# Isolation and characterization of lytic bacteriophages against antibiotic-resistant *Klebsiella pneumoniae* from wastewater samples

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## Abstract

**Introduction**: One of the most important global health problems in the last decade is the increasing prevalence of antibiotic-resistant *Klebsiella pneumoniae*. Phage therapy is considered a highly efficient alternative to treat antibiotic-resistant bacteria. Therefore, this study aimed isolate lytic phages against clinical strains of antibiotic-resistant *Klebsiella pneumoniae* from wastewater samples.

**Materials and methods:** In this study, different samples were collected and the presence of phage was confirmed by a double layer test. The protein and genomic structure were verified using SDS PAGE and RAPD PCR techniques**.** Transmission electron microscopy (TEM) was used for the evaluation of morphology. The isolated phage's stability was assessed by subjecting it to different temperature and pH conditions. An adsorption assay test was conducted to the duration of phage absorption to corresponding host bacteria.

**Result:** In this study, we isolated three lytic phages (k.8, k.22, and k.34) that target *K .pneumoniae.* Transmission electron microscopy (TEM) results indicated that all three phages belong to the *Siphoviridae* family. Additionally, our investigation demonstrated that these phages remain stable

within a temperature range of 4°C to 50°C and a pH range of 5.0 to 9.0. The adsorption assay tests showed that the isolated phages were able to attach to their corresponding host bacteria within 6 to 9 minutes.

**Conclusion:** The present study demonstrated that the isolated phages targeting *K. pneumoniae* exhibited a favorable survival rate when subjected to pH and thermal treatments. Furthermore, the isolated phages exhibited a short latent period, indicating rapid adsorption to the host surface. These findings suggest the potential application of these phages in phage therapy. However, further studies in the field of phage therapy are required.

**Keywords:**
*Klebsiella pneumoniae*, Lytic phage, Phage therapy, Wastewater

## 1. Introduction

The spread of hospital infections, including gram-negative bacteria, has led to a significant crisis within the healthcare community. *Klebsiella pneumoniae* (*K. pneumoniae*) is a significant gram-negative, encapsulated, and non-motile bacterium belonging to the *Enterobacteriaceae* family. It is extensively found in the natural surroundings, encompassing sewage, soil, plant surfaces, and the surfaces of medical devices (1). *K. pneumoniae* can colonize mucosal surfaces, such as the nasopharynx and the gastrointestinal (GI) tract. It has the potential to lead to various illnesses and fatalities in individuals, particularly when the immune system is compromised or disrupted microbiota. These infections include pneumonia, urinary tract infections, abdominal infections, meningitis, purulent liver abscess, septicemia, hospital-acquired pneumonia and community-acquired pneumonia (2).

The rise of multidrug-resistant (MDR) strains of *K. pneumoniae* has become a significant public health concern due to the widespread use of broad-spectrum antibiotics in hospitals. These resistance mechanisms pose challenges for treatment, leading to increased morbidity and mortality among infected patients. The World Health Organization (WHO) has identified *K. pneumoniae* as one of three pathogens of significant concern due to its antibiotic resistance (3). It exhibits several mechanisms of antibiotic resistance, including the production of β-lactamases that inactivate β-lactam antibiotics, the use of efflux pumps to expel antimicrobial agents, and the formation of biofilms that protect bacterial communities from the immune system and antibiotics. Resistance

can also be enhanced by enzymatic modifications of drugs and the loss of porins, which are channels that allow drug entry into the bacterial cell (4). These mechanisms contribute to the resistance seen against a range of antibiotics such as carbapenems, cephalosporins, and aminoglycosides (3).

Phage therapy is a treatment method that uses bacteriophages, which are viruses that infect bacteria. It is a promising approach that is widely regarded as a natural, safe, and highly effective solution to address the growing challenges posed by MDR bacterial infections through the exclusive use of lytic phages (5). The reason for the high success and safety of phage therapy compared to antibiotics is due to their specific action against specific bacteria and the ability to infect only one species, serotype or strain. This mechanism does not destroy the body's natural bacterial flora and only destroys the target pathogenic strain (6). As a result, phages are introduced as suitable candidates to combat antibiotic-resistant infections effectively. Phage therapy, while promising, has several shortcomings, including a narrow host spectrum, where specific phages may only target certain bacterial strains, limiting their effectiveness. Additionally, the human immune system can clear phages, reducing their therapeutic potential. It's essential to consider these limitations alongside the potential benefits when evaluating phage therapy (7). In light of the challenges encountered in treating *K. pneumoniae*, the objective of this study was to identify lytic phages specifically targeting *K. pneumoniae*.

## 2. Materials and methods

### 2.1. Isolation of bacteria

In this study, 100 clinical isolates of *K. pneumoniae* were obtained from hospitals in Ilam and Hamadan provinces. In addition, a standard strain obtained from the Center of Genetic Resources of Iran was utilized as a standard isolate. The Bacterial isolates were cultured in Blood agar and MacConkey agar and placed in a 37°C incubator for 24 hours, and were confirmed by gram staining and biochemical tests including triple sugar iron (TSI), citrate and sulfide-indole-motility (SIM) tests. To determine antibiotic resistance, an antibiogram test was conducted using a clinical and laboratory standards institute (CLSI) established protocol by disc diffusion method on Mueller Hinton's medium. *K. pneumoniae* was tested with eight known antibiotics including Ceftriaxone, Imipenem, Gentamicin, Ciprofloxacin, Cefotaxime, Amikacin, Nitrofurantoin, Levofloxacin finally antibiotic-resistant isolates were chosen for phage isolation.

**2.2. Phage isolation**

Several samples were collected from different sources, including municipal wastewater, hospital wastewater, and also samples of animal feces. In the next step, the samples were passed through a strainer and centrifuged at 5000 rpm for 15 minutes, and then the supernatant was passed through a 0.45µm filter. The samples were mixed with 10 mL of bacteria that had reached the stationary phase (from an overnight culture), and 100µl of calcium chloride was added. The mixture was then placed in a 37 ◦C incubator with a shaker at 125 rpm for 24 hours (8). Subsequently, 100µl chloroform was added to the mixture of, bacteria and filtered wastewater and centrifuged at 5000 rpm for 15 minutes. The supernatant was filtered with a 0.45µm filter and then was diluted by serial dilution method and checked for the presence of phage and plaque formation by double-layer agar method (9). One separated plaque was purified three times by picking in SM buffer (5.8 g/L NaCl, 2.0 g/L MgSO4, 50 mL/L of 1 M Tris, pH 7.5, 5 mL/L pre-sterilized 2% gelatin) (10) and re-plating. Plaque characteristics were recorded and the phage titer was determined. Then the purified phages were stored in glycerol and placed at -80 ◦C.

**2.3. Genome analysis**

To determine the genetic polymorphism of the isolated phages, phage DNA was extracted using Norgen's phage DNA isolation Kit (Norgen Biotech, Canada) following the manufacturer's protocol. The purity and concentration of the DNA obtained were determined through 260/280nm absorbance measures using the NanoDrop spectrophotometer (Microdigital, South Korea).

**2.4. Genomic fingerprinting by randomly amplified polymorphic DNA (RAPD) PCR**

RAPD-PCR was carried out using four primers including OPL5 (5′-ACGCAGGCAC-3′), RAPD5 (5′-AACGCGCAAC-3′), P1 (5′-CCGCAGCCAA-3′), P2 (5′-AACGGGCAGA-3′) were used (11). PCR was performed in a thermocycler under the following thermal cycling conditions: one cycles at 94 °C for 120 s; 40 cycles at 92°C for 60 s,36°C for 60 s, and 72°C for 120 s; and a final step of 10 min at 72°C.

**2.5. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS PAGE)**

To determine the protein structure of the isolated phages, SDS-PAGE was performed. To prepare the bacterial sample, the bacteria were cultured in 15 mL of TSB medium and incubated for 24 hours in a shaker incubator at 37°C. Subsequently, the sample was centrifuged for 10 minutes at 5000 rpm. To the sediment, 50µl of sterile distilled water was added and dissolved. Following that, 250µl of lysate buffer (consisting of 100mM NaCl and 25mM Tris HCl in 10ml of distilled water)

was added and the mixture was kept on ice for 10 minutes. The samples were then subjected to sonication in a sonicator bath (JeioTech, South Korea) for 15 minutes, followed by centrifugation at 14,000 rpm for 30 minutes at 4°C. To prepare the phage sample, the phages concentrated by PEG 8000 were centrifuged for 30 minutes at 13,000 rpm at 4°C. The remaining precipitate was then mixed with 96% ethanol in equal proportions and stored in a freezer at -20°C for 24 hours. Following this, another centrifugation was performed at 13,000 rpm at 4°C for 1 hour, and the supernatant was once again discarded. Finally, all the samples were loaded on a 12% acrylamide gel.

**2.6. Host Range**

In this step, 100 *K. pneumoniae* bacteria collected from different hospitals were evaluated to determine the host range of the isolated phages. In addition to *K. pneumoniae* isolates, eight other reference isolates including *Listeria monocytogenes*, *Escherichia coli*, *Serratia marcescens*, *Salmonella typhi*, *Acinetobacter baumannii*, *Shigella*, *Staphylococcus aureus* and *Streptococcus agalactia* were evaluated to determine the host range. The susceptibility of the bacterial strains was evaluated by spot tests. The plates were incubated at 37°C and were checked 18h later for the presence of plaques and compared with an uninfected as negative control.

**2.7. Transmission electron microscope (TEM)**

At this stage, the morphological characteristics of the isolated phages were investigated using (TEM). 400 mesh formvar coated copper grid and 2% phosphotungestic (PTA) were used to prepare the negatively stained samples. Negative stained sample was examined by50 KV Transmission electron microscope (Zeiss EM 900, Germany). The electron microscopy results were utilized to classify the isolated phage according to the guidelines set by the International Committee on Taxonomy of Viruses (ICTV).

**2.8. Thermal and pH Stability of the isolated phages**

The thermal stability of the isolated phages was evaluated by incubating phages with specific concentrations ($10^8$ PFU/mL) at different temperatures ranging from 4 to 100°C (4, 25, 37, 40, 50, 60, 80, 70, 90, and 100 °C) for one hour. After 1 hour, the survival of the treated phages was checked by the soft agar overlay method. The pH stability was checked at different pH values ranging from pH 3 to 11 (3, 4, 5, 6, 7, 5/7, 8, 9, 10, 11). 1 ml of phage ($10^8$ PFU/mL) with 9 ml of sterile SM buffer (adjusted using NaOH or HCl) was mixed and incubated for 1 h at 37 ◦C. The

determination of phage survival was conducted using the soft agar overlay method for each treated sample after 24 h of incubation at 37°C.

### 2.9. Phage Adsorption Assay

Absorption is the first stage of phage infection of host bacteria. This test was performed for the isolated phages k.22, k.34, k.8. Approximately $5 \times 10^5$ PFU of the isolated phages in 500 µL was mixed with a 500 µL of host bacteria grown to OD600 ($7.5 \times 10^7$) in TSB Broth. For the control group, 200 µL sterile TSB was added. The infected cells were incubated at 37 ◦C for up to 18 min. After 0, 3, 6, 9, 12, and 18 min, two sample batches were withdrawn for each bacteriophage and centrifuged 3 times at 13000 rpm for 5 minutes. The un-adsorbed phage concentration was determined by assaying the supernatants after sterilization through a 0.22µm filter. All the experiments were performed in triplicate, and the average result was taken. Phage titer was compared with 100% control supernatant.

### 2.10. Statistical analysis

All experiments in this study were repeated three times. Differences were analyzed using Chi-squared tests. A difference with $P<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Phage isolation results

Several samples including urban sewage, hospital sewage, and various animal wastes were examined for the presence of phage against *K. pneumoniae*. Overall, three phages were isolated by the double-layer method. Plaques of three phages against *K. pneumoniae* along with control groups are shown in Figure 1.

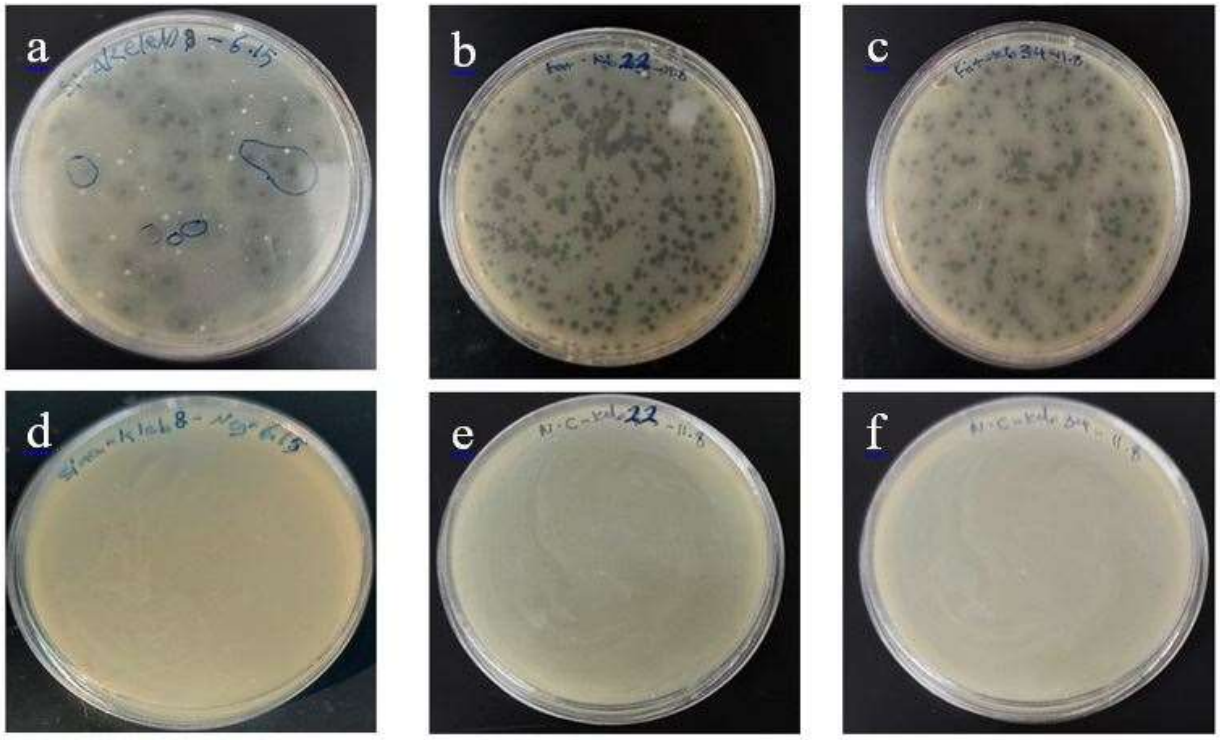


**Fig.1**. **Isolated phage by double layer method.** Plaques of 3 phages: **a:** K.8 phage; **b:** K.22 phage; **c:** K.34; **d:** K.8 negative control; **e:** K.22 negative control; **f:** K.34 negative control.

### 3.2. RAPD PCR results

To investigate of genome polymorphism of isolated phages and bacteria hosts, RAPD PCR was performed with P1, P2 Rapd5, and OLP5 primers for all 3 isolated phages. The results of RAPD PCR are shown in the Figure 2.

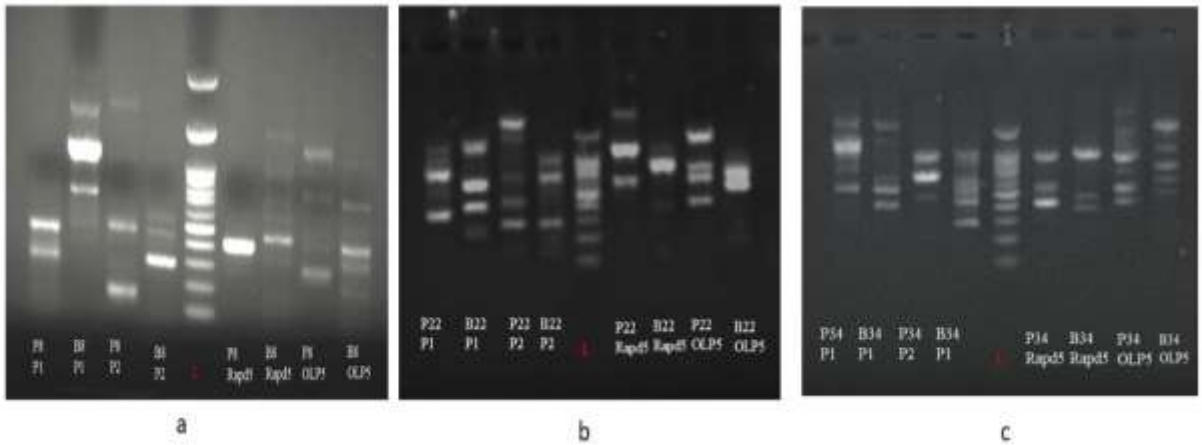


**Fig.2. RAPD band patterns obtained from 3 phages and their host as control group. a:** phage K.8 (P8) along with their host (B8); **b:** phage K.22 (P22) along with their host (B22); **c:** phage K.34 (P34) along with their host (B34). (P bands related to phage and B bands related to bacteria).

### 3.3. SDS-PAGE results

In this study, phage and bacterial protein patterns were evaluated simultaneously. The results of SDS-PAGE electrophoresis revealed that the protein patterns of the isolated phages were different from their host. The results of SDS-PAGE are shown in the Figure 3.

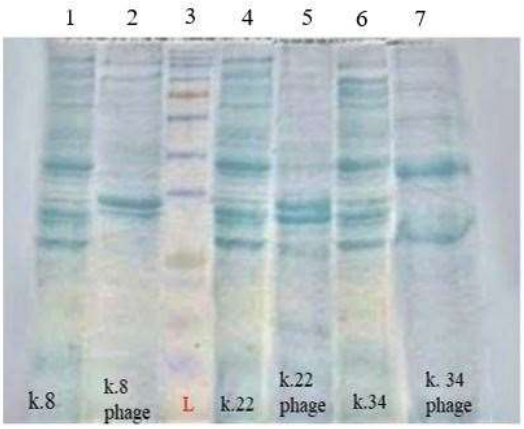


**Fig.3. Protein patterns of the isolated phages and their host as control group**. **Lane 1:** k.8 host bacteria; **Lane 2:** phage k.8; **Lane 3:** protein ladder; **Lane 4:** k.22 host bacteria; **Lane 5:** phage k.8; **Lane 6:** k.34 host bacteria; **Lane 2:** phage k.34.

### 3.4. Host range results

The host range of the isolated Phages was determined by the spot assay method using 100 isolates of pathogenic *Klebsiella*. Among them, 77% (77 of 100) strains were susceptible to K.34 phage; 51% (51 of 100) strains were susceptible to K.22 phage, and only 15% (15 of 100) strains were susceptible to K.8 phage. This result indicates the higher efficiency of k.34 phage ($P<0.001$). In addition to *K. pneumoniae* isolates, eight other reference isolates including *Listeria monocytogenes*, *Escherichia coli*, *Serratia marcescens*, *Salmonella typhi*, *Acinetobacter baumannii*, *Shigella*, *Staphylococcus aureus*, and *Streptococcus agalactia* were evaluated to determine the host range of the isolated phages in this study. Interestingly, our results showed that none of the mentioned were sensitive to the isolated phages.

### 3.5. Transmission electron microscope results

The Transmission electron microscope results revealed that all three phages are classified under the *Siphoviridae* family. Negatively stained electron micrographs of three phages revealed presence of an icosahedral head (diameter: 88.9 ± 1.7 nm (K.22), 97.4 ± 2.6 nm (K.8) and

59.7 ± 2.1 nm (K.34)) and long tails [381.1 ± 2.1 nm (K.22), 137.9 ± 3.1 nm (K.8) and 203.1 ± 2.7 nm (K.34). The Morphology of the three phages is shown in Figure 4.

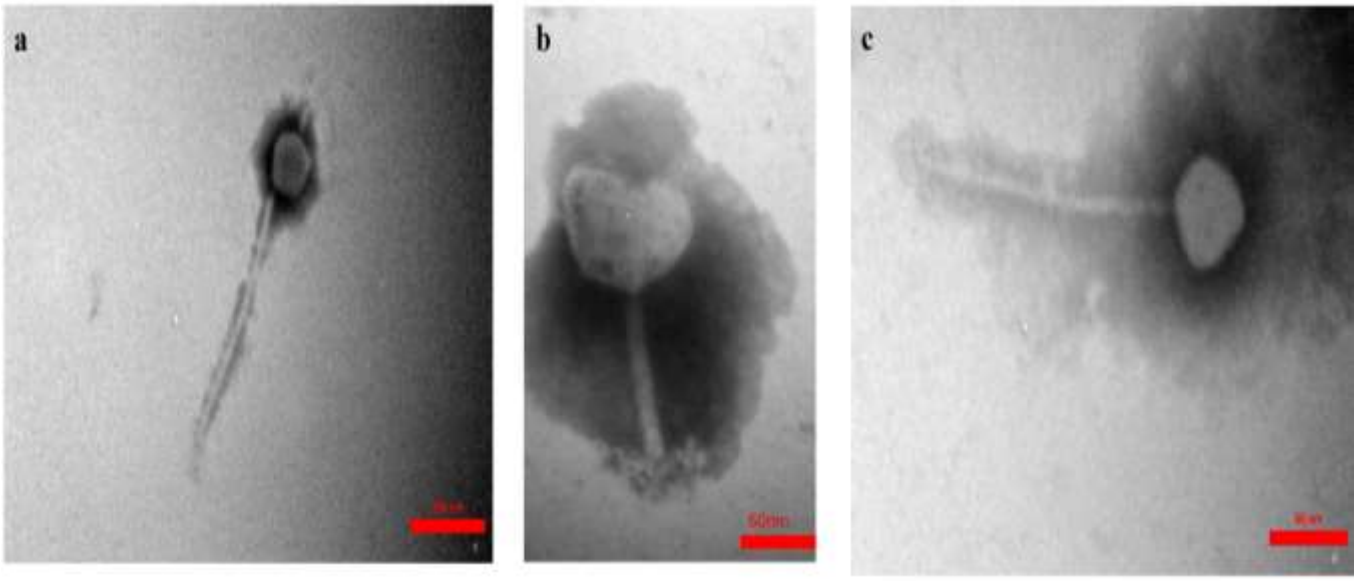


**Fig.4. Images of the Transmission Electron Microscope. a:** K.22 phage; **b:** K.8 phage; **c:** K.34 phage

### 3.5. The results of thermal and pH Stability of the isolated phages

The stability of phages in terms of temperature and pH plays a crucial role in their effectiveness for treatment. The thermal stability test results indicated that phages K.8 and K.34 exhibited activity at temperatures of 4, 25, 37, 40, and 50ºC, while being inactivated at temperatures of 60, 70, 80, 90, and 100 ºC. On the other hand, phage K.22 showed activity at temperatures of 4, 25, 37, 40, 50, 60, and 70ºC, but was inactive at temperatures of 80, 90, and 100ºC. Our investigation of this parameter revealed a high tolerance to a wide range of temperatures, from 4°C to 50°C for all three phages. Our findings indicate that phage K.8 exhibits excellent stability across a broad pH spectrum, remaining active at pH levels 3, 4, 5, 6, 7, 8, and 9, but becoming inactive at pH 10 and 11. On the other hand, phage K.34 demonstrates activity at all pH levels except 3 and 4. Interestingly, phage K.22 shows activity across all pH levels. The results of thermal and pH stability assay of three phages are shown in Figure 5.

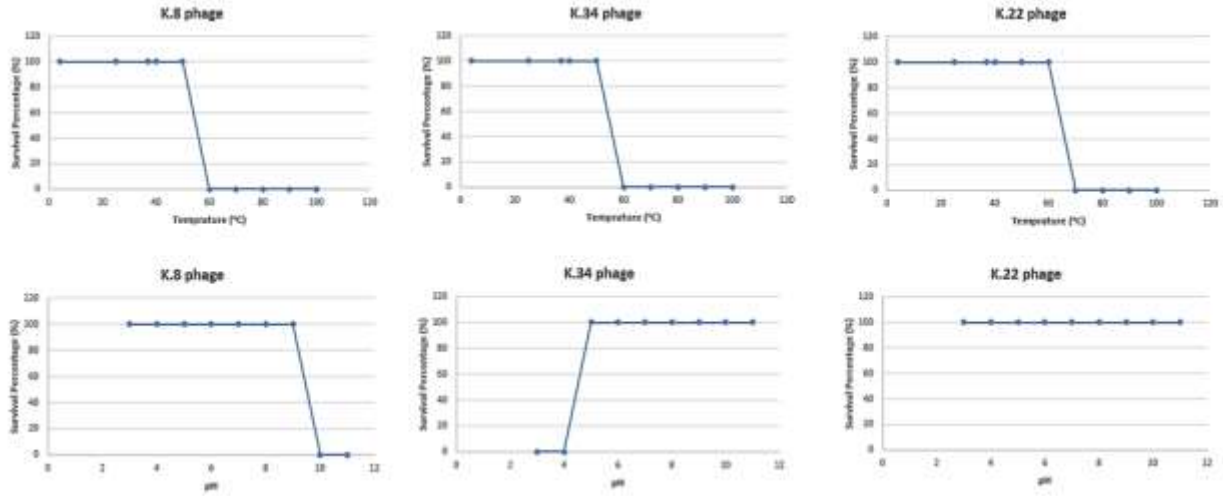


**Fig.5. Phage thermal and pH stability.** Phage thermal and pH stability at different temperature and pH range

### 3.6. Phage adsorption assay results

The initial stage of bacteriophage infection involves the binding of the phage virion to a vulnerable host bacteria, known as the adsorption process. Based on the findings, it was observed that all three phages were rapidly taken up by their respective host bacteria. Specifically, 98% of phage K.8 was absorbed by the host bacteria within 3 minutes, reaching 100% absorption after 6 minutes. On the other hand, 80% of phages K.22 and K.34 were absorbed by their host bacteria within 3 minutes, with complete absorption achieved after 9 minutes. The short latent period of a phage reflects its rapid adsorption to the host surface. The results of the phage adsorption assay of three phages are shown in Figure 6.

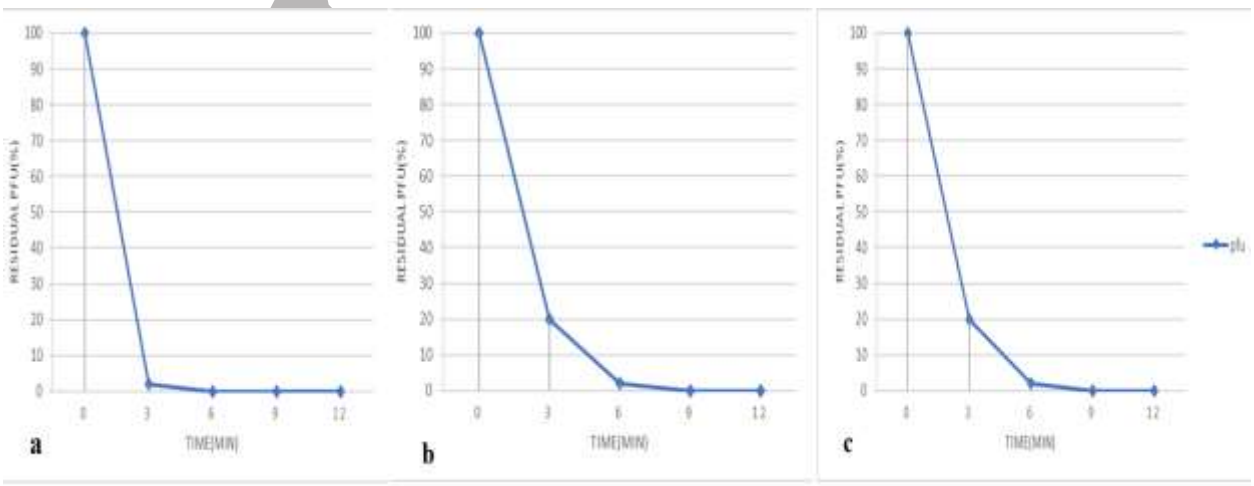


**Fig.5. Phage absorption assay. a:** phage K.8; **b:** phage K.22; **c:** K.34

## Discussion

*K. pneumoniae* is a prevalent bacterium responsible for a wide range of infections, including urinary, respiratory, blood, and soft tissue infections. A recent analysis of the worldwide impact of antibiotic-resistant infections has shown that over 250,000 fatalities are linked to *K. pneumoniae* infections. Furthermore, *Klebsiella* species are a known reservoir for antibiotic-resistant genes, which can be spread to other Gram-negative bacteria (12). The treatment of *K. pneumoniae* infections is severely hindered by antibiotic resistance, particularly with the emergence of carbapenem-resistant *K. pneumoniae* (CRKP). Current therapeutic strategies include the use of polymyxins and tigecycline, but challenges remain due to the bacteria's adaptability and the need for new, more effective treatments (13). Phage therapy is now being considered as a potential alternative treatment option due to antimicrobial resistance. The mechanism of bacterial lysis by phages involves the phages infecting bacterial cells, leading to the production of new phage particles and ultimately resulting in the bursting (lysis) of the bacterial cell. This is fundamentally different from antibiotics, which typically inhibit bacterial growth by targeting specific cellular processes or structures, such as cell wall synthesis or protein synthesis. Because of these differing mechanisms, phages can be effective against MDR bacterial infections where antibiotics may fail Also, Phages minimize the likelihood of antibiotics interacting with other medications, prevent antibiotic toxicity, and sustain their effectiveness even in the presence of biofilm (6). However, there are challenges to the implementation of phage therapy. These include the need for standardized treatment protocols, specific phage preparation for diverse bacterial strains, and patient-to-patient variability in phage response. Furthermore, regulatory issues and the requirement for clinical studies to establish dosages and delivery systems complicate its broader acceptance in clinical practice (14).

Compared to broad-spectrum antibiotics, phages have a specific host. They can survive in the bloodstream, migrate to the infected tissue, and reach the necessary concentration to eradicate the infection in the place, and its placement depends on the presence of bacteria in the place and the way of use does not affect it (15). The utilization of multiple types of bacteriophages simultaneously against the same bacteria significantly reduces the likelihood of resistance. Consequently, phage therapy effectively addresses numerous challenges associated with antibiotic usage (16). Research indicates that the ingestion of phage has proven to be successful in managing dysentery. Phage has also been administered intravenously to treat Staphylococcal septicemia. The

method of phage administration, whether orally or topically, varies based on the seriousness of the infection and can last from 1 to 16 weeks. The treatment success rate has been reported at 92% (17).

In our study, 3 lytic phages were isolated from different samples including hospital and urban wastewater against *K. pneumoniae*. Following the isolation of phages, the protein and genomic structure were verified using SDS PAGE and RAPD PCR techniques in this research. These three phages formed clear plaques on the lawns of host cells. Despite originating from various environmental samples, the TEM results revealed that all three phages are classified under the *Siphoviridae* family. In agreement with our study, 96% of the phages isolated over the past 45 years belonged to the *Siphoviridae*, *Myoviridae*, or *Podoviridae* families (18). Therefore, it is not surprising that the isolated phages belong to one of these three morphological families.

The bacteriophage's host range is determined by the specific genera and species of bacteria that can be targeted and lysed by the phages. This feature is a key aspect of bacteriophage biology. Certain phages are capable of infecting only one or a few bacterial strains, whereas others can infect a wide range of species or even bacteria from various genera (19). In this study, the spot assay method was employed to determine the host range of phages. The spot assay results indicated that none of the three phages had an impact on bacteria including *Listeria monocytogenes*, *E.coli*, *Serratia*, *Salmonella typhi*, *Acinetobacter baumannii*, *Shigella, Staphylococcus aureus*, and *Streptococcus agalactia*. It seems that the isolated phages exhibited a restricted host range and affected only *K. pneumoniae*. This result is similar to that of another lytic *K. pneumonia* phage in the study conducted by *Zhang et al.* (20). The limited host range can provide benefits in phage therapy by reducing the risk of impacting other members within the natural microbiota. Nevertheless, the issue at hand can be resolved by employing a combination of diverse phages as a cocktail, thereby expanding the scope of bacteriolytic effectiveness against both the intended and unintended bacterial pathogens (21).

The host range of the isolated Phages was determined by the spot assay method using 100 isolates of pathogenic *Klebsiella*. Among them, 77% (77 of 100) strains were susceptible to K.34 phage; 51% (51 of 100) strains were susceptible to K.22 phage, and only 15% (15 of 100) strains were susceptible to K.8 phage. This result indicates the higher efficiency of k.34 phage ($P<0.001$). Similarly, in a study conducted by *Wu et al.*, they investigated the lytic activity of Kpp95 phage

against 107 strains of *K. pneumonia*. The findings indicated that phage Kpp95 successfully lysed only 40% (47 strains) of the *K. pneumoniae* strains (22).

The stability of phages in terms of temperature and pH plays a crucial role in their effectiveness for treatment. Properties like heat and pH stability are key factors that can greatly impact the success of phage therapy (23). The thermal stability test results indicated that phages K.8 and K.34 exhibited activity at temperatures of 4, 25, 37, 40, and 50ºC, while being inactivated at temperatures of 60, 70, 80, 90, and 100ºC. On the other hand, phage K.22 showed activity at temperatures of 4, 25, 37, 40, 50, 60, and 70ºC, but was inactive at temperatures of 80, 90, and 100ºC. Our investigation of this parameter revealed a high tolerance to a wide range of temperatures, from 4°C to 50°C for all three phages. Our findings indicate that phage K.8 exhibits excellent stability across a broad pH spectrum, remaining active at pH levels 3, 4, 5, 6, 7, 8, and 9, but becoming inactive at pH 10 and 11. On the other hand, phage K.34 demonstrates activity at all pH levels except 3 and 4. Interestingly, phage K.22 shows activity across all pH levels. This finding was in agreement with earlier observations of *Kim et al.* and *Qadri et al.* (24, 25). These findings suggested that the three phages possess the potential to serve as stable biological agents that could be significant in various aspects of phage therapy.

The initial stage of bacteriophage infection involves the binding of the phage virion to a vulnerable host bacteria, known as the adsorption process. This process is commonly explained using mass-action kinetics, which assumes that host density and adsorption rate have an equal impact on the adsorption process. Consequently, an environment with a high host density can be regarded as having a phage with a high adsorption rate, and vice versa (26). Based on the findings, it was observed that all three phages were rapidly taken up by their respective host bacteria. Specifically, 98% of phage K.8 was absorbed by the host bacteria within 3 minutes, reaching 100% absorption after 6 minutes. On the other hand, 80% of phages K.22 and K.34 were absorbed by their host bacteria within 3 minutes, with complete absorption achieved after 9 minutes. The short latent period of a phage reflects its rapid adsorption to the host surface. Those with short latency have proven to be effective in lysing a higher number of bacterial cells within a specific timeframe, suggesting their potential for biocontrol purposes (27).

Research has indicated that the utilization of diverse samples for phage isolation enhances the likelihood of isolating a greater number of phages that can combat antibiotic-resistant bacteria (8). This study utilized various samples, and the findings indicated that among the 3 isolated phages,

2 were associated with hospital sewage and 1 was isolated from seawater. Our results showed that most phages were isolated from samples collected from hospital sewage. This issue can be due to the possibility of more contamination of hospital wastewater with host bacteria. Similarly, *Beaudoin et al* succeeded in isolating phage from wastewater using the double-layer agar method. They considered the presence of this bacteriophage as the cause of the presence of the host and attributed it to water contamination with human feces (28).

## Conclusion

The present study demonstrates that the isolated phages targeting *K. pneumoniae* exhibited a favorable survival rate when subjected to pH and thermal treatments. Furthermore, the isolated phages exhibited a short latent period, indicating their rapid adsorption to the host surface. These findings suggest the potential application of these phages for managing infections brought about by MDR *K. pneumoniae* strains and prevention and control of hospital-associated infections, especially among immunocompromised patients as they are more vulnerable to such infections. However, additional research is required on evolution of phage resistance, phage biology, phage preparation dosage, and specificity of phage-host interactions.

## Acknowledgments

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## Authors' Contribution

SM and FAJ designed and conceptualized the survey; SB and SS performed the experiments and drafted the manuscript; AT analyzed and interpreted the data; SM and FAJ supervised and critically reviewed the manuscript. All the authors read and approved the final manuscript.

## Ethics

We hereby declare that all experimental procedures were carried out with the utmost respect for the principles of ethical research (ethical approval IR.UMSHA.REC.1402.334).

## Data Availability

The data that support the findings of this study are available on request from the corresponding author.



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