

# Characterization and Lytic Activity of Isolated *Escherichia Coli* Bacteriophages against *Escherichia Coli in Vitro*

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## What's Known

- *Escherichia coli* (*E. coli*) is responsible for 75% of the urinary tract infections and typically treated with antibiotics.
- Unrestricted use of antibiotics has led to the emergence of a new class of drug resistance enzymes called extended-spectrum beta-lactamases (ESBL) and ESBL producing strains.

## What's New

- Due to the global rise in antibiotic resistance, phages have attracted attention as an alternative to antibiotics.
- *E. coli* bacteriophages were isolated from infected urinary specimens, and their lytic activity against *E. coli* was characterized *in vitro*.

## Abstract

**Background:** *Escherichia coli* (*E. coli*) is the most common cause of urinary tract infection (UTI) and typically treated with antibiotics. Unrestricted use of antibiotics may lead to the emergence of antibiotic-resistant bacteria. The present study aimed to isolate and characterize phages against *E. coli* from infected urine samples and to determine the lytic activity of phages against *E. coli in vitro*.

**Methods:** The present experimental study was conducted in the Laboratory of Bouali Sina Hospital (Sari, Iran) in May 2018. *E. coli* was identified from nine urine samples of patients with UTI using conventional microbiological methods. Bacteriophages were isolated from the infected urine specimens, and their lytic activity was determined using the spot test. The titer of the bacteriophages was measured using the double-layer agar technique. The morphology of the bacteriophages was revealed using transmission electron microscopy, and the latent time period and burst size were determined. Data were analyzed using the SPSS software package.

**Results:** *E. coli* was isolated from nine infected urine samples. The lytic activity of bacteriophages against *E. coli* was determined using the spot test by observing the formation of inhibition zones. Transmission electron microscopy showed *E. coli* phages belonging to the *Myoviridae* family. The latent time period was 20 minutes with a burst size of 1,200 plaque-forming unit (PFU) per infected cell. The results of the double-layer agar assay showed that the titer of bacteriophages was  $20 \times 10^8$  PFU/mL.

**Conclusion:** The *E. coli* bacteriophage was isolated from infected urine samples and characterized, and their lytic activity against *E. coli* was determined *in vitro*.

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**Keywords** • *Escherichia coli* • Bacteriophages • Urinary tract infections

## Introduction

Urinary tract infection (UTI) is the most common cause of nosocomial infections and the second most common infection in humans. Most UTIs are caused by normal colon bacteria such as *Klebsiella pneumoniae*, *Escherichia coli* (*E. coli*), *Proteus*, and *Pseudomonas aeruginosa*. Annually, about 150 million people are infected with UTI caused by *E. coli*.<sup>1</sup>

*E. coli* strains that cause UTIs (uropathogenic *E. coli*) are responsible for 75% of UTI, including 40-50% of UTI prevalence in women and 5% in men. *E. coli* is a Gram-negative bacteria of the *Enterobacteriaceae* family causing several human infections such as sepsis, gastroenteritis, neonatal meningitis, gallbladder and bile duct infections, wound infection, pneumonia, peritonitis, and especially UTI and kidney failure.<sup>2-5</sup>

Typically, antibiotics such as aminoglycosides, fluoroquinolones, and beta-lactam are used to treat *E. coli* infections. Up to date, unrestricted use of antibiotics has led to the emergence of a new class of drug resistance enzymes called extended-spectrum beta-lactamases (ESBL) and ESBL-producing strains. A global rise in antibiotic resistance, resulting from the physicians' tendency to prescribe newer drugs, has also undermined empirical treatment.<sup>6-8</sup> Therefore, it is essential to consider alternative medicine to prevent and treat UTI instead of resorting to antibiotics as the first choice. Recently, phages have attracted the attention of physicians as an alternative to antibiotics.<sup>9-11</sup> Phages, used as an individual phage or as a cocktail of several phages, have lytic activity without affecting normal flora. The main advantage of phage therapy over the standard antibiotics therapy is that phage cocktail reduces the possibility of developing phage-resistant bacteria.<sup>12, 13</sup>

In previous studies, phages against *E. coli* were isolated (from sewage, hospital sewage, polluted rivers, and human and animal feces) with the aim of developing a phage therapy to target pathogenic species of *E. coli*.<sup>14-16</sup> Another study reported the effect of oral application and topical treatment of phage cocktail on patients infected with UTI.<sup>17</sup> Chibeu and colleagues investigated and confirmed phage activity against biofilms of uropathogenic *E. coli*.<sup>18</sup> A study also confirmed the lytic activity of commercial phages against *E. coli* isolated from patients with UTI.<sup>19</sup> Some other studies have suggested that the pattern of antibiotic resistance of *E. coli* and its specific phages vary in different regions.<sup>20, 21</sup>

The present study aimed to isolate and characterize the lytic phages against *E. coli* from the urine samples of patients with UTI. In addition, lytic phage activity against *E. coli* with different antibiotic resistance patterns was determined *in vitro* and compared to *E. coli* phages in other regions.

## Materials and Methods

The present experimental study was conducted in the Laboratory of Bouali Sina Hospital (Sari, Iran) in May 2018.

### Isolation of *E. coli* from Infected Urine Samples

*E. coli* was isolated from nine urine samples of patients with UTI and identified using conventional microbiological methods (culture on blood agar, MacConkey agar, and eosin-methylene blue agar; all from QUELAB, USA). The plates were incubated at 37 °C for 24 hours. The pure isolates were characterized and identified through Gram-staining and with biochemical tests such as catalase, Simmons citrate agar, indole production, methyl-red and Voges-Proskauer (MR-VP), and triple sugar iron agar (TSI).<sup>22</sup> Sensitivity to antibiotics agents was determined using the disk diffusion method in accordance with the guidelines from the Clinical and Laboratory Standards Institute (CLSI). The antibiotic discs used were: Nalidixic acid (30 µg), Cefixime (5 µg), Piperacillin (100 µg), Amikacin (30 µg), gentamicin (10 µg), ceftriaxone (30 µg), Nitrofurantoin (300 µg), Ampicillin-Sulbactam acid (10/10 µg), and ceftazidime (30 µg); all from Rosco, USA.<sup>22</sup>

### Isolation of *E. coli* Bacteriophage

Initially, the urine samples were stored at 4 °C. An equal volume of 2X Luria-Bertani (LB) broth (QUELAB, USA) containing *E. coli* was added to each urine sample and incubated overnight at 37 °C with shaking. The culture was then centrifuged at 10,000 ×g for 10 minutes at 4 °C and the supernatant was filtered through Millipore filters with 0.22 µm pore size (Millipore, USA).<sup>23, 24</sup>

### Determination of the Host Range using Spot Test

The overnight *E. coli* cultures (100 µL) from the nine samples were individually mixed with 3 mL top agar and poured into Petri dishes containing bottom agar, and subsequently 10 µL of isolated phages was added. The dishes were incubated at 37 °C overnight. The following day, the dishes were checked for inhibition zones.<sup>23, 24</sup>

### The Titer of Bacteriophages

A double-layer agar assay was performed to determine the titer of bacteriophages. The *E. coli* in the LB medium was inoculated and shook at 37 °C until the optical density at 600 nm was reached. The serial dilutions of phages were prepared in seven tubes and two additional tubes (8 and 9) were used as negative and positive controls, respectively. The diluted phages (100 µL) were added to 100 µL *E. coli* and top agarose (3 mL) was added onto the LB agar plate. The plates were inverted and incubated at room temperature overnight, after which the plaques were counted.<sup>23, 24</sup>

### Electron Microscope Observation of Phages

Bacteriophages were concentrated by

centrifugation at 25,000 ×g for 60 minutes using a high-speed centrifuge. The purified phages were deposited on carbon-coated copper grids (Sigma-Aldrich, Germany) and stained with 2% uranyl acetate (pH=4-4.5). After staining, phages were observed using a Philips CM 300 electron microscope (Philips, USA) at 150 kV.<sup>23, 24</sup>

**The Single-Step Growth Curve**

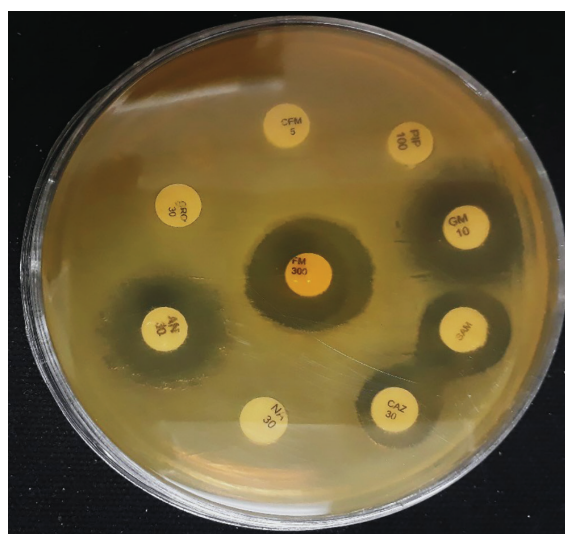
The phage lysate (200 µL) was added to 200 µL of LB broth (containing bacteria) and pre-incubated for 10 minutes at 37 °C to allow adsorption of the phages. A row of 16 sterile capped tubes were divided into four groups and aseptically added 900 µL LB broth to each tube. The phages were diluted in the LB broth by adding 100 µL to the first tube, mixed, and 100 µL was transferred to the second tube in the series (“1” to “4”). Then, 200 µL of an overnight culture of the bacteria was mixed with 200 µL of the diluted phages (“1” to “4”). The tubes were pre-incubated at 37 °C for 10 minutes to 40 hours to allow adsorption of the phages. At intervals, a sample was removed from the mixture and the number of free phages counted using a plaque assay. Petri dishes were incubated overnight at 37 °C. The following day, the number of plaques was counted and PFU/mL was calculated; equal to the number of plaques times the dilution factor (inverse of the dilutions).<sup>23, 24</sup>

**Results**

The results of the conventional microbiological method confirmed the isolation of *E. coli* strains from the infected urine samples (table 1). The sensitivity to antibiotics agents was determined in accordance with the CLSI guidelines. As shown in figure 1, the results revealed that four *E. coli* isolates were resistant to Nalidixic acid (30 µg), Cefixime (5 µg), Piperacillin (100 µg), ceftriaxone (30 µg). These isolates were however sensitive to Amikacin (30 µg), Gentamicin (10 µg), Ampicillin-Sulbactam acid (10/10 µg), and Nitrofurantoin (300 µg); but semi-sensitive to ceftazidime (30 µg). Three other *E. coli* isolates were resistant to Nalidixic acid (30 µg), Cefixime (5 µg), Piperacillin (100 µg), ceftriaxone (30 µg), and ceftazidime (30

µg); but sensitive to Amikacin (30 µg), Gentamicin (10 µg), Ampicillin-Sulbactam acid (10/10 µg), and Nitrofurantoin (300 µg). The other two *E. coli* isolates were resistant to Nalidixic acid (30 µg), Piperacillin (100 µg), and ceftriaxone (30 µg); however, sensitive to Amikacin (30 µg), Ampicillin-Sulbactam acid (10/10 µg), Nitrofurantoin (300 µg), and ceftazidime (30 µg), but semi-sensitive to Gentamicin (10 µg) and Cefixime (5 µg).

The host range of isolated phages was determined using spot testing and inhibition zones were observed in all samples. The results indicated that bacteriophages have lytic activity against nine *E. coli* strains with antibiotic susceptibility patterns (figure 2). The results of the double-layer agar assay showed that the titer of bacteriophages was 20×10<sup>8</sup> PFU/mL. Electron microscopy was performed by negative staining with 2% uranyl acetate (ACROS, Belgium) (pH=4-4.5) at 150 kV. The results showed one isolated phage group with an icosahedral head (60 nm) and a long non-contractile tail (200 nm). The isolated bacteriophages belonged to the *Myoviridae* family (order: *Caudovirales*); figure 3. The results of the single-step growth curve showed that the latent time period of isolated *E. coli* bacteriophages was 20 minutes with a burst size of 1200 PFU per infected host.

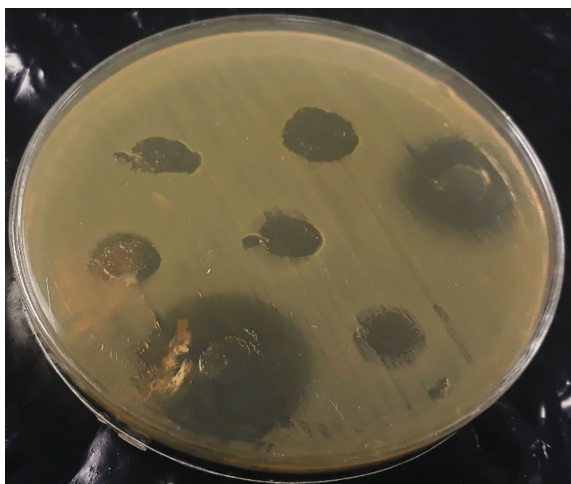


**Figure 1:** Disk diffusion method showed antibiotic susceptibility of nine isolated *Escherichia coli* bacteria from urinary culture of patients with urinary tract infection.

**Table 1:** The results of conventional microbiological methods identified *E. coli* strains from infected urine samples

Test	Result
Gram staining	Gram-negative, rod-shaped bacteria
Growth on MacConkey agar	Positive
Growth on Eosin methylene blue agar	Positive
TSI test	A/A
Citrate test	Negative
Methyl-red and Voges-Proskauer tests	+/-
Indole test	Positive

TSI: Triple Sugar Iron agar

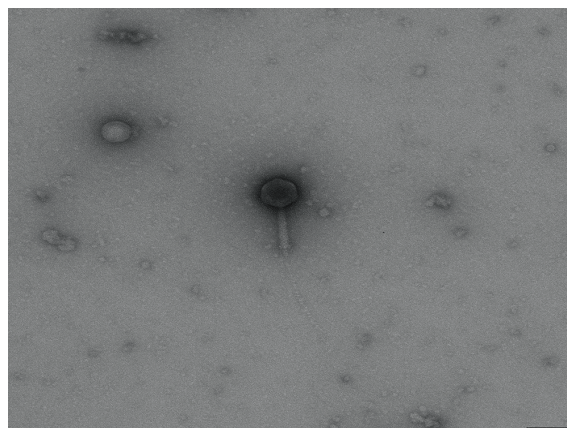


**Figure 2:** Spot test confirmed the lytic activity of *Escherichia coli* bacteriophages against *Escherichia coli* bacteria.

## Discussion

In the present study, the phage against *E. coli* was isolated from the urine sample of patients with UTI and the lytic activity of phages was confirmed with the spot test. The images obtained from the electron microscope revealed the separation of a phage type with a phenotypic attribute belonging to the *Myoviridae* family, with an icosahedral head and a 200-nm tail. Phages related to T7 have been classified into four groups in terms of amino acid of major capsid proteins, including Exo-T-even, Schizo-T-even, Pseudo-T-even, and T-even.<sup>25</sup> Our results showed that the lytic activity of this phage on different strains of *E. coli* was different in terms of their resistance pattern to antibiotics. A previous study showed that the lytic phage T4 and T6 had a limited hosting range, but phage KEP10 displayed lytic activity against a wide range of hosts.<sup>25</sup> Phages which were separated from different sources had different lytic activity. Ghasemian and others isolated 32 phages from the rivers in 32 cities in Iran. They showed that only the phage isolated from the city of Nowshahr had lytic activity against *E. coli*.<sup>26</sup> Moreover, they reported that even the isolated phages from one region had different sources and lytic activities. Another study conducted in the northwest of Iran showed that the phages isolated from urban sewage had a higher impact against *E. coli* compared with those isolated from the rivers.<sup>27</sup>

In a study by Galtie and others, AL505\_P1, AL505\_P2, AL505\_P3 phages were isolated from sewage which belonged to the *Podoviridae*, *Myoviridae*, and *Siphoviridae* families, respectively. The lytic activity of these phages against the *E. coli* AL505 strain, caused by uropathogenic *E. coli* isolated from a patient



**Figure 3:** Electron micrographs showed a phage from the *Myoviridae* family. Negatively stained with 2% uranyl acetate (pH=4-4.5), TEM HT (V)=80,000, TEM magnification=52,000, scale bar=100 nm.

with pyelonephritis, was confirmed.<sup>28</sup>

In the present study, the latent time period of isolated *E. coli* bacteriophages was 20 minutes, and the number of viable progenies per infected host was 1200 PFU per infected host. However, another study reported a latent time period of 24 minutes in phages isolated for sewage water, which belonged to the *Myoviridae* family.<sup>29</sup> Moreover, Pouillot and others reported the latent time period of *Podoviridae* against *E. coli* strain as 25 minutes.<sup>30</sup> Dufour and others<sup>31</sup> used bacteriophage LM33-P1 to infect the *E. coli* O25b strain, which is highly resistant to beta-lactams and fluoroquinolones. They showed a latent time period of 9 minutes *in vitro* against this phage strain. Also, its lytic activity *in vivo* against UTI, septicemia, and meningitis caused by *E. coli* was confirmed. Their findings indicated the potential of lytic phages for the treatment of UTI caused by *E. coli*.

The main limitation of the present study, due to financial constraints, was that genome sequencing of the isolated bacteriophages was not performed.

## Conclusion

*E. coli* bacteriophages were isolated from infected urinary specimens, and their lytic activity against *E. coli* was characterized by different antibiotic resistance patterns *in vitro*. These bacteriophages differed in terms of features and lytic activity depending on the source of the phage.

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**Conflict of Interest:** None declared.

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