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Note

Isolation and characterisation of *Vibrio alginolyticus* lytic bacteriophage φ*Va*-1 from brackishwater clam *Meretrix meretrix* in India

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ABSTRACT

The Gram negative bacterium *Vibrio alginolyticus* is generally found in marine and brackishwater systems. A lytic bacteriophage capable of specifically infecting *V. alginolyticus* was isolated from the brackishwater clam (*Meretrix meretrix*) using agar overlay technique. The phage produced plaques 3 mm in dia, which increased to 5 mm overnight on tryptone soya agar (TSA) plates and the optimum temperature and pH was found to be 32°C and 7.5 respectively. The phage was designated as φ*Va*-1 and nucleic acid characterisation confirmed that the phage has double stranded DNA. Transmission electron microscopic observations revealed that the bacteriophage had hexagonal structure with a long contractile tail and the phage was found to belong to the family *Myoviridae.*

Keywords: Bacteriophage, *Meretrix meretrix, Myoviridae, Vibrio alginolyticus*

The genus *Vibrio* consists of more than 100 species grouped under 14 clades that are widely distributed in estuarine and coastal waters as well as sediments (Alsina and Blanch 1994; Reen *et al*., 2006). A large number of species of this genus are associated with marine organisms like fish, molluscs and crustaceans, in commensal or pathogenic relation (Thompson *et al*., 2004; 2006).

Vibrio alginolyticus is reported as an etiological agent of gastroenteritis worldwide and is isolated from seafood, as well as estuarine, neritic and brackishwaters (Thompson *et al*., 2006). This species is identified as pathogenic to marine animals as well as humans (Blake *et al.*, 1980; Lee*,* 1995; Rikelme *et al.*, 1996; Sudeehsh *et al.*, 2002).

The yellow clam *Meretrix meretrix* is found in marine and brackishwater environments and the meat is widely used for human consumption (Narasimham, 1991). Vibrios associated with infection in bivalve molluscs belong to *V. tapetis, V. atlanticus*, *V. celticus*, *V. artabrorum*, *V. crassostreae, V. cortegadensis, V. alginolyticus* and *V. pectenicida* (Gomez-Leon *et al*., 2005; Beaz-hidalgo *et al.*, 2010; Dieguez *et al.*, 2011; Lasa *et al.*, 2014). Bacteriophages affecting *Vibrio* spp. such as *V. paraheamolyticus, V. cholerae, V. fluvials*, *V. vulnificus* and *V. harveyi* have been isolated from seafoods. However, published reports on bacteriophages from *V. alginolyticus* is rare (Spencer 1960; Kellogg *et al*., 1995; De Paola *et al*., 1998; Pasharawipas *et al*., 2005; Vinod *et al*., 2006; Chrisolite *et al*., 2008; Busico-Salcedo and Owens, 2013). The present study reports isolation and characterisation of a *V. alginolyticus* lytic bacteriophage from the brackishwater clam *M. meretrix*.

About 25-30 nos. of brackishwater yellow clam, *M. meretrix* were collected from Muttukadu Estuary located 35 km south of Chennai in Tamil Nadu, India. The clams were packed in sterile plastic bags and transported to the laboratory in icebox. Outer shells of the clams were removed using sterile forceps and 30 g of tissue was collected under aseptic conditions and homogenised. Isolation and identification of *V. alginolyticus* was carried according to the Bacteriological Analytical Manual (BAM, USFDA, 2004). Twenty five gram of homogenised clam tissue was suspended in 225 ml of sterile alkaline peptone water (APW) (10 g peptone, 10 g NaCl in 1l distilled water (pH 7.5) and incubated at 30°C for 24 h. After incubation, one loopfull of enriched culture was streaked onto the Thiosulpite Citrate Bile Sucrose agar (TCBS) plates (HiMedia, Mumbai) and incubated at 35°C. Presumptive colonies of *V. alginolyticus* were purified on Nutrient agar (NA) plates supplemented with 3% NaCl. All isolates were subjected to a series of phenotypic and biochemical tests such as Gram staining, motility, salt tolerance (0, 3, 6, 8 and 10%), amino acid decarboxylation (arginine, lysine and ornithine), production of indole, acid and acetyle methyl carbinol and sugar fermentation (glucose, sucrose, arabinose, mannitol and cellobiose) (Smibert and Krieg, 1991). The isolates were identified using the keys

described by Alsina and Blanch (1994) and Baumann and Schubert (1984). Purified isolates were stored in tryptone soya agar (TSA) soft (Himedia, Mumbai, India) with 3% glycerin for further use.

Isolation and purification of bacteriophage was carried out using the agar overlay technique described by Adams (1959) and Carlson (2005). Five gram of tissue was collected from 7-10 nos. of clams (*M. meritrix*) and homogenised further in 45 ml phage buffer (9.5 g. Na_2HP0_4, 3 g KH_2P0_4, 2.5 g NaCl, 10 ml. of 0.1 M $MgS0_4$ and 10 ml of 0.1 M $CaC1_2$ pH 7.5). The mixture was incubated on a shaker incubator for 2 h at 32°C and 120 rpm. The mixture was allowed to settle down and tissue particles were removed by centrifugation at 12,500 g for 15 min at 4°C in a refrigerated centrifuge (Sorvall RC 5B). Supernatant was filtered through sterile 0.45 µm syringe filter (Millipore, USA) and 1 ml of the filtrate was used as phage inoculum. Phage inoculam (1 ml) was added to broth culture (0.1 ml) (OD_{600} = 0.3) of *V. alginolyticus* host bacteria grown on tryptone soya broth (TSB, Himedia, India) and incubated at 30°C for 30 min. This was then mixed with 5 ml of molten soft agar (TSB with 1.5% NaCl and 0.7% agar) held at 46°C in a water bath and overlaid on TSA agar plate supplemented with 1.5% NaCl. The plates were incubated at 30°C for 18-24 h and observed for formation of plaques. After incubation, plaques were counted and expressed as plaque formation units (*pfu* ml^{-1}). In order to purify the phages, a single plaque was picked with a sterile scalpel and suspended in 20 ml of log phase culture of *V. alginolyticus* strain and incubated at 120 rpm in an incubator shaker for 18 h at 30°C. After incubation, the culture broth was centrifuged at 15,000 rpm for 15 min. The supernatant was collected and filtered through 0.45 µm filter and aliquots were preserved with 50% glycerol at -70°C for further studies.

Agar overlay technique was performed to detect the optimal conditions for the growth of bacteriophage (Hazem *et al.,* 2006). Optimum temperature for the growth of bacteriophage was determined by incubation of cultures on TSA plates at various temperatures *viz*., 20, 25, 30, 35, 40 and 42°C. Plates were observed for *pfu* at various temperatures. Similarly agar overlay technique was performed to detect the optimum pH and salinity of the bacteriophage. Soft agar and TSA plates were prepared with various pH values of 3.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 and examined for *pfu*.

In order to detect the specific host susceptibility, phage lysate with 10^8 *pfu* ml^{-1} was used for spot assay (Carlson, 2005) on *Vibrio* species such as *V. logei* (2), *V. fischeri* (1), *V. splendidus* (1), *V. paraheamolyticus* (24), *V. anguillarum* (1), *V. cholerae* (Non-O1) (22), *V. fluvialis* (1), *V. mimicus* (14), *V. ordalii* (3), *V. vulnificus* (20), *V. metschnikovii* (1) and *V. alginolyticus* (74).

A 10 µl suspension of purified phage with a titer value of 10^8 pfu ml^{-1} was placed on 200 mesh carbon coated copper grids and stained with potassium phosphotungstate (pH7.2) for 20 seconds. Excess stain was removed by placing the grids on blotting paper. Bacteriophage morphology was examined by transmission electron microscopy (TEM, Philips, CM12 STEM, Netherlands).

Nucleic acid of the bacteriophages was extracted as described by Santos (1991). The phage nucleic acid was treated with DNase I, RNase A (Genei, Bangalore, India) and S1 nuclease (New England Biolabs, MA) according to the manufacturer's instructions to confirm the nature of the nucleic acid of the bacteriophage (Sambrook and Russel, 2001). Nucleic acid was examined by gel electrophoreses using 1% agarose gel containing ethidium bromide (0.5 µg ml^{-1}) and the gel was visualised using UV transilluminator (Biorad, USA).

A total of 74 *V. alginolyticus* isolates were characterised and used for the study. *Vibrio alginolyticus* colonies appeared yellow mucoid, circular colonies, 2 to 3 mm dia in size on TCBS agar plates. They formed swarming colonies on TSA and Zobell marine agar (ZMA), and were found to be short, Gram negative rods and were positive for Voges-Proskauer, oxidase, decarboxylase, catalase and motility tests. They fermented glucose, mannitol, arabinose but none of them fermented salicin, lactose and inositol. All the isolates were highly tolerant to 3-6% NaCl but none of them grew in 0, 8 and 10% NaCl.

Plaques observed on TSA plates after 24 h of incubation were about 1-2 mm dia, with transparent center and well defined edges (Fig.1). The phage was designated as φ*Va*-1.

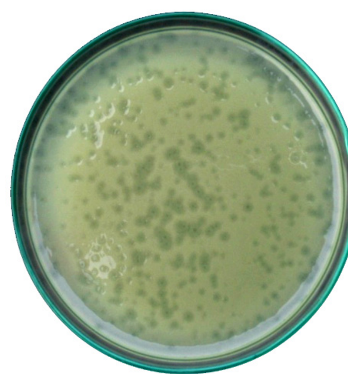

Fig. 1. *V. alginolyticus* bacteriophage plaques on TSA agar plates obtained by agar overlay techniques

Formation of maximum numbers of plaques on TSA plates was considered as the optimum condition of growth of bacteriophage in terms of temperature and pH. Bacteriophage generated maximum *pfu* in the temperature range of 30 to 35°C with a titer of 7.3 × 10^2 *pfu* 100 µl^{-1} at 35°C. Plaque formation was comparatively low at 25 and 40°C while no plaques were formed at 15, 20 and 42°C (Fig. 2).

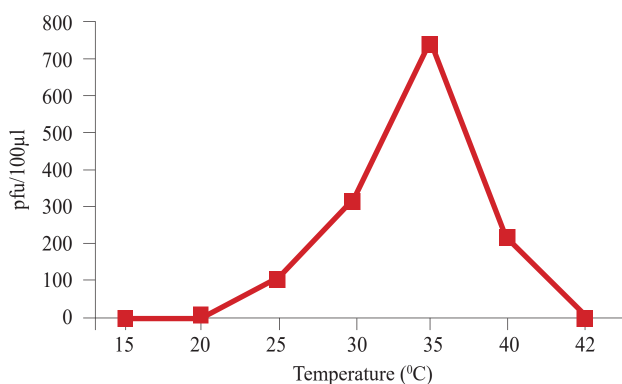

Fig. 2. Growth of *V. alginolyticus* bacteriophage (φ*Va*-1) at different temperatures. *pfu* = plaque forming unit

The optimum pH for the growth of φ*Va*-1 was found to be at 7.5 where the maximum numbers of plaques were observed. No plaques formed at pH 3.0, 4.0 and 9.0. Maximum number of plaques (6.3 ×10^2 *pfu* 100µl^{-1}) was recorded at pH 7.5 (Fig. 3).

Sixty three of the 74 *V. alginolyticus* isolates were found to be susceptible to the phage φ*Va*-1 (84%) in the spot assay. However, this particular bacteriophage was found to be non-infective to other *Vibrio* species such as *V. logei, V. fischeri, V. splendidus, V. paraheamolyticus, V. anguillarum, V. cholerae* (Non-O1), *V. fluvialis, V. mimicus, V. ordalii, V. vulnificus* and *V. metschnikovii*, indicating that the φ*Va*-1 phage is specific to *V. alginolyticus*.

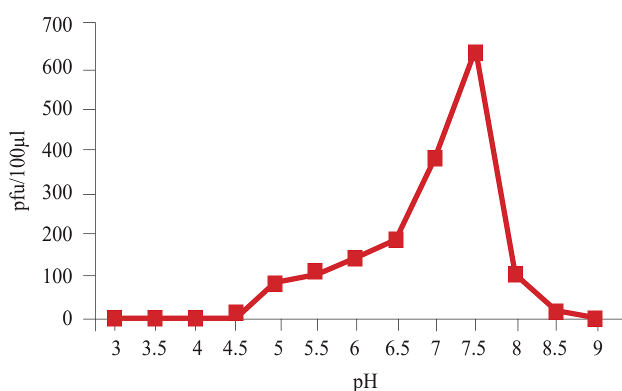

Fig. 3. Growth of *V. alginolyticus* bacteriophage (φ*Va*-1) at different pH. *pfu* = plaque forming unit

Transmission electron microscopy revealed that *V. alginolyticus* bacteriophage has hexagonal shape with distinct tail and thus belonged to the order Caudovirales (Ackermann, 1984). The phage has an icosahedral head with an approximate diameter of 100-120 nm. A tubular tail of approximately 20-30 nm length and having a contractile sheath with a collar was also observed. Based on their morphology especially the presence of sheath with a collar signified that φ*Va*-1 belonged to the family *Myoviridae* (Ackermann, 2007) (Fig. 4).

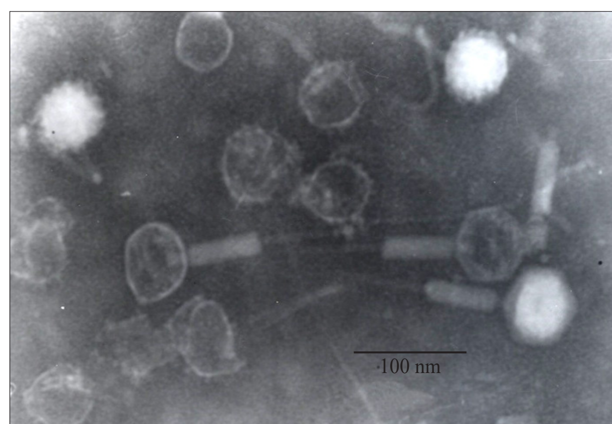

Fig. 4. TEM analysis of φ*Va*-1 shows (x1,000000) a short neck separating the head from the tail

It was observed that the nucleic acid of phage could be completely digested on treatment with DNase I but not with RNase A and S1 nuclease, confirming that the genetic material of the bacteriophage was double-stranded DNA.

Bacteriophages are common in water and a number of reports exists on isolation, characterisation and bacteriophage-host relationships (Kellogg *et al*., 1995; Oakey and Owens., 2000; Payne *et al*., 2004). Marine bacteriophages infecting *Vibrio* spp. are reported from water, sediments and marine animals (De Paola *et al*., 1998; Ping *et al*., 2013). However, bacteriophages particularly infecting to *V. alginolyticus* are rarely reported. The first isolation of bacteriophage of *V. alginolyticus* was reported by Nakamura (1978) and Lin (1993). However those reports did not describe more regarding the characterisation of bacteriophage. Hence, the present study assumes significance by extending the information available on bacteriophages of *V. alginolyticus*.

Assessing growth conditions are very critical in the characterisation of bacteriophages. Temperature is an important factor for bacteriophage survival. It plays a fundamental role in attachment, penetration and multiplication of the bacteriophage in host (Nasser and Oman, 1999; Olson *et al*., 2004). We have investigated

the effects of temperature and pH on the multiplication of this phage. Bacteriophage φ*Va*-1 was tested with various temperature conditions and found that phage grew better at 35°C with a plaque formation of 7.3 $\times 10^2$ *pfu* 100 μl^{-1}. It was also observed that φ*Va*-1 was able to grow better in a temperature range of 35-37°C. It has been reported by Jonczyk *et al.* (2011) that there was 15 and 72% decrease in plaque numbers after maintaining the phages in soft agar at 45°C for 2 and 10 min, respectively. At lower than optimal temperatures, fewer phage genetic materials can penetrate into bacterial host cells. In another report, Tey *et al*. (2009) described that higher temperatures can prolong the length of the latent stage of bacteriophages. Likewise, Lee *et al*. (2014) reported that a phage of *V. vulnificus* has good stability in the broad temperature range of 20-60°C. It is evident that the temperature ranging from 30-37°C is a favourable condition for φ*Va*-1 and 35°C will possibly be the optimum temperature at optimum pH of 7.5. Other studies regarding *Vibrio* phages also indicated that generally, the phages were stable in a broad pH range of 5-9, with optimum range being 5-6 (De Paola *et al*., 1998).

Host susceptibility of lytic *Vibrio* phages was well described earlier (Koga *et al*., 1982; De Paola, 1998). In this study, φ*Va*-1 was lytic to 63 isolates of *V. alginolyticus* with a lysis rate of 84%. Muramatsu and Matsumoto (1991) reported a transduction phage common to *V. parahaemolyticus* and *V. alginolyticus*. Lin *et al.* (1993) reported that, their *V. alginolyticus* phage had 72.22% lytic property to *V. alginolyticus* and 39% cross-lysis rate to *V. parahaemolyticus*. But surprisingly φ*Va*-1 was found to be non-lytic to any of the *V. parahaemolyticus* and *V. anguillarum* isolates*.* Based on their morphological features revealed by TEM analysis, it was confirmed that φ*Va*-1 belongs to the family *Myoviridae*. The family *Myoviridae* are characterised by those having icosahedral or elongated heads and contractile tails that are more or less rigid, long and relatively thick (ICTV, 2012). Our finding is similar to other reports of tailed vibriophages reported by Kellogg *et al.* (1995) who stated that, Myovirus vibriophages are found with head diameters up to 65 nm and tails up to 100 nm. De Paola *et al.* (1998) demonstrated the presence of *Podoviridae* and *Myoviridae* from *V. vulnificus* in oysters. *V. harveyi* bacteriophages isolated from coastal aquaculture systems like shrimp farms, hatcheries and tidal creeks along the east and west coast of India were found to have the typical head and tail morphology and are grouped under Siphoviridae and one under Myoviridae (Shivu *et al.,* 2007).

To our knowledge, φ*Va*-1 is the first report of *V. alginolyticus* phage isolated from a bivalve from India. The finding of this phage provides valuable information that must be considered during the isolation of bacteriophages from other *Vibrio* spp. However, further research is needed to assess more regarding the genomic characteristics of this phage. In terms of the importance of pathogenicity of *V. alginolyticus*, this study has a significant role in phage therapy.

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