J Med Microbiol Infect Dis, 2019, 7 (1-2): 6-11
DOI: 10.29252/JoMMID.7.1.2.6

Original Article

Isolation and Characterization of a *Klebsiella pneumoniae* Specific Lytic Bacteriophage from a Hospital Waste-water Treatment Plant

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Received May 13, 2019; Accepted Jun. 17, 2019

Introduction: Phage therapy has gained interest as a potential alternative for treatment of infections caused by multidrug-resistant (MDR) pathogens. This study aimed to isolate a lytic bacteriophage with the potential to lyse clinical isolates of *Klebsiella pneumoniae*. **Methods:** Water samples were collected from a hospital waste-water treatment plant in Tehran. The samples were filtered and mixed with an overnight grown culture of *K. pneumoniae*. (ATCC 10031) followed by incubation at 37°C overnight. Phage titration, latent period, and burst size measurements were carried out by the double-layer agar method using the *K. pneumoniae* ATCC strain. The isolated phage was characterized by transmission electron microscopy (TEM), thermal, pH, and chloroform stability. Susceptibility of *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* ESBL producing *K. pneumoniae* and 51 MDR *K. pneumoniae* isolates was measured by placing 20 µl of the phage suspension (10^8 PFU) onto bacterial lawns followed by incubation at 37°C overnight. Formation of clear zones indicated susceptibility. **Results:** The isolated lytic bacteriophage formed small clear plaques with a latent period of 40 min and a burst time of 52 min, corresponding to 35-40 phage particles per infected cell. TEM results showed that the phage resembled the tailed *Siphoviridae* family and was designated vB_KpnS-Teh.1. The phage vB_KpnS-Teh.1 was most stable at 37°C, pH 7 and was resistant to chloroform. **Conclusion:** The isolated lytic phage showed specificity towards *K. pneumoniae*. Further research will determine its potential in the treatment of *K. pneumoniae* infections. *J Med Microbiol Infec Dis, 2019, 7 (1-2): 6-11.*

Keywords: *Klebsiella pneumoniae,* Bacteriophage, Waste-water, *Siphoviridae.*

INTRODUCTION

The Gram-negative opportunistic pathogen, *Klebsiella pneumoniae* is responsible for severe infections such as septicemia, pneumonia, urinary tract infections (UTI) and soft tissue infections in hospitalized and immune-compromised patients with underlying diseases [1]. Emergence and spread of multidrug-resistant (MDR) *K. pneumoniae*, specifically the extended spectrum β-lactamase producing strains, is often the cause of antibiotic treatment failure [2, 3].

Lytic bacteriophages are obligate parasites which replicate within a particular bacterial host and cause rapid lysis and cell death within a short period. Therefore, the potential use of lytic phages as alternative antimicrobial agents has received much attention for the treatment of multidrug-resistant clinical isolates of bacteria [4-6]. Several studies have reported isolation and characterization of lytic phages with specific activity against *Klebsiella* spp. and MDR *K. pneumoniae* isolates [7-10]. Most of these bacteriophages are tailed and belong to the order *Caudovirales,* which include the families *Myoviridae, Siphoviridae,* and *Podoviridae* [11, 12]. The efficacy of some of these phages was also studied in *K. pneumoniae* biofilms as well as animal models of *K. pneumoniae* infections [12-14]. This study aimed to isolate and characterize a lytic bacteriophage from raw waste-waters with activity against *K. pneumoniae* and possible potential for future therapeutic applications.

MATERIAL AND METHODS

Bacteriophage isolation and purification. Water samples were collected from a hospital waste-water treatment plant in January 2017 in Tehran. To flasks containing 40 ml of wastewater, 5 ml nutrient broth (10 x, Liofilchem, Italy) and 5 ml of overnight grown *K. pneumoniae* (ATCC10031) in nutrient broth were added and incubated at 37°C overnight. The culture was centrifuged at 2000 × g for 15 min, and the recovered supernatant was filtered through 0.22 µm pore size filters followed by dilution of the filtrate (1:100) in phosphate buffered saline (PBS, pH 7). An aliquot of 100 µl of the phage preparation and 500 µl of *K. pneumoniae* ATCC 10031 (adjusted to 0.5 McFarland standard) were added to 3 ml soft nutrient agar (0.75% agar at 45°C). The mixture was then layered onto a nutrient agar plate and allowed to solidify before incubation at 37°C overnight. The

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appearance of clear plaques indicated the presence of lytic phages. A single clear plaque was isolated for further purification.

Transmission Electron Microscopy (TEM). A high-titer phage preparation (10^{11} PFU/ml) was deposited on a formvar-carbon coated grid Cu Mesh 300, fixed with 1% glutaraldehyde, stained with the standard negative staining using 2% uranyl acetate, and examined by an EM 10C (Zeiss, Germany) transmission electron microscope at 100 kV [12].

Phage adsorption assay. An overnight culture of *K. pneumoniae* ATCC in Mueller Hinton broth (MHB, Liofilchem, Italy) was adjusted to 10^8 CFU/ml. Equal volumes of the bacterial culture and phage suspension (10^7 PFU/ml) were mixed and incubated at 37°C for 5 and 10 min respectively. The cultures were centrifuged at 10000 × g for 5 min, filtered through a 0.22 µm filter, and the numbers of free phages were determined using the double-layer agar method. The reduction in phage titer showed the number of phages adsorbed to the bacterial cells [8].

One-step growth curve. One-step growth curve of the isolated phage was performed using the method of Pajunen *et al.* (2014) with some modifications [15]. Initially, an overnight culture of *K. pneumoniae* ATCC in MHB was adjusted to 10^8 CFU/ml. For phage adsorption, 0.9 ml of bacterial suspension was mixed with 0.1 ml of the phage (10^8 PFU) (multiplicity of infection, MOI=0.1) and incubated at 37°C for 5 min. The mixture was diluted, and samples (0.1 ml) were taken at 5 min intervals up to 30 min, followed by 15 min intervals up to 90 min for determining the number of phages by the double-layer agar method. The latent period, burst time, and burst size were calculated from the one-step growth curve [8]. All assays were carried out at three different times.

Thermal, pH and chloroform stability tests. Thermal stability of the isolated phage was determined by incubation of different phage suspensions (10^8-10^{12} phage particles/ml) at 50-70°C for 2 h and 24 h, followed by determining the number of phages by the double-layer agar method. For pH stability tests, 10 µl of 10^{11} phage suspension was added to 0.99 ml of MHB at a pH range of 3-12 and incubated at 37°C for 18 h before phage titration. Chloroform susceptibility was measured by mixing 1 ml of 10^9 phage particles with 1 ml chloroform (Merck, Germany) and the mixture was incubated at 37°C for 18 h before determination of the phage particle numbers [8].

Determination of optimal multiplicity of infection (MOI). *K. pneumoniae* ATCC strain was grown in MHB at 37°C to an optical density (OD) of 0.6 at 600 nm corresponding to around 10^8 CFU. Bacterial cultures (1 ml) were inoculated with 10^9, 10^8, and 10^7 PFUs at the MOIs 10, 1 and 0.1, respectively. Bacterial growth was monitored by recording OD_{600} at 30 min intervals up to 360 min [8].

Determination of host range. Spot test was employed to determine the bacterial host range for the isolated phage using *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC BAA-747), *Pseudomonas aeruginosa* (ATCC 27853) and ESBL producing *K. pneumoniae* (207 L) [16]. Bacteria were grown in MHB for 6-8 h at 37°C to reach the turbidity of 0.5 McFarland. Bacterial lawns were formed on MHA plates, and 20 µl of phage suspensions (10^8 PFU) were placed on the bacterial lawns. Plates were incubated at 37°C and observed after 6, 18, and 24 h for phage-mediated lysis [6]. Also, 51 MDR *K. pneumoniae* clinical isolates were employed in the spot test.

RESULTS

Morphology. A lytic phage was isolated which formed small clear plaques (≤ 1mm) in *K. pneumoniae* ATCC host. TEM results revealed an isometric headed phage with a capsid size of ~40 ± 2 nm in diameter and a long non-contractile tail of ~300 ± 3.0 nm (Fig. 1), resembling the double-stranded DNA phages of the family *Siphoviridae* [17]. According to the scheme for the nomenclature of bacterial viruses, the phage was designated vB_KpnS-Teh.1 [18].

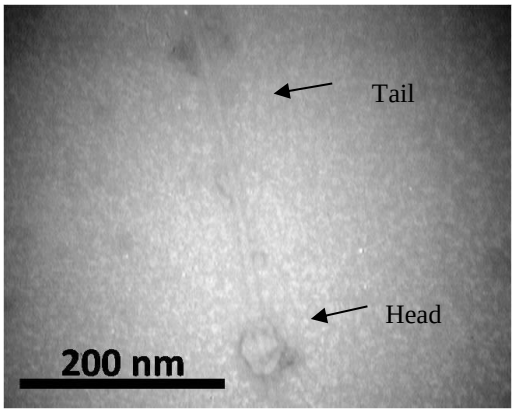


Fig. 1. Transmission electron micrograph of the negatively stained phage vB_KpnS-Teh.1. Black arrows show the head and tail of the phage

Phage adsorption and one-step growth curve. The phage (vB_KpnS-Teh.1) particles were adsorbed to the bacterial host after 5 min (Fig. 2A). The one-step growth curve revealed a latent period of 40 min and a burst time of 52 min, corresponding to about 35-40 phage particles per infected cell (Fig. 2B).

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Thermal, pH and chloroform stability. The vB_KpnS-Teh.1 phage was sensitive to high temperatures. PFU numbers decreased 1 log at 50°C, 3-logs at 60°C after 18 h and 5-6 logs after 2 h at 70°C (Fig. 3A). As shown in Figure 3B, the phage was stable at pH 7, but PFU decreased 4 logs at pH values 5 and 9. At pH 11 and 3, the number of the phages reduced 6 and 7 logs, respectively (Fig. 3B). Phage vB-KpnS-Teh.1 was resistant to chloroform at 37°C for 18 h.

Optimal multiplicity of infection (MOI). As observed in Figure 4, all tested MOIs were similarly effective against the host strain. Hence, we used the MOI 0.1 in further experiments.

Determination of the phage host range. The spot test results showed that phage vB_KpnS-Teh.1 had no lytic activity against the tested bacteria, *E. coli*, *A. baumannii* or *P. aeruginosa*. The lytic activity of the phage was specific to *K. pneumoniae* clinical isolates among which, 8 MDR isolates (15.7%) were sensitive to the vB_KpnS-Teh.1 phage.

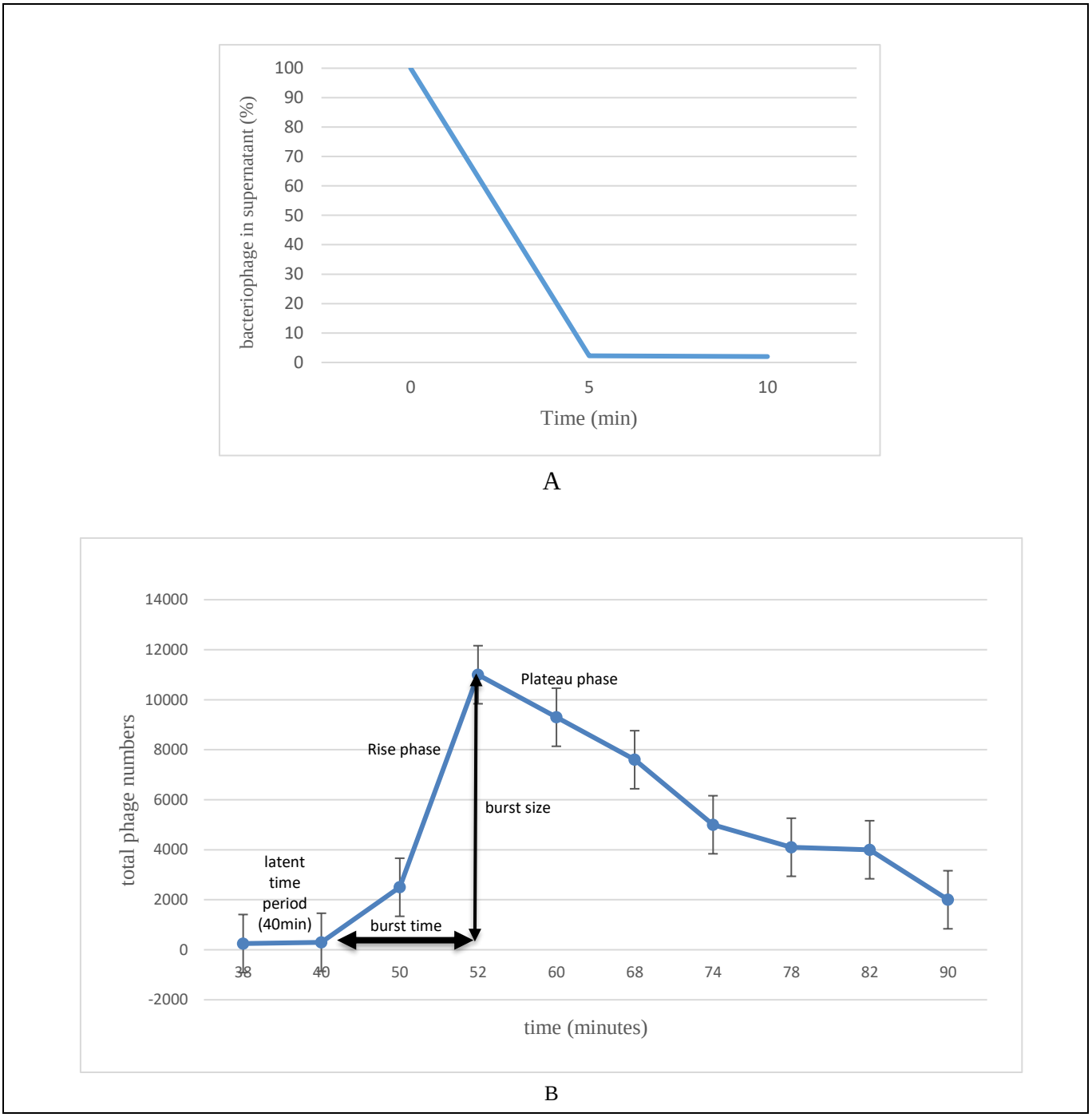


Fig. 2. Adsorption (A) and one-step growth curves of phage vB_KpnS-Teh.1 (B) in *K. pneumoniae* ATCC strain. Latent period, burst time, and size were calculated from the one-step growth curve.

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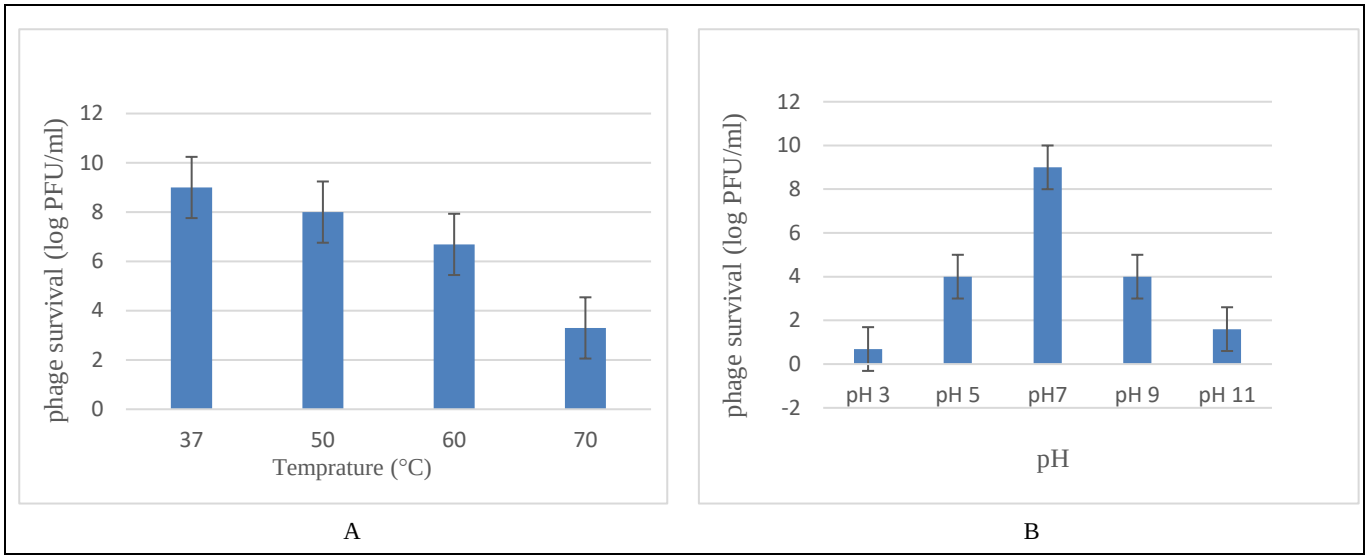
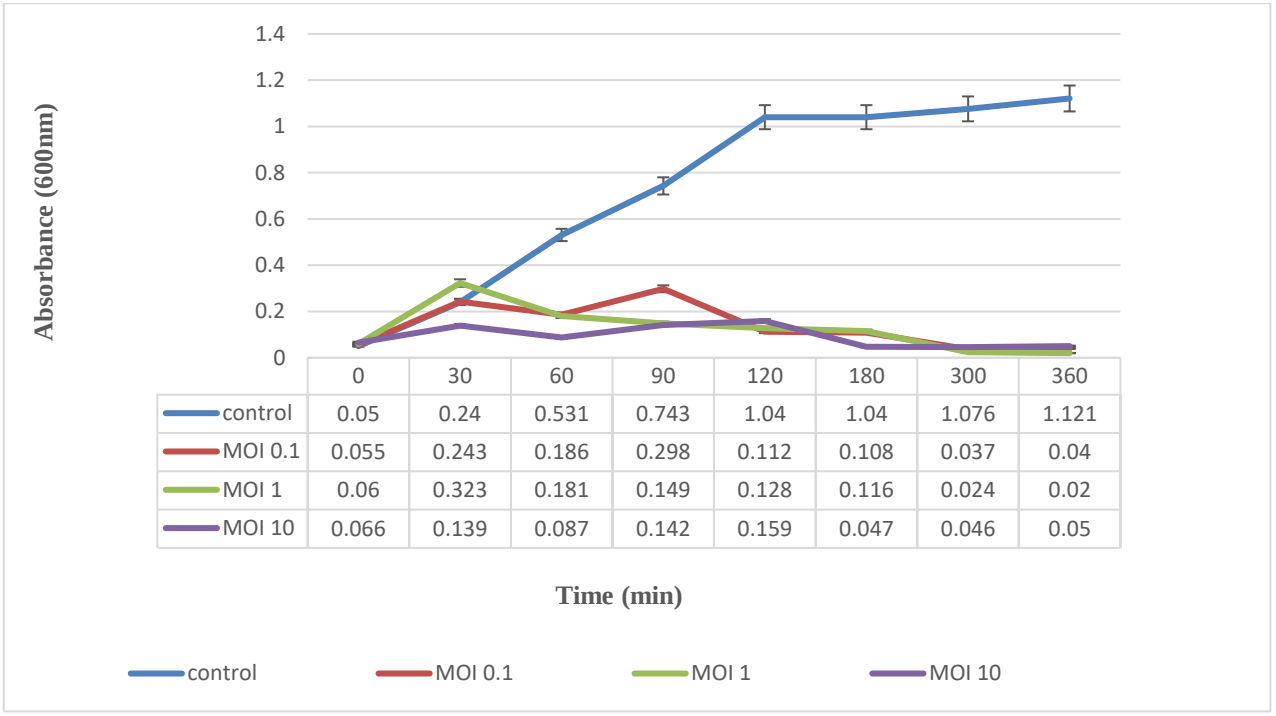


Fig. 3. Thermal (A) and pH stability of phage vB_KpnS-Teh.1 (B)



	0	30	60	90	120	180	300	360
control	0.05	0.24	0.531	0.743	1.04	1.04	1.076	1.121
MOI 0.1	0.055	0.243	0.186	0.298	0.112	0.108	0.037	0.04
MOI 1	0.06	0.323	0.181	0.149	0.128	0.116	0.024	0.02
MOI 10	0.066	0.139	0.087	0.142	0.159	0.047	0.046	0.05

Fig. 4. The efficiency of vB_KpnS-Teh.1 MOI (0.1, 1 and 10) used to infect the *K. pneumoniae* ATCC strain measured by absorbance of the cultures at 600 nm.

DISCUSSION

In this study, we isolated a lytic bacteriophage from a hospital waste-water treatment plant which had specific activity against *K. pneumoniae*. TEM results showed that the isolated phage, vB_KpnS-Teh.1, resembled the double-stranded DNA phages belonging to the family *Siphoviridae*. Other studies have also reported the isolation of *K. pneumoniae* specific lytic phages belonging to the *Siphoviridae* family [7, 10, 12, 17]. The characteristic features of our phage, *i.e.*, the latent period of 40 min, burst time of 52 min and a burst size of 35-40 were similar to the phages reported by others [7, 10]. The isolated phage of this study showed resistance to chloroform, was partially stable at high temperatures (50-70°C) and pH 5-9, and retained residual activity at pHs 3 and 11. Interestingly, about two-thirds of tailed phages, including the members of *Siphoviridae* family are chloroform-resistant [10]. Similar to our results, Jamal *et al.* (2015) reported a *Siphoviridae* phage, designated Z, with stability at 37-70°C and activity at a wide pH range of 5-11 [12]. In another study, an isolated *K. pneumoniae* lytic phage showed thermostability at 30-40°C but lost total activity at 60°C. This phage was

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stable at pH values of 6 to 10, but retained partial activity at pH 5 and 11 [19]. Karumidze *et al.* (2013) also isolated a *K. pneumoniae* specific phage, which was stable at 37°C and 50°C and pH values of 3 to 9 after 24 h of incubation [7].

The vB_KpnS-Teh.1 phage showed a narrow range of lytic activity against *K. pneumoniae* and lysed only 15.7% (8 out of 51) ESBL-positive isolates. In other studies, members of the *Siphoviridae* were reported to exhibit similar activity against *K. pneumoniae*, lysing 7-15% of MDR isolates [10, 12, 19, 20]. These phages multiply more efficiently and elicit faster elimination of their bacterial hosts [10]. Characterization of phages provides us a better understanding of their biology, including host specificity, adaptation to bacterial defense systems, and propagation dynamics in natural systems. The knowledge can assist us in exploiting them as therapeutic agents against MDR bacterial pathogens. Also, even though an isolated phage may act specifically against a particular pathogen, the use of phage cocktails consisting of several known lytic phages can increase their efficacy as therapeutic agents against MDR isolates. The therapeutic use of phage cocktails could also prevent the development of resistance among bacterial pathogens [21].

The isolated *K. pneumoniae* phage vB_KpnS-Teh.1 resembled the double-stranded DNA phages belonging to the family *Siphoviridae*. Our recovered phage had a narrow host range and could lyse only 15.7% of the ESBL producing MDR clinical isolates of *K. pneumoniae*. The narrow host range could be advantageous in phage therapy since it lowers the possibility of affecting other members of the normal flora.

ACKNOWLEDGMENT

This research was approved and funded by Shahid Beheshti University Research Council (Grant No. 600/1724).

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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