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Research Note

Characterization of tailed phages against *E. coli* using DNA restriction digestion analysis

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In the context of emerging newer forms of antimicrobial resistance multidrug resistance (MDR), extensively drug resistance (XDR) and pan drug resistance (PDR) in pathogens, bacteriophages remain a promising alternative antimicrobial weapon to control it (Falagas & Karageorgopoulos, 2008, Magiorakos et al., 2012, Ghajavand et al., 2017). Bacteriophages are viruses that infect and kill bacteria and have many advantages when used alone or in combination with antibiotics *viz*., host specificity, self-limiting replications, harmlessness to the native microflora, longer period for resistance development, are significantly safer and are better tolerated without infecting mammalian cells (Principi et al., 2019). Bacteriophages can disperse a bacterial biofilm, which is very difficult to eradicate with standard antibiotic therapy (Lu & Collins, 2007). Bacteriophages enter the host bacteria through the receptors, replicate inside the host with the host cellular machinery and enter either lytic or lysogenic mode (Ofir & Sorek, 2018). Owing to these unique characteristics, bacteriophages are being freshly evaluated taxonomically (Sharma et al., 2017). Various aquatic environments such as sewage, canals, rivers, lakes, mangroves, coastal waters, aquaculture ponds, etc. remain a sink for the presence of diverse bacteriophages with variations in the host range of activities (Flu & Flu, 1947; Jin et al., 2019; Topka et al., 2019). Bacteriophages are extremely diverse which is based on different bacterial hosts they infect and it is estimated that there could be at least 10^{31} virions in the Earth's biosphere (Bergh et al., 1989; Hambly & Suttle, 2005). More than 96% of them are tailed phages which belong to the order *Caudovirales* (Ackermann, 2007). Bacteriophages in these environments mainly belong to *Caudovirales* (Tailed phages) which consists of *Myoviridae, Podoviridae* and *Siphoviridae* (Fokine & Rossmann, 2014; Iwasaki et al., 2018). The order *Caudovirales* has been expanding dramatically as newer family, genus, or species are being added every year with taxonomical variance (Krupovic et al., 2016). These viruses are isolated using different methods *viz.*, microplate-based method, bioluminescence-based method, colorimetric microtitre method, particle-based electropositive silica gel method, mixed host, drop cast method, and multiple host enrichment (McLaughlin, 2007; Knezevic & Petrovic, 2008; Kim et al., 2009; Liu et al., 2017; Chhibber et al., 2018; de Melo et al., 2019; Vaiyapuri et al., 2021).

Diversity of bacteriophages are predicted by morphometric analysis using electron microscopy, or by analysing of biological properties of the phages such as determination of host range, size, morphology of plaques, growth at different temperatures, pH, osmotic stress, survival in the presence of organic solvents and detergents or by *in silico* analyses of nucleotide sequences of phage genomes. Such analyses provide extremely interesting information about the genetic variability of phage genomes (Jurczak-Kurek et al., 2016). There are various molecular biology tools employed to study the diversity of phages *viz.* PCR identification of genes, restriction enzyme analysis, whole-genome sequence analysis, pulsed-field gel electrophoresis,

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etc. (Thiyagarajan et al., 2011; Cortes et al., 2015; Damnjanovic et al., 2020). Among these, the restriction digestion analysis of the phage genomic DNA is comparatively simple and cost-effective (Adhikary et al., 2014). However, the restriction enzyme digestion analysis has its limitation in the selection of the restriction enzyme for the analysis, level of concentration, and getting pure genomic DNA of phages. In the present study, tailed lytic phages isolated from various aquatic water samples of different geographical locations against *E. coli* host were evaluated for the suitability of restriction enzymes for the future selection of tailed phages.

Bacteriophages were isolated and identified from pooled samples (n=3) of sewage (Kadavanthra and Perumbavoor, Kerala) and water from the canal (Perumbavoor, Kerala) and Lake (Vembanad, Kerala). The isolated phages were compared and characterized by restriction enzyme digestion. About 100 mL of water samples were centrifuged at 8000 rpm at 4ºC for 20 min in order to settle all the debris in the samples. The supernatant was collected and filtered through a 0.45 µm syringe filter (Sartorius, Germany) to make them bacteria-free. Twenty-five *E. coli* strains obtained from the repository of MFB division of ICAR-CIFT isolated from retail fish markets, aquaculture farms, Vembanad Lake were used in this study. Among these, 24 strains were multidrug-resistant (MDR), and one strain NCIM 2089 was the universal *E. coli* host. The *E. coli* strains were inoculated into Tryptic Soya Broth (15 g Pancreatic digest of casein, 5 g peptic digest of soybean meal, 5 g sodium chloride in 1000 mL distilled water; pH 7.3) and the tubes were incubated at 37ºC overnight. The phages were subjected to an enrichment procedure (Van Twest & Kropinski, 2009) i.e., 10 mL of environmental samples were combined with 5 mL of host bacteria and incubated for 4-6 h at 37ºC. Then, the bacteria were removed from the mixture by centrifugation and filtration, and the filtrate was assayed for the presence of phages. One mL of the enriched sample filtrate was mixed with 2 mL culture of *E. coli* and the mixture was poured into 7 mL molten soft agar (1% Agar in Nutrient broth), mixed well and plated (Rao & Surendran, 2003). The plates were subjected to overnight incubation at 37ºC. Individual plaques were picked aseptically and inoculated into 10 mL of bacterial culture in nutrient broth, then incubated for 4 h with shaking at 37ºC. The phage suspension was then centrifuged at 8000 rpm at 4ºC for 20 min and filter sterilized through a 0.45 µm syringe filter (Sartorius, Germany). Fifty-eight phages were isolated, purified, and stored at -80ºC. Host range analysis was carried out using these multiplied individual phages.

For concentrating bacteriophages, 5 mL of respective phage samples were added to 20 mL of specific bacterial culture in nutrient broth, and incubated with shaking at 37ºC for 4 h. Then, the bacterial cells were separated by centrifugation and filtration and DNase I (25 µl-1U μl^{-1}) and RNase A (10 µl, 20 mg ml^{-1}) were added to the supernatant and incubated at 37ºC for 1 h. The phage suspension was added with 10% PEG-8000 / 1M NaCl (final concentration) followed by incubation at 4ºC overnight (Yamamoto et al., 1970; Malik et al., 1990). The pellet was precipitated by centrifugation at 10,000 x g for 30 min at 4ºC. The bacteriophage pellet obtained was resuspended in a small amount of buffer, and allowed to increase by 100-fold concentration of the original lysate. Concentrating bacteriophages prior to DNA extraction procedure yields sufficient DNA mass, volume, concentration, DNA purity, and increase the proportion of phage-derived sequences. The pellet was resuspended in 2 mL Saline-magnesium (SM) buffer (8 mM MgSO4.7H2O, 50 mM Tris-Cl, pH 7.5, 100 mM NaCl). About 500 µl of the phage sample was treated with 1µl of DNase I (1U μL^{-1}) and 0.5 µl of RNase A (20 mg ml^{-1}) and the incubation was carried out at 37ºC for 1 h, followed by treatment with 1.25 µl Proteinase K (20 mg ml^{-1}) and 25 µl of SDS, then incubated at 56ºC for 1h. Phage DNA was extracted using traditional phenol-chloroform method (Malik et al., 1990). The method is cost-efficient, good in purity, and the DNA concentration was found to be more than commercial kits. After phenol-chloroform treatment, the phage DNA was precipitated using 3M sodium acetate pH 5.4 (0.1 volume) and cold ethanol (2.5 volume), centrifuged at 10,000 rpm for 5 min followed by washing in 70% ethanol.

Comparison of restriction digestion for characterization of phages was performed with ten different restriction enzymes *viz*., *Eco*RI, *Pst*I, *Pvu*II, *Mlu*I, *Asc*I, *Xho*I, *Sma*I, *Avr*II, *Apa*I, and *Spe*I (New England Biolabs). The reaction was carried out using 1 unit of restriction enzyme, 1X NEB buffer, and 500 ng of DNA. The tubes were swirled gently and incubated at 37ºC for 1 h. For *Sma*I and *Apa*I the incubation was carried out at 25ºC for 1 h. After one hour, each reaction tube was heated at 65ºC for 5 min to prevent re-annealing of the restriction

fragments. The resulting restriction fragments were separated using agarose gel electrophoresis (1h, 90V) on 1% agarose (Sigma, USA) gel by loading entire sample.

Many phages displayed a significant under-representation of restriction sites leading to restriction site avoidance, owing to their increased probability of escape recognition. Earlier restriction maps of T5 and BF23 bacteriophages were generated using *Sal*l, *Bam*HI, *Eco*RI, *Bal*I, and *Hpa*I restriction endonucleases (Lange-Gustafson & Rhoades, 1979). *Eco*RI, *Pst*I, and *Xho*I have been used to study coliphages P2 and 186. It was found that for coliphage 186, *Eco*RI produced 3 fragments, *Pst*I produced 22 fragments, *Xho*I produced 1 fragment. While for coliphage P2, *Eco*RI produced 3 fragments, *Pst*I produced 3 fragments while *Xho*I did not yield any fragment (Saint & Egan, 1979). Studies on the restriction sites from the genomes of all completely sequenced DNA phages *viz*., T7, lambda, phi X174, G4, M13, f1, fd, and Ike showed that most phages undergo selection to eliminate recognition sites for these enzymes from their genomes (Sharp, 1986). *Pvu*II has been used in experiments to examine coliphages by enzymatic restriction (Hilbert et al., 2017, Zechner et al., 2020). PCR-based DNA fingerprinting studies carried out with coliphages isolated from sewage polluted seawater showed no *Eco*RI and *Pst* I sites (Fattouh et al., 2002). In our present study a total of 58 bacteriophage isolates that were lytic to 25 *E. coli* strains were treated with ten restriction enzymes *viz*., *Eco*RI, *Pst*I, *Pvu*II, *Mlu*I, *Asc*I, *Xho*I, *Sma*I, *Avr*II, *Apa*I, and *Spe*I (New England Biolabs). *Eco*RI cleaved the DNA of eight phages and produced 4 different patterns. *Pvu*II was able to cleave the phage DNA of 27 isolates and yielded 14 patterns. *MluI* produced cleavage in 8 isolates and formed 4 different patterns. *Sma*I and *Spe*I produced only two bands upon restriction, which could not be proved useful in differentiating strains. *Avr*II produced a restriction pattern in two isolates *viz.,* ÕEC-29 and ÕEC-34 with a single pattern (Fig.1, Table 1). It was found that *Pvu*II was able to produce restriction patterns that helped to distinguish about 47% of phages studied (Fig. 2, Table 1). The phages studied were identified as member of Kayfunavirus family based on the sequencing of specific major coat protein (MCP) genes. Thus it could be inferred that *Pvu*II works well for the members of the Kayfunavirus family belonging to the *Caudovirales* (tailed phages).

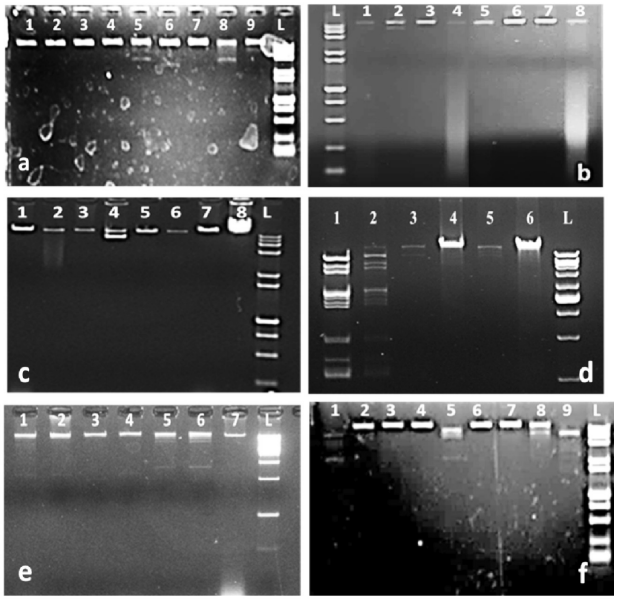

Fig. 1. Restriction pattern produced by enzymes (a) *Asc*I (5,6,8) (b) *Spe*I (1,2) (c) *Sma*I (4) (d) *MluI* (1,2)(e) *Avr*II (5,6)(f) *Eco*RI (1,5,8,9) on 1% agarose gel (L:Ladder) (The figure projects the portion of the gel harbouring phage DNA samples with restriction patterns, that are indicated in brackets)

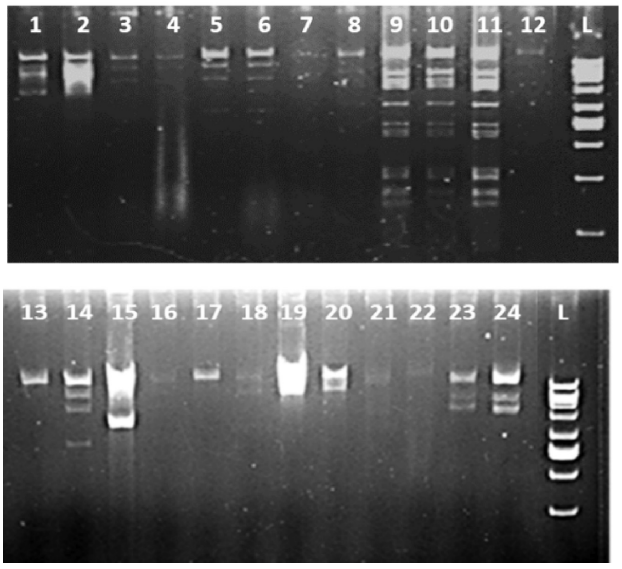

Fig. 2. Restriction pattern produced by the enzyme *Pvu*II on 1% agarose gel (L: Ladder, 1-24: Coliphage DNA samples

Despite the extensive isolation of bacteriophages, the research related to the use of restriction enzymes to characterize phages is limited. In the present work, a direct comparison of the restriction patterns of ten restriction enzymes revealed that *Pvu*II was

Table 1. Table showing the restriction enzymes used, total number of isolates of Coliphages (ϕEC: *E.coli* phage) showing restriction patterns and the total patterns produced by them.

	Isolates with restriction sites	Strain name	No of restriction patterns
*Eco*RI	8	ϕEC-33, ϕEC-35, ϕEC-23, ϕEC-24, ϕEC-44, ϕEC-45, ϕEC-49, ϕEC-50	4
*Pst*I	0	NIL	0
*Pvu*II	27	ϕEC-3, ϕEC-4, ϕEC-13, ϕEC-14, ϕEC-15, ϕEC-16, ϕEC-19, ϕEC-20, ϕEC-21, ϕEC-22, ϕEC-23, ϕEC-24, ϕEC-27, ϕEC-28, ϕEC-29, ϕEC-33, ϕEC-34, ϕEC-35, ϕEC-37, ϕEC-38, ϕEC-39, ϕEC-42, ϕEC-43, ϕEC-44, ϕEC-45, ϕEC-49, ϕEC-50	14
MluI	8	ϕEC-3, ϕEC-4, ϕEC-15, ϕEC-16, ϕEC-23, ϕEC-24, ϕEC-55, ϕEC-56	4
*Asc*I	4	ϕEC-11, ϕEC-12, ϕEC-17, ϕEC-18	2
*Xho*I	0	NIL	0
*Sma*I	2	ϕEC-7, ϕEC-8	1
*Avr*II	2	ϕEC-29, ϕEC-34	1
*Apa*I	0	NIL	0
*Spe*I	2	ϕEC-17, ϕEC-18	1

comparatively better than others. About 14 distinct patterns were discriminated by *Pvu*II alone, followed by *Eco*RI (4 patterns) and *MluI* (4 patterns), *Asc*I (2 patterns), *Sma*I (1 pattern), *Avr*II (1 pattern), and *Spe*I (1 pattern). *Apa*I, *Pst*I, and *Xho*I did not produce any restriction patterns in any of the samples.

The results indicate that restriction analysis can be used as the initial test to discriminate the bacteriophages into different categories and can be adopted along with host range determination studies for further categorizing the phages. The study also confirms the use of *Pvu*II as the potential restriction enzyme that can categorize the tailed phages compared to the other enzymes. Since, the procedure is faster and cost-effective with high degree of reproducibility, it improves the detection of variability among phages.

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