | -                |
| *K. pneumoniae* TM07                        | Clinical samples; Culture collection | -                |
| *K. pneumoniae* VRC10                       | Clinical samples; Culture collection | -                |
| *K. pneumoniae* ATCC 10031                  | Institute Passasure 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 PɸBw-Kp1, PɸBw-Kp2 and PɸBw-Kp3, on ESBL producing *K. pneumoniae* laufa_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 PɸBw-Kp1, PɸBw-Kp2, and PɸBw-Kp3 in various pH values for 1 h at 37°C (One-way ANOVA P ≥ 0.05; F = 0.120) (A). Adsorption rate of the bacteriophages PɸBw-Kp1, PɸBw-Kp2, and PɸBw-Kp3 at various times for 1 h (P ≥ 0.05; F = 0.0003) (B). Temperature stability of bacteriophages PɸBw-Kp1, PɸBw-Kp2, and PɸBw-Kp3 at several temperatures for 1 h (P ≥ 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 Iaufa_lad2. One-way ANOVA displayed that a significant difference was observed in each chart (P < 0.0001). The latency period of PɸBw-Kp1 was 20 min and the burst time was 80 min. The latency period of PɸBw-Kp2 was 55 min and the burst size was 80 min. The latency period of PɸBw-Kp3 was 40 min and the burst time was 90 min. The data show the mean ± SD (P value < 0.0001 for all phage groups). Identical results were obtained in 3 repetitions.
the phage PbBw-Kp1 was digested by *Hind III*, *Bam HI*, and *KpnI* enzymes, the DNA of the phage PbBw-Kp2 was digested by all used restriction enzymes, and the phage PbBw-Kp3 was digested by *Hind III* and *EcoRI* enzymes (Fig. 6).

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

**DISCUSSION**

The present study attempted to evaluate the effectiveness of phages to inhibit burn wound infecting *K. pneumoniae* including an ESBL producing strain, lauf0_lad2, which showed significant sensitivity to three phages including PbBw-Kp1, PbBw-Kp2, and PbBw-Kp3. TEM results showed that PbBw-Kp3 to the *Siphoviridae* (lambda-like phages), and that both phages PbBw-Kp1 and PbBw-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. Kesik-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 found in T7-like phage (KP32). KP34 genome was digested by all used endonucleases in their research (*EcoRV, EcoRI, HindIII, Nsil, Ncol, Pael*, 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...
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 belonging 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 was 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 adsorption 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 aero-

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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, BgIII, PvuI, and Smal. 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 HindIII, BamHI 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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