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Detection and Characterization of a Novel Lytic Bacteriophage (vB-KpneM-Isf48) Against *Klebsiella pneumoniae* Isolates from Infected Wounds Carrying Antibiotic-Resistance Genes (*TEM*, *SHV*, and *CTX-M*)

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Abstract

Background: Approximately 80% of nosocomial infections are caused by strains of *Klebsiella pneumoniae*. Resistance to β-lactam antibiotics is a result of expression of extended-spectrum β-lactamase (ESBL) genes. Recently, phage therapy has gained increasing attention due to its many advantages over chemotherapy.

Objectives: The aim of this study was to isolate ESBL-positive *Klebsiella pneumoniae* strains from different types of wounds, and a lytic bacteriophage against them.

Methods: During a two-year period from January 2013 to February 2015, in a cross-sectional study, 41 *K. pneumoniae* strains were isolated from 193 categories of infected wounds at three hospitals in Isfahan, Iran. Phenotypic and genotypic methods were used to detect the ESBL-positive strains. A lytic phage against *K. pneumoniae* was isolated, and its host range, morphology, thermal and pH stability, saline stress, and estimated genome size were determined.

Results: Of the 41 *K. pneumoniae* isolates, 18 were ESBL-producing and 36 carried antibiotic-resistance genes. A total of 36 out of 41 isolated samples carried one or more resistance genes. The results showed that the differences between phenotypic and genotypic identification methods were significant (P = 0.0001). The *SHV*, *CTX-M*, and *TEM* genes were detected in 29, 10, and 9 isolates of the tested bacteria, respectively. No bacteria contained both the *SHV* and the *CTX-M* genes. The frequency of the *SHV* gene was significantly higher than that of the other genes (P = 0.0001). The phage's morphology features placed it in the *Myoviridae* family. Only 38 out of 41 clinical isolates were susceptible to the phage. Phage titers were completely preserved after one hour of incubation at 30°C and 40°C, and they were stable at different pH values. The phage's survival decreased when the salt concentration was increased.

Conclusions: The high rate of isolation of antibiotic-resistant strains of *K. pneumoniae* was consistent with other studies. As the phage was virulent and specific for *K. pneumoniae*, and was stable and active at different pH values, salt concentrations, and temperatures, its application in phage therapy of infected wounds is suggested.

Keywords: Antibiotic Resistance, Bacteriophage, *Klebsiella pneumoniae*

1. Background

Klebsiella pneumoniae is an opportunistic nosocomial pathogen belonging to the *Enterobacteriaceae* family. It causes a variety of infections, including urinary tract infections and septicemia, in intensive care units. It also causes wound infections (1).

Approximately 80% of nosocomial infections are caused by multidrug-resistant (MDR) strains of *K. pneumoniae* (2). Since 1980, third-generation cephalosporins, e.g. cefotaxime, ceftazidime, and ceftriaxone, have been introduced as useful and effective drugs against most nosocomial infections (3). However, the excessive use of cephalosporins in clinical practice has resulted in increased bacterial resistance to these antibiotics, especially by *Enterobacteriaceae* (4). Resistance to β-lactam antibiotics among the *Enterobacteriaceae* family is a result of the expression of extended-spectrum β-lactamase (ESBL) genes (5). Among the members of *Enterobacteriaceae*, *K. pneumoniae* has the highest level of ESBLs (6). Moreover, studies have shown that the most prevalent ESBL-producing bacterium in Pacific Asia, Europe, and North America is *K. pneumoniae*. ESBLs arise mainly due to mutations in β-lactamases encoded by the *SHV*, *TEM*, and *CTX-M* genes (3, 7). The genes encoding β-lactamase enzymes are located on transferable plasmids (8). ESBL-producing *K. pneumoniae* are usually resistant to common antibiotics. Therefore, treatments for these infections are limited to a few expensive antibiotics, which in most cases are not available in developing countries (4).

Recently, researchers have been searching for alternative methods to control these infections, including combination therapy with herbal extracts, natural compounds, small molecules, and bacteriophages, in an attempt to reduce the incidence of antibiotic resistance in

ESBL-producing bacteria (9-11).

Phage therapy has garnered increasing attention due to its many advantages over chemotherapy. Phages have a high specificity for their target bacteria, indicating that they do not disturb or harm the normal flora; they also lack any side effects and are reproducible (12-14). Carson et al. (2010) isolated lytic bacteriophages and used them for the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli* with 99% efficacy (13). Lytic phage 1513, which was isolated using a clinical isolate of *K. pneumoniae*, was evaluated in the treatment of pneumonia induced by MDR *K. pneumoniae* in mice; this protected the mice against lethal pneumonia (15). Jensen et al. (2015) isolated a new lytic phage against a methicillin-resistant isolate of *Staphylococcus aureus* (MRSA), and used it as an effective tool to decontaminate human MRSA from both fabrics and hard surfaces (16).

2. Objectives

The aim of this study was to isolate and identify ESBL-producing *K. pneumoniae* strains from different types of wounds using phenotypic and genotypic methods, and to isolate and characterize a lytic phage against them.

3. Methods

In this cross-sectional study, 193 infected wounds (with viscous, yellowish-white fluid, i.e., pus), including 58 diabetic foot ulcers, 56 bedsores, 41 surgical wounds, and 38 miscellaneous wounds, were tested for the presence of *K. pneumoniae* at three hospitals in Isfahan, Iran, from January 2013 to February 2015. Burn and clean wounds were excluded. The basic clinical data for each patient, including wound source, gender, and age, were recorded. Written informed consent was obtained from each participant before inclusion in the study. This study was approved by the ethics committee of the Isfahan University of Medical Sciences (November 2015; Code: 494084).

Sterile swabs were placed into sterile enrichment broth medium (Scharlau, Spain), and transferred to the laboratory. The samples were cultured in blood and EMB agars (Scharlau, Spain), and incubated at 37°C for 24 hours. *K. pneumoniae* strains were identified by individual colony morphology, gram staining, and biochemical assays (urease, indole, citrate, methyl red, and TSI, all from Scharlau, Spain), Voges-Proskauer, and motility (17).

3.1. Antimicrobial Susceptibility

All isolated *K. pneumoniae* strains were tested for susceptibility to amikacin (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), cefazolin (30 μg), tetracycline (30 μg), nalidixic acid (30 μg), and nitrofurantoin (300 μg), by the Kirby-Bauer disk diffusion method (Mast, United Kingdom) according to CLSI (Clinical and Laboratory Standards Institute, 2012) guidelines. After overnight incubation at 37°C, the diameters of the zones of inhibition around the discs were recorded and compared with the interpretive criteria recommended in the CLSI guidelines (18).

3.2. Detection of ESBL-Producing K. pneumoniae

ESBL-producing bacteria were detected using the method of combined antibiotic disks. Briefly, ceftazidime (30 μg) and ceftazidime + clavulanic acid (30 μg/10 μg) discs were placed at a distance of 25 mm on a Mueller-Hinton agar (Scharlau, Spain). The plates were inoculated with a bacterial suspension of 0.5 McFarland turbidity standard, and incubated overnight at 37°C. A $\geq$ 5 mm increase in the diameter of the inhibition zone for the combination disc versus the ceftazidime disc confirmed ESBL production (3).

3.3. DNA Extraction

Bacterial genomic DNA extraction was performed using the boiling method. Briefly, two colonies of pure isolated bacteria were placed into a tube containing 100 μL of double-distilled water, and were then heated at 100°C for 10 minutes. The cells were then pelleted by centrifugation, and the supernatant containing DNA was stored at -20°C until tested (19).

For phage DNA extraction, 150 mL of enriched phage suspension (10^8 PFU) was centrifuged at 20,000 g for 90 minutes. The concentrated phage suspension was treated with DNaseI (CinnaGen, Iran). The phage DNA was extracted using the SDS/proteinase K method. Briefly, 300 μL of the concentrated phage suspension was added to 0.5 mg/mL of proteinase K (Fermentas, Germany) in the presence of 0.2 M NaCl (Merck, Germany) and 0.25% sodium dodecyl sulfate (SDS) (Merck, Germany), then incubated at 65°C for 30 minutes. The DNA was then extracted with phenol (CinnaGen, Iran), chloroform (Merck, Germany), isoamyl alcohol (Merck, Germany), and ethanol (Merck, Germany) precipitation. The pellet was air-dried and dissolved in double-distilled water (20).

3.4. Primers Used

Previously designed primers for detection of *SHV* (F: GATGAACGCTTTCCCATGATG, R: CGCTGTTATCGCTCATGGTAA, 214 bp) (21), *TEM* (F: ATGAGTATTCAACATTTCCG, R: CTGACAGTTACCAATGCTTA, 867 bp) (22), and *CTX-M* (F: TTTGCGATGTGCAGTACCAGTAA, R: CGATATCGTTGGTGGTGCCATA, 590 bp) (23) genes were used to identify

K. pneumoniae. Also, by comparing the full genome sequences of *K. pneumoniae* in the NCBI database (http://www.ncbi.nlm.nih.gov/), the transcriptional regulator *HdfR* gene was recognized as a specific marker for the molecular detection of *K. pneumoniae*. Oligo7 software was used to design new primers for this region, and the most appropriate primers were selected by standard scales (melting temperature, GC content, primer secondary structures, etc.). The specificity of the chosen primers (F: ATCATCGCTTCCTATCAGTCCC, R: ACCCGTCATCGTAATAACATCCG, 456 bp) was analyzed by online primer-BLAST in the NCBI database.

3.5. PCR

HdfR, *SHV*, *TEM*, and *CTX-M* β-lactamase genes were detected by PCR. Thermal cycling conditions for all PCR primer sets were as follows: 95°C for 5 minutes; 30 cycles at 95°C for 30 seconds, 58°C for 60 seconds, and a 72°C for 45 seconds; then 72°C for 5 minutes. DNA was amplified by PCR performed in a total volume of 25 μL, containing 10 pmol of each primer, 25 μmol of dNTPs (CinnaGen, Iran), 1 μL of template DNA, 2.5 μL of 10X Taq buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3)) (CinnaGen, Iran), 2 mM of $MgCl_2$ (CinnaGen, Iran,) and 2 units of Smar Taq DNA polymerase (CinnaGen, Iran). The PCR products (10 μL) were then analyzed by agarose gel electrophoresis (Sigma-Aldrich, USA).

3.6. DNA Sequencing and Analysis

Sequencing was performed on a randomly selected *HdfR* amplification product (Macrogen, South Korea). A WU-BLAST-2 search of the determined sequences against a nucleotide sequence database (EMBL, European Bioinformatics Institute) was performed. This sequence was recorded with accession number KT321530 in GenBank.

3.7. Phage Isolation, Purification, and Preparation of Concentrated Stocks

To isolate and enrich the bacteriophage, a standard method was carried out with some modifications. Briefly, 50 ml of wastewater (from an urban wastewater treatment plant in Isfahan, Iran) was clarified by centrifugation at 8,000 g for 10 minutes at 4°C. Next, 50 mL of the supernatant was added to 50 ml of overnight-cultured *K. pneumoniae*, and the mixture was incubated at 37°C for approximately 24 hours with constant shaking (20 rpm). After incubation, to remove the residual bacterial cells, centrifugation (8,000 g for 10 minutes at 4°C) and filtration with a 0.22 μm syringe filter (Sartorius, India) was performed. To confirm the presence of bacteriophages, 100 μL of overnight-cultured *K. pneumoniae* was mixed at 45°C with 10 mL of soft BHI agar (Scharlau, Spain), and then overlaid on the surface of the solidified BHI agar (1.5% agar) and allowed to solidify for 30 minutes. Next, 10 μL of the filtrated medium was added to the surface with the spotting method, and incubated overnight. Following the appearance of a transparent zone (plaque), in order to purify the isolated phage, a single plaque was picked with a Pasteur pipette and dissolved in 1 mL of SM buffer (100 mM NaCl, 50 mM Tris (CinnaGen, Iran), and 10 mM of $MgSO_4$ [Merck, Germany]). For purification and determination of the phage titer, the phage suspension was serially diluted (10^{-1} - 10^{-10}) in SM buffer, and processed by the double-layer agar method, as described above. This procedure was repeated three times to ensure the purity of the isolated phage. The final phage suspension was stored at 4°C and used for further characterization (24).

3.8. Host Range

The host range of the phage was assessed within a range of gram-positive and gram-negative standard strains or clinical isolates. The standard and the isolates from the clinical case strains included *Escherichia coli* (ATCC 25922 and isolates from wound, urine, and diarrhea), *Shigella sonnei* (ATCC 12022 and isolates from a Shigellosis case), *Shigella flexneri* (isolated from a Shigellosis case), *Klebsiella pneumonia* (ATCC 7880 and isolates from wounds), *Pseudomonas aeruginosa* (ATCC 27853 and an isolate from a wound), *Enterobacter cloacae* (ATCC 23355 and an isolate from a wound), *Enterococcus faecalis* (ATCC 29212), *Proteus mirabilis* (an isolate from a wound), *Staphylococcus saprophyticus* (ATCC 15305), *Staphylococcus epidermidis* (an isolate from urine), *Staphylococcus aureus* (ATCC 35933 and an isolate from a wound), *Acinetobacter baumannii* (an isolate from a wound), *Enterobacter aerogenes* (ATCC 13048), and *Streptococcus pyogenes* (ATCC 19615). To test the susceptibility of the bacterial strains, spot testing was used. The spot tests were performed for each strain, and the plates were incubated overnight at 37°C, then checked for any plaque formation against an uninfected negative control.

3.9. Phage Characterization by Transmission Electron Microscopy (Phage Morphology)

Phage morphology was examined with transmission electron microscopy (Philips, Netherlands) of the purified phage particles. A very high titer (approximately 10^{10} PFU/mL) was added to the surface of a carbon-coated copper grid before negative staining with 2% (w/v) uranyl acetate (Sigma-Aldrich, USA). After air-drying, the stained grids were visualized at an accelerating voltage of 100 kV (24).

3.10. Determination of Phage Thermal Stability

The phage lysis activity against *K. pneumoniae* was assessed by preparing serial dilutions of the phage, and spot-testing at various temperatures (30, 40, 50, 60, and 70°C). Phage suspensions (10 μL) of each dilution (10^4 - 10^8 PFU) were spotted onto a bacterial lawn in soft agar after incubation at the respective temperatures for 1 hour. The plates were incubated overnight to examine the host cell lysis. According to Jin et al. (2012), the observation of a completely transparent zone of inhibition is considered the optimal temperature for antibacterial activity of the phage (25).

3.11. Determination of Phage Stability at Different pH Values

To evaluate the stability of the phage at different pH values (3, 4, 5, 6, 7, 8, 9, 10, and 11), phage suspensions were incubated at 37°C for 1, 3, 9, 12, and 24 hours. Next, 100 μL of phage suspension (10^8 PFU) was inoculated into 900 μL of each pH-adjusted SM buffer. Then, the titer of the active phage was determined with the overlay method (26).

3.12. Saline Stress

The stability of the free phage particles in a hypersaline environment was estimated by the incubation of 100 μL of phage (10^8 PFU) in various concentrations (1%, 3%, 6%, 9%, 11%, 15%, and 18%) of NaCl (900 μL). Then, serial dilutions of each NaCl concentration were prepared after 1, 3, 6, 12, and 24 hours, and the titer of the active phage was determined by the overlay method (27).

3.13. Adsorption Rate

The adsorption rate of the phage to the host bacterium was determined by adding 1 mL (10^8 PFU) of phage to 9 mL of overnight-cultured host bacterium (OD600). The phage-host mixture was kept at 37°C, and aliquots were collected at 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, and 30 minutes. Each aliquot was centrifuged at 8,000 g for 10 minutes to sediment the phages attached to the bacteria. The titer of the unabsorbed phages in supernatant was then measured with the double-layer method (28).

3.14. Estimation of Phage Genome Size

The purified nucleic acid was treated with EcoRI, BamHI, and HindIII restriction enzymes (Promega, Korea), based on the manufacturer's guidelines. The phage DNA was mixed with each of the endonuclease enzymes. After overnight incubation at 37°C, the cleaved nucleic acids were analyzed by electrophoresis in 0.7% (w/v) agarose gel. The approximate sizes of the digested DNA fragments of the genome were estimated with SequentiX Gel Analyzer software (Klein Raden, Germany).

3.15. Statistical Analyses

The data were analyzed using GraphPad Prism 6.1 software (GraphPad Software Inc., USA). Fisher's exact test was used for statistical analyses. P values of < 0.05 were considered to be significant.

4. Results

4.1. Bacterial Isolation and Characterization

Based on phenotypic and biochemical features, amplification, and sequence analysis of the *K. pneumoniae*-specific gene (*HdfR*), 41 bacterial isolates were identified as *K. pneumoniae*.

4.2. Antimicrobial Susceptibility Test

On antibiogram tests, resistance to amikacin (41.46%), cefepime (21.95%), cefotaxime (75.6%), ceftazidime (73.17%), cefazolin (87.8%), tetracycline (34.14%), nalidixic acid (24.39%), ciprofloxacin (26.82%), and nitrofurantoin (21.95%) was observed (Table 1). Most of the bacteria were resistant to cefazolin (87.8%), while most were sensitive to nitrofurantoin and cefepime (21.95%). Four out of 41 bacterial strains were resistant to all antibiotics tested in this study.

4.3. Phenotypic and Genotypic ESBL Detection

Of the 41 *K. pneumoniae* isolates, 18 were ESBL-producing and had been obtained from 18 clinical samples, including three from diabetic feet, three from bed sores, 10 from surgical wounds, and two from miscellaneous wounds. All 18 of the ESBL-producing *K. pneumoniae* isolates carried one, two, or three ESBL genes. Eighteen ESBL-negative samples carried antibiotic-resistance genes.

A total of 36 out of 41 isolated samples carried one or more resistance genes. The results showed that the differences between phenotypic and genotypic identification methods were significant (P = 0.0001).

No bacteria had both the *SHV* gene and the *CTX-M* gene. The *SHV*, *CTX-M*, and *TEM* genes were detected in 29, 10, and 9 bacterial isolates tested, respectively (Table 2). The frequency of the *SHV* gene was significantly higher than the other genes (P = 0.0001).

4.4. Phage Isolation

A virulent phage was recognized based on observation of clear plaques on double-layered plates. The isolated phage was designated as vB-KpneM-Isf48, according to Kropinski et al. (2009) (29).

Table 1. Patterns of Antimicrobial Susceptibility and ESBL Screening of the *K. pneumoniae* Isolates[a]

Antibiotic	Pattern of Antimicrobial Susceptibility			β-Lactamase Inhibitor	
	Sensitive	Intermediate	Resistant	ESBL$^+$	ESBL$^-$
Amikacin	10 (24.39)	14 (34.14)	17 (41.46)	-	-
Cefotaxime	5 (12.19)	5 (12.19)	31 (75.6)	-	-
Ceftazidime	7 (17.07)	4 (9.75)	30 (73.17)	-	-
Cefepime	31 (75.6)	1 (2.43)	9 (21.95)	-	-
Cefazolin	3 (7.31)	2 (4.87)	36 (87.8)	-	-
Ciprofloxacin	23 (56.09)	7 (17.07)	11 (26.82)	-	-
Nalidixic acid	29 (70.73)	2 (4.87)	10 (24.39)	-	-
Nitrofurantoin	27 (65.85)	5 (12.19)	9 (21.95)	-	-
Tetracycline	23 (56.09)	4 (9.75)	14 (34.14)	-	-
Ceftazidime/clavulanate	-	-	-	18 (43.9)	23 (56.09)

[a]Values are expressed as No. of isolates (%).

Table 2. Frequency of *SHV*, *CTX-M*, and *TEM* Genes in *K. pneumoniae* Isolates From Infected Wounds

Source of Bacteria	Number of Samples	ESBL Genes						
		SHV	*CTX-M*	*TEM*	*SHV* and *CTX-M*	*SHV* and *TEM*	*CTX-M* and *TEM*	*SHV* and *CTX-M* and *TEM*
Diabetic foot ulcer	8	4	0	1	0	0	0	1
Bedsore	10	3	1	1	0	0	0	3
Surgical wound	18	12	1	0	0	1	1	1
Miscellaneous	5	4	2	0	0	0	0	0
Total	41	23	4	2	0	1	1	5

4.5. Phage Morphology

Transmission electron microscopy showed that the isolated phage had a head 96 nm in width and 118.5 nm in length, a 55 nm tail, and a 54 nm endplate. Its morphological features placed it in the *Myoviridae* family (Figure 1).

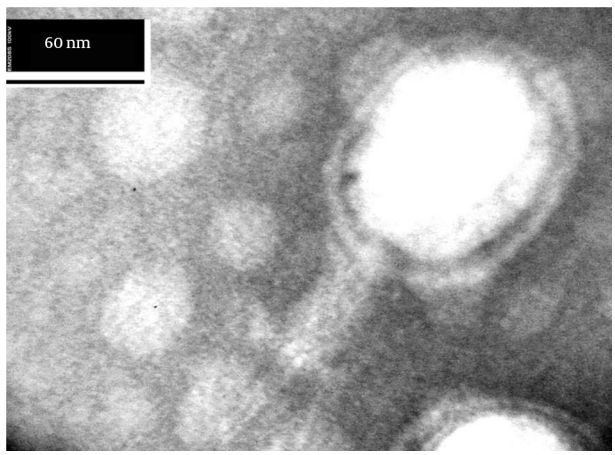


Figure 1. Electron Micrograph of the Phage vB-KpneM-Isf48 of the *Myoviridae* Family, With a Head Size of 95 × 118.5 nm, a Tail of 55 nm, and an Endplate of 54 nm, With Attached Fibers

4.6. Determination of Phage Host Range

Using the spot-test method, among all standard strains of the bacteria and clinical isolates of *K. pneumoniae* used to determine the host range of the phage, only *K. pneumoniae* (ATCC:7880) and 38 out of 41 clinical isolates were susceptible.

4.7. Thermal Stability

In spite of considerable reductions in the titer of the phage, full inactivation was observed only at 60°C and 70°C. High levels of activity of the phage were observed after 1 hour of incubation at 30°C to 40°C. Phage titers were completely preserved after 1 h of incubation at 30°C and 40°C.

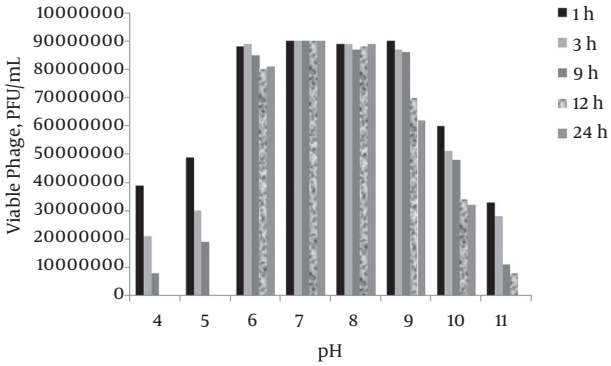


Figure 2. Stability of the Phage vB-KpneM-Isf48 at Different pH values at Different Time Intervals

4.8. pH Stability

The stability of the phage at different pH values (3, 4, 5, 6, 7, 8, 9, 10, and 11) is shown in Figure 2. The fewest changes in the titer of the phage were observed at pH values of 6 - 8. After 12 hours of incubation at pH 4 and 5, and also after 24 hours of incubation at pH 11, the titer of the phage reduced to zero.

4.9. Saline Stress

The stability of the phage at various saline concentrations was investigated by calculating the titer change over a period of 24 hours. Although full inactivation was not observed at different concentrations, the rate of phage survival decreased when the salt concentration was increased.

4.10. Adsorption Rate

In the first 4 minutes, the phage exhibited a very rapid adsorption to the bacteria (approximately 78%). The maximum adsorption (approximately 89%) was observed after incubation of the mixture for 25 minutes.

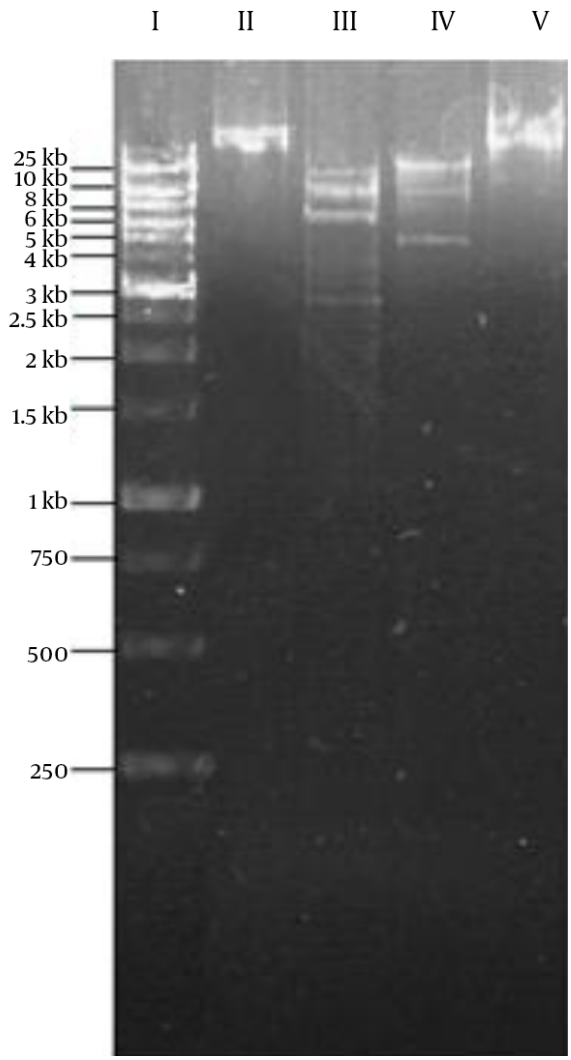
4.11. Estimation of the Phage Genome Size

The phage genomic DNA digestion patterns showed that the genomic DNA was digested by EcoRI and BamHI restriction enzymes, but not by HindIII. According to the data analyzed by the SequentiX Gel Analyzer, the size of the phage genome was approximately 39 kb (Figure 3).

5. Discussion

The uncontrolled use of chemical agents, especially antibiotics, for the treatment of infectious diseases has resulted in the emergence of highly antibiotic-resistant bacteria in the last few decades (30). Third-generation

Figure 3. Restriction Profile Analysis of the Phage vB-KpneM-Isf48 Genome



I, ladder; II, untreated phage DNA; III, EcoRI; IV, BamHI; V, HindIII.

cephalosporins are some of the most effective antibiotics used against bacterial infections (5). ESBLs play a very important role in the resistance to β-lactam antibiotics, via the destruction of the β-lactam cycle in the β-lactam antibiotic structure. The *CTX-M*, *SHV*, and *TEM* genes are the most common genes that encode ESBLs (5). The detection of ESBLs is based on phenotypic and molecular methods (31). In the current study, both phenotypic and molecular methods were examined for 41 *K. pneumoniae* isolates from different wounds. Among them, 18 (43.9%) and 36 (87.8%), respectively, were ESBL-positive by phenotypic and molecular methods. The difference observed between these two methods might be a result of the higher sensitivity of the molecular method compared to the phenotypic method, or the lack of expression of ESBL genes.

The pattern of antimicrobial susceptibilities was examined in all of the isolates, and indicated that 87.8%, 75.6%, 73.17%, 41.46%, 34.14%, 26.82%, 24.39%, 21.95%, and 21.95% of the isolates, respectively, were resistant to cefazolin, cefotaxime, ceftazidime, amikacin, tetracycline, ciprofloxacin, nalidixic acid, cefepime, and nitrofurantoin. Therefore, the highest resistance was observed for cefazolin and cefotaxime, while the highest sensitivity was recorded for cefepime and nitrofurantoin. Different antimicrobial susceptibility patterns have been reported in other studies; for example, Doosti et al. (2015) reported that 64.3%, 83.2%, and 85.1% of their isolates, respectively, were resistant to nalidixic acid, ceftazidime, and amikacin. Meanwhile, 60%, 63.3%, 40% and 6.7% of isolates were resistant to amikacin, cefotaxime and ceftazidime, cefepime, and ciprofloxacin, respectively, in a study by Ahmed et al. (2013) (3). In another study by Feizabadi et al. (2010), 65.1% and 61.7% of isolates were resistant to cefotaxime and ceftazidime, respectively (32). Moreover, the rates of resistance to antibiotics described by Nasehi et al. (2010) were 18% for ciprofloxacin and 17.5% for amikacin (33). In the present study, ESBLs were detected based on phenotypic and molecular methods by combined antibiotic disks and PCR, respectively. Among 41 isolates, 18 (43.9%) were positive with the combined antibiotic disc method. On the other hand, 36 (87.8%) were positive for at least one of the resistance genes using PCR. The dissimilarity (43.9%) between the results of these two different methods might be related to the higher sensitivity of the molecular method, as well as to the effect of environmental factors or culture conditions, which can temporarily downregulate some of the resistance genes. In addition, in the current study, the frequency of the genes encoding ESBLs were 29 (60.4%), 10 (28.8%), and 9 (18.7%) for *SHV*, *CTX-M*, and *TEM*, respectively. However, among 18 ESBL-positive isolates detected by combined antibiotic disks, the frequencies of *SHV*, *CTX-M*, and *TEM* were 13 (72.2%), 7 (38.9%), and 6 (33.3%), respectively. In a study by Wang et al. (2013), 84.3% and 50.4% of ESBL-positive *K. pneumoniae* carried *SHV* and *TEM* genes, respectively, which was nearly the same as our results (34). On the other hand, in a study by Nasehi et al. (2010), the rates for *SHV*, *CTX-M*, and *TEM* were 26%, 24.5%, and 18%, respectively, which were much lower than our results, especially for the *SHV* gene (33). The differences between our phenotypic and genotypic results and the frequency of resistance genes, and the results obtained in other studies, may be due to different geographical locations and to the number and source of the isolated bacteria. On the other hand, our results, showing a high rate of isolation of antibiotic-resistant strains of *K. pneumoniae*, was consistent with other studies. This shows that the rates of resistant *K. pneumoniae* from different sources, especially in wounds, are high enough to interrupt effective antibiotic therapy. This also draws attention to the use of alternative ways to treat these bacterial infections.

Due to the emergence of MDR bacteria, the use of bacteriophages has recently been considered as an alternative strategy for the treatment of bacterial infections. In this study, a novel lytic phage infecting *K. pneumoniae* was isolated from a mixture of urban and hospital wastewater, and characterized in order to investigate its lytic activity against the host bacterium. The host range study of the isolated vB-KpneM-Isf48 phage (most probably belonging to the *Myoviridae* family) was performed on a number of gram-positive and gram-negative bacterial species. The results showed that the vB-KpneM-Isf48 phage was *K. pneumoniae* species-specific. In other words, the vB-KpneM-Isf48 phage did not demonstrate any lytic activity against other bacteria, such as normal flora, compared to antibiotics that affect both the normal flora and infectious bacteria.

The phage was able to lyse 100% (18 out of 18) and 94.4% (34 out of 36) of ESBL-positive isolates detected by phenotypic and genotypic methods, respectively. Overall, 92.68% (38 out of 41) of all of the bacteria investigated in this study were susceptible to this phage. Among them were four isolates that were resistant to all of the tested antibiotics. This narrow host range is an advantage for this phage's use in phage therapy.

Due to different strains and types of structural proteins, varied thermal, pH, and saline stabilities have already been reported for different phages (10). Therefore, it was of interest to investigate the stability of the phage under conditions mimicking wounds. In almost every case, the temperature of a wound increases by approximately 2°C when it is infected (35). According to our results, the detected phage was stable and active in temperatures ranging from 40°C to 50°C (for 60 minutes). Therefore, the phage can survive in infected wounds with temperature ranges from 38°C to 41°C (36). This makes the phage a

good candidate for phage therapy of wound infections. The phage also showed acceptable pH stability over a broad range of pH values, ranging from 5 to 11. The maximum stability was observed at pH 7, but it was completely inactivated after 12 and 24 hours of incubation at pH 4 and 5. Unlike the pH of healthy skin, which adjusts itself between 4 and 6, the pH of wounds might be neutral or a little basic (7.15 - 8.9) (37, 38). The optimal pH for the highest activity of the isolated phage was nearly the same as the pH of wounds. Therefore, it can be declared that due to this property as well, this phage is an appropriate candidate for the treatment of wound infections. In addition, the saline-tolerance experiment showed that the phage retained its activity in NaCl concentrations of up to 18% after a 24 hours incubation period. As normal saline with the NaCl concentration of 0.9% is routinely used for wound-cleaning (39), the results suggest that the addition of the phage in normal saline could be a suitable way to apply the phage for phage therapy. The phage was adsorbed to the host bacterium very quickly, which is an advantage for antibacterial activity, and is again a positive property for use in phage therapy.

To the best knowledge of the authors, this is the first time in Iran or worldwide that a virulent phage specific to *K. pneumoniae* strains that carry antibiotic-resistance genes (*TEM*, *SHV*, and *CTX-M*), isolated from infected wounds, is identified and characterized. As the phage is virulent, stable, and active at different pH values, salt concentrations, and temperatures, it can tolerate wound conditions and remain active when used for the treatment of infected wounds, and its application for the phage therapy of infected wounds is suggested.

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Footnotes

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