CHAPTER 5

ISOLATION AND IDENTIFICATION OF BACTERIOPHAGE FROM SEWAGE WATER

“All organisms are nothing but a bag of other organisms walking around”

-Thomas Miller

5.1 OBJECTIVE

To characterize phage isolates from sewage water samples in order to determine its potential in transport and disinfection studies.

5.2 INTRODUCTION

Bacteriophages are extremely abundant in the biosphere with remarkable genetic diversity and are a driver of global geo-chemical cycles (Emma and Suttle 2005). Approximately, 6000 million phage species have reported globally, but only small proportion of them has been characterized so far (Ackermann 2001).

Bacteriophages are obligatory intracellular bacterial parasites which lack an independent metabolism, exploiting bacterial cells for their reproduction. They are highly specific, often being restricted within a single bacterial species. However, some bacteriophages have a relatively broad host range, infecting multiple species within a genus closely related to their host
(Singh et al. 2016). A phage particle or virion consists of single or double stranded (ss or ds) DNA or RNA molecules encapsulated inside a protein or lipoprotein coat. According to the International Committee on the Taxonomy of Viruses (ICTV), over 95% of the phages in the literatures belong to the tailed dsDNA phages (Ackermann 2007).

Scientific studies, involving phage-derived technologies can guarantee bacterial detection, industrial, therapeutic, nanotechnology and other fields yet to be explored. Their exploration has already become as an interesting tool to fight against antibiotic resistant bacteria (Dabrowska et al. 2005). Moreover, the spontaneous occurrence of phage resistant mutants has challenged the phage treatment efficacy (Carlton et al. 2005)

Enteric bacteria are normal inhabitants of the intestines of humans and other animals (Davis 2005) but are often isolated from aquatic ecosystems after sewage has been introduced into it. Aquatic environments contaminated with enteric bacteria possess a potentially serious threat to both human and animal health (Beaudoin et al. 2007). Among them, the major water-borne pathogens are the strains of E. coli associated with illnesses such as diarrhea, dysentery, and increases mortality rate worldwide (World Health Organization 2005).

Thus, with reference to the above stated facts, the present study aimed to isolate and characterize phages that are active against E. coli KP005067.
5.3 MATERIALS AND METHODS

5.3.1 Bacterial strain and growth conditions

*E. coli* KP005067 isolated from Canoli canal, Kozhikode district, Kerala, India was selected as the host bacteria for phage isolation. For experiments, the strain was cultured on EMB agar, MacConkey agar and Nutrient agar and incubated at 37°C for 24 hours. For enrichment of the strain, a colony from EMB agar plate was inoculated into alkaline peptone water (5-10 mL) and incubated at 37°C for 6-8 hours.

5.3.2 Sample collection

Sewage samples were collected from stagnant water at a depth of 3 meters, using sterile containers from 11 sites of the canal. Precautions were taken while collecting the samples by wearing sterile gloves and masks. Approximately, 100 mL of sewage samples were collected and transported immediately to the laboratory and stored at 4°C, until further use.

5.3.3 Epifluorescence Microscopy

The viral and bacterial abundance in the sewage samples were assessed using Epifluorescence Microscopy by SYBR staining method. Bacteria and virus like particles were enumerated from sewage samples (Patel 2007) using 0.8 μm nitrocellulose membrane support filters (Millipore) and 0.02 μm filters (Whatman) respectively.
5.3.4 Bacteriophage isolation

Bacteriophages were isolated from sewage samples by enrichment technique (Cerveny et al. 2002). Under sterile conditions, 4.5 mL of sewage sample was emulsified with 0.5 mL of tryptone broth and 0.5 mL of log-phase broth culture (optical density measured at 600 nm) of *E. coli* KP005067. They were incubated overnight at 37°C, in shaker incubator and later centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was filtered using a 0.22 μm syringe filter (Nupore, India) to obtain a bacteria free filtrate and analyzed for the presence of phages through spot test method (Chang et al. 2005). Double layer plaque technique was performed for bacteriophage isolation (Santos et al. 2009). Three milliliter of 0.6% of trypticase soy agar (TSA) was inoculated with 100 μL of 3-5 hours old bacterial culture and 100 μL of bacteria free filtrate. The suspension was plated on fresh 1.5% TSA plate and incubated in an upright position at 37°C overnight. Formation of plaques or zones of clearance indicated the presence of phages.

5.3.5 One-step growth kinetics

One step growth experiments were carried out based on the method described by Leuschneret al. (1993) with some modifications. The experiment was initiated at a multiplicity of infection (MOI) 0.1 in a 15 mL tube containing phage (approximately 4 x 10^6 PFU/mL) and its host *E. coli* KP005067 (approximately 2 x 10^8 CFU/mL) in 10 ml TSB. After incubation in water bath at 37°C for 10 minutes (to allow phage adsorption), the tube was centrifuged at 13,000 rpm for 15 minutes. The supernatant was collected and subjected to plaque assay to determine the titre of the unabsorbed phage. The pellet containing (partially) infected cells were immediately re-suspended in 10 mL of pre-warmed TSB. After taking the first sample, the tube was returned to the
water bath (37°C). At every 5 minutes (up to 60 minutes) interval, 100 μL of sample was collected, immediately diluted and subjected to plaque assay. Latent period was defined as the time interval between the end of adsorption and the beginning of first burst, as indicated by the initial rise in phage titer. Burst size was calculated as the ratio of the final number of liberated phage particles to the initial number of infected bacterial cells during the latent period.

5.3.6 Electron Microscopy

The morphological examination of bacteriophage was performed as described by Oliveira et al. (2009) with minor modifications. Phage particles were sedimented by centrifugation at 25,000 rpm for 60 minutes. Phages were washed twice in 0.1 M ammonium acetate (pH 7), filtered through a 0.22 μm pore size membrane filter, deposited on copper grids provided with carbon-coated format films and examined in a Zeiss EM109 electron microscope.

5.3.7 Quantification of phage DNA

Phage DNA was extracted from the purified phages by using sodium dodecyl sulfate (SDS), EDTA and proteinase K and the DNA was precipitated by adding 3 M sodium acetate and absolute ethanol (Petrovski et al. 2011). The pellet was dried and resuspended in an appropriate volume of sterile high-purity water and stored at -20°C. The DNA samples were quantified on 0.8% agarose gel along with DNA ladder digested with EcoRI/HindIII.
5.3.8 UV irradiation studies

Purified phages were diluted using SM buffer to concentrations of $10^{-3}$, $10^{-4}$, $10^{-5}$ and $10^{-6}$. Four 100 μL of viral drops were placed close together on an empty Petri dish under the UV lamp. After every 15 minutes, one drop was removed and transferred into a microcentrifuge tube along with 400 μL of *E. coli* KP005067. The tubes were mixed well and kept at 37°C for 10 minutes. After incubation, 250 μL of virus and bacterial mixture were added to 11 mL of warm soft agar and poured onto a petri dish. The dishes were incubated overnight, and the number of clear zones on the plate was counted.

5.4 RESULTS

SYBR green stained double stranded DNA more effectively and the probability of observing phage particles was high. By using 0.015 μ pore size nucleopore filter, numerous small DNA particles together with large DNA particles were recognized from the sewage samples (Figure 5.1). The criteria for the distinction between larger DNA-associated particles (possibly bacteria) and other smaller particles (phage) were to analyze the abundance of phage and its host bacteria in the water samples. The results showed a large number of phage in the water samples.
The results of plaque assay and spot assay are shown in table 5.1. Out of eleven sewage samples, only 63.6% (7) were positive for spot assay. When the phage filtrate was spotted onto the lawn culture of the host bacteria clear circular zone (plaque) with average diameter of 5-7 mm was observed. This indicated the lytic nature of the isolated phage. Out of these seven samples, 71.4% were positive for plaque assay. Among which sample 4 (S4) showed highest plaque assay with 86 PFU/mL, while sample 7 (S7) had the lowest (21 PFU/mL). All the other six samples were found to negative for plaque assay.
Table 5.1: Plaque and spot assay in water samples

<table>
<thead>
<tr>
<th>Samples #</th>
<th>Plaque assay in PFU/ml</th>
<th>Spot assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>48</td>
<td>+</td>
</tr>
<tr>
<td>S2</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>S3</td>
<td>42</td>
<td>+</td>
</tr>
<tr>
<td>S4</td>
<td>86</td>
<td>+</td>
</tr>
<tr>
<td>S5</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>S6</td>
<td>39</td>
<td>+</td>
</tr>
<tr>
<td>S7</td>
<td>21</td>
<td>+</td>
</tr>
<tr>
<td>S8</td>
<td>ND</td>
<td>+</td>
</tr>
<tr>
<td>S9</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>S10</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>S11</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

ND- not detected, +: positive, -: negative

Figure 5.2 shows the one step growth curve of bacteriophage. The latent period was only 15 minutes (excluding 10 minutes for adsorption), which is shorter than the typical latent periods (21-120 minutes). The rise period began after 20 minutes, with host cell lysis completed after 30 minutes releasing 35 progeny virions per infected cell.
Figure 5.2: One step growth curve of bacteriophage

Analysis of phages using transmission electron microscopy aids in the morphological classification of phage into viral family and order. In the current study, the isolated phage belonged to the family Siphoviridae and order Caudovirales due to the presence of a non-enveloped head and long contractile tail (Figure 5.3). The phage heads were measured between opposite apices with an approximate size of 49.7 ± 5.37 nm and the length of the tails was 143 ± 3.25 nm.

Figure 5.3: Transmission electron microscope image of phage
The isolated phage DNA was electrophoresed on 0.8% agarose gel with *EcoRI/HindIII* marker (BangloreGenei, Banglore, India). Molecular weight of the phage DNA was found to be 31 kb. The result revealed that the phages were sensitive to the marker, and exhibited different banding patterns confirming that the phages harbored double stranded DNA as genetic material (Figure 5.4).

![Genomic DNA of phage run on an agarose gel](image)

**Figure 5.4:** Genomic DNA of phage run on an agarose gel

In the UV irradiation study, the number of plaques were counted for each length of time and compared to the control group. The averages were taken and plotted as percentages (Figure 5.5). In the present study, the control group had a significantly higher survival rate than any of the groups that were exposed to UV. Upon comparison with non-irradiated phage, irradiated phage showed a decrease in lytic activity. There was a minor difference in lytic activity between 15 and 30 minute trials, but thereafter, a clear downward trend was observed in groups exposed for longer than 30 minutes. Table 5.2 showed the calculated standard deviation of each time segment. Thus, if the UV exposure time exceeds 30 minutes, there can be a steady decline in the lytic activity of the phage.
Table 5.2: Error in percentage compared using standard deviation

<table>
<thead>
<tr>
<th>Time</th>
<th>Average plaque count</th>
<th>% of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>149.41 ± 28.63</td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>120.50 ± 28.71</td>
<td>80.65</td>
</tr>
<tr>
<td>30</td>
<td>117.33 ± 21.13</td>
<td>78.53</td>
</tr>
<tr>
<td>45</td>
<td>101.33 ± 15.44</td>
<td>67.82</td>
</tr>
<tr>
<td>60</td>
<td>84.41 ± 20.56</td>
<td>56.50</td>
</tr>
</tbody>
</table>

Figure 5.5: Trend of phage survival to UV exposure

\[
y = -9.983x + 106.65 \\
R^2 = 0.9503
\]
5.5 DISCUSSION

The objective of this study was to isolate and characterize phage that is specific to *E. coli* KP005067, which is a water-borne pathogen. The isolation was carried out from sewage waters as it was considered to be a good source for isolation of phage (Kumari et al. 2009). In this study, different samples of stagnant water from Canoli canal were collected, for phage isolation and characterization. By using the sewage water samples, lytic bacteriophages were isolated by employing double agar overlay method using *E. coli* KP005067 as representative strain. Oliveira et al. (2009) and Bao et al. (2011) also employed similar method for the isolation of bacteriophages. Plaque activity was observed within 12 hours against host bacteria and found to be lytic in nature with plaque size of about 5-7 mm. These findings were in correlation with Flynn et al. (2006); Higgins et al. (2007); Sillinkorva et al. (2008); Mahadevan et al. (2009); Oliveira et al. (2009) and Jammalludeen et al. (2012).

About 6000 species of bacterial viruses have been described so far (Ackermann 2007). Bacteriophage classification and identification was done initially on the basis of tailed, filamentous and cubic phages (Kasweka 2009), whose genetic material is either DNA or RNA. Plaque morphology is one of the foremost criteria for characterization of phages (Shukla and Hirpurkar 2011). Even in this study, only double stranded DNA phages were isolated. Moreover, isolated phages possessed a long non contractile tail and icosahedral capsid head, which is confirmed to be a member of the family *Siphoviridae*. These features indicated that the bacteriophage belonging to family *Siphoviridae* and order *Caudovirales* which is the commonly isolated bacteriophage group against *E. coli* (Goodridge et al. 2003, Careysmith et al. 2006, Flynn et al. 2006). In contrast, Jothikumar and Reddy (2000) found that plaque morphology
was not affected by addition of cations and also, did not find any relationship between coliphage family and specific plaque morphology.

The phage genome size was about 31.2 kb as compared to the wild type double stranded DNA phage whose genome size was 33.5 kb (Singh et al., 2016). Goodridge et al. (2003), Flynn et al. (2006), Lappe et al. (2009) also reported that the molecular weight of phage DNA in range of 40-50kbp belonging to the family Siphoviridae of order Caudovirales. Phage can also be classified based on the conservation of gene arrangement, but the phage must be completely sequenced (Kovalyova and Kropinski 2003). Bacteriophage, with larger genome size of 16 kb or greater was found to be in the order of Caudovirales, with greater infectivity and virion sophistication than with smaller genome size (Abedon 2011).

The biggest challenge in characterizing phage is to understand the replication of its genetic material. On UV exposure, there was no effect found on phage up to 30 minutes. But the lytic nature of phage decreased comparatively with increased exposure to UV (Singh et al. 2016). Because of stress, phage may also have less impact on bacterial populations and more trouble in replicating. Somatic phages and bacteriophage f2 were also found to be completely inactivated (3 log unit reduction) on exposure to less than 3 hours of UV irradiation (Davies-Colley et al. 2005). Also the persistence of somatic coliphages were found to be longer when compared to fecal coliforms, enterococci and F-RNA phages when exposed to UV light (Muniesa et al.1999). The UV radiation can cause 5% loss in viable phage per hour in water sources due to the formation of thymine dimers (Wommack et al. 1996).
Many phages are known to be highly specific for their receptors and show little or no interaction with receptors even at slightly different structure. This specificity forms the basis of numerous phage typing methods for the identification of bacterial species or subspecies. The current study suggests that, the phage for *E. coli* KP005067 was highly specific to its respective host. The study also convey that, host specific bacteriophage against human pathogens are prevalent and can be readily isolated from sewage ecosystems.