CHAPTER - III
5. Chapter - III: Morphological Identification and Molecular Characterization of Bacteriophages

5.1. Introduction

Bacteriophages are existed in many varieties that have either single or double-stranded RNA or DNA genomes that range in size from a few thousand to half a million base pairs (bp). Bacteriophages are the first complete genomes to be sequenced, beginning with the 5,386bp single-stranded DNA (ssDNA) phage φX174 in 1977 due to its small size and simplicity of isolation (Sanger, 1977).

Bacteriophages are present in thirteen different families which was includes Myoviridae, Siphoviridae, Podoviridae, Microviridae, Corticoviridae, Tectiviridae, Leviviridae, Cystoviridae, Inoviridae, Lipothrixviridae, Rudiviridae, Plasmaviridae and Fuselloviridae. The diversity of bacteriophages is reflected by their diversity in genome size, which ranges from 4 to 600 KB. In general, 96% of bacteriophages having tail, which comes under Myoviridae, Siphoviridae and Podoviridae have long, short non-contractile tail respectively (Brussow and Hendrix, 2007) and these phages are belongs to Caudovirales order. These Caudovirales order viruses have 18 – 500kb dsDNA as their genomes. They have both icosahedral head and a tail. In that, head contains the DNA and tail is responsible for binding of the target on the host cell and for delivering the DNA into the binded host cell. Although members of the tailed-phage group have many things in common, they nonetheless represent an extremely diverse collection of viruses.

However, all phage have a chromosome encased in a capsid that is composed of phage-encoded proteins. For many phage types, the capsid is attached to a tail structure that is also made from phage-encoded proteins. Phage genome encodes DNA packaging, head, tail and tail fibers, DNA replication, transcription regulation and lysis genes. These functions are squeezed into a 20kb DNA genome (Casjens, 2005).
5.2. Objectives

- To study the morphological characteristic features of phages; and
- to analyses the phage DNA and structural proteins;

5.3. Materials and Methods

5.3.1. Morphological Identification of Phages

Negative staining was performed to visualize phage morphology using transmission electron microscope (TEM). Ten microliters of phage suspension ($10^8$ PFU/ml) was applied to the surface of a collodium - coated 200 mesh, copper grid and negatively stained with 1% phosphotungstic acid for 1 min. After drying, photographs were taken under a TEM (TECHNAI10-Philphs, Netherland). Size was determined from the average of three independent measurements. The phage was classified according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV, 1995) based on their morphological features (Mazaheri et al., 2010).

5.3.2. Isolation of DNA

Bacteriophage nucleic acid was extracted as described by Maniatis et al. (1982). Purified phage particles were treated with SDS (10 %) and proteinase K (20mg/ml) at 50°C for 1 h. Proteinaceous materials were removed by adding an equal volume of phenol-chloroform. The extraction was repeated again with chloroform, and the nucleic acids were precipitated with 0.25 volumes of 3 M sodium acetate and 2.5 volumes of absolute ethanol. The final pellet was washed twice with 70% ethanol, air dried, and then resuspended in TE buffer. The whole lysate was loaded on the 0.8% TAE agarose gel (Tris Base, Boric Acid, EDTA, pH 8.3) containing 0.5 mg/ml of ethidium bromide along with DNA ladder (A DNA digested with EcoRI/HindIII). The gel was run at a voltage of 90V and the bands were visualized using a BioRad gel documentation system (Maniatis et al., 1982).
5.3.3. Analysis of Structural Proteins

The purified phage solution was precipitated with 4 volumes of ice cold acetone and it was pelleted by centrifugation at 12000 rpm for 10 min at 4°C. The pellet was air-dried and resuspended in PBS buffer (8g NaCl, 0.2g KCl, 0.2g KH₂PO₄, 1.44g Na₂HPO₄ x 2H₂O, pH 7.5). An aliquot of the concentrated phage proteins was boiled for 3 min in sample buffer (0.175 M Tris-HCl (pH 6.8), 15% w/v glycerol, 5% w/v SDS, 4.65% w/v DTT, a trace of bromophenol blue). Vertical electrophoresis was performed on 10% separating and 4% stacking acrylamide gels in running buffer (0.025 M Tris- HCl, 0.192 M glycine, 0.1% w/v SDS). Wide range Sigma markers were used as protein molecular weight markers. Proteins were stained with the Bio-Safe Coomassie G-250 and documented (Eyer et al., 2007).

5.3.4. Restriction Digestion Analysis

For genome size estimation, isolated phage DNA was digested with the restriction endonucleases EcoRI, HindIII, BamHI and PstI (Takara Bio, Shiga, Japan) according to the manufacturer’s instructions. 1µg phage genome was digested by using the above mentioned restriction enzymes and digested DNA fragments were separated by agarose gel electrophoresis using 2% ultrapure agarose (Invitrogen, USA). The DNA bands were visualized by ethidium bromide (10mg/ml) nucleic acid stain (Hi-Media, India), and the sum of each band size was calculated as the phage genome size. 1kb DNA ladder (Takara Bio, Shiga, Japan) was used as the molecular size markers (Yamaki et al., 2014).

5.4. Results

Bacteriophages have morphological characterized by using TEM for the identification and classification. According to the TEM micrograph phage TXP1 has shown icosahedral head with long tail (Fig. 3.1A) Which was non contractile, phage DXP2 has shown icosahedral head with a short tail (Fig. 3.1B) and phage MXP2 has shown icosahedral head with a long contractile tail (Fig. 3.1C).
Figure 3.1. Transmission electron micrographs of phages

Note: (A) TXP1; (B) DXP2 and (C) MXP2
Phage capsid diameter, tail length and tail width were measured and capsid diameter was recorded as 60, 55 and 42 nm, tail length was recorded as 161, 12 and 102 nm and tail width were also recorded as 6, 4 and 8nm of phages TXP1, DXP2 and MXP2 respectively (Table 3.1). Based on the phage morphological features obtained from TEM image taxonomical identification was performed by using the international committee on taxonomy of viruses (ICTV) classification system. In this taxonomical identification phages TXP1, DXP2 and MXP2 were belonging to the Caudovirales order and phage TXP1 was Myoviridae family, Bcep78 like virus which was non enveloped linear double stranded DNA as their genome. Phage DXP2 belongs to Podoviridae family T7 like virus which is enveloped virus with linear double stranded DNA. Similarly, phage MXP2 having double stranded DNA as its genome and it under comes Siphoviridae family XP10 like virus (Table 3.2).

Table 3.1. Morphological features of phages

<table>
<thead>
<tr>
<th>Phage</th>
<th>Capsid Diameter (nm)</th>
<th>Tail length (nm)</th>
<th>Tail Width (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXP1</td>
<td>60</td>
<td>161</td>
<td>6</td>
</tr>
<tr>
<td>DXP2</td>
<td>55</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>MXP2</td>
<td>42</td>
<td>102</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3.2. Taxonomical features of phages

<table>
<thead>
<tr>
<th>Phage</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Characteristics</th>
<th>Genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>TXP1</td>
<td>Caudovirales</td>
<td>Myoviridae</td>
<td>Bcep78 like virus</td>
<td>Non-enveloped, with long tail</td>
<td>Linear dsDNA</td>
</tr>
<tr>
<td>DXP2</td>
<td>Caudovirales</td>
<td>Podoviridae</td>
<td>T7 like virus</td>
<td>Non-enveloped, with short tail</td>
<td>Linear dsDNA</td>
</tr>
<tr>
<td>MXP2</td>
<td>Caudovirales</td>
<td>Siphoviridae</td>
<td>XP10 like virus</td>
<td>Non-enveloped, with long tail</td>
<td>Linear dsDNA</td>
</tr>
</tbody>
</table>

Phage genomic DNA analysis could be possibly allows the approximate determination of genomic size of the phages. All the three bacteriophages (TXP1, DXP2 and MXP2) yielded its genome at 23 kb in size (Fig. 3.2). The size
of the phage genome was determined by the comparison with two different
markers which were included \( \lambda \)DNA/Hind III and 1 kb markers. In addition,
phage structural proteins were analyzed by SDS PAGE. In this SDS PAGE
results were observed three distinct bands were in all the three phages (TXP1,
DXP2 and MXP2) at 125, 44 and 40 kDa (Fig. 3.3).

The genomic variability in these three phage genomes has been identified
by comparing their restriction pattern between the individual bacteriophage
through restriction fragment length polymorphism (RFLP) patterns with EcoRI,
BamHI, HindIII and PstI restriction enzymes. The digested genomic DNA of
phages can be resolved in 2% agarose gel electrophoresis and it reveals the
result as restriction analysis with the four restriction enzymes (EcoRI, BamHI,
HindIII and PstI) showed an identical restriction pattern of DNA extracted from
the phages TXP1, DXP2 and MXP2 which were recovered from the phage lysate.
Phages TXP1 and DXP2 were digested by the enzyme EcoRI and BamHI,
digestion pattern of these two phages were similar at 600 and 800 bp. Though,
1000bp fragment was observed in phages TXP1 and MXP2 by EcoRI digested
(Fig. 3.4A).

Similarly, phage TXP1 and DXP2 were digested by the HindIII and PstI
enzymes and enzyme digestion was observed at 1000 and
500 bp and there were none of digestion was observed in MXP2 phage
(Fig. 3.4B). The overall restriction analysis was indicates that phages TXP1 and
DXP2 shows more similar restriction pattern. But the digestion pattern of
phage TXP1 by all the enzymes are very clear, where it was very less in phage
DXP2. Though, phage MXP2 doesn’t show any restriction pattern by these four
restriction enzymes. These differences in sensitivity and difference in restriction
pattern reveals the phages TXP1, DXP2 and MXP2 isolated from the samples
belongs to different families.
**Figure 3.2. Phage genomic DNA**

Note: M1 – λDNA/Hind III Ladder; M2 – 1kb Ladder; 1- Phage TXP1; 2- Phage DXP2; 3 – Phage MXP2

**Figure 3.3. Phage structural proteins**

Note: M – Protein ladder; 1- Phage TXP1; 2- Phage DXP2; 3 – Phage MXP2
Figure 3.4. Restriction fragment length polymorphism of phages

Note: M -ladder; 1- Phage TXP1; 2- Phage DXP2 and 3- MXP2 digested with EcoRI; 4- Phage TXP1; 5- Phage DXP2 and 6- MXP2 digested with BamHI; 7- Phage TXP1; 8 - Phage DXP2 and 9- Phage MXP2 digested with HindIII; 10- Phage TXP1; 11-Phage DXP2 and 12-Phage MXP2 digested with PSt1.
5.5. Discussion

For this present study, all the three phages (TXP1, DXP2 and MXP2) were used at $10^8$ concentrations for TEM identification by negative staining with phosphotungstic acid. But, the standard virus preparation procedure still used today, known as “negative staining” was developed in the late 1950s and has a sensitivity limit of $10^6$ viruses per ml (Brenner and Horne, 1959; Beniac et al., 2014). Further, the present study results reveal that the phage TXP1, DXP2 and MXP2 were tallied and contained double stranded DNA, which is typical of phages belonging to the order Caudovirales. Phages TXP1 DXP2 and MXP2 were belong to Myoviridae, Podoviridae and Siphoviridae family respectively.

The present study phage morphological identification was carried out by the international committee for taxonomy of viruses (ICTV) classification system and all of these three phages having an icosahedral head. Liew and Alvarz (1980) have reported that the X. campestris phage HP1, HP3, HT7, HT3h, OH2 and OK2 which are having a polyhedral head with contractile tail. Phages DXP2 and MXP2 were belong to Siphoviridae and Podoviridae family. Similarly, Ahern et al. (2014) have been reported that Siphoviridae phages Sano and Salvo, Podoviridae phages Prado and Paz, which were isolated against Xylella fastidiosa with a host range that includes Xathomonas sp. Further, Ahmad et al. (2014) have observed that the Xanthomonas phages Cp1 and Cp2 were belonging to Siphoviridae family, which resembles the morphology of phage MXP2 for this present study.

For comparative genomic analysis of tailed bacteriophages along with environmental study gives an elaborate representation of their size and genetic structure (Hendrix, 2003). These comparisons of the phage genomes have brought their highly mosaic nature into sharper focus. Casjens (2005) had reported that the complete sequences of about 150 bacterial genomes have shown that the many prophage and parts thereof that reside in these bacterial genomes must comprise a significant fraction of earth’s phage gene pool.
analysis of these phages at the DNA level provides a basis for the characterization of future phage isolates.

Currently, bacteriophages are classified on the basis of their genome single stranded versus double stranded and RNA versus DNA. Their diversity is also reflected by the diversity of genome sizes, which ranges from barely 4 kb to up to 600 kb (Brussow and Hendrix, 2002). The present study reported that the genomic DNA of phages TXP1, DXP2 and MXP2 were found to be 23kb in size, which were compared by both λDNA/Hind III and 1 kb marker. The present study correlates with the results of Gasic et al. (2011) who reported that the phages KΦ1, KΦ2, KΦ3, KΦ4, KΦ5, KΦ6, KΦ7, KΦ8, KΦ9 and KΦ15 have similar genome size approximately 22kb which were isolated against Xanthomonas euvesicatoria from soil and pepper seeds.

Apparently, there were various genomic sizes of bacteriophages reported. Particularly, potato field soil sample bacteriophages Stsc1 and Stsc3 genome was estimated to be approximately 42 and 50 kb respectively. This genomic size was determined by the restriction patterns and the size of their whole genomes in 0.4% agarose gel (Goyer et al., 2005). Similarly, Schnabel and Jones (2001) reported that varied sizes of phage genome were analyzed, where the Erwinia amylovora phages φEA1, φEA7, φEA100, φEA125 and φEA116c genomes were estimated at 46, 35, 35, 35 and 75 kb respectively.

The present study identified phages TXP1, DXP2 and MXP2 has expressed its structural proteins in the range of 125 to 40 kDa. The phages structural proteins are visualized in SDS PAGE and it was found to be 40 kDa coat protein and 125 kDa minor structural protein was present in all of these three phages TXP1, DXP2 and MXP2. Apart from that, phages TXP1 and MXP2 only results 44 kDa tail fiber proteins; it was very thin in phage DXP2. Yuzenkova et al. (2003) have reported that Xanthomonas oryzae phage Xp10 has the tail fiber protein at 45 kDa and they also observed the major head and tail proteins at 150 and 25 kDa. Most relatedly, Fouts et al. (2013) reported
that the structural proteins of *Vibrio cholera* phages ϕJA1 and ϕVchO139-I have been visualized in SDS-PAGE and it indicates that most predominant bands was observed at 47 kDa, which were identified as the major capsid protein, portal proteins at 79 kDa and minor structural protein were observed at 87 codes in both phages. A similar results were observed by Ahamed *et al.* (2014) who have reported that, 45 kDa major head protein was observed in Cp1 phage which were isolated against *Xanthomonas axonopodis* pv. *citri*.

Analysis of phage genomic data is starting to give a clearer view of the great genetic diversity of this population and, on the other hand, the remarkable underlying similarities. The genetic diversity of the bacteriophages can be studied by analyzing its restriction pattern by using restriction enzymes (Brussow and Hendrix, 2002). The present study results were revealed that all bacteriophage genomes are generally sensitive to the restriction enzymes which was includes EcoRI, BamHI, HindIII and PstI exhibited different, but closely related restriction endonuclease patterns, and common bands were appearing in phages TXP1 and DXP2 for the three restriction enzymes (EcoRI, BamHI and HindIII) only. Barrangou *et al.* (2002) who reported that, all the *Myoviridae* phage DNA samples were sensitive to BamHI, EcoRI, and HindIII and exhibited different, but closely related restriction endonuclease patterns, and common bands appeared for the three phages.

Further, phage genome sizes estimated from RFLP patterns were approximately 59, 41, and 46 kb for phages ϕR03, ϕR05 and ϕR12, respectively. In this study the restriction pattern of the phages TXP1 and DXP2 by enzymes BamHI, EcoRI, HindIII and PstI were closely similar but not identical. Similarly, Beilstein and Dreiseikelmann (2006) reported that the DNA of the phages ϕO272, ϕS121 and ϕM164 were hydrolyzed by the same enzymes and the patterns of DNA restriction fragments were similar but not identical. In conclusion, we observed that the Caudovirales group bacteriophages having the most common morphometric characteristic features.