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Characterization of *Pseudomonas aeruginosa* PAO Specific Bacteriophages Isolated from Sewage Samples

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Abstract

The purpose of this study was to isolate and characterize *Pseudomonas aeruginosa* phages with regard to their morphology, growth characteristics, genetic material and structural protein composition. Five phages specific to *P. aeruginosa* PAO were isolated from sewage samples. Adsorption rates and one step growth curves of these phages were determined and on this basis, phages were found to be distinct entities. DNA of all the phages subjected to restriction enzyme digestion, confirmed the presence of double stranded (ds) DNA as well as heterogeneity among these phages. RAPD PCR analysis of the phage DNA also revealed difference among all the 5 phages as distinct banding patterns were seen in each case. Transmission electron microscopy (TEM) however, showed morphological similarity among the phages as all were assigned to family *Podoviridae*, order *Caudovirales* on the basis of their head and tail morphologies, with some size variations. Earlier reports revealed that *Pseudomonas* phages either belonged to family *Siphoviridae*, *Myoviridae or Podoviridae.* However, in the present study, we report isolation of *P. aeruginosa* phages belonging to family *Podoviridae* with icosahederal heads and short stumpy non-contractile tails first time from India.

Keywords: Bacteriophages; Sewage samples; *Pseudomonas aeruginosa;* Transmission electron microscopy; *Podoviridae; Caudovirales.*

1. Introduction

Pseudomonas aeruginosa is a gram negative free living bacterium and an opportunistic pathogen that can inhabit environments including plants, soil, water and other moist locations [1, 2]. *P. aeruginosa* nowadays is commonly isolated from cases of nosocomial infections especially from compromised hosts, such as patients suffering from respiratory diseases, cancer,

children and young adults with cystic fibrosis and burns [3-5]. According to the National Nosocomial Infections Surveillance System, *P. aeruginosa* is the third most common pathogen associated with all hospital acquired infections, accounting for 10.1% of all nosocomial infections and is associated with a high mortality rate [6]. One of the reasons for its increased virulence is its notable resistance to many currently available antibiotics [7, 8].

As a result, novel and most effective approaches for treating infections caused by multidrug-resistant bacteria are urgently required. In this context, one such approach is the possible use of bacteriophages that parasitize and kill the bacteria against which it is targeted [9-12]. The administration of phages as pharmaceutical agents was a common practice in the preantibiotic era. However, this approach was abandoned following the use of antibiotics and interest in this strategy has again resurfaced due to increased drug resistance. Moreover, the relative simplicity and economy of phage therapy makes it an affordable preposition. This has not happened earlier, probably because of a poor understanding of mechanism of bacterial pathogenesis and of the nature of phage-host interactions, absence of animal models of diseases, or due to badly designed and executed experiments and field trials which led to failure of using phage therapy [13-15]. Besides this, scientists concentrated on escalated production of newer and newer antibiotics of commercial importance. However, the increasing incidence of multidrug-resistant bacteria and a deficit in the development of new chemotherapeutics to counteract bacteria, has rekindled the interest in phage therapy [16].

With an aim to explore the possibility of using phages against burn wound infection caused by drug resistant strains of *P. aeruginosa*, we attempted to isolate phages from the environment that were active against *P. aeruginosa* PAO and characterize them before application in animal model, with respect to morphology, structural proteins, growth characteristics and genomic material.

2. Materials and Methods

Bacterial strain and growth media: Standard strain *Pseudomonas aeruginosa* PAO obtained from Dr. Barbara H. Igleski, University of Rochester, New York (U.S.A) and maintained in our laboratory was used in this study. This organism was stored in 60% glycerol at -80 ^{o}C and when necessary, maintained on nutrient agar slants at 4 ^{o}C.

Isolation of bacteriophages: The enrichment method of Cerveny *et al.* [17] was adopted for the isolation of phages specific to *P. aeruginosa* PAO from sewage samples. Samples were collected either from drainage of different localities in the town or from fresh water lakes around Chandigarh. These sites were selected to isolate phages as sewage/ water is known to harbour many different bacteria and hence the likelihood of prevalence of phages against different organisms. Samples were collected from each site and transferred to the laboratory quickly for the phage isolation. All the phages were isolated in separate experiments from separate samples.

In brief, sewage samples were collected; centrifuged (10,000 rpm, 10 min, 4^{o}C) and supernatants were filter sterilized (0.45 µm pore size Millipore filter). 50 ml filtered sewage sample and 50 ml sterile nutrient broth were mixed with 5.0 ml overnight culture of *P. aeruginosa* PAO and incubated at 37^{o}C overnight. The bacteria were removed by centrifugation; supernatant was filter sterilized and checked for the presence of phages.

Spot test and plaque assay: To detect the presence of phages in supernatant, spot test was carried out as described by Chang *et al.* [18]. The phage titer was determined by plaque assay by employing double agar overlay technique. Briefly, each of the phage suspension was serially diluted. 100 µl diluted phage and 100 µl host bacterium (10^{8} CFU/ml) were mixed with 5.0 ml molten soft agar (0.75 % agar, w/v) and poured quickly on top of the solidified nutrient agar plate [19]. The numbers of plaques were counted after incubating the plates overnight at 37^{o}C.

Phage propagation and purification: All the isolated phages were purified by successive single-plaque isolation until homogenous plaques were obtained by the standard procedure described by Sambrook *et al.* [20]. Briefly, one well

separated phage was picked with sterile pasture pipette along with the surrounding cell mass and inoculated into 5.0 ml nutrient broth, in which 1% overnight culture of host strain was added and incubated at 37^{o}C with agitation at 240 rpm. After complete lysis, the mixture was centrifuged (10,000 rpm, 10 min, 4^{o}C), filter sterilized and treated with chloroform (1% v/v) to remove any bacterial contamination. Purified phages were stored in 60 % glycerol at -80^{o}C for long term storage. Short term stock preparations were maintained at 4^{o}C.

Concentration of bacteriophages: Phages were concentrated according to the method of Yamamoto *et al.* [21] with some modifications. Briefly, *P. aeruginosa* PAO host cells were added to phage preparation at an MOI of 0.1 and vigorously shaken for 4-5 h at 37^{o}C, resulting in complete lysis of bacteria. The culture fluid was centrifuged (10,000 rpm for 10 min, 4^{o}C) and filter sterilized. NaCl and polyethyleneglycol (PEG) 8000 were added to filtered lysate to a final concentration of 1 M and 10% respectively and kept at 4^{o}C overnight. The precipitates were collected by centrifugation, resuspended in 2-3 ml PBS (Phosphate buffer saline, pH 7.2) and treated with equal volume of chloroform to remove PEG 8000 and bacterial cell debris from the bacteriophage suspension.

Phage adsorption rate and single step growth curve: Phage adsorption experiment was carried out by the method as described by Adam [19]. Briefly, to the *P. aeruginosa* PAO culture, phage suspension was added at multiplicity of infection (MOI) of 0.1 and incubated at 37^{o}C for 15 min. Aliquots (100 µl each) were taken at 1 min intervals (up to 15 min) and number of free infectious phage particles was calculated by phage titration employing double agar overlay technique.

Single step growth curve of phages was determined according to the method of Pajunen *et al.* [22]. Phages were added at an MOI of 0.1 to the cells of *P. aeruginosa* PAO and allowed to adsorb for 15 min at room temperature. The mixture was then centrifuged (10,000 rpm, 10 min, 4^{o}C) and pellet containing infected cells was suspended in 10 ml nutrient broth followed by incubation at room temperature. Samples were taken periodically in duplicate at 5 min interval for a period of 1 h, immediately diluted and titrated by the double-layer technique. First set of sample was immediately diluted before titration. Second set of sample was treated with 1% (v/v) chloroform to release intracellular phages in order to determine the eclipse period.

Bacteriophage DNA isolation and Restriction enzyme digestion: Purified phage particles (10^{10}-10^{11} PFU/ml) were treated with 1 µg of DNase I and RNase A (Bangalore Genei, Bangalore, India) at 37^{o}C for ½ h. To the mixture, Proteinase K (Bangalore Genei, Bangalore. India) and SDS were added at a final concentration of 0.05 mg/ml and 0.5% respectively and incubated at 56^{o}C. After 1 h incubation, an equal volume of phenol: chloroform was added to remove proteinaceous material. The extraction was repeated thrice with phenol- chloroform- isoamyl alcohol (25:24:1). The nucleic acid was precipitated with chilled ethanol and suspended in 20 µl of TE buffer (10 mM Tris-HCl, pH 7.0, 1.0 mM EDTA, pH 7.0) according to standard procedure [20]. Restriction enzyme digestion of isolated phage DNA was carried out following the instructions provided by suppliers. Type II restriction endonucleases, Hin*f* 1 and BsuRI (MBI Fermentas, USA) were added to purified bacteriophage DNA. The restriction digests were separated on 0.8% agarose gel in 1× TAE buffer (40 mM tris-acetate and 1 mM EDTA, pH 8.0) (Sigma) containing 0.5 µg/ml of ethidium bromide (Hi- Media) at 100 V for 2-3 h.

RAPD PCR analysis: Random Amplified Polymorphic DNA (RAPD) of bacteriophage DNA was carried out following the method of Johansson *et al.* [23]. Six 10-mer primers, Primer 1 (5' GGTGCGGGAA 3'), Primer 2 (5' GTTT CGCTCC 3'), Primer 3 (5' GTAGACCCGT 3'), Primer 4 (5' AAGAGCCCGT 3'), Primer 5 (5' AACGCGCAAC 3') and Primer 6 (5' CCCGTCA GCA 3') (Bangalore Genei, Bangalore, India) were used. PCR mixture (25 µl) consisted of 1µl of phage DNA, 2.5 µl PCR buffer, 1.0 µl dNTP's (stocks of 100 mM dATP, dCTP, dTTP and dGTP), 1 µl primer, 1.0 µl Taq DNA polymerase (3U) (Banglore Genei, Banglore, India) and 19.5 µl distilled water. Reactions were performed at 94^{o}C for 5 min followed by 45 cycles of consecutive primers annealing (26°C and 31 °C

for 2-3 min) extension (72°C for 2 min), denaturation (94°C for 1 min) and final extension (72°C for 10 min) in Mini Cycler™ (MJ Research). Amplified RAPD PCR products were electrophoresed on 2 % agarose gel (Sigma) containing 0.5 µg /ml of ethidium bromide (Hi-Media).

Electron Microscopy: To observe phage morphology, transmission electron microscopy of *Pseudomonas* phages was performed as described by Goodridge *et al.* [24] with some modification. Drops of ultracentrifuged phage samples (1, 00, 000 rpm for 2 h, 4°C; L-80, Beckman Instrument, Switzerland) were dropped on nitrocellulose coated grids (diameter, 3 mm; 300 meshes). After 5 min, the phage particles were stained with 2% (w/v) potassium phosphotungustate (pH 6.8-7.2, Hi- Media) for 10 s. The grids were allowed to dry for 20 min and examined under a transmission electron microscope (Hitachi H 7500, Tokyo, Japan) at 80 Kv.

SDS-PAGE of bacteriophages: For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) separation, ultracentrifuged *Pseudomonas* phages were mixed with equal volume of sample buffer (0.0625 M Tris-HCl; pH 6.8, 1% SDS, 15% glycerol, 1% Beta-mercaptoethanol and bromophenol blue), heated in a boiling water bath for 3-5 min [25] and electrophoresed in an SDS- polyacrylamide gel (12%) by the standard method [26]. Protein bands were visualized after staining the gel with silver stain. Medium range protein molecular weight marker (Bangalore Genei, India) was used for size determination of proteins.

3. Results

Five *P. aeruginosa* bacteriophages were isolated. In the present study, five bacteriophages specific to *P. aeruginosa* PAO were isolated from sewage samples. Once isolated, these phages were screened for their lytic activity on the basis of clear plaque formation. Therefore, five phages exhibiting potent lytic activity with clear plaques (3-4 mm in diameter) were selected for characterization and designated as Pa29, Pa30, Pa31, Pa33 and Pa34.

Table.1: Different properties of *P. aeruginosa* PAO specific bacteriophages.

Phages	Adsorption rate (min) [a]	Eclipse period (min) [a]	Latent period (min) [a]	Burst size (PFU per bacterial cell) [a]
Pa29	8.00 ± 1.00	15 ± 5.00	20 ± 8.66	130 ± 7.07
Pa30	12.00 ± 2.00	20 ± 8.66	25 ± 5.00	120 ± 4.35
Pa31	10.00 ± 3.60	15 ± 8.66	20 ± 13.22	110 ± 12.12
Pa33	5.00 ±1.73	10 ± 5.00	20 ± 5.00	100 ± 5.19
Pa34	9.00 ± 3.00	15 ± 10.00	25 ± 10.00	140 ± 6.24

[a] The values are mean ± standard deviations (n = 3).

Growth Characteristics

Adsorption rate of selected *Pseudomonas* phages (Pa29, Pa30, Pa31, Pa33 and Pa34) was determined by mixing phage with excess of *P. aeruginosa* PAO cells, and then non-adsorbed infectious phages were serially diluted and counted. Results presented in Table 1 show that adsorption of phage particles to bacterial cells, the initial step of phage infection, varied from 5-12 min. Similarly eclipse period was found to vary

from phage to phage (10-20 min) on the basis of single step growth curve experiment. The latent period, defined as the time interval between the adsorption and the beginning of the first burst was comparatively longer (20-25 min) than eclipse period in all the 5 different phages. Burst size per infected cell varied from phage to phage, found to be shortest for Pa33 and longest for Pa34. Difference in adsorption rate, eclipse period, latent period and burst size per infected bacterial cell established that these 5 phages were independent entities.

Restriction digestion of bacteriophage DNA

DNA of all the selected *Pseudomonas* phages was isolated and electrophoresed on 0.8% agarose gel along with a λ Hind /III marker (Banglore Genei, Bangalore, India). Molecular weight of all the phage DNA was found to be ranging from 23.0-24.0 kb. For molecular characterization of phages, the isolated phage DNA samples were subjected to digestion with type II restriction enzymes Hin*f* I and BsuRI. The results revealed that all the *Pseudomonas* phages were sensitive to Hin*f* I and BsuRI and exhibited different banding patterns confirming that all the *Pseudomonas* phages were genetically different and harbored ds DNA as genetic material (Fig.1). However, in lane F, two extra bands were observed when Pa30 phage DNA digested with restriction enzyme BsuRI was analysed on agarose gel.

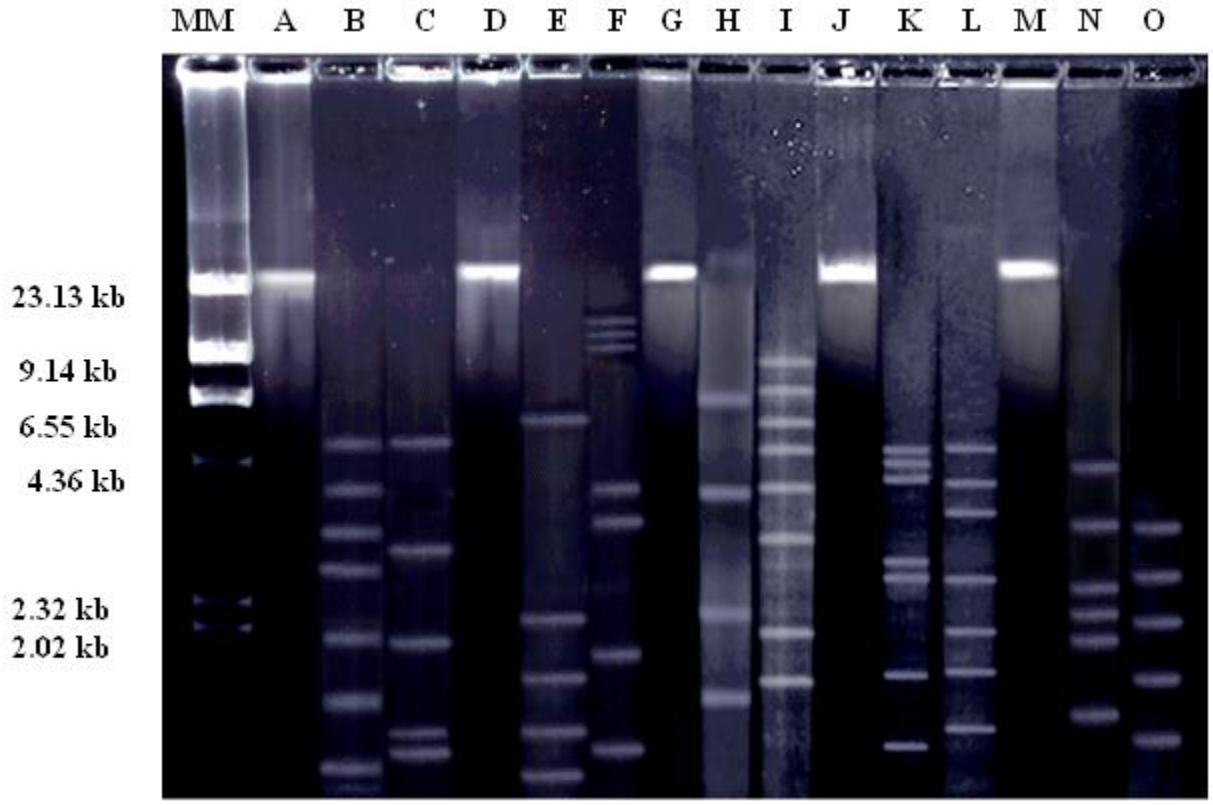

Figure.1. Restriction endonuclease digestion patterns of DNA isolated from five newly isolated *P. aeruginosa* PAO phages. Lane MM, λ phage DNA digested with Hind/ III (kilobases); Lane A, Pa29 uncut DNA; Lane B, Pa29 DNA digested with Hin*f* I; Lane C, Pa29 DNA digested with BsuRI; Lane D, Pa30 uncut DNA; Lane E, Pa30 DNA digested with Hin*f* I; Lane F, Pa30 DNA digested with BsuRI.; Lane G, Pa31 uncut DNA; Lane H, Pa31 DNA digested with Hin*f* I; Lane I, Pa31 DNA digested with BsuRI; Lane J, Pa33 uncut DNA; Lane K, Pa33 DNA digested with Hin*f* I; Lane L, Pa33 DNA digested with BsuRI; Lane M, Pa34 uncut DNA; Lane N, Pa34 DNA digested with Hin*f* I; Lane O, Pa34 DNA digested with BsuRI.

RAPD PCR amplification for finger printing of phages

To provide a fingerprint of the phages isolated from the sewage samples, RAPD PCR was performed with the DNA samples isolated from the five *Pseudomonas* phages. Six different primers were used, out of which only primer 2 (5' GTTTCGCTCC 3') provided bands with all the *Pseudomonas* phages. All the phages exhibited different patterns showing that all the phages are genetically unique and could be distinguished by RAPD PCR analysis (Fig. 2).

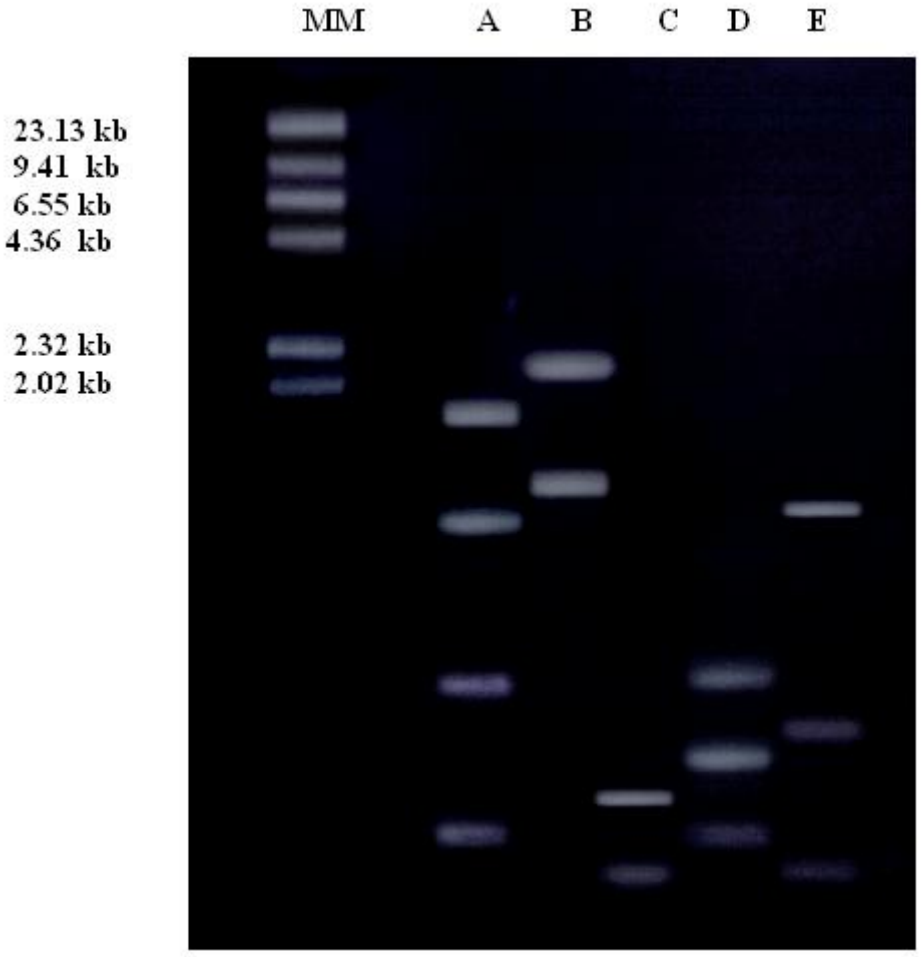

Figure. 2. Agarose gel electrophoresis showing RAPD PCR amplification of DNA isolated from five newly isolated *P. aeruginosa* PAO phages with primer 2. Lane MM, λ Phage DNA digested with Hind/ III (kilobases); Lane A, Pa29; lane B, Pa30; lane C, Pa31; lane D, Pa33; lane E, Pa34.

Phage morphology

Five phages were examined by electron microscopy of negatively stained preparations. All these phages possessed icosahederal heads and very short non contractile tails (Fig. 3). According to International Committee on Taxonomy of viruses, these phages were assigned to family *Podoviridae* order *Caudovirales* [27, 28]. These phages were further classified into subcategory morphotype C, subdivision C1 in the classification proposed by Bradley and Ackermann [29, 30]**.** Measurements taken from these electron micrographs revealed that Pa29, Pa30, Pa31, Pa33 and Pa34 had heads of sizes of approximately 42.13 ± 2.57, 44.28 ± 3.78, 46.05 ± 2.10, 73.32 ± 4.44 and 37.39 ± 2.30 nm respectively while very short stumpy non contractile tails of length 4.76 ± 0.41, 7.14 ± 1.43, 4.84 ± 1.05, 19.25 ± 1.57 and 3.47 ± 0.87 nm respectively, as summarized in Table 2.

Structural protein analysis

Five *Pseudomonas* phages were also analyzed for their structural proteins composition. All the 5 phages assigned to *Podoviridae* family showed

similar structural protein pattern exhibiting 4 major structural protein bands of approximately 20, 22, 27 and 45 kDa and 1 minor structural protein band of molecular weight approximately 67 kDa (Fig.4).

Table.2 Morphological features of different *P. aeruginosa* PAO specific bacteriophages as determined by electron microscopic examination.

Phages	Order	Family	Head Capsid (nm)[a] Diameter	Tail (nm)[a]	
				Diameter	Length
Pa29	*Caudovirales*	*Podoviridae*	42.13 ± 2.57	5.94 ± 0.40	4.76 ± 0.41
Pa30	*Caudovirales*	*Podoviridae*	44.28 ± 3.78	8.09 ± 0.82	7.140 ± 1.43
Pa31	*Caudovirales*	*Podoviridae*	46.05 ± 2.10	15.14 ± 1.05	4.84 ± 1.05
Pa33	*Caudovirales*	*Podoviridae*	73.32 ± 4.44	11.10 ± 2.22	19.25 ± 1.57
Pa34	*Caudovirales*	*Podoviridae*	37.39 ± 2.30	4.63 ± 0.50	3.47 ± 0.87

[a] The values are mean ± standard deviations (n = 3).

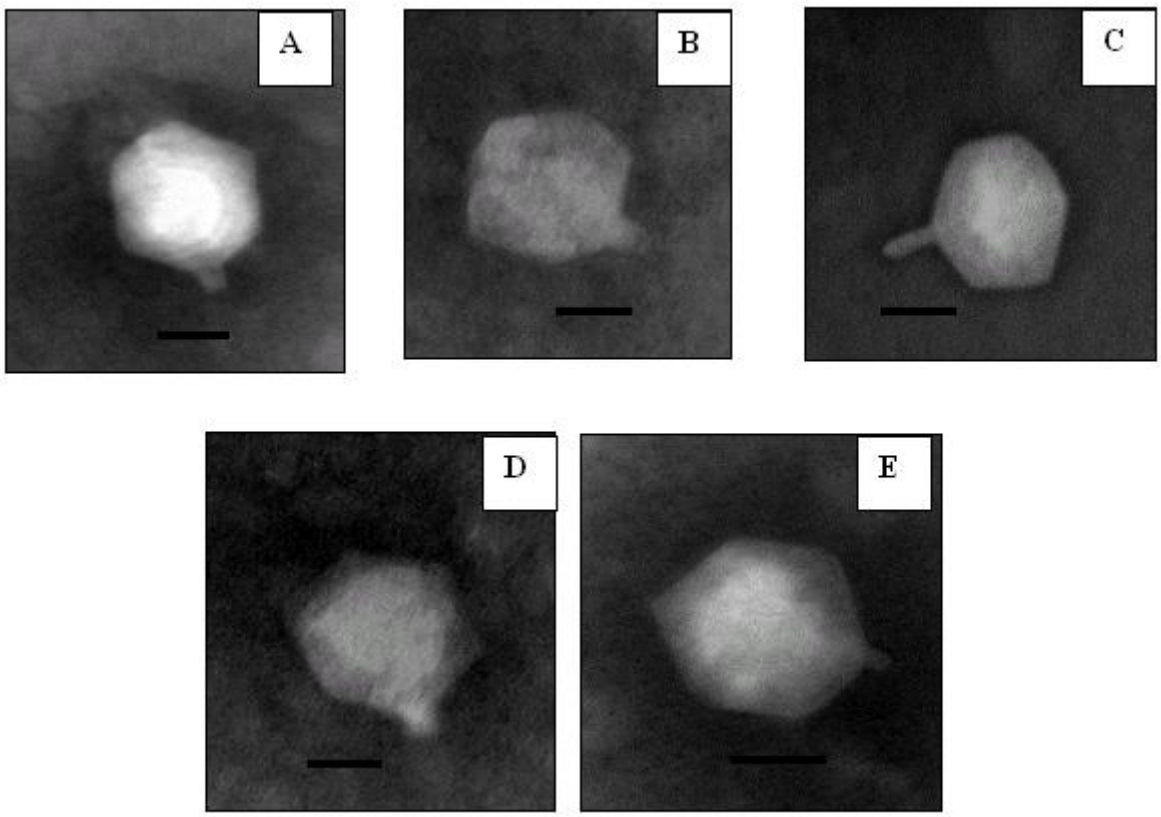

Figure.3. Electron micrograph of negatively stained *P. aeruginosa* PAO phages. (A) Pa29, (B) Pa30, (C) Pa31, (D) Pa33, and (E) Pa34. Bars 20 nm, as determined by TEM.

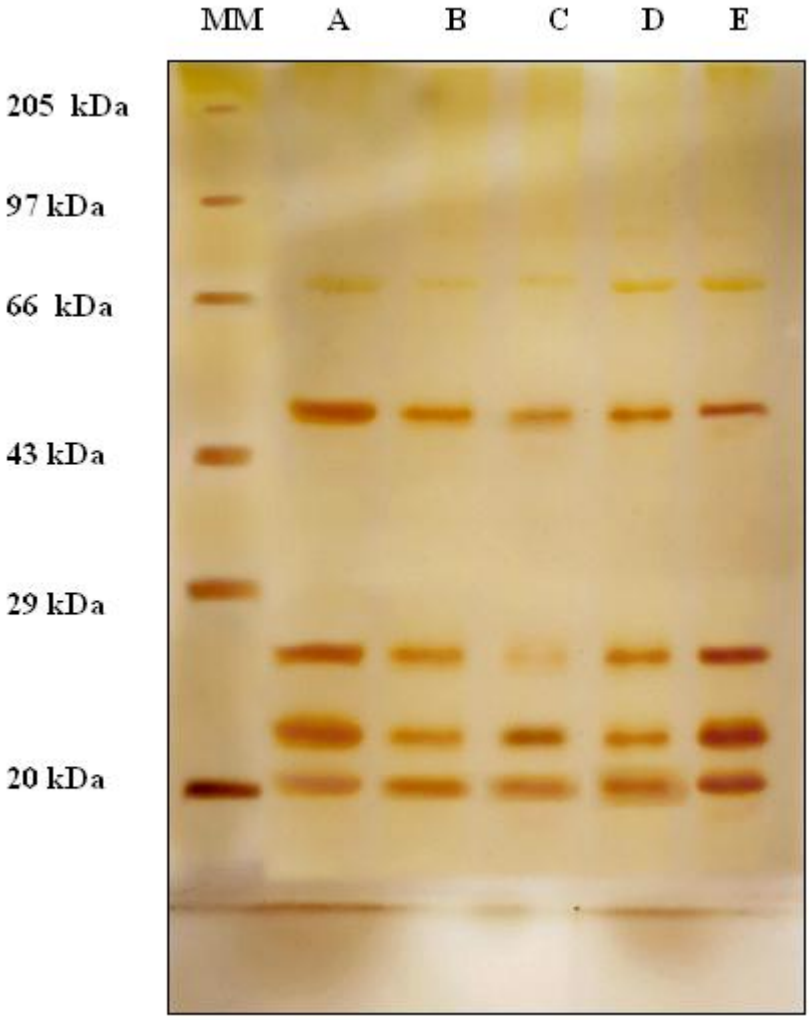

Figure. 4. SDS-PAGE patterns of structural proteins of five newly isolated *P. aeruginosa* PAO phages. Lane MM, Medium range protein molecular weight marker (kilobases); lane A, Pa29; lane B, Pa30; lane C, Pa31; lane D, Pa33; lane E, Pa34.

4.0. Discussion

Bacteriophages are ubiquitous in nature and likely to be present in environments with high densities of metabolically active bacteria [31]. Phages are generally isolated from environments that are habitats for the respective host bacteria e.g. sewage, soil, water [32]. Therefore, in the present study, five bacteriophages specific to *P. aeruginosa* PAO were isolated from sewage samples from different locations in Chandigarh, Northern India. The ability of phage to increase in number during the infectious process makes phages excellent potential diagnostic and therapeutic agents for fighting bacterial diseases [33]. However, temperate phages are of little use in phage diagnostics and therapy as these phages are inappropriate candidates since they can lead to transfer of virulence genes and those mediating resistance to antibiotics [34]. According to Adam [19], a big clear plaque is a characteristic of virulent (lytic) phages. Therefore, five isolated phages showing potent lytic activity with clear plaques were selected for further studies.

Molecular weight of isolated phage DNA was in the range of 23.0 - 24.0 kb. Our results corroborate the findings of Nordeen and co-workers [35], where phages specific to *Pseudomonas syringae* belonging to family *Podoviridae* and morphotype C, had genome size of 25 kb. *Pseudomonas* phages were found to exhibit different banding pattern with Hin*f* I and

BsuRI restriction enzymes which confirmed that all the *Pseudomonas* phages harbored ds DNA and were genetically different. Two extra bands were observed in case of phage Pa30 upon digestion with BsuRI. It is not uncommon to have difficulties in digesting DNA with restriction enzymes. Restriction enzyme BsuRI has recognition site 5'-GGCC-3'. This site sometimes may get mutated (point mutation) or methylated because this sequence is very sensitive to methylation [36-38]. It is likely that it might have happened in phage Pa30 DNA, as it gave ambigious results possibly giving normal bands as well as bands that belonged to changed viral clones.

Earlier reports indicate that all *Podoviridae* phages possessed ds DNA as genetic material [30]. RAPD PCR analysis of phage DNA provides a simple and reproducible method of differentiation of closely related phages in each family. In an earlier study, RAPD analysis has been used to differentiate between closely related six *Leuconostoc fallax* bacteriophages isolated from industrial sauerkraut fermentation [39]. In another study, Jothikumar *et al.* [40] also used RAPD to make fingerprint of 10 isolated phages against *Escherchia coli* (ETEC). In our laboratory also, we were also able to distinguish between five closely related phages of *Klebsiella pneumoniae* B5055 on the basis of this technique (manuscript communicated). Results obtained in this study confirmed these earlier observations that RAPD analysis can be used as rapid method for identification, typing and discrimination of closely related phages.

Morphological characteristics seen under an electron microscope are considered important in phage taxonomy [41] and 96% of all phages investigated in the last 45 years have turned out to be members of the *Siphoviridae*, the *Myoviridae*, or the *Podoviridae* [42]. It was, therefore, not surprising to find that the isolated phages belonged to one of these three morphological families [43]. Head and tail dimensions of these phages closely resembled with some of the earlier reported Vibriophages such as N1, N2, N3, N4, N5 and S5 belonging to *Podoviridae* family [44, 25]. The findings of this study also support the observations made by earlier workers where phages such as ΦPLS743, LK16, LKA1 and ΦKMV against *Pseudomonas aeruginosa* belonging to *Podoviridae* family were isolated [45-47]. To the best of our knowledge, this is the first report on isolation and characterization of bacteriophages specific to *Pseudomonas aeruginosa* PAO, belonging to family *Podoviridae* from India.

Once isolated phages were confirmed to be the members of same family, five *Pseudomonas* phages were also analyzed for their structural proteins composition. The protein patterns showed that the members of the same morphotype have common structural proteins and these protein patterns can be used for phage morphotype characterization and differentiation. This observation supports the findings of an earlier study where three *Leuconostoc fallax* bacteriophages, ØR03, ØR05, and ØR12 assigned to the *Myoviridae* family showed common protein patterns as well [36].

On the basis of the results of this study, we conclude that five *Pseudomonas* phages isolated from environment belonged to family *Podoviridae* order *Caudovirales* showing presence of similar proteins but differing in other characteristics. These results suggest that it is possible to place phages in their respective family morphotype on the basis of their outer membrane protein (OMP) profiles in situations where electron microscopy may not be available. However, further studies on the OMP profile of phages belonging to different families will help in proving this point.

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