



Full Length Article

Identification and *In Vitro* Evaluation of Phage vB_EcoS_P4261 Against UPEC Isolates in Urinary Tract Infections

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Abstract

Uropathogenic *Escherichia coli* (UPEC) is a common causative pathogen in the vast majority of urinary tract infections (UTIs) in hospital. The increasing prevalence of antibiotic-resistant UPEC emphasizes that alternatives to conventional antibiotics are needed. Here, we described phage vB_EcoS_P4261 isolated from UTI patient in affiliated hospital of Jinggangshan university. Phage characteristics including MOI, one-step growth curve, stability and bacteriolytic activity *in vitro* were determined and evaluated. Biological analysis of the isolated phage revealed that vB_EcoS_P4261 can form round, transparent, edge neat plaque. The head of the phage is hexagonal and has a long tail. The incubation period of phage is 10 min, the outbreak period is 60 min, the burst size is as high as 117PFU/infected cell, and it has good thermal stability and certain resistance to ultraviolet rays. Related evaluation of the isolated phage *in vitro* indicated ideal lysis effect against the corresponding multiple-drug-resistance UPEC isolates and standard *E. coli* stains. In this study, an efficient lytic phage vB_EcoS_P4261 against UPEC was identified. The microbiological properties of P4261 suggest that it may be useful for controlling multi-drug resistance bacterial populations, which also provides a potential therapeutic option for the study and therapy of UTIs caused by UPEC. © 2020 Friends Science Publishers

Keywords: UPEC; Bacteriophage; Identification; Bacteriolytic activity

Introduction

Escherichia coli (*E. coli*) is an opportunistic pathogen in both human and animals, which is frequently isolated and identified in clinic. UPEC is a common pathogen of human pyelonephritis and other urinary tract infections. It can result in persist infections in bladder tissues and cause recurrent urinary tract infections (UTIs). Hemolytic uremic syndrome was also caused by these pathogens in severe cases (Shah *et al.* 2019). With the fast outbreak of antibiotic-resistant bacteria, especially in UTIs, the application of bacteriophages has restore attention as an efficient alternative therapy method for *E.coli* infection control (LaVergne *et al.* 2018).

Phage is a type of virus that infects bacteria and affects the biology and evolution of the host bacteria (Cowley *et al.* 2015; Fu *et al.* 2015). Phages can target and lyse host bacteria. Phages are the most ordinary microbes on earth and show huge diversity in the host range. Phages are cost-effective, have no severe negative consequences and are highly toxic to

antibiotic-resistant bacteria compared to conventional drugs (Borysowski *et al.* 2011; Wang *et al.* 2016). Currently, the problem of bacterial resistance is becoming more and more prominent. Clinical studies have found that phages have shown good therapeutic effects on a variety of bacterial infections. Meanwhile, due to their strong specificity, rapid proliferation and wide source, phages are expected to be used as a new antibacterial agent to supplement or effectively replace antibiotic therapy of drug-resistant bacteria (Sarker *et al.* 2016). The results of related studies show that phage therapy can reduce the amounts of bacteria in animals, reduce the mortality rate, and even completely cure the pathogenic bacterial infections (O'Flaherty *et al.* 2009; Peng *et al.* 2018).

Here, a virulent phage isolated from the urine sample of patients who diagnosed with UTI was identified, and also the biological characteristics of *E. coli* bacteriophages were analyzed. This work may lay a solid foundation for the application of bacteriophages in the therapy of uropathogenic *E. coli* infections.

Materials and Methods

Sample collection

For this study, both bacteriophages and the bacteria were collected from the urine samples of patients with UTIs in affiliated hospital of Jinggangshan university. Strains MC1061, MG1655, DM1187 and strain Min27 of *E. coli* K-12 were donated by Professor Yaxian Yan of Shanghai Jiao Tong University. 22 strains of *E. coli* U4372 and U4261 (Table 1) were isolated from urine samples, and *staphylococcus aureus* ATCC43300 was donated by associate professor Xianqing Deng of Jinggangshan university. The strains were cultured in THB or LB liquid medium at 37°C.

Isolation and identification of phages

Phages were isolated from urine samples obtained in hospital and transported under 4°C. The urine samples were filtered with the 0.22 μ m filter membrane, and the obtained filtrate was successively added to SM buffer for 10-fold dilution. The supernatants were subjected to lytic phage evaluation using different *E. coli* isolates on LB plates. The commonly used double-layer agar plate method was applied to perform the plaque formation assay. After overnight (8–12 h) incubation of phage and indicator bacteria, plaque forming unit on the plate of bacteria culture indicated the existence of lytic phage. Single plaque of the phage was purified three times until the edge of the plaque is neat and uniform in size. A single plaque was selected for amplification and culture. Phage supernatant was deposited at 4°C for following experiments.

Host range evaluation

Spot test as well as double agar overlay methods were carried out to evaluate the host range of phage P4261. In the first place, the phages were isolated using the indicator bacterium *E. coli* K-12 MC1061 and the wide host range of P4261 was evaluated with the clinical isolates. In the spot test, the bacteriophage lysate was spotted onto the bacterial lawn and its lysis activity was determined by the bacteriophage removal. This study was performed in triplicates.

Morphological characterization by transmission electron microscopy (TEM)

To identify the morphological characters of P4261, the phage sample was stained negatively by 2% phosphotungstic acid (PTA) and observed under TEM. The phage particles were stained negative by 2% phosphotungstic acid (PTA) and observed under transmission electron microscope. Briefly, the purified phage supernatants were added onto a copper grid and dry in RT for 8 min. 10 μ L of 2% PTA was added to

the dried phage particles waiting for 5 min. Lastly, the copper grid was washed for three times with ultrapure water in order to clean the redundant stain and observed under TEM (FEI, Hillsboro, USA).

Phage genomic type analysis

Purified phage genome was extracted and prepared as described previously (Niu *et al.* 2014). In order to identify the genome type of the phage genome, purified phage genome was treated with nuclease of DNase I (20 U/ μ L), RNase A (5 U/ μ L), and Mung bean nuclease (20 U/ μ L) at 37°C for 1 h. Products of digested phage nucleic acid were analyzed using 1% agarose gel electrophoresis.

Assay of optimal multiplicity of infection (MOI)

Overnight bacteria cultures of *E. coli* K-12 strain MC1061 were diluted with LB and incubated in a shaker at 37°C until reaching an early logarithmic growth phase. The bacteria were counted as CFU/mL, and mixed with phages according to different MOIs (0.001, 0.01, 0.1, 1, 10 and 100). After 8 h incubation, the culture was centrifuged with 6000 rpm for 10 min in a refrigerate centrifuge. After that, the supernatants were then filtered using 0.22 μ m filter. The titer of the phage in the supernatant was directly evaluated.

One-step growth curve

One-step growth experiments of the phage were carried out using a modified method described previously (Pajunen *et al.* 2000). Briefly, vB_EcoS_P4261 phage was added to a log phase *E. coli* K-12 MC1061 bacteria culture at a MOI of 0.1, and allow for incubation for 15 min. The phage and bacteria mixture were subjected to centrifuge at 12,000 rpm for 60 seconds. The supernatants were discarded, and the sediment of phage and bacteria cells were suspended with fresh LB and then incubated in a shaker of 180 rpm at 37°C. A small amount of samples were collected at 10 min or 30 min intervals (0, 10, 20, 30, 40, 50, 60, 90, 120, and 150 min) and the phage titer of each sample were immediately determined. This assay was repeated at least in triplicate.

Phage stability

The stability of phage P4261 was evaluated at different temperatures (40, 50, 60, 70 and 80°C). After incubated in the water of different temperature for 30 min, the phage aliquots were subject to plaque forming assay to detect the phage titer. The pH stability of the phage at different pH-values was also detected. To analyze the chemical stability of the phage, the P4261 was incubated with different concentration of chloroform (1, 2, and 5%, vol/vol) for 30 min at RT. Furthermore, P4261 was also exposed to ultraviolet ray for 0, 15, 30, 45, 60, 75, 90, 105 and 120 min, and the sample titer was determined immediately through a double-layer agar plate method.

Bacteriolytic activity of P4261 *in vitro*

The fresh *E. coli* bacteria culture were diluted 1:100 with LB and incubated at 37°C until reaching OD₆₀₀ Value 0.4–0.6. Phage P4261 was added at a relatively high, medium and low MOI of 100, 1, 0.01 and an identical *E. coli* (U3872 and U3519) culture with equivalent volume phage was tested. The mixture was cultured at 37°C in an incubator shaking of 160 rpm. The bacteria lytic activity of phages was evaluated by observing OD₆₀₀ Value of culture medium at an interval of 30min for 4 h.

Statistical analyses

The data points were drawn by GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, U.S.A.). Data are expressed as mean ± standard deviation (SD).

Results

Phage isolation and determination of host range

Here, a lytic *E. coli* phage designated as P4261 from urine sample of UTI patient in affiliated hospital of Jinggangshan university was isolated. Using *E. coli* K-12 strain MC1061 as the indicator strain, the phage plaques of round, smooth and tidy edge and with 1–2 mm in diameter were observed (Fig. 1A). The phage P4261 had a strong ability to form plaques on *E. coli* strains and UTIs isolates, suggesting a potential application of therapeutic effect in UTIs (Table 1). In addition, the P4261 phage showed relative strong bacteriolytic activity against a MRSA (methicillin-resistant *S. aureus*) strain ATCC43300 (Table 1).

The phage morphology was observed through TEM analysis and the results demonstrated that the phage P4261 having the icosahedral head of approximately 40 ± 5.0 nm as well as long non-contractile tail of approximately 115 ± 5.0 nm in length (Fig. 1B). Based on the classification of International Committee on Taxonomy of Viruses (ICTV) (Adams et al. 2014), the phage P4261 was likely to belong to the family *Siphoviridae*, *Caudovirales* considering the morphological characteristics.

Genome type analysis

The purified phage genome was digested by three kind of different enzyme of nucleases. The electrophoresis results revealed that P4261 genome was entirely digested by DNase I, while RNase A or Mung bean nuclease had no effect on the phage genome (Fig. 2), which indicating that P4261 was a double-stranded DNA virus.

Determination of MOI

The results showed that when MOI=0.1, the titer of the phage lysates was the highest (7.26×10^{14} PFU/mL; Table 2), indicating that the optimal MOI of the phage was 0.1.

Table 1: Lytic activity of phage vB_EcoS_P4261

Strain Type	Strains	Lytic Activity
<i>E. coli</i> strain K-12	MC1061	+++
	MG1655	+++
	DM1187	+++
UPEC isolates	U3872	++
	U3519	++
	U4469	+
	U4261	-
	U129	-
	U4196	-
<i>E. coli</i> strain O157	Min27	-
<i>P. aeruginosa</i> isolates	U955	-
Isolates of <i>enterobacter cloacae</i>	U547	-
<i>Staphylococcus aureus</i>	ATCC43300	++

Note: +++: Very strong lytic ability; ++: lytic ability; +: weak lytic ability; -: No lytic ability

Table 2: Determination of optimal multiplicity of infection (MOI) of phage P4261

Number	Bacteria (CFU/mL)	Phage (PFU/mL)	MOI	Phage Titer (PFU/mL)
1	10^9	10^6	0.001	1.34×10^8
2	10^9	10^7	0.01	4.42×10^{10}
3	10^9	10^8	0.1	7.26×10^{14}
4	10^9	10^9	1	1.48×10^{11}
5	10^8	10^9	10	7.52×10^{10}
6	10^7	10^9	100	3.21×10^9

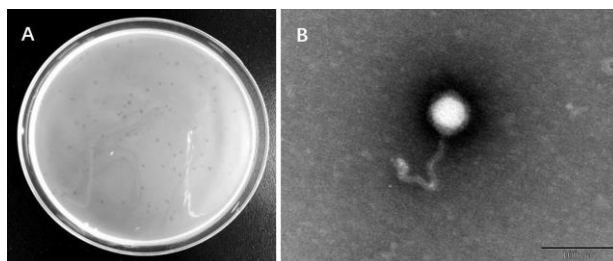


Fig. 1: Morphological characterization and life cycle of *Escherichia* phage vB_EcoS_P4261

(A) Transmission Electron Microscopic (TEM) image of *Escherichia* phage vB_EcoS_P4261 shows that the phage belongs to *Siphoviridae* family, (B) Observed plaques of *Escherichia* phage vB_EcoS_P4261 infecting *E. coli* K-12 MC1061 using double agar overlay method

One-step growth curve

The phage life cycle is one of the important criteria for evaluating therapeutic phage lysis activity and determining its infectivity. Accordingly, one-step growth assay with the presence of indicator *E. coli* MC1061 was carried out to evaluate the latency time and burst size of the phage. The phage P4261 was found to have a short latency time of 10 min and the calculated burst size was 117 PFU/ infected cell (Fig. 3) according to Burst Size=phage titer (PFU/mL) at the end of burst period/host bacteria concentration at the initial stage of infection (CFU/mL). It is well known that when phages have a short latency time and a large amount of phage release from infected host, they are characterized as virulent phages. The results indicated that *E. coli* phage P4261 is a virulent phage with very adaptive therapeutic application.

Phage stability

The potential therapeutic application of phage P4261 was determined by evaluating the phage stabilities in different conditions. The thermal stability was detected under different temperatures. It was demonstrated that the activity of phage P4261 was relative stable under 50°C. Higher temperatures led to a progressive inactivation of phage. Phage P4261 was totally inactivated when it was heated to a high temperature of 70°C (Fig. 4A).

The pH stability was evaluated with different pH (2–13) prepared in SM buffer. The phage showed a relatively high survival rate under pH value of 6 to 10. However, under other pH scale, the activity of phage decreased dramatically (Fig. 4B).

In addition, the survival rate of phage was affected in the presence of 1, 2 and 5% chloroform. The results showed that chloroform had a significant effect on the titer of phages. As the concentration of chloroform increased, the survival rate of phages decreased, suggesting that the phages were sensitive to chloroform (Fig. 4C).

Ultraviolet irradiation assay showed that the phage titer dropped sharply after sampling at an interval of 15 min. With the extension of the irradiation time, the phage titer decreased, indicating that the phage was sensitive to ultraviolet radiation, but also had certain resistance (Fig. 4D).

The study indicated that phage structure might be related to survival under adverse conditions. Previous studies reported probable correlations between phage morphology character and its survival rate under adverse circumstances (Wang *et al.* 2016). It was showed that tailed phages can be stable under harsh environment (Rabitsch *et al.* 2004). From the above, the phage stability results showed a wide range of thermal and pH stability and also strong resistance to ultraviolet.

Bacteriolytic activity of P4261

The phage P4261 bacteriolytic activity was determined in a log-phase culture of Uropathogenic *E. coli* (UPEC) strains U3872 and U3519. *E. coli* K-12 was used as a control. We added the phage lysate into the culture with a relative high MOI of 100 to avoid the bacteria re-proliferation under low MOI infection status. Results showed that the growth of these strains was substantially suppressed directly after phage invasion (Fig. 5). These results suggested that P4261 was highly effective against UPEC *in vitro* and indicated a therapeutic potential *in vivo*.

Discussion

This study described the isolation, identification and potential application of bacteriophage against UPEC isolates from UTI patients. According to the phage morphology determined by TEM analysis, it was named as vB_EcoS_P4261. This phage was homologous to

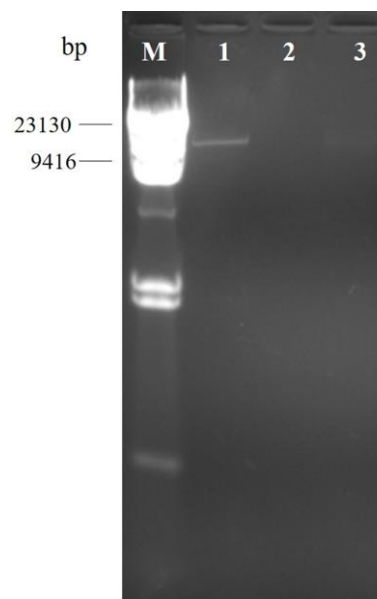


Fig. 2: Agarose gel electrophoresis image of phage vB_EcoS_P4261 genome digested with nuclease. Lane M: λ -Hind III digest DNA Marker, Lane 1-3: phage vB_EcoS_P4261 genome digested with RNase A, DNase I, and Mung Bean Nuclease, respectively

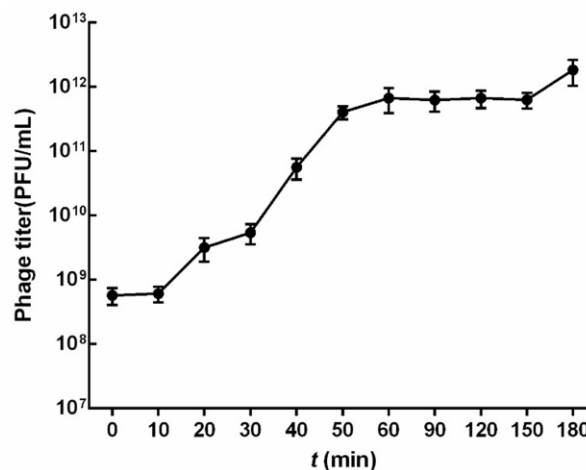


Fig. 3: One-step growth curve of phage vB_EcoS_P4261 in *E. coli*. Phage vB_EcoS_P4261 was co-incubated with *E. coli* strain cultured at a MOI of 0.1 for 15 min at 37°C. Results are shown as means $\pm$ SD from triplicate experiments. The latent period was short as 10 min which represents interval between the absorption and the beginning of the initial burst. The burst size was estimated at 117 PFU/infected cell, which was the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells

phages which belong to the family *Siphoviridae* in morphology based on the classification rule of ICTV. Therefore, here, vB means viruses Bacteriophage; Eco: *Escherichia coli*; S: *Siphoviridae*. 4261 is the urine sample number of isolated phages.

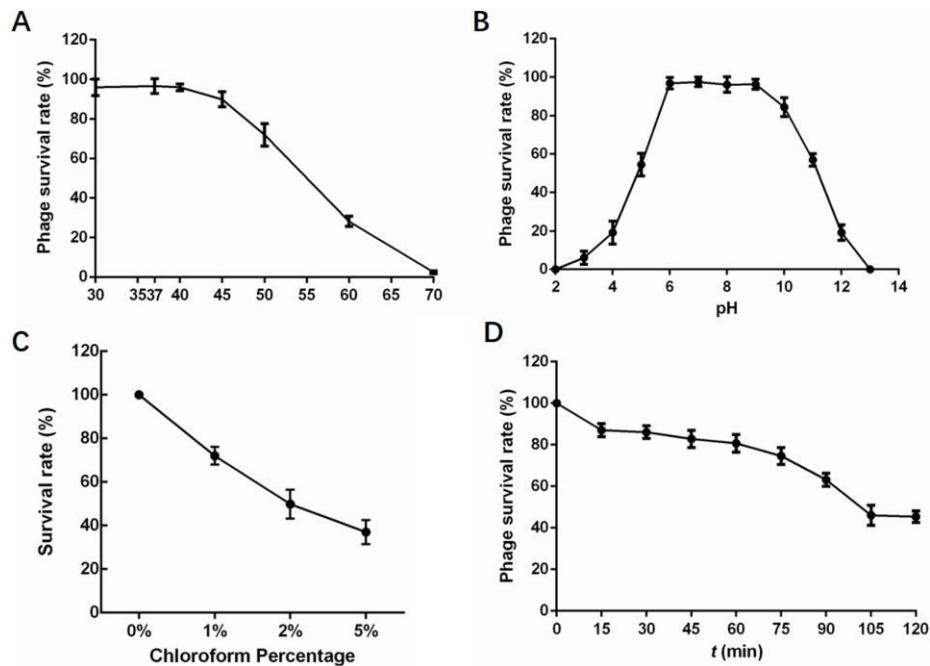


Fig. 4: Stability evaluations of phage vB_EcoS_P4261. (A) Thermostability: Phage vB_EcoS_P4261 was incubated at various temperatures as indicated. an aliquot sample of phage P4261 were collected after 30 min; (B) pH stability: Phage vB_EcoS_P4261 was incubated under different pH conditions for 3 h; (C) Chloroform stability: Phage vB_EcoS_P4261 was treated with chloroform (1, 2, 5%, vol/vol) for 30 min; (D) Ultraviolet light stability: Phage vB_EcoS_P4261 was exposed to UV light for 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. The overall results were expressed as survival rates, and were titrated immediately using double-layer agar plate method. Results are shown as means $\pm$ SD from triplicate experiments

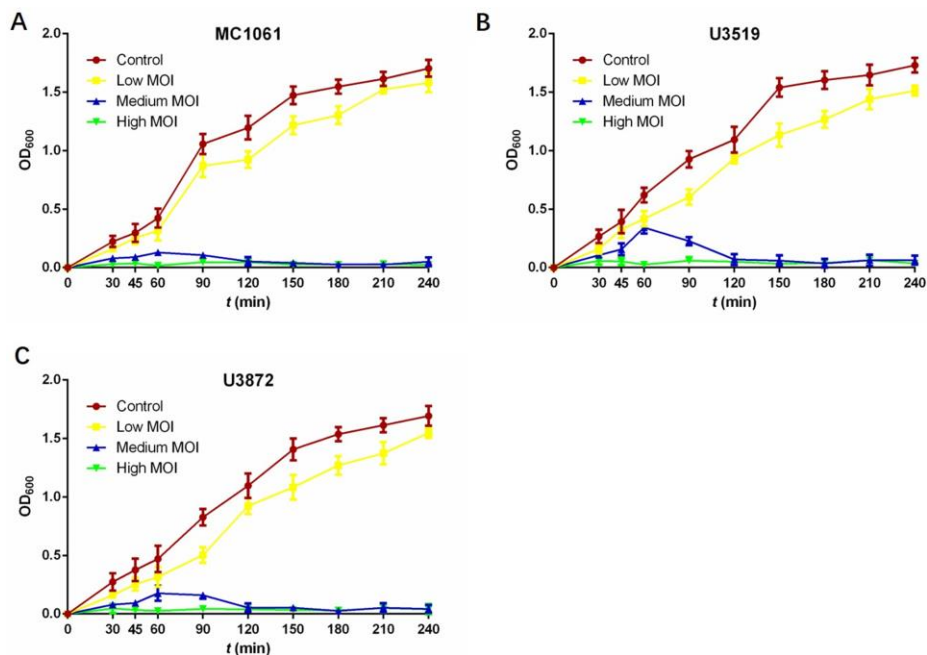


Fig. 5: Bacteriolytic activity of phage vB_EcoS_P4261 against *E. coli* K-12 strain and UPEC isolates *in vitro*. Early exponential cultures of *E. coli* K-12 strain MC1061 (A) and UPEC isolates U3519 (B) and U3872 (C) strains were co-cultured with vB_EcoS_P4261 phage at different MOIs: Low MOI of 0.01, Medium MOI of 1, and High MOI of 100, respectively. *E. coli* cultured with a similar volume of phage diluent was used as a control. Results are shown as means $\pm$ SD from triplicate experiments

Our study investigated virulent phage vB_EcoS_P4261, which was found to be capable of infecting *E. coli* K-12 strains, *S. aureus*, and UPEC isolates. Characterization experiments showed that P4261 has many biological features suitable for therapy applications in UTIs including short latent periods, large burst sizes, wide host range and high stability. UTIs are considered to be one of the most common bacterial infection cases in community hospitals (Malik *et al.* 2020), accounting for about 40% of nosocomial infections. As a result, the proliferation of drug-resistant isolates of UPECs has refocused researchers' attention on antimicrobial approaches to phage therapy. The emergence of broad-spectrum lactamase (ESBL) and strains which can produce biofilm adds another barrier to the traditional antibiotic treatment of urinary pathogens, further promoting the exploration of new treatment measures. Many researchers have reported *in vivo* and *in vitro* bacteriolytic activities of phages, which indicating a potential therapy application as an antibacterial agent (Capparelli *et al.* 2006; Watanabe *et al.* 2007; Gu *et al.* 2012; Yen *et al.* 2017).

In this study, in order to evaluate the application potential of phage vB_EcoS_P4261 *in vitro*, bacteriolytic activity experiments were performed by apply different MOIs to infect the *E. coli* strains. Results demonstrated that the phage substantially inhibit and lysed the bacteria under a relative medium and high MOI, which indicating a potential phage therapy candidate for antibiotics-resistance UPEC infections. Since bacteriophages have strong lysis activity against the pathogen responsible for diseases, the isolation and identification of phages which can target pathogenic bacteria is of great importance. The application of bacteriophages in therapy has been reported as a dramatic method for treating *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *staphylococcus aureus* infections under different conditions, especially in UTIs (Pallavali *et al.* 2017). What's more, antibiotic resistance has reached dangerous levels for public health, therefore, alternative methods, for example, phage therapy, are urgently needed. Our study is part of an ongoing effort by the science behind phage therapy to promote better understanding behind the use of phages as therapeutic tools (Manohar *et al.* 2018).

Conclusion

In the present study, a phage named as vB_EcoS_P4261 against UPEC, and with efficient lytic ability was identified. The biological characteristics of P4261 suggest that it is of great potential for its therapeutic application in bacterial infections, especially in UTIs.

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Author Contributions

Bin Liu, Xiaolong Qu and Qiang Fu designed the study. Weiye Wang, Huimin Li, Zijun Tang, Wei Zhang performed experimental work. Li Guo, Yangming Chen collected the samples. Caihua Dai, Yuqing Wang and Yating Xu analyzed the data. Bin Liu and Qiang Fu wrote the article.

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