Trop J Nat Prod Res, April 2022; 6(4):488-493

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

A Novel Broad-Host-Range Phage for Treatment of Mouse Model of *Escherichia coli* Urinary Tract Infection

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ARTICLE INFO

Article history:
Received 28 August 2021
Revised 24 January 2022
Accepted 01 April 2022
Published online 03 May 2022

ABSTRACT

The increasing emergence of drug resistance in pathogens of urinary tract infections (UTIs) has resulted in a pressing need to develop new approaches to control this infection. This study is aimed at isolating broad-host-range phage active against isolates of uropathogenic *Escherichia coli* and other bacterial pathogens causing UTIs and to study its therapeutic potential on mouse model of chronic UTIs. The broad-host-phage was isolated through enrichment of *E. coli* isolates with sewage water and detected by spot lysis method, growth inhibition assay and top plaque assay. A novel broad-host-range phage, PEC34, was isolated and showed a 100% lytic activity towards UPEC, whereas other isolated phages showed a 12-30% lytic activity. The phage PEC34 showed lytic activity against Gram-negative pathogens of UTI like *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella oxytoca* and *Enterobacter cloacae*, but showed no lytic activity against *Pseudomonas aeruginosa* nor *Staphylococcus aureus*. The phage therapy of mouse model of chronic UTI by the broad-host-range phage PEC34 through transurethral and intraperitoneal injection of 100 µL (10^{11} plaque forming units (PFU) of phage preparation on day 10 after the establishment of the infection resulted in clearance of pathogenic bacteria from the urine of mice and homogenates of bladders and kidneys of sacrificed mice after only 24 h, whereas therapy by a narrow host-range phage PEC80 showed no such effects. The phage P34 could be a strong candidate for treatment of UTIs and other infections caused by bacteria sensitive to lytic action of the phage.

Keywords: Phage therapy, Urinary tract infections, broad-host-range phage, Chronic UTI mouse model, Alternative therapy.

Introduction

Urinary tract infections (UTIs) are common infections affecting mostly women of reproductive age. The most prevalent etiological agent in UTIs is *E. coli* which is responsible for 80-90% of community acquired UTIs, and 30% of nosocomial UTIs including pyelonephritis, prostatitis, asymptomatic bacteruria and cystitis.[1] The extensive and uncritical use of antibiotics in the treatment of UTIs and other bacterial infections has resulted in development of drug resistant uropathogenic *E. coli*.[2,3] Thus, there is a pressing need for development of an alternative therapeutics for bacterial pathogens of UTIs and other bacterial infections.

Phage therapy is defined as using particles of lytic phages and their enzymes to combat bacterial pathogens.[4] The advantages of phages are their rapid action on targeted bacteria that could results in the control of bacterial infection within 24 hours with little or no undesired effects related with antibiotics therapy as the impact on bacterial normal flora and other undesired effects like cytotoxic, nephrotoxic, hepatotoxic, ototoxic, mutagenic and carcinogenic effects, or the problem of hypersensitivity associated with administration of many antibiotics.[5] The phage therapy is an outstanding alternative therapy for cases of drug-resistant bacterial infections including UTIs.[6]

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Citation: Abed SY, Alsakini AH, Mohammad MK, Mohammed SQ, Kaabi SAG. A Novel Broad-Host-Range Phage for Treatment of Mouse Model of *Escherichia coli* Urinary Tract Infection. Trop J Nat Prod Res. 2022; 6(4):488-493. doi.org/10.26538/tjnpr/v6i4.6

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Most of the phages exert narrow host-range against their bacterial host, but phages that exert broad host-range against many intergeneric bacterial species are known and considered to be the effective candidates for phage therapy of various human infections.[7-10] The objectives of this this study were to isolate broad host-range phage active against all isolates of *E. coli* and other bacterial pathogens of UTIs and to study the therapeutic potential of the broad host-range phage in the treatment of mouse model of chronic UTIs.

Materials and Methods

Bacterial isolates

Eighty-four drug-resistant isolates of uropathogenic *E. coli* (UPEC) were obtained from culture bank of biology department- College of Science- Mustansiriya University and employed as host-bacteria for isolation of broad-host-range phage.

Sewage water sample collection

Sewage water samples were collected from 10 water plants in Baghda d. Water samples were membrane filtered (0.22 µm) to obtain filtrate f ree of bacteria and other particles.[11]

Phage isolation

The phages lytic for each of the 84 UPEC were isolated through enrichment of each of UPEC with sewage water by mixing a volume of 40 mL of sewage water with 5 mL of overnight broth culture of UPEC plus 5 mL of Luria Bertani (LB) broth (10X). After 18 h of incubation of the mixture at 37°C in a shaker incubator (180 rpm), the culture was centrifuged at 10,000 rpm. The supernatant was filtered through 0.22 µm membrane filter and tested for the presence of lytic phage against the UPEC by the spot lysis assay.[12]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Spot lysis method
A volume of 10 µL of tested supernatant was applied on the surface of a lawn of host bacterium on LB agar. The plates were incubated at 37°C for 18 h. The development of a plaque on the place where supernatant dropped is indicative of the presence of lytic phage for the host bacterium. The plaque was picked up by sterile loop, placed on 1.5 mL of SM buffer (Sigma-aldrich, USA) and hand shaken for 5 min. The lysate was transferred to a sterile eppendorf tube. Any remaining bacterial host cells were killed by addition of chloroform to the supernatant in a ratio of 1/10 (v/v) with shaking for 5-7 min. Host cell debris were pelleted by centrifugation at 10000 rpm for 5 min and the supernatant containing phages was transferred to sterile eppendorf tube.[13]

Phage titration
A host bacterium was cultured in LB broth and incubated till development of absorbance of culture (O.D. 600 nm) to 0.5. Top agarose was prepared (0.7%), $MgCl_2.6H_2O$ (0.1%), Yeast extract (0.5%), Bacto-tryptone (1%), divided into 3 mL tubes and equilibrated in a water bath at 45°C. Serial dilution was made on phage preparation by using equilibrated top agarose tubes, mixed well and a volume of 1 mL of each dilution was poured over bacterial lawn of host bacterium on LB agar. The concentration of phage preparation was calculated and expressed as plaque forming units per mL (PFU/mL) of phage preparation.[12]

Study of morphological properties of plaques
The morphological properties of phage plaques were specified through the top layer plaque assay described previously. The properties of plaque diameter (mm), plaque shape, plaque margin cut (regular or irregular) and plaque turbidity or clarity were determined for each phage against each of the 84 isolates of UPEC.[13]

Growth inhibition assay
A volume of 50 µL of indicator bacterium grown in Luria Bertani broth (Optical density of 0.4 at 625 nm), 50 µL of Luria Bertani broth (2X), 100 µL of phage(s) preparation and 50 µL of membrane filtered 0.1% diphenyl tetrazolium chloride (TTC) (Himedia, India) were aliquoted into each well of microtiter plate. The plate was incubated at 37°C for 24 h. The micro-titer plate was read via micro-titer plate reader and the percentage inhibition was calculated using the equation below:[14]

$$\%inhibition = 100\left(\frac{The\ absorbace\ of\ controls - The\ absorbance\ of\ treated\ wells}{The\ absorbace\ of\ controls}\right)$$

Animals
Female albino mice were employed for experimental UTI model. The animals were acclimatized in their cages for 24 h before administration of bacterial infectious dose and left to feed and drink water freely.

Ethical approval
We confirmed that the experiments on mouse model of UTIs were carried out according to the recommendations of Committee of the ethics of Laboratory experiments on animals in Ministry of higher education and scientific research in Iraq under the approval numbered 541 issued in March 3 2019.

Inoculum preparation
The host bacterium was cultured in human urine (membrane filter sterilized) and passaged three times to enhance the adaptation of pathogenic bacteria to urine conditions. [15] The bacterial culture in urine was incubated in shaker incubator at 37°C for 18 h (200 rpm) and centrifuged for 10 min at 7000 rpm. Bacterial pellet was resuspended in phosphate buffered saline to about 10^{10} Colony forming units (CFU)/mL.

The induction of a mouse chronic UTI
Before initiation of transurethral injection bacterial inoculum, mice were anesthetized by injection with sodium pentobarbital at a dose of 0.05 mg/g body weight. Prior to injection of bacterial inoculation, the periurethral area was sterilized by 70% ethanol. Bacterial inoculation was injected transurethrally via a Teflon catheter of 24 gauge (Outer diameter of 0.7 mm and length of 19 mm). For induction of chronic UTI in mice, the bladder mucosa was traumatized before injection of bacterial inoculum through injection of 0.1 N HCL solution (100 µL) for 45 seconds, followed by neutralization of the acidic urinary tract by injection of 0.1 N KOH (100 µL) and flushing by injection of normal saline via tuberculin syringe.[16] After 24 h of bladder mucosa traumatization, the mice were injected through a microsyringe (Sigma-Aldrich, USA) with a dose of 1 $x10^6$ CFU (20 µL) for 30 seconds. A model of chronic UTI induced by two strains of *E. coli* 53 and *E. coli* 208 via transurethral injection of bacterial suspension in normal saline of 1 x 10^6 organisms for each of bacterial isolates. Starting from day 1 of infection up to 30 days after infection, 3 mice were randomly selected at intervals of 2 days for culturing of urine. The same 3 selected mice were sacrificed and the homogenates of urinary bladders and kidneys were prepared for calculation of bacterial CFU/ organ.[17]

The phage therapy of mouse model of chronic UTI
The phage therapy for mouse chronic UTI was done through injection of 100 µL of phage preparation transurethrally and intra-peritoneally after 10 days of infection. Two phages preparation were used for treatment of mouse model of UTI; the phage preparation of the broad host-range phage PEC34 and the phage preparation of narrow host-range phage PEC80. Groups of 30 mice were used for treatment with each phage preparation. A number of 3 mice were randomly selected from each treatment group daily starting from day 10 up to day 20 after infection for detection of UPEC. The same selected 3 mice on each day were dissected and homogenates of urinary bladders and kidneys were used for calculation of CFU/organ.[18]

Bacterial tests
For confirmation of diagnosis of UPEC recovered from culture of homogenates of urinary bladders and kidneys of sacrificed mice, recovered *E. coli* was tested for specific O and K antigen, P fimbriae and type 1 fimbriae. The specific O and K antigens were detected by agglutination test with goat polyclonal antibodies to *E. coli* O + *E. coli* K antigens (Abcam, England). The type P fimbriae and type 1 fimbriae were detected by hemagglutination with human erythrocytes with or without mannose.[19]

Bacteriological analysis
The urinary bladders and kidneys were removed from sacrificed mice aseptically and placed in grinding tubes (Sigma-Aldrich, USA) containing 1 and 5 mL of sterile normal saline for each urinary bladder and kidney, respectively. The organs were homogenized by using a Teflon grinder (Sigma-Aldrich, USA). The homogenates were serially diluted in normal saline and a volume of 50 µL from each dilution was streaked on DHL agar (Sigma-Aldrich, USA) for calculation of CFU/organ and incubated at 37°C for 24 h. The number of CFU of bacteria was calculated using the equation below:

$$\text{Number of CFU/organ} = \text{mean nubmer of bacteria for each entire organ} \pm \text{the standard deviation (SD)}$$

The minimum limit for detection of bacteria by this procedure was 100 CFU for kidney and 20 CFU for urinary bladder. [17]

Statistical analysis
Data were evaluated using Statistical Program For Social Science (SPSS version 20.0), as well as Data Analysis via Microsoft Excel 2010. Quantitative data were expressed as mean ± standard deviation (SD) Qualitative data were stated as frequency and percentage. The following tests were done: Independent-samples t-test of significance was used to compare between two means; a one-way or two-way analysis of variance (ANOVA) when comparing between over than two means, Least significant difference-LSD test was used to significant compare between over than three means, probability (P-value) was considered as below; P-value ≤ 0.01 was considered significant and P-value >0.05 was considered non-significant.

ISSN 2616-0684 (Print)
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Results and Discussion

Isolation of broad host- range phage

A total of 120 phages lytic for the 84 isolates of UPEC were isolated through enrichment of each UPEC isolate with sewage water from different sewage water processing plants. Each of the 120 phages was tested for its lytic activity against all UPEC isolates. Only one phage isolate, the phage PEC34, showed broad host- range towards all UPEC isolates, whereas other phages showed very narrow host- range and were lytic only to 12-30% of UPEC isolates or less. The percentage inhibition for the phage PEC34 was 75-100% against UPEC isolates, whereas those of the narrow-host-range phage, PEC86, was 0-75% (Figure 1).

Host spectrum of broad-host range

The host spectrum of the broad host-range phage, PEC34, was detected against other bacterial pathogens isolated from UTIs. The phage PEC34 showed lytic activity towards isolates of *P. mirabilis*, *C. freundii*, *Klebsiella oxytoca*, *E. cloacae*, but showed no lytic activity against *P. aeruginosa* or *S. aureus*.

Establishment of mouse chronic UTI

Daily urine culture showed positive culture of *E. coli* in mouse urine beginning from day 1 after infection. Culture results of bladders and kidneys homogenates of sacrificed mice at intervals of days 1, 3, 5, 7, 10, 14, 24 and 30 after infection showed slight variation in the number of bacteria. Figures 2 and 3 shows the mean of culture results of urinary bladders, and kidneys of 3 mice at interval during the period of mouse chronic UTI.

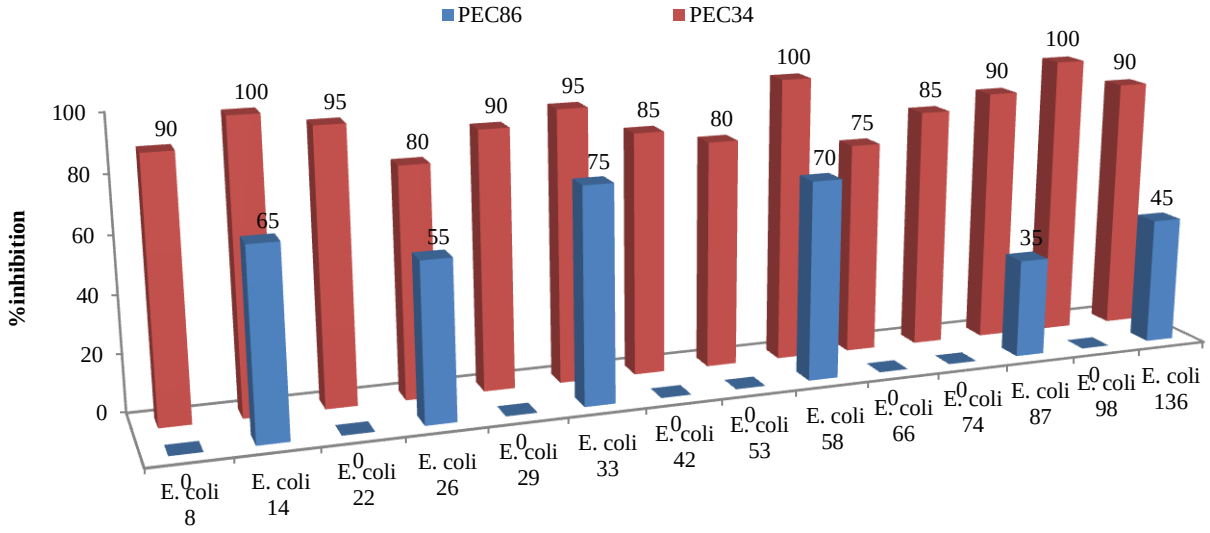

Figure 1: The growth inhibition for the broad host-range phage PEC34 versus the narrow host-range phage PEC86 against isolates of UPEC.

The induction of mouse model of chronic UTI

The results of bacterial culture of homogenates of bladders and kidneys of sacrificed mice taken from models of traumatized and non-traumatized urinary bladder mucosa at intervals of 2 days starting from day 1 up to day 30 after transurethral injection of UPEC and the homogenates of urinary bladder and kidney were cultured and the number of CFU/organ of UPEC was calculated (Figures 2 and 3).

The phage therapy of mouse model of chronic UTI

The phage therapy of a mouse model of chronic UTI by the broad host-range phage PEC34 resulted in clearance of pathogenic bacteria from the urine of mice and homogenates of urinary bladder and kidneys of sacrificed mice after only 24 h of transurethral and intraperitoneal injection of phage preparation, whereas therapy by a narrow host-range phage PEC80 showed no effect on positive culture result for the presence of UPEC in daily urine culture infected mice nor culture results of homogenates of urinary bladder and kidneys of sacrificed mice (Figures 4,5, 6 and 7).

The mouse model of chronic UTI is specified by the existence of bacterial pathogen in a concentration of at least 1 x 10^6 CFU in the urinary tract (urinary bladder and kidney) after 3 weeks of challenge with the pathogen.[17] The traumatization of urinary bladder mucosa with hydrochloric acid (HCl) (45 sec.) and subsequent neutralization by Potassium hydroxide (KOH) [16] resulted in induction of the chronic infection that last for 1 month and even more with no complication of systemic infection. [20]

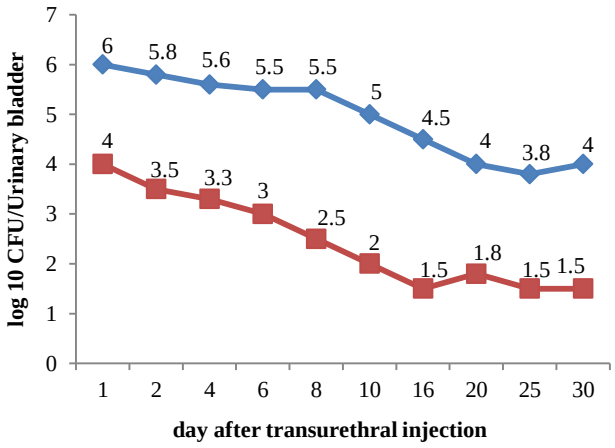

Figure 2: The bacterial culture results of Urinary bladder homogenates of mice sacrificed on days 1, 2, 4 , 6, 8, 10 , 16, 20, 25, 30 after transurethral injection of UPEC for both groups of mice with traumatized bladder mucosa (♦), and non-traumatized bladder mucosa (■).

ISSN 2616-0684 (Print)
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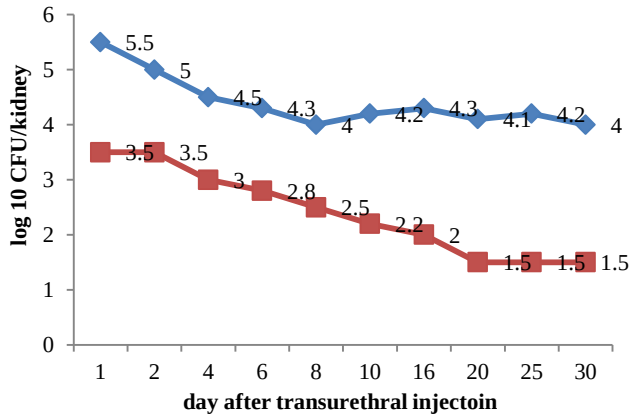

Figure 3: The culture results of kidney homogenates of mice sacrificed on days 1, 2, 4, 6, 8, 10 , 16, 20, 25, 30 after transurethral injection of UPEC for both groups of mice with traumatized bladder mucosa (♦), and non-traumatized bladder mucosa (■).

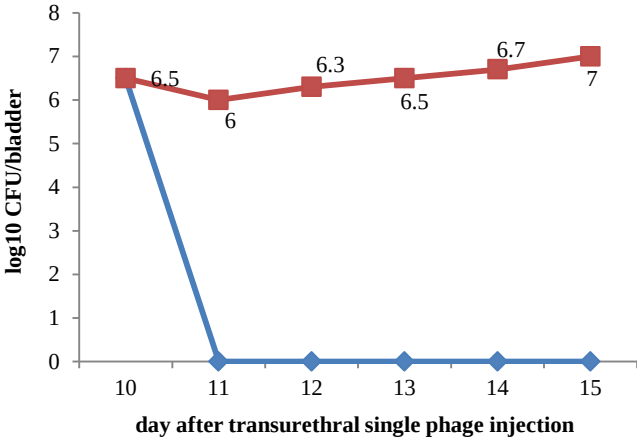

Figure 4: The bacterial culture results of urinary bladder homogenates of mice sacrificed on days 10, 11, 12, 13, 14, and 15 of infection establishment after transurethral injection of phage preparation on day 10. Mice injected with preparation of broad host- range phage PEC34 (♦), Mice injected with preparation of narrow host- range phage PEC80 (■).

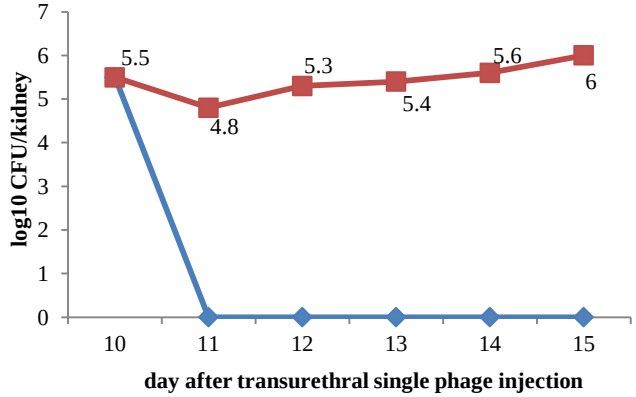

Figure 5: The bacterial culture results of kidney homogenates of mice sacrificed on days 10, 11, 12, 13, 14, and 15 of infection establishment after transurethral injection of phage preparation on day 10. Mice injected with preparation of broad host- range phage PEC34 (♦), Mice injected with preparation of narrow host- range phage PEC80 (■).

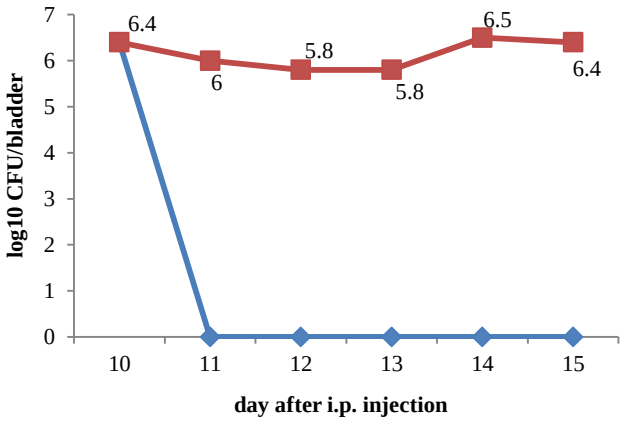

Figure 6: The bacterial culture results of urinary bladder homogenates of mice sacrificed on days 10, 11, 12, 13, 14, and 15 of infection establishment after intraperitoneal injection of phage preparation on day 10. Mice injected with preparation of broad host- range phage PEC34 (♦), Mice injected with preparation of narrow host- range phage PEC80 (■).

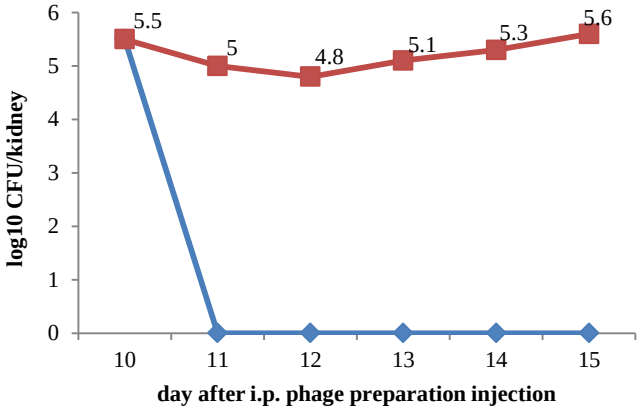

Figure 7: The bacterial culture results of urinary bladder homogenates of mice sacrificed on days 10, 11, 12, 13, 14, and 15 of infection establishment after intraperitoneal injection of phage preparation on day 10. Mice injected with preparation of broad host- range phage PEC34 (♦), Mice injected with preparation of narrow host- range phage PEC80 (■).

The induction of UTI in mice with non-traumatized urinary bladder mucosa resulted in a transient UTI that resolved spontaneously within 7 days without any treatment (Figures 2 and 3). The spontaneous resolution of UTI in infected mice without traumatization of bladder mucosa made such model improper for evaluation of therapeutic potential of phage preparation in the treatment of this infection, whereas the chronic model of UTI induced through traumatization of bladder mucosa made the evaluation process successful and the results accepted clinically.

The success of both transurethral and intraperitoneal route of administration of phage preparation in the treatment of mouse model of chronic UTI was indicated for the potential of such preparations to be an easy and potent alternative method of treatment as chemotherapy. [21-23]

The direct clearance of UPEC from the urine of homogenates of sacrificed mice just within 24 h of injection of preparation of phage PEC34, transurethrally or intraperitoneally, is indicated for the high activity of this broad host-range phage in the treatment of UTIs beside all other infection caused by various pathotypes of *E. coli* as enteropathogenic *E. coli*, enteroinvasive *E. coli* (EIEC), enterhomorrhagic *E. coli* (EHEC) and enteroinvasive E. coli (EIEC)

that cause very serious and life-threatening infection especially that caused by multi-drug and extensive-drug resistance *E. coli*. [24, 25]
The importance of phage therapy in the cases of UTIs is not confined to the cases of UTIs caused by drug-resistant *E. coli*, but also to the cases of infection in women during pregnancy and perinatal period owing to risks of antibiotic administration on health status of embryo, fetus or newborn baby. [26-30]

Conclusion

The intergeneric activity of the broad host-range of the phage PEC34 against *Proteus mirabilis*, *Citrobacter freundii*, *Klebsiella oxytoca* and *Enterobacter cloacae* that all considered as a common etiological agent of human UTIs beside its high activity against all isolates of *E. coli* made it an optimal phage for treatment of UTIs resulted from *E. coli* and other Gram-negative pathogens. Furthermore, this phage could be an optimal therapy for all other infections caused by all other Gram-negative bacterial pathogens that are sensitive to lytic activity of this phage.

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We would like to thank the head of biology Department - College of Science - Mustansiriya University and the Presidency of Mustansiriya University for providing all the requirements for completing this research.

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