Journal of Cell and Molecular Research (2021) 13 (1), 81-91 DOI: 10.22067/jcmr.2021.70313.1006

Research Article

Isolation and Characterization of Lytic Bacteriophages Infecting *Escherichia coli* Antibiotic-Resistant Isolates from Urinary Tract Infections in North-west of Iran

Raheleh Majdani*

Department of Biology, Faculty of Basic Sciences, University of Maragheh, Maragheh, Iran

Received 8 May 2021 Accepted 15 August 2021

Abstract

One of the most prevalent bacterial infections, urinary tract infection (UTI), affects millions of people yearly worldwide. To control the increasing antibiotic-resistant infections, it is essential to introduce alternative approaches such as phage therapy. In this study, isolation, purification, and enrichment of eight lytic bacteriophages, which are active against antibiotic-resistant *Escherichia coli* strains from human urinary tract infections, were carried out. Molecular analysis of the bacteriophages was performed using two endonuclease enzymes (*Eco*RV and *Xba*I). Then, two of eight isolated bacteriophages with the highest host range were further characterized to determine their morphology, one-step growth, latent period, burst size, and stability under different environmental conditions. Allbacteriophage isolates (n=8) showed genome variation as it was evidenced by the enzyme digestion process (*Eco*RV). Both phages with the broadest host ranges (PEcMa2/17 and PEcMa3/17) showed an efficient lytic activity against five bacterial isolates. Electron microscopy confirmed that selected phages belong to S*iphoviridae* and *Myoviridae* families. The latent period of both propagated phages was determined as 15min. The burst size was estimated to be 100pfu/ml and 120pfu/ml in PEcMa2/17 and PEcMa3/17, respectively. Both phages showed more than 50% stability at 37ºC and lower investigated temperatures, and they were survived efficiently in pH=7. It was while their genome properties were different. The introduced bacteriophages showed high stability and strong antibacterial potential against *Escherichia coli* strains from UTIs. As candidates for phage therapy, more characterization steps, such as molecular analysis and experimental assays are needed before the therapeutic application.

***Keywords*:** Bacteriophage; Urinary tract infection; *Escherichia coli*; Antibiotic resistance; Phage therapy

Introduction

Urinary tract infection (UTI), as one of the most prevalent bacterial infections, affects millions of people yearly worldwide (Stamm and Norrby, 2001). Due to the notable role of UTIs in the morbidity of all aged adults, significant economic and public health problems have resulted. As a result of these problems, the quality of life in afflicted individuals is changing consequently (Flores-Mireles et al., 2015; Kostakioti et al., 2012). Some complex situations such as frequent recurrences and high-levels of antibiotic resistance are reported as severe challenges in UTIs (Flores-Mireles et al., 2015). The most common causative agent for uncomplicated and complicated UTIs is uropathogenic *Escherichia coli* (UPEC) (Flores-Mireles et al., 2015). Antibiotic resistance in uropathogenic bacteria such as *Escherichia coli* (*E.coli*) could be explained as the major cause of community and nosocomially acquired UTIs. The incidence of multi- drug resistant strains of *E. coli*, especially those producing extended-spectrum beta-lactamases (ESBL) such as CTX-M-type enzymes, may cause more challenges for the prognosis of urinary tract infections (Pouillot et al., 2012).

Bacteriophages (phages), viruses that infect bacteria, are the most abundant entities on the earth. They are non-hazardous and self-replicating natural agents which increase in number with the lysis of their bacterial targets. Also, virulent phages could be effective agents in removing bacterial biofilms (Jassim., 2012). Phage therapy could be a more appropriate approach to treat urinary tract infections. Comparing with the last generation of antibiotics, using bacteriophages is not expensive, and it can be used with a catheter under the control of medical personnel several times a day. Also, systemic therapy is not required, and bacteriphages will be applied locally. The efficiency of phage therapy

* Corresponding author's e-mail address: *rahelehmajdani@yahoo.com*

Licensed as Open Access CC-BY

combatting UTIs increases because of no physical and metabolic barriers in this kind of therapeutics, and also the occurrence of adverse effects will be reduced (Sybesma et al., 2016). Some previous studies also investigated the phage therapy potential to treat UTIs. The effect of oral and topical application of a phage cocktail was evaluated on patients with UTI. The study confirmed the lytic effects of phages on reducing the biofilms of uropathogenic *E.coli* (Chibeu et al., 2012). In addition, the effectiveness of commercial phages targeting an *E.coli* isolate from UTI patients was reported (Sybesma et al., 2016). Interestingly, the antibiotic resistance pattern of *E.coli* and its specific phages were variable in different regions (Tuem et al., 2018). Considering the advantages of phages over antibiotics, the lower rate of discovering effective antibiotics against antibiotic-resistant bacterial strains, and the high prevalence of UTIs, phages could be explained as a promising and highly potentiated alternative to antibiotics to control the urinary tract infections.

In this study, isolation, purification, and enrichment of eight lytic bacteriophages against antibiotic-resistant *E.coli* strains from human urinary tract infections were performed. Also, to suggest effective phages for phage therapy procedures of UTIs, their antibacterial efficacies and host ranges were determined. After analyzing the variety of genomes using endonuclease enzymes, two phages with thebroadest host ranges were selected for more characterizations as proper candidates for future phage therapy eperiments.

Materials and Methods

Bacterial strains and characterization

A number of 55 *E.coli* isolates of human urinary tract infections were obtained from clinical diagnostic centers in theEast Azerbaijan province of Iran between January- August 2017. Confirmation of all isolates as *E.coli* was performed in the microbiology laboratory of the University of Maragheh by applying biochemical standard tests (Quinn et al., 1994). Afterward, preservation and storage of *E. coli* isolates were done at the temperature of -20°C.

Antibiotic susceptibility and bacterial selection

Evaluation of antibiotics sensitivity of identified *E. coli* isolates was conducted using the disk diffusion method (Bauer et al., 1966). 13 different common antibiotic discs were applied in the present study, including nitrofurantoin (300 micrograms), tetracycline (30 micrograms), amoxicillin (25 micrograms), ciprofloxacin (5 micrograms), nalidixic acid (30 micrograms), trimethoprim/sulfamethoxazole (1.25/23.75 micrograms) gentamicin (10 micrograms), ceftriaxone (30 micrograms), amikacin (30 micrograms), ceftazidime (30 micrograms), cefpime (30 micrograms), cefotime), chloramphenicol (30 micrograms) (Padan Teb, Iran). *E coli* ATCC 25922 and *E.coli* ATCC 35218 were used as quality controls. According to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines the isolates were reported as resistant, intermediate, or sensitive to different antibiotics . Finally, eight multi-drug resistant bacterial isolates were subjected to phage isolation assay, and stored at -20°C.

Isolation and purification of phages

Samples To isolate bacteriophages against eight multi-drug resistant human *E.coli* bacteria samples were collected from rivers and an urban sewage treatment center in Maragheh (The East Azerbaijan province, north-west of Iran). Samples were immediately transported to the microbiology laboratory of the University of Maragheh under cold conditions (4ºC). They were centrifuged and then filtered using a 0.45µm millipore membrane filter. Concentrated sterile trypticase soy broth (TSB) and antibiotic-resistant bacterial suspension (host-bacteria) were added to sterile water samples. After adding 1% $MgSO_4$ (v/w), the mixture was shaked (130rpm) overnight at 37ºC. Then, 3 ml chloroform was added, and the mixture was shaked for another 24h at 37ºC. After 2h at room temperature, it was centrifuged (25min, 1500*g*), and the supernatant was filtered with a 0.45µm membrane. Phage activity of the filtrate against 10 *E. coli* isolates was determined using the double-layer agar overlay technique (Adams, 1959) and the spot test (Chang et al., 2005). Purifying the isolated phages was performed according to the previous study (Jun et al., 2013). Moreover, single plaque isolation steps were repeated three times.

Titration and precipitation of bacteriophages

The double-layer agar overlay method was carried out to evaluate the titre of the bacteriophage suspensions (Adams, 1959). Also, 10X dilutions in TSB containing tubes were used, and the plates were incubated at 37°C for 24h. The bacteriophage titer of each solution is expressed as pfu/ml. One-step filtration of phages was done, and the solution was further concentrated and purified using polyethylene glycol (PEG) precipitation (Ahmadpour et al., 2016).

Determination of host range of bacteriophages

In this study, we used eight isolates of multi-drug-resistant *E. coli* originated from the human urinary tract infections. To determine the host range of the phages, spotting 10µl of phage suspension (10^9pfu/ml) was carried out onto the bacterial isolate lawn cultures. All tests were repeated three times. According to the host range, two isolated bacteriophages were suggested for effective phage therapy to combat antibiotic-resistant *E.coli* bacteria from human urinary tract infections (East Azerbaijan, Iran). Then, characteristics of selected phages were further studied.

Morphological characterization of bacteriophages

Two selected phages with the highest host range were used for morphological examination via transmission electron microscopy (TEM) following the negative staining. Briefly, a drop of purified phages (10^{10}pfu/ml) was loaded on the surface of a formvar coated grid (copper grid size 200 mesh), and after staining with 2% uranyl-acetate, it was examined using a Zeiss Leo 906 TEM instrument operating at 100kV (Carl Zeiss Company, Germany).

The efficiency of bacteriophage adsorption

Bacteria were grown in the TSB medium at 37ºC and the OD was adjusted to 0.3 (Wavelenght 600nm). Samples were then centrifuged, and the resultant pelletswere washed with 1ml of 0.85% NaCl . Then, the pellet was re-suspended in 1.5ml of TSB medium, and incubated at 37ºC for 15min. Bacteriophages were added to the bacterial suspension with the lysate multiplicity of infection (MOI) of 0.1. Samples were withdrawn periodically through out the incubation time with 5min intervals to be titrated. The first sample collected immediately following the addition of the phage lysate to the bacterial suspension (zero time point) was considered as the reference with 100% of nonadsorbed phages.

One-step growth experiment

Determination of the latent period and burst size of selected phages (PEcMa2/17 and PEcMa3/17) was carried out using one-step growth curve experiment, as it was described previousely with some modifications (Pajunen et al., 2000). The bacteriophages at MOI of 0.01 were added to *E.coli* cultures (10^8pfu/ml). Then, the mixtures were incubated for 15min at 37ºC, which allows the bacteria to adsorb phages. After incubation, the mixture was centrifuged for one min at 4ºC to remove any nonabsorbed phages. Pellets were resuspended in the fresh TSB medium, and incubated at 37ºC. Then, the samples were obtained at 5-min intervals, and the phage titer (pfu/ml) was evaluated using the double-layer agar method, and one-step growth curves were plotted. All experiments were performed at least in triplicate.

Stability studies

The thermal stability of phages was tested in 10^9pfu/ml of phage lysate that was subjected to different temperatures, including -20, 4, 22, 37, 60, and 90˚C for 1 and 24h. After incubation in a temperature-controlled water bath, the phage activity was determined using the agar overlay method. Investigated pH ranges were from 3 to 11. To determine the stability the phage lysates were incubated at a pH-controlled environment for 1 and 24h. Also, the phages were exposed to U.V. radiation according to the method of Ramirez et al. (Ramirez et al., 2018). Briefly, phages were placed in sterile plates and irradiated for 15 and 30min with a U.V. lamp in a laminar flow cabinet (λ=254 nm) at a distance of 0.6m, and phage survival was calculated in each case. The stability results were represented as viability percentages at different pH ranges. All experiments were repeated in triplicates.

DNA extraction and enzymatic digestion

Isolated phage suspensions (n=8) were treated with DNase I (1U/µl; Jena Bioscience, Germany) and RNase A (5ug/µl; Jena Bioscience, Germany) to degrade bacterial nucleic acids, and the mixtures were incubated for 40min at 37ºC. After inactivation of the enzymes, purified phage genomic DNA was prepared using QIAamp DNA Mini Kit (Qiagene, Germany) with some modifications. Briefly, after mixing the solution with the lysis buffer, it was vortexed, and placed at 65ºC for 30min. Then, ethanol was added, and the resultant mixture was transferred to a particular column, and centrifuged. After twice washing, DNAs were eluted. In the next step, obtained genomic DNA solutions of bacteriophages were subjected to a round of nuclease treatment using *Xba*I (Jena Bioscience, Germany) and *Eco*RV (Jena Bioscience, Germany) enzymes according to the manufacturer's instructions. Fragments were visualized on 2% agarose gel using an ultraviolet transilluminator (ECX-20-M, Syngen, USA).

Results

Antibiotic sensitivity test

In the current study, 55 bacteria (from 63 samples) were confirmed as *E.coli* based on

standard biochemical tests. The antibiotic susceptibility test was conducted for all samples. According to the results, the highest antibiotic resistance rate among the isolates was related to amoxicillin (51.2%). 64.6% of bacteria were resistant to three or more investigated antibiotics, among which 18.2% of isolates showed resistance to five or more antibiotics.

Eight multi–drug (5 or more) resistant bacteria were selected as bacterial references to isolate bacteriophages which were active against these bacteria as the host cells. Obtained results, including the titre of bacteriophages are shown in Table 1.

Isolation of lytic bacteriophages

Table 1. Titration results obtained forphage isolates which are active against 8 multi-drug resistant *E.coli* isolates.

Phages	*E.coli* isolates (resistant to five or more antibiotics)	Phage titer (pfu/ml)
P1	Isolate 1	1.2×10^{8}
P2 (PEcMa2/17)	Isolate 2	3.2×10^{9}
P3	Isolate 3	10^{10}
P4	Isolate 4	1.8×10^{7}
P5	Isolate 5	5.6×10^{7}
P6	Isolate 6	1.1×10^{8}
P7	Isolate 7	2.8×10^{9}
P8 (PEcMa3/17)	Isolate 8	10^{8}

Based on the results, 2 of 8 phages indicated the most lytic effects against bacterial isolates. They effectively lysed five *E.coli* bacterial strains. Although, the bacterial isolates that each of the two bacteriophages lysed were not identical. These bacteriophages were selected for studying their morphology and other biological characteristics. Selected phages with the highest hostrange value were named PEcMa2/17 and PEcMa3/17. Resultant plagues of PEcMa2/17 and PEcMa3/17 using double-layer agar method are shown in Figure 1a and b (Figure 1). They showed clear plaques with 2.5-3 and 1.5-2 mm in diameter on lawn cultures of bacteria, respectively. All isolated phages (n=8) were purified and enriched. Precipitated phages (n=8) were stored at 4ºC for further experiments.

The morphology of the bacteriophages PEcMa2/17 and PEcMa3/17 was examined by TEM (Figure 2a and b). PEcMa2/17 had a head with a diameter of 70±5nm, a hexagonal outline, a long noncontractile tail of 10nm in diameter, and a length of 120±5nm which belongs to order *Caudovirales*, family *Siphoviridae* (Figure 2a). PEcMa3/17 has a head of about 80±5nm in diameter, a hexagonal outline, contractile tail with a diameter of 10nm, and length of 100±5 nm that was classified as a member of order *Caudovirales*, family *Myoviridae* (Figure 2b).

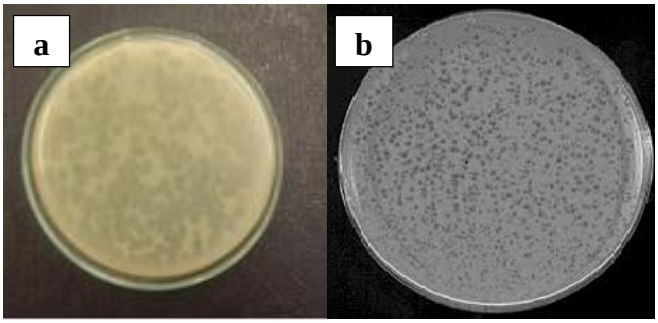

Figure 1. Plaque morphology of selected bacteriophages, PEc2-Ma17 (a) and PEc3-Ma17 (b), in bilayer agar method.

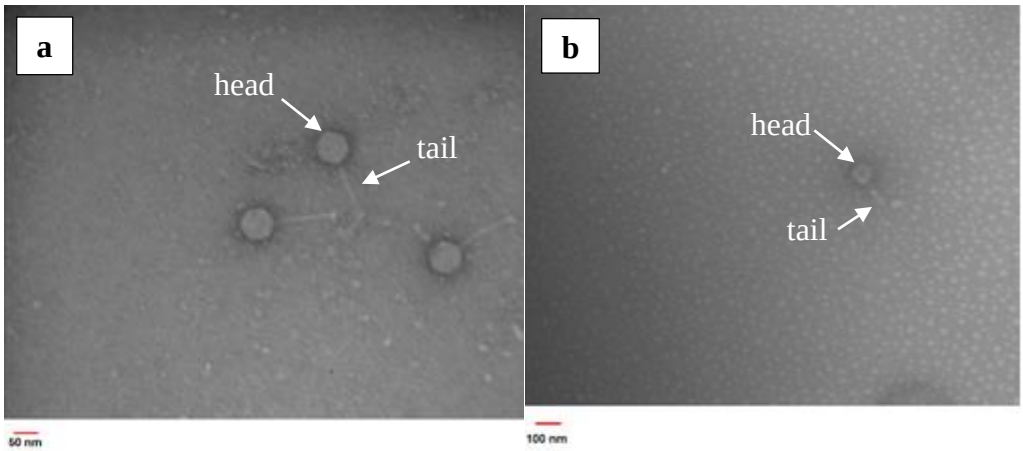

Figure 2. Transmission electron microscopy images of the bacteriophages PEcMa2/17 and PEcMa3/17 following the negative staining with uranyl acetate. Electron micrographs of PEcMa2/17 virions showthe typical morphology of phages within the family *Siphoviridae* with a head (diameter 70±5nm) and a hexagonal outline, a long noncontractile tail of 10nm,and the length of 120±5nm. (a). PEcMa3/17 virions showed the typical morphology of phages within the family *Myoviridae* with a head of about 80±5nm in diameter, a hexagonal outline, and a contractile tail with a diameter of 10nm and length of 100±5nm (b).

One-step growth experiment

According to the one-step growth experiment, the latent period, which is defined as the time intervals between the absorption and the beginning of the first burst, was 15min for both of the propagated phages. The burst size was estimated to be 100pfu/ml after 40min and 120pfu/ml after 45min in PEcMa2/17 and PEcMa3/17, respectively (Figure 3).

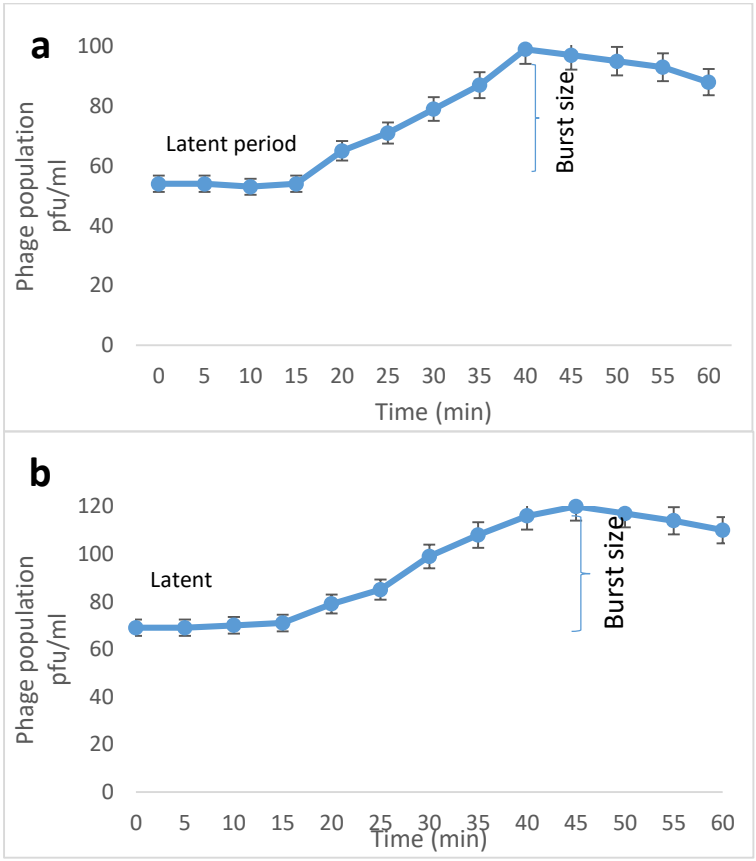

Figure 3. One-step growth curves of the PEcMa2/17 (a) and PEcMa3/17 (b) phages. The vertical axis showspfu per infected cell in the cultures at different time points. Each data point represents the mean from three independent experiments, and the error bars indicate standard deviations. (a) The latent period of PEcMa2/17 is 15min, and burst

size was estimated to be 100pfu/ml. (b) The latent period of PEcMa3/17 is 15min, and burst size was estimated to be 120pfu/ml.

Stability results

The sensitivity of virions of PEcMa2/17 and PEcMa3/17 bacteriophages was tested under different environmental conditions, including temperature, pH, and U.V. radiation. Results are shown in Table 2, Table 3, and Table 4, respectively.

Table 2. Resistance of two phage virions to different temperatures (percent of surviving phages under certain conditions is shown).

Phage name	-20°C		4°C		22°C		37°C		60°C		90°C	
	1h	24h	1h	24h	1h	24h	1h	24h	1h	24h	1h	24h
PEcMa2/17	96	95.4	65	60	68.4	56.7	75	70.7	22.1	3.6	17.3	0
PEcMa3/17	97	86.5	84.6	61.9	76.3	52.5	76	56.3	31.7	7.9	18.6	0

Table 3. Resistance of two phage virions to different pH rates (percent of surviving phages under certain conditions are shown).

Phage name	pH=3		pH=5		pH=7		pH=9		pH=11	
	1h	24h	1h	24h	1h	24h	1h	24h	1h	24h
PEcMa2/17	23.4	10	47.6	34.1	97	88	53.5	42	32.9	13.4
PEcMa3/17	16.7	10.5	84.1	74.5	99	91.2	57.8	37.1	40.5	34.3

Table 4. Resistance of two phage virions to U.V. radiation (percent of surviving phages under certain conditions are shown).

Phage name	U.V. radiation	
	15min	30min
PEcMa2/17	35	0
PEcMa3/17	11	0

DNA extraction and enzymatic digestion

Extracted genomic DNAs of all phage isolates, including PEcMa2/17 and PEcMa3/17, are shown in Figure 4 (a). Eight different RFLP (restriction fragment length polymorphism) patterns were determined in isolated bacteriophages through the digestion of their genomic DNA using the *EcoR*V enzyme (Figure 4b). The resultant fragments of genomic digestion was distinct for PEcMa2/17 and PEcMa3/17. One of the bacteriophage genomes (in lane 7) was not digested with the *EcoR*V enzyme, and eight obtained patterns were different. The digestion results of the eight bacteriophage genomes using *XbaI* are shown in the Figure 4 4c). As it was mentionedno phage genomes were digested. The genomes of two bacteriophages PEcMa2/17 and PEcMa3/17 were not digested with *Xba*I. Molecular analysis of two selected bacteriophages using *EcoR*V enzyme determined the genome size of about 33,600bp for PEcMa2/17, and more than 36,200bp for PEcMa3/17, respectively.

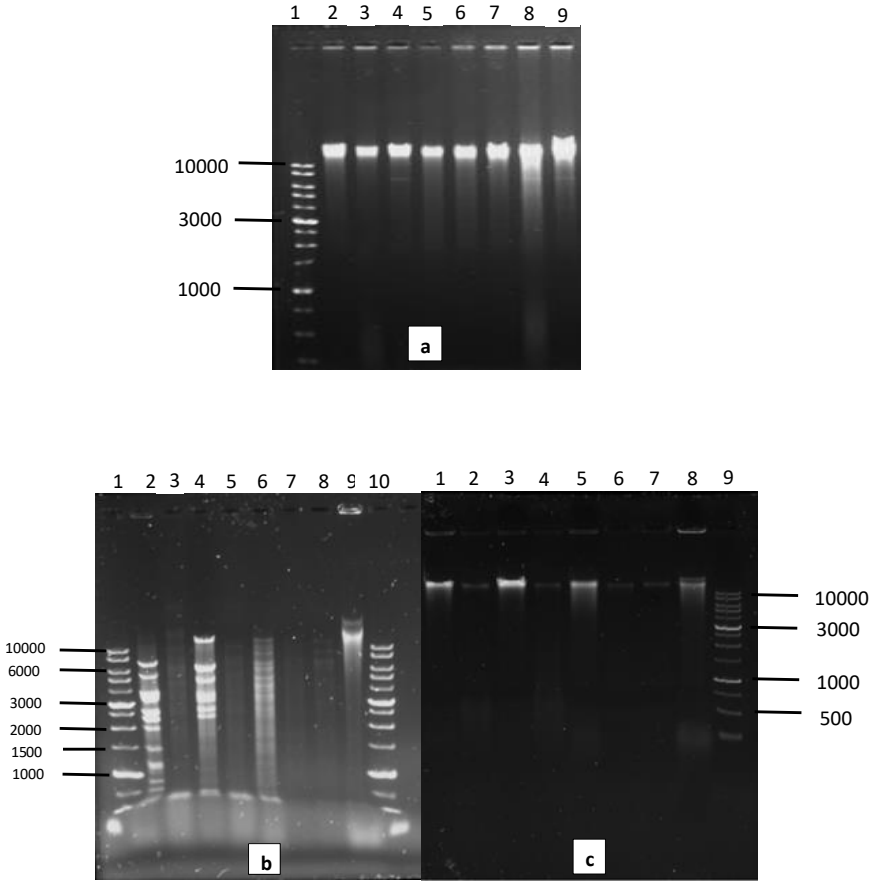

Figure 4. Genomic DNAs of 8 phage isolates were run onagarose gel 1%. (a) lane1: 1Kb DNA Ladder (Cinaclon, Iran), lane 2: bacteriophage PEcMa2/17, lane 3: bacteriophage PEcMa3/17, lanes 4-9: extracted genomic DNAs from other isolated bacteriophages, (b) Phage genomes digested with *Eco*RV; Lane 1: 1Kb DNA Ladder (Cinaclon, Iran), lane 2: bacteriophage PEcMa2/17, lane 4: bacteriophage PEcMa3/17, lanes 3 and 5-9: extracted genomic DNAs from other isolated bacteriophages, lane 10: 1Kb DNA Ladder (Cinaclon, Iran). (c) Phage genomes digested with *Xba*I; lane 1: bacteriophage PEcMa2/17, lane 2: bacteriophage PEcMa3/17, lanes 3-8: extracted genomic DNAs from other isolated bacteriophages, Lane 10: 1Kb DNA Ladder (Cinaclon, Iran). The molecular size of the DNA marker is shown (bp) in a, b and c.

Discussion

The emergence of antibiotic-resistant strains of bacteria has become a severe concern over recent decades, and it can be a significant threat to human healthcare in the future years. Then appropriate alternative therapies should be founded to fill the gap of infections treatment. Phage therapy as an effective and biological approach is highly regarded by researchers today.

In the current study, the antibiotic sensitivity of *E.coli* isolates from urinary tract infections was determined. The highest rate of antibiotic resistance was observed to amoxicillin, and 64.6% of isolates were resistant to three and more used antibiotics, and also 18.2% of isolates showed resistance to five and more antibiotics. In a previous study, the highest rate of antibiotic resistance of urinary *E.coli* bacteria was shown to ampicillin (50%), according to our results (Rfalskiy et al., 2020). Also, in a survey in Tabriz (north-west of Iran), the rate of resistance to tetracycline, SXT, nalidixic acid, gentamycin, ceftriaxone, ciprofloxacin, and nitrofurantoin was 80.9%, 63.9%, 43.98%, 43.11%, 33.01%, 26.96%, and 10.98% respectively (Molaabaszadeh et al., 2013). In other recent studies, more antibiotic resistance of urinary *E.coli* isolates was reported as Yazdi et al. revealed that 78.8% of *E.coli* isolates were MDR (multi-drug resistant), and the most resistance was observed to ampicillin and ciprofloxacin with the rate of 92.3% and 61.9% respectively (Yazdi et al., 2020). Also, 40% of *E.coli* bacteria isolated from urinary infections in Sari (north of Iran) were resistant to nalidixic acid and

ceftriaxone while were sensitive to amikacin, gentamycin, and nitrofurantoin (Rahimzadeh et al., 2020).

Our results showed a wide host range of selected bacteriophages about 62%. Also, using two isolated bacteriophages together could be more efficient against uropathogenic *E.coli* bacteria. In a survey on lytic effectiveness of 29 phage suspensions and four commercial phage cocktails on different *E.coli* isolates from urinary cultures of patients with UTIs, the highest lytic activity of the phage cocktails were 66%-93%, while used phage suspensions had the highest lysis on 42%, 39% and 24% of *E.coli* isolates respectively (Sybesma et al., 2016). Necel and colleagues evaluated the host range of phages vB_Eco4M-7 and ECML-117 against 97 *E.coli* strains. They reported that the phages had a lytic effect on 34 and 39 strains, respectively The majority of the *E.coli* strains were lysed by these bacteriophages belonged - to the O157 serotype (Necel et al., 2020). Phage VB_EcoS-Golestan, isolated from Iran, showed lytic activity against 56% of *E.coli* from urinary infections. Also, the phage had a lytic effect on 15 of 25 isolates resistant to ten used antibiotics, and it could lyse four of six *E.coli* bacteria resistant to all (n=17) used antibiotics (Yazdi et al., 2020). In another study, one bacteriophage isolated from Sari, Iran, had a lytic effect on all of nine used *E.coli* isolates from urinary infections (Rahimzadeh et al., 2020). In China, the ability of one isolated bacteriophage, vB_EcoS-B2, was tested against some MDR strains of *E.coli* that could lyse only seven of 35 clinical MDR strains. Despite a relatively narrow host spectrum of the bacteriophage, authors suggested it as a biocontrol agent against *E.coli* due to its strong lytic effect (Xu et al., 2018).

In the current study, selected bacteriophages against *E.coli* from urinary tract infections belonged to *Caudovirales, Siphoviridae,* and *Caudovirales, Myoviridae* that was by other studies (Yazdi et al., 2020; Rahimzadeh et al., 2020). Three other coliphages from the human gut (ϕAPCEc01, ϕAPCEc02 and ϕAPCEc03) were also classified in *Caudovirales*, family *Myoviridae* and *Siphoviridae* (Dalmasso et al., 2016). Reports of some other works refer to locate the phage with lytic activity against urinary *E.coli* bacteria (myPSH) in *Caudovirales*, family *Podoviridae* (Manohar et al., 2018). Also, in another study, lytic phage vB_EcoS-B2 with an isometric head with a mean diameter of 48 nm and the long noncontractile tail targeted *E.coli* clinical strains belonged to the family of *Siphoviridae* (Xu et al., 2018). Two isolated bacteriophages against pathogenic *E.coli* strains with 66 nm diameter heads and contractile tails belonged to the *Myoviridae* family (Necel et al., 2020).

Two important characteristics for phage therapy candidate bacteriophages are large burst size and short latency period. The burst size is closely related to phage propagation (Amarrillas., 2017). Then a phage with a large burst size may have a selective advantage as an antibacterial agent since phages with a large burst size can increase the initial dose of phages several 100-fold in short periods. Bacteriophages with these desirable features are more appropriate candidates for therapeutic purposes. Therefore, high burst size (100 and 120 pfu/ml) and short latent period (15 min) of both isolated phages, PEc2-Ma/17 and PEc3Ma/17, would indicate their strong potential application for phage therapy after more characterization. Bacteriophages vB_Eco4M-7 and ECML-117 had a strong lytic effect on O157 *E.coli* strains, showed a latent period of 10 min, with a burst size of approximately 100 phages per cell (Necel et al., 2020). The burst size and latent period of coliphages in other studies were determined 40 min and 100 pfu/ml (Yazdi et al., 2020), 20 min and 1200 pfu/ml (Rahimzadeh et al., 2020), and 10 min and about 90 pfu/ml (Dalmasso et al., 2016). Also, another phage isolated against MDR (multi-drug resistant) clinical strains of *E.coli* had a latent period of 15min and a burst size of approximately 224.1±10.7 (Xu et al., 2018).

Our results showed that the most viability of both bacteriophages (PEcMa2/17 and PEcMa3/17) was observed at a temperature of -20°C after 1 h. The bacteriophages were totally deactivated after 24h at 90ºC. Their stability was more than 50% at the temperature range of -20 to 37°C. In these temperatures, the survival rate of the bacteriophages was not strongly affected by the length of incubation time, especially about PEcMa2/17. In a study, the biological activity of phage vB_EcoS-B2 had no difference in the temperature ranging from 4 to 50ºC, but increasing the temperature above 55ºC decreased the activity sharply (Xu et al., 2018). Both phages were shown the most survival rate in pH value of 7 with the rate of more than 87% after 1 and 24 h incubation. Both of the bacteriophages showed lytic effects at pH 3 and 11. The lowest stability rate of the bacteriophages was in pH value of 3 after 24h incubation. Survivability of two bacteriophages was similar in pH values of 3, 7, 9, but not in pH value of 5 that survival rate of PEcMa3/17 was more than PEcMa2/17 after 1 and 24h incubation. In a previous study, a coliphage against *E.coli* originated from UTIs showed maximum activity at pH values of 7 and 8, according to our results. The bacteriophage

showed a high lytic effect in the pH values from 5 to 10 after 1 h incubation. However, the bacteriophage stability was decreased significantly at pH values of 3 and 11 after 1 and 24h incubation. In our study, both bacteriophages were deactivated after 30min exposure to U.V. radiation, and after 15min, stability of PEcMa2/17 was more than PEcMa3/17.

Environmental conditions such as temperature and pH level can affect the applications of bacteriophages to combat pathogenic bacteria (Ly-Chatain, 2014). Declining phage titer in lower pH values could have been happened due to high concentration of H+ and hydroxyl ions and consequently denaturation of capsid proteins (Feng et al., 2003). In addition, the concentration of hydrogen ions in an acidic solution can result in a decrease in bacteriophage concentration because of the aggregation process. High temperatures cause irreversible damage or denaturation of the virus particles (Wang tan et al., 2021). Some works suggested the possibility of a relationship between the structure of bacteriophages and their stability under adverse environmental conditions (Lasobras et al., 1997). Tailed phages suffer adverse conditions better than other phages (Ackermann et al., 2004). They generally show a higher ability to adapt to adverse conditions, leading to the applicable phage therapy. Also, other studies reported that strong ultraviolet light and large temperature fluctuations cause phages belonging to *Myoviridae* to protect themselves in biofilms or pseudo-lysogens formed by their bacterial hosts (Jonczyk et al., 2011). Extreme resistance of *Podoviridae* family bacteriophages to dry environments and large temperature fluctuations also was reported (Prigent et al., 2005). Generally, more stability of a bacteriophage in a wide range of environmental conditions is an important advantage to a phage therapy candidate (Jamal et al., 2015). For using phages in phage cocktails of phage therapy, it is essential to consider the stability of each bacteriophage at different environmental conditions. Based on our data, phages PEcMa2/17 and PEcMa3/17 with high stability in a wide range of temperatures and pH conditions could be used as the candidate for phage therapy at different environmental settings. Both phages had approximately similar pH values and temperatures stability, which could be used together in a phage cocktail.

Results of molecular analysis confirmed differences among eight isolated bacteriophages, also in two selected bacteriophages, with the digestion by one of the enzymes (*Eco*RV). Based on resultant fragments from enzyme digestion, the genome size of PEcMa2/17 (approximately 33,200 bp) was shorter than PEcMa3/17. Comparing to other studies, Pacifico and colleagues reported that the genome size of phages against *E.coli* from clinical samples classified to *Myoviridae* was between 33,688 to 33,807bp and isolated *Siphoviridae* phages in the study had more than 44,000 bp length that was following our results (Pacifico et al., 2019). The genome size of other bacteriophages (e VB_EcoS-Golestan) isolated against *E.coli* from urinary tract infection belonged to the *Siphoviridae* family, was 44,829bp in length (Yazdi et al., 2020). Different RFLP patterns of all eight bacteriophages using *Eco*RV suggested the presence of some variations in their genetic characteristics. Unfortunately, no reports were found about enzyme digestion of *Escherichia coli* bacteriophages, but in a study in Poland, 51 genomes of phages isolated against uropathogenic strains of *Proteus mirabilis* showed 34 different RFLP patterns using *Eco*RV enzyme (Maszewska et al., 2016). In another study, six isolated bacteriophages against *Vibrio parahaemolyticus* that belonged to *Siphoviridae* and *Podoviridae* families showed some variations in their RFLP patterns (Wang tan et al., 2021). Variations in the genome of isolated bacteriophages from the same origin can be helpful to access more phages to design effective bacteriophage packages, which can be useful to control antibiotic-resistant urinary tract infections.

Conclusion

PEcMa2/17 and PEcMa3/17 are virulent phages against UTIs *E.coli* bacteria. The large burst size and short latent period of both isolated phages in addition to their high stability could indicate their strong potential as the candidates for phage therapy of UTIs caused by *E.coli*. Further molecular analyses and sequencing of the entire genome are required to characterize the introduced bacteriophages. It is worth mentioning that we need more advanced experimental studies to evaluate their potential for the therapeutic applications.

Acknowledgment

This paper is published as part of a research project supported by the University of Maragheh Research Affairs Office.

References

Adams, M.H., 1959. Bacteriophages, Interscience Publishers, New York.

Ahmadpour, S., Mardani, K., Tukmechi, A. (2016). Culture and molecular characterization of phages

isolated from rainbow trout farms and sewage treatment plants and investigation of their effects on Yersinia ruckeri. Iranian Journal of Fishery Sciences 15: 267-280.

Ackermann, H.W., Tremblay, D., Moineau, S. (2004). Long-term bateriophage preservation. WFCC Newslett 38: 35-40.

Amarillas, L., Rubi-Raangel, L., Chaidez, C., Robles, A., Rojas, Felix, J. L. (2017). Isolation and characterization of phiLLS, a novel phage with potential biocontrol agent against multidrugresistant *Escherichia coli*. Frontiers in Microbiology 8: article 1355.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. American Journal of Clinical Pathology 45(4): 493-496.

Chibeu, A., Lingohr, E. J., Masson, L., Manges, A., Harel, J., Ackermann, H. W. (2012). Bacteriophages with the ability to degrade uropathogenic *Escherichia coli* biofilms. Viruses 4(4): 471-487.

Dalmasso, M., Strain, R., Neve, H., Charles, M. A., Franz, P., Fabien J. and et al. (2016). Three new *Escherichia coli* phages from the human gut show promising potential for phage therapy. PLoS One 9: 0156773.

Feng, Y. Y., Ong, S. L., Hu, J. Y., Tan, X. L., Ng, W. J. (2003). Effects of pH and temperature on the survival of coliphages MS2 and Qβ. Journal of Industrial Microbiology and Biotechnology 30(9): 549-52.

Flores-Mireles, A.L., Walker, J.N., Caparon, M., Hultgren, S.J. (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nature Reviews Microbiology 13 (5): 269-84.

Jamal, M., Hussain, T., Das, C.R., Andleeb, S. (2015). Characterization of *Siphoviridae* phage Z and studying its efficacy against multidrugresistant *Klebsiella pneumoniae* planktonic cells and bioflm. Journal of Medical Microbiology 64: 454-462.

Jassim, S., Abdulamir, A.S., Bakar, F.A. (2012). Novel phage-based bio-processing of pathogenic *Escherichia coli* and its biofilms. World Journal of Microbiology and Biotechnology 28(1): 47-60.

Jonczyk, E., Klak, M., Miedzybrodzki, R., Gorski, A. (2011). The influence of external factors on bacteriophages-review. Folia Microbiologica 56: 191-200.

Jun, J.W., Kim, J.H., Shin, S.P., Han, J.E., Chai, J.Y., Park, S.C. (2013). Characterization and complete genome sequence of the *Shigella* bacteriophage pSf-1. Research in Microbiology 164: 979-986.

Kostakioti, M., Hultgren, S.J., Hadjifrangiskou, M. (2012). Molecular blueprint of uropathogenic Escherichia coli virulence provides clues toward the development of anti-virulence therapeutics. Virulence 3(7): 592-3.

Lasobras, J., Muniesa, M., Frias, J., Lucena, F., Jofre, J. (1997). Relationship between the morphology of bacteriophages and their persistence in the environment. Water Science and Technology 35: 129-132.

Ly-Chatain, M. H. (2014). The factors affecting effectiveness of treatment in phages therapy. Frontiers in Microbiology 5: article 51.

Manohar, P.T., Tamhankar A.J., Lundborg C.S., Ramesh, N. (2018). Isolation, characterization and *in vivo* efficacy of *Escherichia coli* phage myPSH1131. PLoS one 13(10): e0206278.

Maszewska, A., Wójcik, E., Ciurzyńska, A., Wojtasik, A., Piątkowska, I., Dastych, J., Różalski, A. (2016). Differentiation of polyvalent bacteriophages specific to uropathogenic *Proteus mirabilis* strains based on the host range pattern and RFLP. Acta Biochimica Plonica (63): 303-310.

Merabishvili, M., Vandenheuvel, D., Kropinski, A.M., Mast, J., De Vos, D., Verbeken, G. et al. (2014). Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*. PloS One 9(8): e104853.

Mohamed, M.A., Abdifetah, O., Hussein, F.A. (2020). Antibiotic resistance pattern of *Escherichia coli* isolates from outpatient with urinary tract infections in Somalia. Journal of Infection in Developing Countries 14(3): 284-289.

Molaabaszadeh, H., Hajisheikhzadeh, B., Mollazadeh, M, Eslami, K., Mohammadzadeh Gheshlaghi, N. (2013). The study of sensibility and antimicrobial resistance in *Escherichia coli* isolated from urinary tract infection in Tabriz City. Journal of *Fasa* University of Medical Sciences 3(2): 149-154.

Necel, A., Bloch, S., Nejman-Faleńczyk, B., Grabski, M., Topka, G., Dydecka, A., Kosznik-Kwaśnicka, K., Grabowski, L., Jurczak-Kurek, A., Wołkowicz, T., Węgrzyn, G., Węgrzyn, A. (2020).

Characterization of a bacteriophage, vB_Eco4M-7, that efectively infects many *Escherichia coli* O157 strains. Scientific Reports 10: 3743.

Pacifico, C., Hilbert, M., Sofka, D., Dinhopl, N., Pap, I. J., Aspöck, C., Carriço, J. A., Hilbert, F. (2019). Natural occurance of *Escherichia coli*-infecting bacteriophages in clinical samples. Frontiers in Microbiology 10: article 2484.

Pajunen, M., Kiljunen, S., Skurnik, M. (2000). Bacteriophage φYeO3-12, Specific for *Yersinia enterocolitica* Serotype O:3, Is Related to Coliphages T3 and T7. Journal of Bacteriology 182(18): 5114-5120.

Pouillot, F., Chomton, M., Blois, H., Courroux, C., Noelig, J., Bidet, P., et al. (2012). Efficacy of bacteriophage therapy in experimental sepsis and meningitis caused by a clone O25b: H4-ST131 *Escherichia coli* strain producing CTX-M-15. Antimicrobial Agents and Chemotherapy 56(7): 3568-75.

Prigent, M., Leroy, M., Confalonieri, F., Dutertre, M., DuBow, M. S. (2005). A diversity of bacteriophage forms and genomes can be isolated from the surface sands of the Sahara Desert. Extremophiles 9: 289-296.

Quinn, P.J., Carter, M.E., Markey, B.K., Cartel, G.R. (1994). Clinical Veterinary Microbiology, Mosby-Year book Europe Limited, Wolfe publishing Ltd. London, England.

Rafalskiy, V., Pushkar, A., Yakovlev, S., Epstein, O., Putilovskiv, M. (2020). Distribution and antibiotic resistance profile of key gram-negative bacteria that cause community-onset urinary tract infections in the Russian Federation. Journal of Global Antimicrobial Resistance 21: 188-194.

Rahimzadeh, G., Resch, G., Rezai1, M. S., Nemati Hevelaee, E. (2020). Characterization and lytic activity of isolated *Escherichia coli* bacteriophages against *Escherichia Coli in Vitro*. Iranian Journal of Medical Sciences 45(4): 298-303.

Ramirez, K., Cazarez-Montoya, C., Lopez Moreno, H. S., Castro-del Campo, N. (2018). Bacteriophage cocktail for biocontrol of Escherichia coli O157:H7: Stability and potential allergenicity study. PLoS One 13(5):1-19.

Sabouri Ghannad, M., Mohammadi, A. (2012). Bacteriophage: time to re-rvaluate the potential of phage therapy as a promising agent to control multidrug-resistant bacteria. Iranian Journal of Basic Medical Sciences 15(2): 693-701.

Stamm, W.E., Norrby, S.R. (2001). Urinary tract infections: disease panorama and challenges. The Journal of Infectious Diseases 183(1): S1-S4.

Sybesma, W., Zbinden, R., Chanishvili, N., Kutateladze, M., Chkhotua, A., Ujmajuridze, A. (2016). Bacteriophages as potential treatment for urinary tract infections. Frontiers in Microbiology 7: article 465.

Tuem, K. B., Gebre, A. K., Atey, T. M., Bitew, H., Yimer, E. M., Berhe, D. F. (2018). Drug resistance patterns of *Escherichia coli* in Ethiopia: A Meta-Analysis. BioMed Research International 4536905.

Tan, C. W., Rukayadi, Y., Hasan, H., Abdul-Mutalib, N. A., Jambari, N. N., Hara, H., Thung, T. y., Lee, E., Radu, S. (2021). Isolation and characterization of six *Vibrio parahaemolyticus* lytic bacteriophages from seafood samples. Frontiers in Microbiology 12: article 616548.

Xu, Y., Yu, X., Gu, Y., Huang, X, Liu, G., Liu, X. (2018). Characterization and Genomic Study of Phage vB_EcoS-B2 Infecting Multidrug-Resistant *Escherichia coli*, Frontiers in Microbiology 9: article 793.

Yazdi, M., Bouzari, M., Ghaemi, E., Shahin, Kh. (2020). Isolation, characterization and genomic analysis of a novel bacteriophage VB_EcoS-Golestan infecting multidrug-resistant *Escherichia coli* isolated from urinary tract infection. Scientific Reports 10: 7690.

Open Access Statement:
This is an open access article distributed under the Creative Commons Attribution License (CC-BY), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.