Introduction

The Gram-negative bacteria belonging to Enterobacter spp. are the third leading cause of hospital-acquired infections of blood stream and respiratory tract after Staphylococcus aureus and Pseudomonas aeruginosa [1]. The E. aerogenes and E. cloacae are among the two most common Enterobacter species, responsible for causing human infections (90–99% of Enterobacter infections) [2]. The Enterobacter cloacae mainly causes urinary and respiratory tract infections. The majority of Enterobacter spp. are resistant to antibiotics of common use and present a global clinical problem [1]. The Enterobacter cloacae is associated with different types of outbreaks in hospital settings [3-5].

The bacteriophages were first discovered by Félix d’Herelle in 1917 [6]. The therapeutic potential of bacteriophages in controlling the bacterial infections in humans were first employed in 1919 [6]. Consequently, the anti-plague activity of bacteriophages was reported by d’Herelle in 1925 [7]. The concept of phage therapy ceased in western medicine due to the emergence of antibiotics, while it is still researched and applied in Russia and neighboring countries [8]. The West remained reluctant in using the bacteriophages as therapeutic agents due to early unreliable trials; however now phage therapy has also gained attention in the developed world.

Increasing emergence of antibiotic resistance poses a challenge among the scientific community to identify potential modalities for controlling the antibiotic resistant Enterobacter spp. The bacteriophages against the Enterobacter spp. can be utilized as an alternative therapy for controlling the antibiotic resistant isolates. The bacteriophages selectively infect their bacterial host down to individual strains. The bacteriophages are thought of as a potentially valuable tool in controlling bacterial infections, which in certain cases of antibiotic resistance, might be the only available effective modality. Additionally a single dose of phage can destroy a specific bacterium, while multiple doses of antibiotics are needed to treat bacterial infections. Currently no harmful effects of phages to eukaryotic cells have been reported. The United States Food and Drug Administration have approved the use of Listeria phage in foods, which has re-established the use of phages as antimicrobial agents [9]. The current effort was done to isolate and characterize the lytic phages against Enterobacter cloacae to be used in phage therapy.
2 Materials and Methods

2.1 Host bacterial strains

A characterized environmental isolate of *Enterobacter cloacae* (accession no: KF975427) is used as the bacteriophage host (donated by Dr. Yasir Rehman, Assistant Professor in the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan) in this study. The host bacterial strain was streaked on L-agar plates and cultured in L-broth for use after 24 hours incubation at 37°C.

2.2 Isolation and purification of bacteriophages

The bacteriophage was isolated from sewage outlet of Tech Housing Society, Lahore. Isolation and purification of the bacteriophages was performed according to an already published protocol by our group [10]. Purified bacteriophage lysate was stored at 4°C when storing for short time or further processing, while stored at -20°C for longer time. The viral titer in each phage lysate is expressed as plaque forming units per millilitre (PFU/mL).

2.3 Determination of host specificity

The host infectivity of each isolated phage against *Aeromonas caviae* (accession no: KF975428), *Pseudomonas stutzeri* (accession no: KF975434), and *Klebsiella pneumoniae* (accession no: KF975429) (gift from Dr Yasir Rehman, Assistant Professor at the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore Pakistan) was determined through the double layer agar technique. The host specificity against two hospital isolates of *Enterobacter cloacae* (strain number EC11 and EC12) was also tested.

2.4 Examining bacteriophage stability at wide range of pH and temperature

The viability of *Enterobacter cloacae* phages at varying pH and temperatures was investigated. Briefly, known concentrations of purified bacteriophages were incubated in L-broth with different pH values (5-10), incubated for 1 hour at 37°C. Similarly, to study the temperature stability of bacteriophages, a known concentration of bacteriophages was incubated at varying temperatures (28, 35, 37, 40, 45, 50, 55, 60 and 65°C) for 1 hour in L-broth (pH 7.0) followed by calculating phage titer by the double layer agar method [11].

2.5 Analysis of bacteriophage growth parameters

The one step growth curve of the bacteriophages was performed in duplicates following an already reported method [10]. Actively growing cells of host strain (O.D600 of 0.4-0.6), were suspended in 500 μL of L-broth and mixed with bacteriophages, and incubated for one minute at 37°C. Centrifugation of the mixture was done at 11000 x g for 30 seconds and the pellet was re-suspended in 100 mL fresh broth followed by incubation at 37°C with shaking. Viral titer was counted by double layer agar technique from samples taken at 5 minute intervals for an hour.

2.6 Assessment of bacteriophage antibacterial activity

The potential of the bacteriophages against the respective host bacterial strain was checked according to already published work [10]. Briefly, a 24 hour bacterial culture (1 x 10⁸ CFU) and respective bacteriophage (1 x 10⁹ PFU) were added in one flask, while the other flask containing the same concentration of bacteria but no phages was taken as control. The optical density was taken at intervals of 2 hours for a 24 hour period after incubation at 37°C with shaking (120 rpm).

2.7 Stability of bacteriophages during storage

The purified phages (1 x 10¹⁰ CFU/ml) were incubated for one month at different storage temperatures (-20, 4, 37°C) followed by assay for phage infectivity.

2.8 Isolation of bacteriophage genomic DNA

The DNA of the bacteriophages was extracted as previously mentioned [10] followed by electrophoretic analysis in 0.7% TBE agarose gel. The isolated bacteriophage genome was treated with endonuclease I to assess either it is single or double stranded DNA.
3 Results

The bacteriophages TSE1, TSE2 and TSE3 against *Enterobacter cloacae* were isolated from the sewage exhaust of the Tech housing society Lahore and are shown in Figure 1 and Figure 2. The plaque morphology along with the PFU/mL were recorded for each stock of the virus. The plaque morphology of TSE1 and TSE3 phages appeared as pinpoint, while morphology of the TSE2 was small circular on double layer agar plates (Figure 2). The plaque size of TSE1 and TSE3 appeared less than 1mm, while it was 1mm for the plaques of TSE2. The virus titer of TSE1, TSE2 and TSE3 were determined as $2.0 \times 10^8$, $4.8 \times 10^6$ and $2.5 \times 10^8$ PFU/mL respectively.

The host range of the isolated phages was checked against different bacterial strains, including *Aeromonas caviae*, *Pseudomonas stutzeri*, and *Klebsiella pneumoniae* and hospital isolates of *Enterobacter cloacae*. All the TSE phages exhibited a narrow host range as they were unable to infect any tested bacteria other than *Enterobacter cloacae*. The purified phages ($1 \times 10^{10}$ CFU/ml) were incubated for one month at different temperatures followed by analysis of viral titer. All the TSE phages remained stable at both tested storage temperatures (-20°C and 4°C), while none remained stable at 37°C (Figure 3).

The phage survival in the adverse environment is also a desired characteristic for their use in phage therapy. The PFU of all the phages was maximum at pH 7 ($2.2 \times 10^7$ for TSE1, $4.6 \times 10^4$ for TSE2 and $2.3 \times 10^4$ for TSE3), while reduction of PFU was observed at both pH 6 ($2.8 \times 10^5$ for TSE1, $7.0 \times 10^2$ for TSE2, $1.4 \times 10^3$ for TSE3) and at pH 8 ($2.8 \times 10^5$ for TSE1, $3.6 \times 10^1$ for TSE2 and $1.3 \times 10^1$ for TSE3). No plaques were observed at pH 5, 9 and 10 (Figure 4). The PFU was highest after treatment at 37°C while fewer plaques were observed at other temperatures (28°C to 60°C), however no lytic activity was observed after treatment at 65°C (Figure 5).

The latent period, and burst size of all the TSE phages was calculated from a one-step growth curve. The latent period for all TSE bacteriophages was calculated as 20 minutes. However the burst size of TSE1, TSE2 and TSE3 was 360, 270 and 311 particles per cell respectively (Figure 6).

The potential of the isolated TSE phages to reduce the growth of their host bacteria and development of phage resistant bacteria were analyzed by plotting the bacterial growth reduction curve. The OD$_{600}$ of non-infected *E. cloacae* increased throughout the 24 hours incubation which indicates their normal growth pattern. The bacteriophages reduced the growth of target bacteria during 8-18 hours post infection. However the development of resistant bacteria occurred after 18 hours post infection, and attained the optical density similar to uninfected control at 24 hour post infection (Figure 7), which indicates the development of phage resistant bacterial cells.
K. Ameer Khawaja, et al.

The genome of all the bacteriophages was isolated and observed under UV after running the 0.7% agarose gel for one and half hours. The genome of all the phages appeared double stranded DNA as it was not digested by endonuclease I enzyme, indicative of double stranded DNA. The genome of all the phages appeared more than 12 kb in size (Figure 8).

4 Discussion

Hospital acquired infections (HAIs) are global, but are less studied in developing nations like Pakistan [12]. In the present study, bacteriophages against the Enterobacter cloacae which usually show marked antibiotic resistance were under investigation. The bacteriophages against an environmentally isolated Enterobacter cloacae (accession no: KF975427) strain were isolated and characterized. The TSE phages were isolated from sewage exhaust which has been reported as a good source for bacteriophage isolation [13]. The TSE phages under study showed a narrow host range, a characteristic of majority of bacteriophages already reported [15]. The ability of phages to infect specific host(s) makes them suitable for phage therapy [16].

The stability of studied TSE phages at varying temperatures and pH was also investigated, this data is helpful in determining their suitability for use in phage therapy through the oral route. The TSE phages showed their viability at pH of 6-8, while at neutral pH (7.0) maximum phage titer was recorded (Figure 4). The ability of phages to tolerate at varying pH levels indicates that...
Isolation and characterization of lytic phages TSE1-3 against Enterobacter cloacae

The bacteria become resistant to phages by downregulating the expression of the phages receptors (flagella and capsules), which make them less virulent [22]. The genome of the studied phages was larger than 12 kb, and it was DNA evident by digestion with the DNase. According to the size of the genome, we can deduce that the genome is double-stranded, as the size categorization placed them among medium-to large-sized genomes, which are mostly double-stranded [23]. The reported small size phage genomes are around 5.5 kb, while the larger size genomes may be greater than 250 kb [24].

Currently, there are great number of studies on bacteriophage isolation and characterization against E. cloacae. The emergence of phage resistant strains poses a great challenge for phage therapy, while there is need to investigate its role in decreasing the bacterial virulence. The concept of using bacteriophages in contrast to antibiotics for controlling bacterial infections looks promising. The financial investment for phage therapy is considerably less compared with identification of a novel antibiotic. Further work on bacteriophage genome characterization, dose and lytic activity against more clinical isolates might lead to utilization of these phages in therapy.

Conflict of Interest: Authors declare to have no conflict of interest for this work.

References


