Bacteriophages can control bacterial biofilms

BACTERIOPHAGE COCKTAIL EXHIBITED SUPERB ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY AGAINST SELECTED UROPATHOGENS

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ABSTRACT

Urinary tract infections (UTIs) are most abundant bacterial infection worldwide. Approximately 150 million urinary tract infections occur every year globally. The predominant pathogens involved in etiology of UTI are Escherichia coli followed by Staphylococcus spp., Pseudomonas aeruginosa, Proteus spp., Klebsiella spp., Acinetobacter spp., Enterobacter spp., Citrobacter spp., and Enterococcus spp. It is estimated that uropathogenic E. coli (UPEC) cause 80% of UTIs cases. The over use of anti-microbial agents has extraordinarily amplified the rise of multidrug resistant (MDR) uropathogens. Biofilm formation by UPEC is also a major cause of antimicrobial resistance. To curb harmful ripple effects of antibiotic resistance we need to identify alternative treatment procedures. Bacteriophages are bacterial viruses that can invade and infect bacteria. Phage therapy is considered as best alternate to combat several antimicrobial resistant pathogens, including MDR uropathogens. The aim of this research was to isolate and characterize lytic bacteriophages from sewage water and to identify antibacterial and antibiofilm activity of isolated bacteriophage against UPEC. Bacterial samples of UPEC were collected from Ayub Teaching Hospital Abbottabad and Punjab University Lahore. Various biochemical tests including Gram staining, oxidase and catalase test were performed to identify UPEC. The isolated bacteriophages were confirmed by spot assay and plaque assay against the host bacterial strain. The phages were purified by serial dilution up to 5-6 times repeatedly. For morphological studies electron microscopy was performed. Both the isolated phages were tested for the antibiofilm activity. These two phages showed excellent antibiofilm activity against UPEC. The specificity, lytic activity and efficient bacterial growth reduction ability of isolated phages in this study emphasize that these phages could be a suitable therapeutic agent for treatment of MDR infections.

Keywords: Bacteriophages; uropathogenic E. coli; phage therapy; urinary tract infections; sewage sample.
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**Highlights**

The aim of study was

- To isolate and characterize lytic bacteriophage against UPEC.
- To determine the antibacterial and anti-biofilm potential of isolated bacteriophage against uropathogenic bacteria.
- To utilize isolated bacteriophage as therapeutic agent against UTI.

1. INTRODUCTION

Urinary tract infections (UTIs) are one of most common bacterial infections in both community and healthcare settings. UTIs are a major public health issue as they affect large population of humans. Approximately 150 million UTIs occur every year globally which places a major economic burden on the health-care system with societal costs of more than 10 billion US$ across the world (Ullah et al., 2018). UTIs are caused by abnormal growth of pathogens in the urinary tract. UTIs may be asymptomatic or symptomatic. The primary symptoms of UTI include fever, dysuria, abdominal pain, suprapubic discomfort, itching, malodor, increased frequency of urination, ulcer and blister formation in genital area (Odoki et al., 2019). It is reported that 20-40% of females experience recurrent episodes of UTI within 3-4 months while children develop recurrence within 3 years. More than 50% of females experience at least one episode of UTIs during their lifetime.

UTIs are commonly caused by Gram negative (80-85%) as well as Gram-positive (15-20%) bacterial pathogens. The predominant uropathogens are *Escherichia coli* followed by *Staphylococcus* spp, *Pseudomonas aeruginosa*, *Klebsiella* spp, *Enterobacter* spp, *Acinetobacter* spp, *Proteus* spp, *Citrobacter* spp, and *Enterococcus* spp. It is estimated among Gram negative uropathogens, uropathogenic *E. coli* (UPEC) is responsible for 80% of UTIs worldwide (Chooramani et al., 2020). UPEC may lead to serious bloodstream infections (Mann et al., 2017).

UPEC possess variety of virulence and antibiotic resistance factors. Microbial biofilm is the main cause of antibiotic resistance. Biofilm is a community structure of bacterial cells encased in extracellular polymeric matrix and adheres to various surfaces. The ability of microorganisms to grow as biofilm is considered as major requirement for their survival under hostile environment and stress conditions. Production of biofilm is a major issue as it leads to treatment failure and difficulty in recovery from infection. The biofilm formation ability of UPEC is significant as formation of biofilm contribute to persistent and recurrence of UTIs. According to NIH (National Institutes of Health), over 60% of microbial infections are because of biofilms (Mena Viveros, 2014). The prevalence of biofilm among UPEC ranges from 60-70%. Treatment of biofilm associated infections is challenging as they show increased multi drug resistance (Karigoudar et al., 2019). Microbes inside biofilm become more resistant to antibiotics than planktonic cell, hence make their eradication difficult. Biofilm associated pathogens which are resistant to antimicrobial agents are commonly responsible for recurrence and chronicity of infections. Hence, eradication and treatment of biofilm associated infections remains challenging in clinical settings (Behzadi et al., 2020).

The rise of antimicrobial resistance among Gram-negative uropathogens is a significant global challenge which results in difficult eradication of infection. Antimicrobial resistance (AMR) usually emerged due to mutation or transfer of genetic material among pathogens. Effective antibiotics are required for the treatment of UTIs, which
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are now threatened by antibiotic resistant Gram-negative uropathogens. As a result, AMR among uropathogens is one of the major causes of treatment failure for UTIs (Khoshnood et al., 2017). To curb harmful ripple effects of antibiotic resistance we need to identify alternative therapeutic options. Scientists throughout the world are trying to develop potential alternative treatment methods to overcome the threat of AMR (Giacomini et al., 2021). Among these alternative options, bacteriophages have gained major attention as an alternative therapeutic agent against MDR pathogens. In early 20th century, phages were generally used in treatment of human and animal infections. It has been reported that phages and phage encoded proteins have potential for treating resistant bacterial infection.

Bacteriophages are viruses that can infect and kill bacteria without any damage to human or animal cells. Bacteriophages are most diverse and abundant biological entities on the earth. It has been estimated that there are more than 1031 phage population on the planet. They can be found in different environments including soil, water, air, terrestrial surfaces and extreme environments. Moreover, they have been detected in environments populated by bacteria including animal and human intestines (Principi et al., 2019). Even phages are the best known phages made completely of proteins and DNA (Ahmed et al., 2020). It is estimated that 96% of phages belong to one of the three families of tailed phages and contain ds DNA. These three families are Myoviridae, Siphoviridae and Podoviridae belong to the order Caudovirales (Ye et al., 2019). Myoviruses have long contractile tail, Siphoviruses possess long non-contractile tail while Podoviruses have short non-contractile tail (Niu et al., 2014).

Phage therapy is emerging rapidly and has resulted in several clinical trials and cases of life-saving therapeutic use. Phage therapy is advantageous than antibiotic due to less or no side effects and self-replication ability. Phage lytic enzymes, monophage and cocktail bacteriophage preparation are used to reduce the chances of bacterial resistance (Samir, 2021). In this research work; isolation, characterization and antibiofilm activity of two bacteriophages, isolated from the indigenous environment, was determined against UPEC.

2. MATERIALS AND METHODS

Isolation and Identification of Bacterial Sample

Bacterial samples of UPEC were collected from Ayub Teaching Hospital Abbottabad (ATH) and Punjab University Lahore. All the bacterial isolates used in this study were streaked on the MacConkey agar, Eosin methylene blue (EMB) agar and Nutrient agar plates using a sterilized wire loop. Strains were identified as uropathogenic E. coli by various biochemical assays including catalase, oxidase, and Gram staining. Two bacterial isolates (E. coli ATD and E. coli 8640) were used as host for bacteriophages.

Bacterial Growth Curve

Growth curve describes the density of bacterial cells in broth media over time. The UPEC strains were grown in LB broth at 37°C with shaking. Optical density (O.D) and bacterial count was used to develop growth curve for E. coli strains. Bacterial strain was inoculated in flask containing 50 mL of LB broth and (O.D600) was measured after every 30 min. At the same time aliquots of culture were plated on LB agar plates through spread plate technique using different serial dilutions. The O.D600 and colony forming units (CFUs) were plotted on graph.

Isolation, Enrichment and Confirmation of Bacteriophages
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Sewage samples were collected from different sources including DHQ Mansehra, sewage exhausts of Ayub Teaching Hospital, Baffa sewage canal and CPEC sewage canal near Hazara University Mansehra. Collected sewage samples were transported to the laboratory and left undisturbed to sediment the solid debris. For enrichment of bacteriophages, sewage sample (40 mL) was mixed with 10 mL LB broth (5 X) and 1 mL of log phase bacterial culture in a 100 mL flask followed by overnight incubation at 37°C in a shaking incubator at 120 rpm. After overnight incubation, sample was centrifuged at 10,000 rpm for 10 min and supernatant was filtered through a 0.22 μm sterile syringe filters. Spot assay and double layer agar method was used to examine the supernatant (lysate) for the presence of lytic phage. Overnight incubated log phase bacterial culture (100 μL) was spread on LB agar plates with the help of sterile glass spreader. After few seconds, phage filtrate (5 μL) was spotted on the plates. After 24 hours incubation at 37°C the plates were examined for the transparent zone. The clear zone indicated the presence of lytic bacteriophages. However, double layer agar method was used to examine phage concentrations and plaque morphology (Khawaja et al., 2016).

Purification of Bacteriophages

For purification of bacteriophages, double layer agar assay was performed. Serial dilutions ($10^{-1}$-$10^{-10}$) of phage lysate were prepared by adding LB broth (900 μL) and phage filtrate (100 μL) in microcentrifuge tubes. To each dilution overnight grown bacterial culture (100 μL) was added. The mixture was poured onto the labeled LB agar plate followed by addition of semisolid LB agar (3-5 mL). For proper mixing of the lysate, Petri plates were swirled in circles. Plates containing semisolid agar were left for 10 min followed by overnight incubation. After incubation, lytic activity was observed on each plate (Kropinski et al., 2009). Based on transparency and size different plaques were observed and highlighted. Single plaque from the agar plate was picked using a sterile pipette tip and dropped in log phase broth culture of host bacteria in 50 mL flask. The flasks were incubated overnight at 37°C in a shaking incubator. The process was repeated 8-10 times to get purified phages.

Determination of Phage Titer

After purification of bacteriophages, the titer of bacteriophages was determined in samples in terms of plaque forming units / mL (pfu/mL) (Khawaja et al., 2016; Kropinski et al., 2009). To determine the phage titer, plates having phage dilution i.e. $10^{-7}$-$10^{-9}$ were selected through which the plaques were easily countable (20-300 pfu). After that plaques were observed and counted. The standard formula for phage titer determination is

\[(\text{pfu/mL}) = (\text{number of plaques} / D) \times V\]

\[(D= \text{dilution factor, } V= \text{volume used})\]

Characterization of Bacteriophages

Plaque Morphology

Plaque morphology (size and shape of plaque) is an important variable to evaluate the cytopathic effect of bacteriophages on bacterial colonies. Bacteriophages produced plaques with different types of morphology. Some bacteriophages produced small, transparent circular plaques while others produced large, irregular and semitransparent plaques. Lytic bacteriophages usually produce clear transparent plaques.
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**Morphological Study of the Isolated Bacteriophages**

Transmission electron microscopy was performed at, Leicester University UK to determine morphological characters of isolated phage. Following a lab standard protocol bacteriophage preparation for TEM examination was prepared. Briefly, centrifugal filter column (Amicon Ultra-4 centrifugal filter unit, Cat # C7719) was used for concentration of phage lysates. Concentrated phage filtrate (10 μL) was applied to carbon coated grids, followed by addition of phosphor-tungstic acid (15 μL) before obtaining images.

**Bacterial Growth Reduction Assay**

Growth reduction assay was used to measure the ability of bacteriophages to reduce *in vitro* growth of specific bacteria (Asif *et al.*, 2023). Two flasks containing 50 mL LB broth were inoculated with log phase bacterial culture of (O.D600=0.23 containing 6.2× 106 CFUs). Phage lysate (2.3 × 106 pfu/mL) at MOI (Multiplicity of infection) of 1.0 were added into one flask while other flask was considered as a control (bacterial culture only). Both flasks were incubated at 37°C with shaking at 120 rpm. After every 2 hours bacterial growth in the flasks was checked by measuring the O.D600 of the culture in a spectrophotometer till 24 hours. The isolated phages were checked for their ability to inhibit bacterial growth. Bacterial growth reduction was tested for either single phages or cocktail phages (Bibi *et al.*, 2016).

**Determination of Anti-biofilm Potential**

To evaluate anti-biofilm ability of isolated phages, the biofilm of *E. coli* ATD was developed in 96-well polystyrene microtiter plate. In the wells of pre-sterilized microtiter plate, LB broth (160 μL) and 10 μL of bacterial culture (O.D 600=0.23, containing 6.2 × 106 CFUs) were added. Then, 26.9 μL of phage (2.3 × 109 pfu/mL) was added simultaneously, after 1, 6, 9, 24 and 72 hours in the wells of microtiter plate at different MOIs (100, 10, 1, 0.1 and 0.01). After treatment with phage, microtiter plate was incubated for 5 days at 37°C. In each experiment, sterility control (sterile LB broth) and untreated control (wells containing bacterial strain only) was also used. After 5 days of incubation, wells of microtiter plates were washed three times with normal saline. Crystal violet (CV) assay was used for qualitative measurement of biofilm. In CV assay, 0.1% of crystal violet (200 μL) was added in all wells for 10 min. CV stain was then decanted by washing three times with normal saline (0.85%) to remove the excess stain. After the plates were air dried, 33% of glacial acetic acid (200 μL) was added. Microplate reader was used to examine biofilm growth at O.D580 nm (Shafique *et al.*, 2017). Each experiment was performed three times.

**Host Range Determination of Bacteriophage**

The host range of isolated bacteriophages against different pathogenic bacteria was determined by using spot assay (Alvi *et al.*, 2021a). The host range indicates whether a bacteriophage has broad or narrow range of activity. The isolated phages were tested against 10 clinical isolates of *E. coli* and five pathogenic bacteria including *S. aureus*, *Acinetobacter*, *P. aeruginosa*, *K. pneumoniae* and *Salmonella* spp.

3. RESULTS

**Identification of Bacterial Strain**
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The clinical isolates of UPEC were grown on differential and selective media including MacConkey’s agar, nutrient agar and EMB agar. The colonies of UPEC were examined on each media (Table 1.)

Table 1: Colonies of UPEC on different media.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Media</th>
<th>Colony Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>MacConkey agar</td>
<td>Dark pink colonies</td>
</tr>
<tr>
<td>3.</td>
<td>Nutrient agar</td>
<td>Greyish-white colonies</td>
</tr>
<tr>
<td>4.</td>
<td>EMB agar</td>
<td>Green metallic sheen colonies</td>
</tr>
</tbody>
</table>

**Gram’s Staining and Biochemical Identification**

All bacterial isolates obtained from UTI patients were Gram negative, catalase positive, lactose fermenters and oxidase negative and were confirmed as UPEC (Figure 1).

![Figure 1: Results of (A) Gram staining (B) Catalase test (C) Oxidase test. *E. coli* isolates were found Gram negative, catalase positive and oxidase negative.]

**Bacterial Growth Curve**

Optical density and bacterial count were used to develop growth curve for bacterial strains (*E. coli* ATD and *E. coli* 8640) (Figure 2).

**Isolation and Purification of Bacteriophages**

All four sewage samples were enriched for *E. coli* specific phages and only two of them showed positive results with clear zones. The bacteriophage isolated from ATH Abbottabad was named as ECAC whereas phage isolated from CPEC canal was named as ECCC (Figure 3, Table 2).

**Determination of Phage Titer**

Serial dilution and double layer agar method was used to determine the phage titer. The titer of phage ECAC was \((2.3 \times 10^9\) pfu/mL) while ECCC phage titer was \((4 \times 10^9\) pfu/mL) (Table 3).
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Figure 2: Bacterial growth curve using Optical Density and CFU/mL of (A) E. coli ATD and (B) E. coli 8640.

Figure 3: Results of spot assay (A) Phage ECAC (B) Phage ECCC.

Table 2: Sample source for phage isolation.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Host Bacteria</th>
<th>Phage</th>
<th>Source of Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>E. coli ATD</td>
<td>Phage ECAC</td>
<td>Ayub teaching hospital sewage</td>
</tr>
<tr>
<td>2.</td>
<td>E. coli 8640</td>
<td>Phage ECCC</td>
<td>CPEC Canal</td>
</tr>
<tr>
<td>3.</td>
<td>E. coli 8</td>
<td>No phage isolated</td>
<td>Baffa Sewage canal</td>
</tr>
<tr>
<td>4.</td>
<td>E. coli 6</td>
<td>No phage isolated</td>
<td>DHQ Mansehra</td>
</tr>
</tbody>
</table>

Table 3: Titer of isolated phages.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Isolated Phages</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECAC</td>
<td>(2.3 x 10^9 pfu/mL)</td>
</tr>
<tr>
<td>2.</td>
<td>ECCC</td>
<td>(4 x 10^9 pfu/mL)</td>
</tr>
</tbody>
</table>

Characterization of Bacteriophages

Plaque Morphology

The ECCC produced transparent plaques with irregular edges having diameter of 2.5 mm, whereas the plaques of ECAC were small pin point, transparent and circular with 1.5 mm diameter (Figure 4). The titer of phage ECAC was (2.3 x 10^9 pfu/mL) while ECCC phage titer was (4 x 10^9 pfu/mL).
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Figure 4: Plaque morphology of (A) Bacteriophages ECAC produced by infection in *E. coli* ATD, (B) Bacteriophages ECCC produced by infection *E. coli* 8640 by double layer agar overlay method.

**Morphological Features of Isolated Phages**

TEM of bacteriophages is important to study the bacteriophage morphology and revealed that the phage ECAC was a member of family Siphoviridae with average head diameter of 60 nm and tail length of 70 nm. While phage ECCC was placed in the family Myoviridae with long contractile tail of 120 nm and a head with about 30 nm in diameter, respectively. The morphology of both the isolated phages suggested that both these phages have tail and belong to the order Caudovirales (Figure 5, Table 4).

Table 4: Bacteriophage morphological features and classification.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Name</th>
<th>Tail</th>
<th>Head diameter (nm)</th>
<th>Tail length (nm)</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>ECAC</td>
<td>Yes</td>
<td>60nm</td>
<td>70nm</td>
<td>Siphoviridae</td>
</tr>
<tr>
<td>2.</td>
<td>ECCC</td>
<td>Yes</td>
<td>30nm</td>
<td>120nm</td>
<td>Myoviridae</td>
</tr>
</tbody>
</table>

Figure 5: Electron Micrograph of (A) phage ECAC (B) phage ECCC.

**Reduction Assay**

Bacterial growth reduction by lytic bacteriophages is an essential criterion for selection of bacteriophages as therapeutic tool (Pallavali *et al*., 2017). The lytic activity of the bacteriophages was performed at MOI 1 for 24 hours with bacteria *E. coli* ATD and *E. coli* 8640 in LB broth. The control contained *E. coli* ATD and *E. coli* 8640 only without bacteriophage. The reduction in bacterial growth in test group was compared with the control group.
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Bacterial growth was checked by measuring the (O.D600) of the culture after every 2 hours, for 24 hours. Bacteriophage ECAC demonstrated the ability to reduce growth of \textit{E. coli} ATD for initial 18 hours of infection while a slight increase in growth was observed after 18 hours of observation (Figure 6). It was found that ECCC inhibited the growth of \textit{E. coli} 8640 till 24 hours of infection. The cocktail of these two phages was found to have strong ability to inhibit bacterial growth till 24 hours as compared to single phages (Figure 6).

![Figure 6](image)

\textbf{Figure 6:} Bacterial reduction assay (A): Reduction assay of phage ECAC (B): Reduction assay of phage ECCC (C): Reduction assay of cocktail phage.

\textbf{Determination of anti-biofilm assay}

Biofilm of UPEC was grown on a 96-well microtiter plate and its growth kinetics were studied using CV assay. Efficiency of phage therapy depends on the efficient invasion of phages into biofilm. The age of biofilms might influence the efficiency of phage infection. Based on findings phage ECAC has diverse capacity to reduce bacterial biofilm at MOI 0.01, 0.1, 1, 10 and 100 at different intervals of time. While phage ECAC exhibited significant bacterial biofilm reduction at MOI 0.01 at 24 hours (Figure 7).

\textbf{Host Range of Phage}

The lytic capability of phages was tested against 10 clinical MDR isolates of \textit{E. coli} and five pathogenic bacteria including \textit{P. aeruginosa}, \textit{S. aureus}, \textit{S. typhi}, \textit{K. pneumoniae} and \textit{Acinetobacter}. The spot test showed that some strains of \textit{E. coli} were sensitive to ECAC and ECCC phages whereas no infectivity was seen for other genera, tested. Our results revealed that isolated phage has narrow host range (Table 5).
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![Graph showing OD (CV) variation over time for different MOIs](image)

**Figure 7:** Antibiofilm Potential of Phage ECAC.

**Table 5:** Results of host range of phage ECAC and ECCC.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Bacterial isolates</th>
<th>Spot test ECCC</th>
<th>Spot test ECAC</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>E. coli 1</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>2.</td>
<td>E. coli 2</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>3.</td>
<td>E. coli 3</td>
<td>+</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>4.</td>
<td>E. coli 4</td>
<td>-</td>
<td>+</td>
<td>Clinical</td>
</tr>
<tr>
<td>5.</td>
<td>E. coli 5</td>
<td>+</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>6.</td>
<td>E. coli 6</td>
<td>-</td>
<td>+</td>
<td>Clinical</td>
</tr>
<tr>
<td>7.</td>
<td>E. coli 8500</td>
<td>+</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>8.</td>
<td>E. coli 8518</td>
<td>-</td>
<td>+</td>
<td>Clinical</td>
</tr>
<tr>
<td>9.</td>
<td>E. coli 8475</td>
<td>+</td>
<td>+</td>
<td>Clinical</td>
</tr>
<tr>
<td>10.</td>
<td>E. coli 8587</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>11.</td>
<td>K. pneumoniae</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>12.</td>
<td>Salmonella Spp.</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>13.</td>
<td>Acinetobacter Spp.</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>14.</td>
<td>P. aeruginosa</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
<tr>
<td>15.</td>
<td>S. aureus</td>
<td>-</td>
<td>-</td>
<td>Clinical</td>
</tr>
</tbody>
</table>

### 4. DISCUSSION

Antibiotic resistance in bacteria has now become a big threat to global public health. The emergence of antibiotic resistant strains gains interest to develop new alternative approach of treating these infections. The therapeutic use of phages was first time started in 1919. First successful trial of phage therapy was conducted in Paris for the treatment dysentery, while all the treated patients successfully recovered. However, success of phage was just for short time. Treatment of MDR uropathogens become challenging due to rise of antibiotic resistance, therefore alternative therapeutic methods are required. Our study highlights the use of bacteriophage as a possible alternative solution.
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In the present study we carried out isolation of bacteriophages that would be specifically active against MDR uropathogenic *E. coli*. In the first step, uropathogenic *E. coli* isolates were collected to be used as host. Further characterization was carried out for confirmation of bacterial strain. Bacteriophage isolation, purification, morphological characterization, reduction assay, antibiofilm assay and host range determination were the next steps. In current study, we successfully isolated lytic phages from sewage water against uropathogenic *E. coli*. Our results are in accordance with a recent study in which lytic phage was isolated from hospital sewage against EPEC (Vahedi *et al.*, 2018). Similarly another study reported isolation of VB_EcoS-Golestan phage against *E. coli* from wastewater with high lytic activity (Yazdi *et al.*, 2020). Our study is supported by the fact that many researchers have reported presence of lytic phages in sewage water against *E. coli*.

The titer of phage ECAC was $2.3 \times 10^9$ pfu/mL and phage ECCC was $4 \times 10^9$ pfu/mL; therefore sewage water seems to be a good source for the isolation of lytic phage. This suggests wastewater may have diverse phage populations that could be used to treat MDR infections. By observation of plaque morphology only transparent plaques were considered for further study because lytic phages produce clear transparent plaques. Phage ECAC formed small transparent plaques while phage ECCC produced clear plaques with irregular edges. In a recent study researcher found that SaPL bacteriophage produced transparent, circular plaques with diameters of 4-5 mm (Alvi *et al.*, 2021b). Morphological characteristics of phages can be used for their classification. Electron microscopy of bacteriophages revealed both phage ECAC and ECCC belong to the order Caudovirales (Figure 5). The head and tail morphology showed that these phages were a member of family Siphoviridae and Myoviridae, respectively. Over 95% of reported phages belong to Caudovirales in which 62% phage belong to family Siphoviridae (Amarillas *et al.*, 2017).

Bacterial growth reduction assay helps to predict the feasibility of bacteriophage for phage therapy. In this study, phage ECAC reduced bacterial growth initial 18 hours of infection while phage ECCC inhibited the growth of bacteria till 24 hours of infection (Figure 6). Comparatively to single phage, the cocktail of both phages was found to have strong reduction ability till 24 hours (Figure 6). Phage cocktail used in our study has higher potential to inhibit bacterial growth as compared to other studies, hence could be used as therapeutic agent. The results of bacterial growth reduction by ECCC and ECAC depicts that both these phages can be utilized in phage therapy, singly or as cocktails to treat UTI infections due to UPEC. A study previously reported that bacteriophage TSP inhibited the bacterial growth for initial 12 hours at MOI 1 and 10 (Tabassum *et al.*, 2018).

The efficiency of isolated phages to reduce UPEC biofilm is an indication that these phages can be used to treat severe and prolonged UTI. Moreover, these phages might be used to clear biofilms from in-animate surface like catheters and other medical devices. Similarly in another study biofilm of *P. aeruginosa* was cleared *in vivo* by the combination of two different phages (Seth *et al.*, 2013). Moreover, researchers isolated AZ1 phage and tested its anti-biofilm activity against MDR *P. aeruginosa*. They suggested that phage AZ1 was able to eliminate biofilms of *P. aeruginosa* (Jamal *et al.*, 2017).

In this study, spot assay was used to test host range of isolated phages against bacterial strains. The results showed that some strains of *E. coli* were sensitive to both phages but no activity was seen for other genera, tested. Our results revealed that isolated phage has narrow host range. Similarly another study reported narrow range phages restricted to maximum of two bacterial species (Carey-Smith *et al.*, 2006). Broad host range phages showed great benefit in fighting against multiple pathogens but narrow host specificity have the benefit of target specificity.
Bacteriophages can control bacterial biofilms which is a key feature of phage therapy as compared to antibiotics. Both the phages ECCC and ECAC can be used specifically to treat UPEC, whereas the normal microbial flora will not be affected.

5. CONCLUSION AND RECOMMENDATIONS
Bacteriophage ECCC and ECAC are potent antibiofilm agent against UPEC. Due to antibiotic resistance in UPEC, urinary tract infections are difficult to treat and alternative treatment options need to be discovered. Consequently, the introduction of alternative methods to eradicate UPEC would be an important step towards phage therapy. On the basis of this study, it is concluded that both phages ECAC and ECCC have lytic activity against uropathogenic E. coli. Moreover, these phage cocktail also exhibited efficient bacterial growth reduction ability. Based on all these characteristics, phage ECAC and ECCC can be used as potential agent for phage therapy. Nevertheless, further in vivo trials are required to ensure safety for phage use.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgement
The authors acknowledge the contributions and support from the Department of Microbiology, Hazara University Mansehra and Institute of Microbiology and Molecular Genetics University of the Punjab.

Authors Contribution
Atia Ajaz conducted major portion of the research, Iqbal Ahmad Alvi and Zeeshan Niaz supervised and designed the research project. Dr. Shafiq Ur Rehman has provided the resources for this research and helped to draft the initial draft and the revision of the research article. Qazi Ajaz Ahmed has provided the bacterial samples and assisted in manuscript writing. Zahid Ahmad and Saman Saqib assisted in the phage characterization.

6. REFERENCES


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