# Characterization of a Novel Lytic Bacteriophage φEC14 that Infects *Enterobacter cloacae* Clinical Isolates

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***Abstract:*** *Enterobacter cloacae, an important agent associated with nosocomial infection, usually involves expanded-spectrum cephalosporin resistance. The therapeutic potential of bacteriophage is a possibly alternative chemotherapy against bacterial infection. In this study, we have characterized one newly isolated bacteriophage φEC14, which is lytic to E. cloacae specifically. Transmission electron microscopy revealed that phage φEC14 had an icosahedral head and long contractile tail, morphologically similar to phages belonging to family Siphoviridae. Pulsed-Field Gel Electrophoresis (PFGE) showed that the size of φEC14 virion DNA was in rang of 23.0-48.5 kb. Restriction analysis showed that lytic phage φEC14 was a double-stranded DNA virus, which might be cut by some restriction endonucleases. SDS-PAGE of phage proteins exhibited one major band and six minor bands with molecular weight ranging from 6.5 to 66.4 kilo-Dalton. In one-step experiment, phage φEC14 had a short latent period of 10 minutes and a burst size of 50 PFU/cell. The best understanding of the biological features of lytic bacteriophage φEC14 would facilitate the development of an alternative agent to control the spread of multidrug-resistant E. cloacae.*

***Keywords:*** *Phage therapy, Enterobacter cloacae, Antibiotic resistance.*

## Introduction

*Enterobacter cloacae*, a member of the *Enterobacteriacea* family, is fermentative, usually motile, gram-negative bacterium distributed in soil, water, and found in clinical settings. *E. cloacae* is a well-recognized opportunistic pathogen that causes significant infections, especially in neonate, elderly and immunocompromised patients, such as bacteremia, pneumonia, skin/soft tissue infection, urinary tract infection, and intra-abdominal infection [2]. This microbe exhibits intrinsically resistant to ampicillin and narrow-spectrum cephalosporins, due to a chromosomal cephalosporinase [12], furthermore, high prevalence of mutators among *E. cloacae* isolates associated with antimicrobial resistance is considered to related to the over production of the species-specific cephalosporinase, or production of plasmid-mediated extended-spectrum β-lactamases [14]. In fact, patients with infection by antibiotic-resistant *E. cloacae* are likely to develop antibiotic therapy failure and usually need a more prolonged course of antibiotic therapy. Currently, the increasing incidence of antibiotic-resistant bacterial strains has stimulated resurgence in interest into bacteriophage therapy [10, 11]. Bacteriophages, often referred to as phages, are viruses that infect bacteria. Basically, there are three types of phages, lytic, temperate and chronic [11]. Application of bacteriophages as antibacterial agent usually involves in lytic phage due to their ability to kill bacterial host, following infection, by lysing infected cell to release progeny phages.

Recent years, a series of reports of phage efficacy has been published in experimental infections including *Staphylococcus aureus* [3], vancomycin-resistant *Enterococcus faecium* [1], *Pseudomonas aeruginosa* [23], and *Escherichia coli* [22] including extended-spectrum β-lactamase strains. However, to our knowledge, very few descriptions of lytic phages of *E. cloacae* have been documented. In this article, clinical isolates of *E. cloacae* were utilized as indicator hosts for the isolation of lytic phages from sewage. A novel virulent phage, designated φEC14, was characterized and its basic biological features were carried out.

## Materials and methods

### *Bacterial strains and culture conditions*

Sixteen clinical isolates of *E cloacae* obtained from Department of Clinical Laboratory, China-Japan Union Hospital, Changchun, China, and identified using an API 32 GN (Biomerieux Ltd., France) system, were used for phage isolation and identification. Other bacterial strains, such as *Pseudomonas aeruginosa* and *Escherichia coli*, were employed for phage host range investigation. All bacterial strains were grown on Nutrient agar and Luria-Bertani broth at 37 °C.

### *Isolation of bacteriophages*

Procedures of phage isolation was conducted as previous description [6, 7]. Several specimens from raw sewage were collected from a local hospital. Specimens were immediately brought to the laboratory and centrifuged at 10,000 × *g* for 15 min at 4 °C to remove debris. The supernatants were filtered through a 0.45-μm-pore-size membrane. Five milliliter of the filtered supernatant was mixed with 6 ml of double-strength modified Luria-Bertani broth (containing 0.03% $CaCl_2$ and 0.04% $MgSO_4$) and 1 ml ($5 \times 10^8$ CFU/ml) of each strain of *E. cloacae* in exponential growth phase. The enrichment culture was incubated in a shaker at 37 °C, 200 rpm. After 18 h, the culture was centrifuged at 12,000 × *g* for 15 min, and supernatant was used to check the presence of lytic phages by the double-layer method. When plaques developed, a clearly single-plaque was added to 150 ml of sterile Luria-Bertani broth inoculated with 2% host cells in exponential growth phase. After incubation with shaking at 37 °C, 200 rpm for 5 h, complete lysis was achieved. The lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C to remove bacterial debris.

### *Morphological features of phage by transmission electron microscope*

Ten μl of purified phage particles ($10^{10}$ PFU/ml) was spotted onto a 400-mesh-size formvar-cabon-coated copper grid, stained with 2% uranyl acetate and then examined by transmission electron microscope (Hitachi Co. Ltd., Tokyo, Japan) at an accelerating voltage of 80 kV.

### *Analysis of phage genome by restriction and PFGE*

Procedures for extraction of DNA was performed according to a method described previously [13, 21]. Phage DNA was isolated from a high-titer phage stock using Amicon Ultracentrifuge tube (100 KD) to remove soluble macromolecules up to 100 KD. Phage genomic DNA was subjected to restriction digestion with eight restriction enzymes (*Eco*RI, *Eco*RV, *Hind*III, *Xba*I, *Nde*I, *Bgl*II, *Bam*HI and *Pst*I). The digested products were analyzed by agarose gel electrophoresis. To determine the size of phage genome, pulsed-field gel electrophoresis (PFGE) was carried out according to the standard protocol [6, 18]. A midrange PFGE marker was used for estimation of genomic size, and the samples were analyzed by electrophoresis using a Bio-Rad CHEF III system at 6 V/cm for 18 hours at 14 °C with pulses ramps from 3.5-4 s.

### *SDS-PAGE analysis of phages*

To determine the proteins of phage particles, SDS-polyacrylamide gel electrophoresis (PAGE) was carried out [26]. Briefly, a whole phage lysate was prepared by mixing 20 µl of purified phage particles ($10^{12}$ PFU/ml) with 20 µl of loading buffer and heating at 100 °C for 5 min. A volume of phage lysate (about $10^{10}$ PFU) was subjected to a 12.5% SDS-PAGE directly and the gel stained with 0.1% Coomassie brilliant blue.

### *Measurement of multiplicity of infection (MOI) and one-step growth assay*

Mid-exponential host bacterial cultures ($6 \times 10^8$ CFU/ml) were mixed with serial dilutions of bacteriophage stock solution. After adsorption, centrifugation at 5,000 g for 8 min was performed for removing free bacteriophages, pellets were resuspended with LB medium, and samples were taken for titration of bacteriophage. One-step growth experiment was determined as described previously [19, 25]. Briefly, to investigate the latent period and burst size, host cells ($3 \times 10^6$ CFU/ml) were mixed with phage particles ($3 \times 10^5$ PFU/ml) for 5 min, washed with LB medium to remove free bacteriophages, and then resuspended in fresh LB medium. An aliquot of the bacterial suspension was collected at 6 min time intervals during incubation at 37 °C for the titration of newly produced phages using the double-layer-agar plate method.

### *Host range analysis*

The host range of the phages was examined by spectrophotometric loss of turbidity on 16 clinical isolates of *E. cloacae* as described earlier [5, 16] with required modifications. All strains were inoculated for 12 h at 37 °C and grown to an $OD_{590}$ of 0.2. One ml aliquot of the bacterial solution ($10^8$ CFU/ml) was incubated with 1 ml of phage stock solution ($10^6$ PFU/ml) at 37 °C for 60 min. A spectrophotometer was used to monitor the lytic activity, measured as a decrease in $OD_{590}$ of tested host cells.

## Results

### *Isolation of virulent bacteriophage φEC14*

Sixteen *E. cloacae* clinical isolates were employed as an indicator strains. Virulent bacteriophages were isolated from sewage samples by the double-layer method, and plaques were allowed to check by incubating at 36 °C for 16 hours. Visible plaques were found, and the plaques had a diameter of 3 to 5 mm (Fig. 1). Further phage purification was carried out by picking single plaques and purifying through three times. A lytic bacteriophage active against *E. cloacae* was isolated and named φEC14.

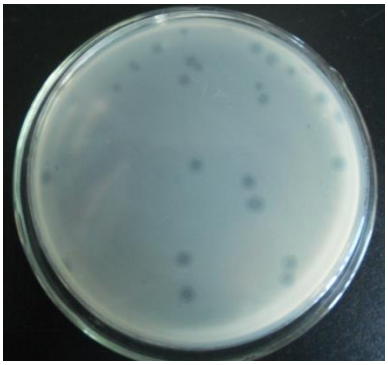

Fig. 1 Development and morphology φEC14 plaques

## *Morphology of phage φEC14 by transmission electron microscopy*

Purified phage lysate was spotted onto a 400-mesh-size formvar-carbon-coated copper grid with 2% uranyl acetate and observed by transmission electron microscopy. The results showed that phage φEC14 had an icosahedral head, about 50 nm in diameter, a 150 nm long tail (Fig. 2). Thus, phage φEC14 was morphologically similar to phages belonging to the family *Siphoviridae.*

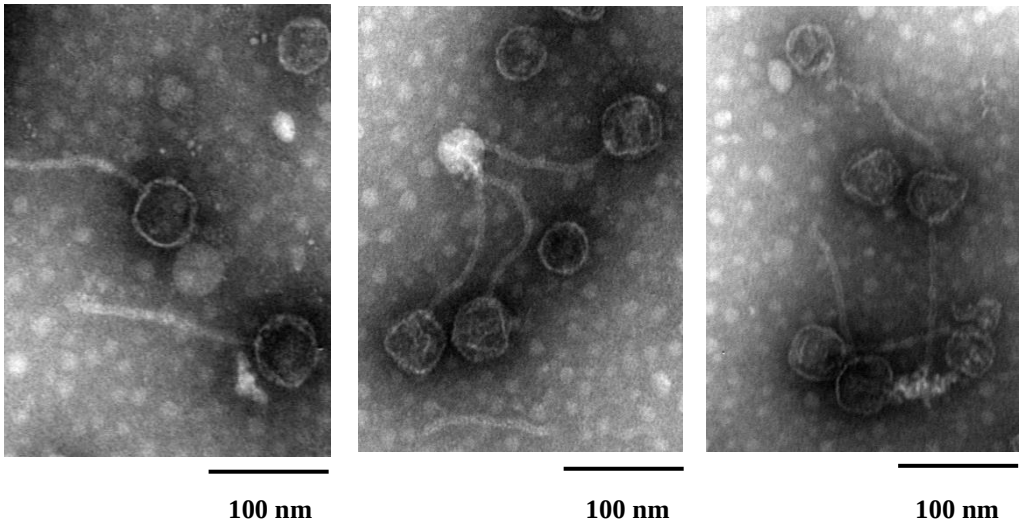


Fig. 2 Electron micrograph of uranyl acetate negatively stained phage φEC14. The bars indicate 100 nm.

## *Phage φEC14 genome size analysis by restriction mapping and PFGE*

After enrichment of phage φEC14, phage DNA was extracted and purified. Genomic DNA was cut by 8 restriction endonucleases (*Eco*RI, *Eco*RV, *Hind*III, *Xba*I, *Nde*I, *Bgl*II, *Bam*HI and *Pst*I). As shown in Fig. 3, the φEC14 DNA was cut by only *Eco*RI, *Eco*RV, *Nde*I and *Xba*I into distinct bands.

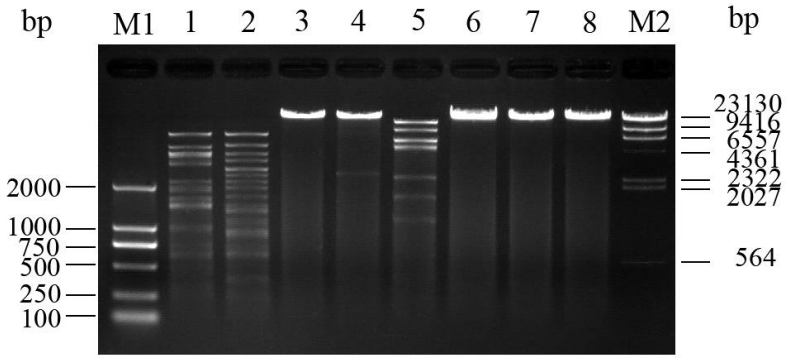


Fig. 3 Restriction digestion of φEC53 genome

Lane 1: Digestion of phage DNA with *Eco*RI; Lane 2: Digestion of phage DNA with *Eco*RV; Lane 3: Digestion of phage DNA with *Hind*III; Lane 4: Digestion of phage DNA with *Xba*I; Lane 5: Digestion of phage DNA with *Nde*I; Lane 6: Digestion of phage DNA with *Bgl*II; Lane 7: Digestion of phage DNA with *Bam*HI; Lane 8: Digestion of phage DNA with *Pst*I. M1: D2000; M2: λDNA/*Hind*III.

The PFGE indicated the genome size was to be 23.0-48.5 kb (Fig. 4). The restriction analysis also demonstrated that phage φEC14 was a dsDNA virus. Sequencing of φEC14 genome has been completed by Beijing Liuhe BGI Technology Corporation, and the size of φEC14 genome, which is precisely determined by Beijing Liuhe BGI Technology Corporation, is 37.8 kb. Further analysis of phage φEC14 sequence is underway.

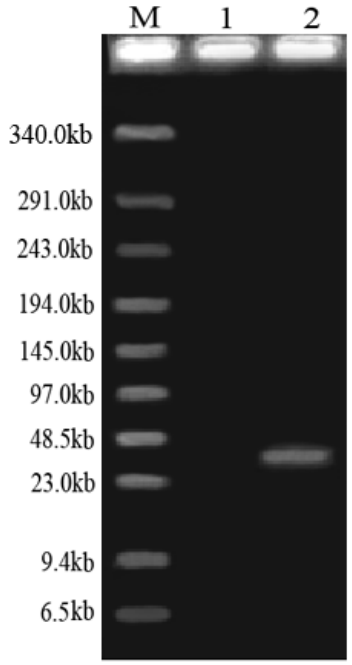


Fig. 4 PFGE of φEC14 genome
Lane 1: φEC14 genome ($10^4$ PFU), Lane 2: φEC14 genome ($10^{12}$ PFU), M: PFGE marker.

## *Proteomic analysis of phage proteins*

Purified phage particles were subjected to analysis by SDS-PAGE after staining with Coomassie brilliant blue and destaining. As shown in Fig. 5, a major protein band and a total of six minor protein bands were observed, with molecular weights ranging from 10 to 29 kilo-Dalton.

## *One-step growth experiment of bacteriophage φEC14*

A one-step growth experiment was carried out, and the latent period and burst size of bacteriophage φEC14 were evaluated. A typical curve, which includes the latent phase, exponential phase, and stationary phase, was drawn. As shown in Fig. 6, the latent period was confirmed to be 10 minutes, and the burst size of bacteriophage φEC14 was 50 PFU/infected cell, which can be indicated as ratio of the final count of phages ($1.5 \times 10^8$ PFU/ml) to the initial count of infected host cells ($3 \times 10^6$ CFU/ml).

## *Host-range determination*

Determination of host range was measured by spectrophotometric loss of turbidity on 16 clinical isolates of *E. cloacae*, one clinical isolate of *Pseudomonas aeruginosa*, and one clinical isolate of *E. coli*. The results showed (Fig. 7) that phage φEC14 could infect 3 of 16 (18.7%) clinical isolates of *E. cloacae* tested, indicating a relatively narrow host range. The phage φEC14 did not infect clinical isolates of *P. aeruginosa* and *E. coli*.

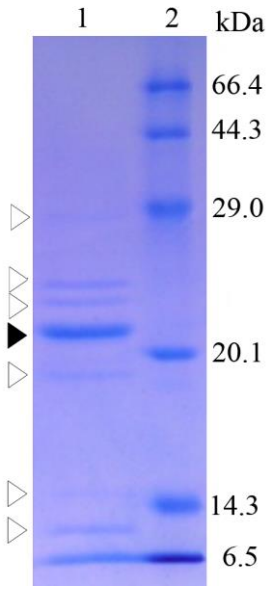


Fig. 5 SDS-PAGE analysis of φEC14 structural proteins (Solid arrow indicates major protein band; blank arrows indicate minor protein bands.)

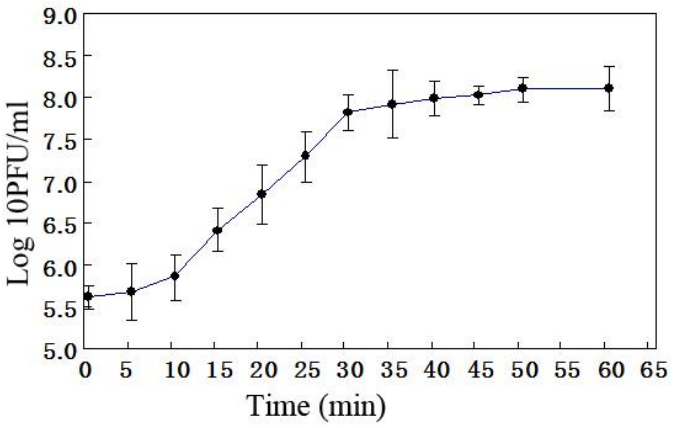


Fig. 6 One step growth curve of phage φEC14 (Results are the means ± standard deviations of three independent experiments.)

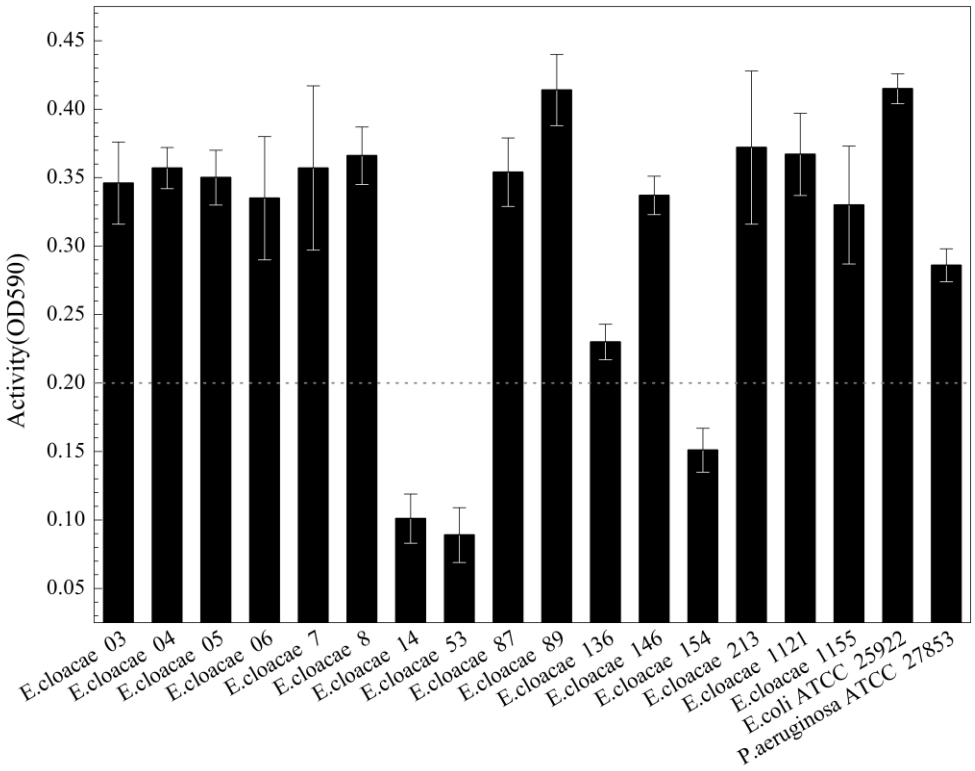


Fig. 7 φEC14 activities against clinical isolates *in vitro*

## Discussion

Recently, the increasing incidence of antibiotic-resistant pathogenic microorganisms has triggered resurgence in interest into bacteriophages [4, 10]. To our knowledge, the basic biological features of lytic phage of *E. cloacae* have rarely been investigated, although bacteriophage therapy has been discussed extensively in some papers [8, 9, 16]. Nowadays,

infections with multidrug-resistant (MDR) *E. cloacae* have become a threat in nosocomial settings [17]. Therefore, the significance of deploying specific bacteriophages against MDR *E. cloacae* is of great concern. The present study deals with the isolation and characterization of a virulent phage, φEC14, which exhibited high specificity for the clinical isolates of *E. cloacae*, an important agent of nosocomial infections.

In this work, we isolated a novel lytic bacteriophage φEC14, which is specific for three clinical isolates of *E. cloacae*, from sewage water. The ability of phage φEC14 to form clear plaques on lawns of three strains of *E. cloacae* is indicative of lytic phage. Actually, few reports of lytic phages specific for *E. cloacae* have been published, and to the best of our knowledge, this is the first report of an *E. cloacae* phage isolated from the continent of China that showed lytic activity against these pathogens. On the basis of transmission electron microscopy, phage φEC14 had an icosahedral head (50 nm in diameter) with a long tail (150 × 10 nm). Thus, phage φEC14 was morphologically similar to phages belonging to the family *Siphoviridae*, the order *Caudovirales*. Based on the analysis of PFGE, the size of φEC14 virion DNA was in rang of 23.0-48.5 kb. Our future study will focus on further genomic sequencing and gene annotation of the whole φEC14 genome to understand the genetic characteristics of this phage.

With respect to the one-step growth experiment, φEC14 proliferates efficiently, with a short latent period (10 min), a burst size of 50 PFU/infected cell, and no more than 60 CFU/ml of the sensitive host cells remained viable after those host cells were incubated with phage φEC14 at a multiplicity of infection (MOI) of 10 at 37 °C for 40 min, suggesting that this phage would be effective when introduced into the body at normal or near normal body temperature. Through the preliminary *in vitro* host range test, the phage φEC14 could lyse 18.7% of the tested clinical isolates of *E. cloacae*, indicating a relatively narrow host range. Therefore, the broad-host range *E. cloacae* bacteriophages are worth further investigation in the future studies. The differences in the lytic activities of phage φEC14 against the strains tested will be the focus of our future research both *in vivo* and *in vitro*. Given that the therapeutic use of bacteriophage in clinical trials has been carried out [20, 24], it is an urgent need for preparation of phage to control bacterial disease. In this paper, a novel lytic phage φEC14 specific to *E. cloacae* was characterized. The viral particle exhibited some distinguished properties including short latent period and small size of genome. Considering these advantages, φEC14 may be a good potential therapeutic/disinfectant agent for controlling the prevalence of nosocomial infections caused by *E. cloacae*.

## Conclusion

In conclusion, we report on the preliminary characterization of a virulent *E. cloaca* bacteriophage with efficient in lysing host cells and genome size. It may be a candidate as therapeutic agent against nosocomial infections caused by *E. cloacae*. Our further work will focus on the experimental therapy of phage φEC14 to control MDR *E. cloacae* infections both *in vivo* and *in vitro*.

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