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Editor's Choice

ORIGINAL ARTICLE

Isolation and characterization of broad spectrum bacteriophages lytic to *Vibrio harveyi* from shrimp farms of Kerala, India

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Significance and Impact of the Study: In sustainable aquaculture, application of antibiotics is prohibited to manage vibriosis, including the one caused by *Vibrio harveyi*. In lieu of antibiotics, an eco-friendly alternative method, phage therapy, is recommended here. To facilitate the same, a set of six broad spectrum *V. harveyi* phages, as cocktail, has been constituted and characterized based on morphological traits and by employing molecular tools. These phages were also found to infect other aquaculture pathogens belonging to *Vibrio* and *Aeromonas*. Subsequent to *in vivo* trials, they can find application in shrimp hatcheries as prophylactics and therapeutics.

Keywords
broad spectrum phages, *Caudovirales*, lytic phages, phage therapy, soft agar overlay, *V. harveyi*, *V. harveyi* phage, vibriophage.

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Abstract

Of 33 phages isolated from various shrimp farms in Kerala, India, six were segregated to have broad spectrum lytic efficiency towards 87 isolates of *Vibrio harveyi* with cross-infecting potential to a few other important aquaculture pathogens. They were further tested on beneficial aquaculture micro-organisms such as probiotics and nitrifying bacterial consortia and proved to be noninfective. Morphological characterization by transmission electron microscopy (TEM) and molecular characterization by RAPD and SDS-PAGE proved them distinct and positioned under *Caudovirales* belonging to Myoviridae and Siphoviridae.

Introduction

Luminous *V. harveyi*, the major pathogenic bacterium involved in the shrimp disease 'vibriosis', has been reported worldwide from aquaculture systems, mainly from shrimp farming areas where shrimp culture is a major industry (Harris and Owens 1999; Ruangpan *et al.* 1999; Oakey and Owens 2000). As the infected larvae of shrimp emit blue-green luminescence, this disease is otherwise known as bacterial luminous disease or luminous bacteriosis (Karunasagar *et al.* 2007). Even though *V. harveyi* is a common aquaculture pathogen which causes diseases in a variety of aquatic animals, vibriosis in *Penaeus monodon*, the giant tiger shrimp, is of great concern because of the animals' high economic significance. Extensive use of antibiotics for the management of *V. harveyi* has led to ban of import of shrimp in many countries due to the presence of antibiotic residues and the concern of emergence of antibiotic-resistant bacteria. This necessitated great deal of investigations worldwide for finding sustainable alternatives to antibiotics. At the very beginning of 20th century, phages were proven to have excellent antibacterial properties by Twort (1915) and d'Herelle (1917). Later, phages lost their importance

as therapeutics when chemical biocontrol agents came to the fore. Recently, phage therapy has re-emerged as an efficient and successful treatment option for those bacterial diseases associated with aetiological agents having antibiotic resistance (Biswas *et al.* 2002; O'Flaherty *et al.* 2005). The host-specificity and self-replicating nature are the major advantages of phages over other alternatives (Smith and Huggins 1982).

Certain *V. harveyi* phages, both lytic and temperate, have been isolated from Australia, Thailand and India (Ruangpan *et al.* 1999; Oakey and Owens 2000; Pasharawipas *et al.* 2005; Shivu *et al.* 2007). For therapeutic use, the phages selected should be lytic and should not revert to lysogeny as in the case of the phages isolated by Oakey and Owens (2000) which was later found to increase virulence in avirulent strains of *V. harveyi* (Austin *et al.* 2003; Munro *et al.* 2003). Vinod *et al.* (2006) isolated a lytic bacteriophage which had broad spectrum biocontrol ability to *V. harveyi*. The phages reported by Shivu *et al.* (2007) were promising therapeutic agents for the control of *V. harveyi*. Karunasagar *et al.* (2007) demonstrated the efficiency of *V. harveyi* phages against biofilm formation in hatchery tanks.

The study reported here was focused on *V. harveyi* phages which infected diverse isolates of *V. harveyi* and other aquaculture pathogens in hatchery environment. As these phages were envisaged to be used in recirculating aquaculture system (RAS), their infectivity towards probiotics and nitrifying bacterial consortia were also investigated. Phages were characterized using transmission electron microscopy (TEM), by way of RAPD profiling and SDS-PAGE.

Results and discussion

Altogether 33 *V. harveyi* phages were isolated by enrichment from six water samples and two sediment samples collected from major shrimp farming regions of Kerala, India (Table S1). The purified lysates were subjected for screening based on host range analyses. Plaques developed by phages on respective host bacteria were categorized as clear, moderately clear and turbid (data not shown). Based on the spectrum of activity (Table S2) and quality of plaques, six phages were segregated as 'broad spectrum phages'. Of them, *Vi ha* 32, *Vi ha* 21, *Vi ha* 15, *Vi ha* 68 and *Vi ha* 6 could lyse more than 50% of *V. harveyi* isolates tested, and *Vi ha* 19 showed more than 70% lytic efficiency (Table 1). The susceptibility of bacterial strains to phage lysis varied and this might be due to variation of receptor molecules, restriction modification system in the host or superinfection exclusion (Duckworth *et al.* 1981). It is evident from the present study that phages which form large and clear plaques have broad spectrum host range as reported earlier by Shivu *et al.* (2007). The wide spectrum activity of these phages suggests that they have potential for application as agents for biocontrol of luminous *V. harveyi* in aquaculture environments.

Table 1 The segregated broad spectrum *Vibrio harveyi* phages based on host range and their percentage of lysis (Percentage is calculated from infection with 87 *V. harveyi* strains)

Phage isolates	Percentage lysis
Vi ha 32	43·7
Vi ha 21	51·7
Vi ha 15	54·0
Vi ha 68	67·8
Vi ha 6	67·8
Vi ha 19	70·1

For successful application of therapeutic phages in aquaculture systems, it is necessary to assess their potential to attack beneficial microbiota in the system. As per cross-infectivity studies, nitrifying bacterial consortia used in recirculating aquaculture systems (RAS) were found not susceptible to the cocktail phages (Fig. S1) as the addition of phage cocktail did not inhibit nitrification. Likewise, the probiotic bacteria viz. *Bacillus cereus* sensu lato (MCCB 101) and *Arthrobacter* sp. (MCCB 104) were also not susceptible to the phages. Meanwhile, they showed lytic potential to other vibrios and aeromonads (Table S3) isolated from aquaculture systems as well. This observation is novel in the sense that the phages segregated are appropriate for recirculating aquaculture systems where they infect *Vibrio* and *Aeromonas* and spare nitrifiers and probiotic *Bacillus* and *Arthrobacter*. Meanwhile, *V. harveyi* phages of Vinod *et al.* (2006) and Shivu *et al.* (2007) were lytic only to *V. harveyi* and not to other species of *Vibrio*.

When the phages are introduced to aquaculture environment, it is mandatory to ascertain that the target bacterial species do not harbour lysogenic phages, as such strains would not be susceptible to the phages. However, such studies have not been conducted with the host strains of *V. harveyi*, and therefore, prior to field application, existence of pathogenic *Vibrio* and *Aeromonas* harbouring lysogenic phages has to be investigated, and in this context, the study conducted here can be considered as precursor experiment for field trials.

Morphology of the phages was revealed by transmission electron microscopy and found to have different structural features and dimensions (Fig. 1a–f). All of them were tailed and grouped under the order *Caudovirales*. Among them, five (*Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21 and *Vi ha* 32) belonged to family Myoviridae (icosahedral head and contractile tail with defined base plates) and one (*Vi ha* 68) to Siphoviridae (icosahedral head with long, flexible,

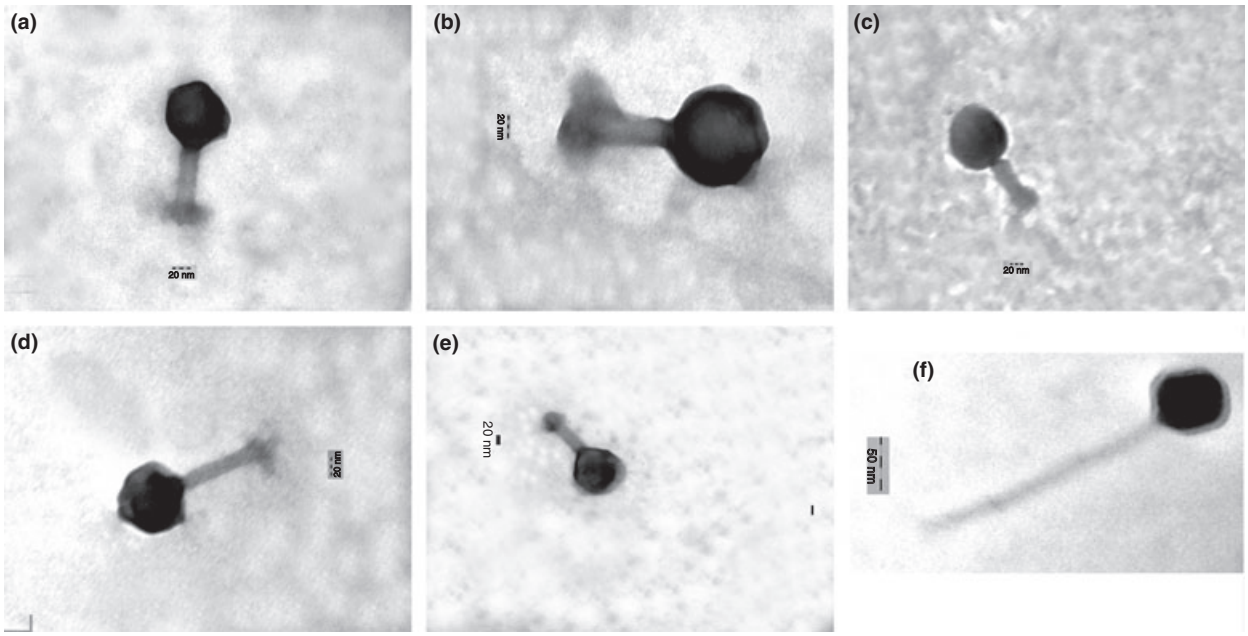

Figure 1 Transmission electron micrographs of *Vibrio harveyi* phages. Panels a to f: phages *Vi ha* 6, *Vi ha* 15, *Vi ha* 19, *Vi ha* 21, *Vi ha* 32 and *Vi ha* 68, respectively.

non-contractile tail). Shivu *et al.* (2007) obtained a majority of *V. harveyi* phages under Siphoviridae in their collection and speculated that Siphoviridae phages might be prevalent in aquaculture environment compared with Myoviridae. Contrary to that, the majority of phages in this study belonged to Myoviridae. All phages under Myoviridae and the single phage under Siphoviridae were with isometric heads and hence brought under A1 and B1 morphotypes, respectively, as per Ackermann (2001). The dimensions and features of the phages are given in the Table 2.

Digestion with RNase and moong bean nuclease did not degrade the phage nucleic acid confirming that all the six phages were having double-stranded DNA and hence confirmed to be under *Caudovirales*. RAPD of the *V. harveyi* phages resulted in differential and reproducible fingerprints. Upon visualizing the dendrogram (Fig. 2), the clusters could be distinguished at ≥0·36r. Cluster 1 consisted of all members of Myoviridae *viz. Vi ha* 6, 15, 19, 21 and 32, and cluster 2 the lone single member of Siphoviridae, *Vi ha* 68, and yielded distinct pattern by both the primers. In cluster 1, five phages showed varying percentages of similarity among themselves, and the maximum similarity (94%) was shown between *Vi ha* 21 and 32. Precisely, RAPD fingerprinting suggested that the phages were distinct from each other, as 100% similarity could not be observed between any. Barrangou *et al.* (2002) and Shivu *et al.* (2007) also used RAPD for molecular differentiation in phages of *Leuconostoc fallax* and *V. harveyi*, respectively.

Protein profile of the six *V. harveyi* phages (Fig. 3) exhibited variations despite the fact that there were two major bands (166 and 78 kDa) common to all the members of Myoviridae, with variations in the minor bands. The minor bands might be responsible for host-specificity or the characteristics specific to a particular phage (Hantke 1978). The Siphovirus *Vi ha* 68 also had two major bands and a number of minor bands, but quite

Table 2 Morphological features of *Vibrio harveyi* phages revealed by transmission electron microscopy

Phages	Characteristics	Morphotype	Total Length (nm)	Head Diameter (nm)	Family
Vi ha 6	Isometric head, contractile tail	A1	152·0	70·8	Myoviridae
Vi ha 15	Isometric head, contractile tail	A1	180·6	84·0	Myoviridae
Vi ha 19	Isometric head, contractile tail	A1	186·1	94·4	Myoviridae
Vi ha 21	Hexagonal head, contractile tail	A1	197·3	78·9	Myoviridae
Vi ha 32	Hexagonal head, contractile tail	A1	221·4	107·1	Myoviridae
Vi ha 68	Isometric head, noncontractile tail	B1	277·1	62·8	Siphoviridae

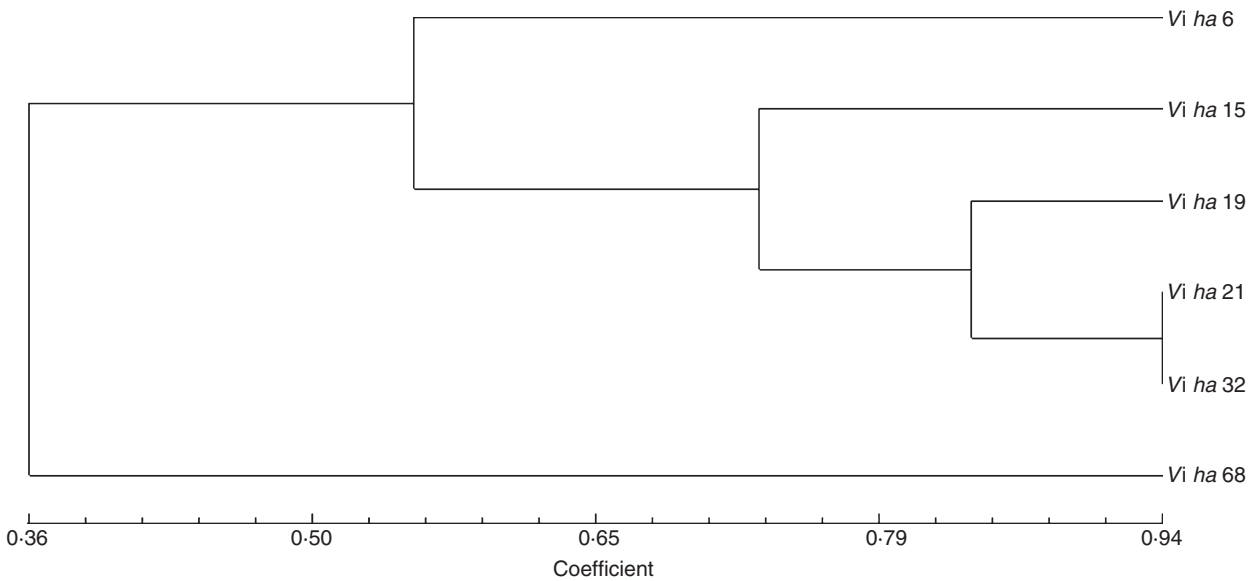

Figure 2 Dendrogram based on RAPD profile.

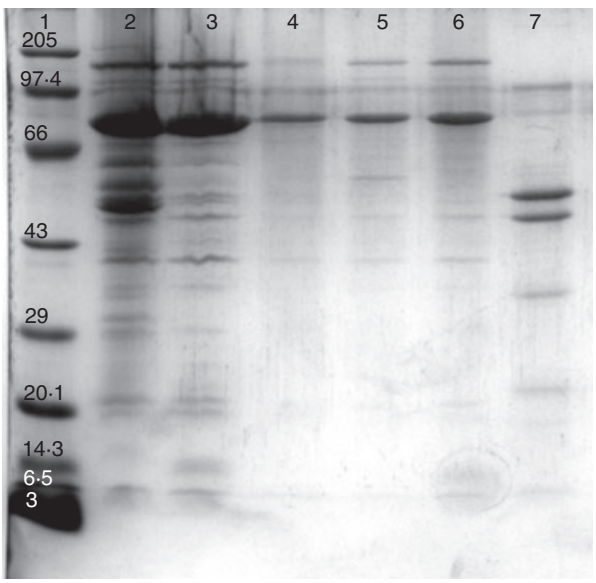

Figure 3 SDS-polyacrylamide gel electrophoretic profiles of phage structural proteins. Lane 1: broad range protein molecular weight marker (in Kilo Daltons); lane 2: *Vi ha* 6; lane 3: *Vi ha* 15; lane 4: *Vi ha* 19; lane 5: *Vi ha* 21; lane 6: *Vi ha* 32; lane 7; *Vi ha* 68.

different compared with others. SDS-PAGE was found to be effective in differentiating phages.

It is common to have wide variations in the banding pattern of proteins of different phages. For instance, Barrangou *et al.* (2002) observed the presence of 2–5 bands in *Leuconostoc fallax* phages, and Nasu *et al.* (2000) found a single 5 kDa band in a phage of *V. parahaemolyticus*. T-phages having 42 bands have been reported by Price and Rooyen (2001), and Chang *et al.* (2005) observed 25 bands in 250-kb giant phage of *Stenotrophomonas maltophilia* ΦSMA5. In this study, the phages showed 8–21 protein bands in SDS-PAGE. The pattern was found in agreement with morphology revealed by TEM, and the dendrogram resulted out of the RAPD pattern. However, Shivu *et al.* (2007) and Barrangou *et al.* (2002) described phages having quite identical protein banding pattern also.

Isolation, screening and characterization of broad spectrum *V. harveyi* phages were the main accomplishments of this study. As an outcome of characterization, the phages segregated turned out to be different in identity. The concept of treatment of vibriosis using a concoction of phages has tremendous potential as an alternative to antibiotics and shall take forward shrimp larval production technology under RAS a long way. The concept of overlapping the host ranges of all the six phages may be advantageous in managing the diverse strains of *V. harveyi* from hatchery point of view. Moreover, the coexistence of the screened phages with probiotics and nitrifying bacteria underlines its suitability in sustainable aquaculture.

Materials and methods

Bacterial isolates and media

Eighty-seven isolates of *V. harveyi* from shrimp hatcheries across east and west coast of India (Table S4) were obtained from the culture collection of National Centre for Aquatic Animal Health, Cochin University of Science

and Technology. The isolates were grown in ZoBell's medium (Himedia, Mumbai, India) at 28°C for 18 h. Their purity was established by plating onto ZoBell's agar plates.

Bacteriophage isolation

Vibrio harveyi phages were isolated from water and sediment samples collected from various shrimp ponds of Kerala, India, following enrichment (Cerveny *et al.* 2002). Sampling locations are shown in Fig. S2. During primary enrichment, coculture of *V. harveyi* isolates was inoculated into a known volume of sample along with ZoBell's broth and incubated overnight at 28°C. The resultant lysate was harvested by centrifugation at 8000 ***g***. Subsequently, isolates of *V. harveyi* were inoculated individually in to ZoBell's broth along with primary lysate and incubated for 6 h in orbital shaker. Subsequent to harvesting, phage titre in the secondary lysate was determined following soft agar overlay method (Adams 1959).

Propagation of phages

Phage propagation was carried out as per the modified method described by Su *et al.* (1998). The plates with confluent lysis were selected and flooded with 5 ml salt–magnesium (SM) buffer (50 mmol l^{-1} Tris-HCl, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgSO_4$, 0·1% gelatin, pH 7·5). The plates were placed on an orbital shaker and subjected to continuous gentle agitation (60–80 rpm) for 6 h. The contents were decanted and saved (without disturbing the bottom agar layer), centrifuged and the supernatant filtered through 0·2 μm PVDF membrane filter to get bacteria-free phage lysates. The phage titres were determined by soft agar overlay method, and the lysates were stored at 4°C (for immediate use) and −80°C as 50% glycerol stocks (for long term storage).

Host range analyses

Host range analyses were conducted to segregate broad spectrum lytic phages from 33 phage lysates generated. All the 33 phages were allowed to infect 87 isolates of *V. harveyi* in the collection. It was performed by spotting 10 μl purified phage lysate on bacterial host lawns prepared on ZoBell's agar plates. The presence of plaques after overnight incubation indicated the lytic efficiency of each phage lysate on the host bacterium. The plaques were categorized as clear, moderately clear and turbid. Based on the performance, six *V. harveyi* phages which produced clear plaques against maximum number of bacterial isolates were segregated as the 'broad spectrum phages'.

Identification of host bacteria

The identity of the bacterial host of screened broad spectrum phages was performed phenotypically (Alsina and Blanch 1994) and confirmed by sequence analysis of 16S rRNA gene. The sequences were deposited in NCBI GenBank under the accession numbers JN990075 (MCCB 153-LB 6), JN990076 (MCCB 154-LB 15), JQ920474 (MCCB 155-LB 19), JN990077 (MCCB 156-LB 21), JN990078 (MCCB 157-LB 32) and JQ920475 (MCCB 158-LB 68).

Cross-infectivity with other bacterial isolates of aquaculture importance

The segregated six *V. harveyi* phages were examined for their infectivity on other aquaculture pathogens and probiotic organisms (Table S1).

Cross-infectivity to nitrifying bacterial consortia

To determine the susceptibility of nitrifying bacterial consortia developed at National Centre for Aquatic Animal Health (Achuthan *et al.* 2006) to the phages, the phage cocktail was inoculated into a stabilized 8-day-old nitrifying bacterial consortia having the salinity optima of 15 ppt in mineral base medium (10 mg l^{-1} $(NH_4)_2$ SO_4 and 2 mg l^{-1} KH_2 PO_4 in 100 ml 15 g l^{-1} salinity seawater) and incubated under obscurity on a rotary shaker for 5 days, while uninoculated consortia under the same conditions served as control. The concentration of total ammonia-nitrogen (Solorzano 1969), nitrite-nitrogen (Bendschneider and Robinson 1952) and nitrate-nitrogen (Strickland and Parson 1968) was determined spectrophotometrically once in every 24 h. Ammonia-nitrogen was supplemented on its consumption until the end of the experiment.

Morphological characterization by transmission electron microscopy

Transmission electron microscopy was used to characterize the phages morphologically. The purified phage lysates were fixed in 0·5% glutaraldehyde, dissolved in 4% paraformaldehyde, positively stained with 2% uranyl acetate and viewed under TEM (Philips CM 10, Amsterdam, the Netherlands).

Nature of nucleic acid in phages

Nucleic acid of bacteriophages was extracted following Su *et al.* (1998). To the pure phage lysates, DNase I (New England Biolabs, Ipswich, MA, USA) was added to

remove any contaminating bacterial DNA, and phage particles precipitated using 2 mol l^{-1} $ZnCl_2$ and pelleted by centrifugation. Pellets dissolved in TENS buffer were treated with proteinase K and deproteinated by phenol–chloroform method. DNA precipitated using 100% ethanol was washed with 70% ethanol and centrifuged. The pellet was resuspended in MilliQ water (Millipore™, Billerica, MA, USA) and DNA quantified spectrophotometrically.

The extracted nucleic acid was digested with DNase-free RNase (Sigma, St. Louis, MO, USA) to determine whether the nucleic acid was either DNA or RNA. To digest 10 μg nucleic acid, 20 U of the enzyme was added and incubated in a water bath at 37°C for 1 h, and the product was electrophoresed through 0·8% agarose gel at 110 V. To determine whether the phage DNA was single/double stranded, 10 μg of DNA was incubated with 20 U of moong bean nuclease and 2 μl NEB buffer 1 (New England Biolabs) at 37°C for 1 h, and the resultant product was electrophoresed through 0·8% agarose gel.

RAPD profiling

RAPD profiling was carried out following the method described by Johansson *et al.* (1995) with few modifications. Two decamer primers P-1 (5′-CCG CAG CCA A-3′) and P-2 (5′-AAC GGG CAG A-3′) reported by Shivu *et al.* (2007) were used in this study to amplify RAPD sequences. An aliquot of 30 μl RAPD-PCR mixture contained 2·5 μl 10X Thermopol buffer (New England Biolabs), 2 μl (250 μmol l^{-1}) each dATP, dCTP, dGTP and dTTP, 10 μm each of decamer primer, 50 ng DNA template and 1 μl Taq polymerase (0·5 U, New England Biolabs) in sterile MilliQ. DNA amplification was performed using a thermal cycler (Master Cycler, Eppendorf, Hamburg, Germany). Thermal cycler programmes were modified as given below: for primer (P-292): initial denaturation of 94°C for 5 min; 35 cycles of 94°C for 20 s, 45°C for 30 s, 72°C for 1 min and a final extension of 7 min at 72°C; for primer (P-293): initial denaturation of 94°C for 3 min; 35 cycles of 94°C for 5 s, 36°C for 45 s, 72°C for 90 s and 5 min at 72°C. The amplified products were analysed on 1·5% agarose gel electrophoresis at a constant current of 60 mA. 1-kb and 100-bp ladders (New England Biolabs) were used as molecular size markers. Clustering and dendrogram construction were carried out using the software NTSYS pc version 2.0 (Exeter Software, Setauket, NY, USA).

Phage Purification and SDS-PAGE

The harvested phage lysates were clarified by centrifugation at 8000 ***g*** for five min and then passed through 0·45 and 0·22 μm PVDF membrane filter. The phages in the supernatant were concentrated by polyethylene glycol (PEG) precipitation (Shukla *et al.* 2009). The resultant pellet was dissolved in 1 ml SM buffer. For purification of the precipitated phages, discontinuous sucrose gradients (30% and 70%) were prepared in SM buffer. The 1 ml PEG pelleted phage suspension was carefully layered on to the top of the sucrose cushion. Ultracentrifugation was carried out at 1 00 000 ***g*** for 2 h. The clear opaque band obtained at the interface was collected, dissolved in SM buffer and stored at 4°C.

The profiles of phage structural proteins were analysed by SDS-PAGE. To 100 μl purified phage suspension obtained by sucrose gradient centrifugation, 5 μl reducing sample buffer (0·5 mol l^{-1} Tris-HCl, 10% SDS, 20% Glycerol, 10 mmol l^{-1} mercaptoethanol, 0·01% bromophenol blue) was added and boiled for 5 min. The resultant solution was subjected to reducing SDS-PAGE following the method of Laemmli (1970) using 5% stacking gel and 15% resolving gel at a constant current of 12 mA. After electrophoresis, gels were stained with 0·025% Coomassie brilliant blue stain R-250 and then destained in a solution of 5% methanol and 7% acetic acid. Molecular weight of the unknown protein bands was determined by comparing with the broad range protein molecular marker (Bangalore Genei, Bangalore, India).

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Conflict of interest

No conflict of interest declared.

References

Achuthan, C., Kumar, V.J.R., Manju, N.J., Philip, R. and Singh, I.S.B. (2006) Development of nitrifying bacterial consortia for immobilizing in nitrifying bioreactors designed for penaeid and non-penaeid larval rearing systems in the tropics. *Indian J Mar Sci* **35**, 240–248.

Ackermann, H.W. (2001) Frequency of morphological phage descriptions in the year 2000. Brief review. *Arch Virol* **146** (5), 843–857.

Adams, M.H. (1959) *Bacteriophages*. New York, USA: Interscience.

Alsina, M. and Blanch, A.R. (1994) A set of keys for biochemical identification of *Vibrio* species. *J Appl Bacteriol* **76**, 79–85.

Austin, B., Pride, A.C. and Rhodie, G.A. (2003) Association of a bacteriophage with virulence in *Vibrio harveyi*. *J Fish Dis* **26**, 55–58.

Barrangou, R., Yoon, S.-S., Breidt, F., Fleming, H.P. and Klaenhammer, T.R. (2002) Characterization of six *Leuconostoc fallax* bacteriophages isolated from an industrial sauerkraut fermentation. *Appl Environ Microbiol* **68**(11), 5452–5458.

Bendschneider, K. and Robinson, R.J. (1952) A new photometric method for the determination of nitrite in sea water. *J Mar Res* **11**, 87–96.

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A.N., Powell, B., Carlton, R. and Merril, C.R. (2002) Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infect Immun* **70**, 204–210.

Cerveny, K.E., DePaola, A., Duckworth, D.H. and Gulig, P.A. (2002) Phage therapy of local and systemic disease caused by *Vibrio vulnificus* in iron-dextran-treated mice. *Infect Immun* **70**, 6251–6262.

Chang, H.-C., Chen, C.-R., Lin, J.-W., Shen, G.-H., Chang, K.-M., Tseng, Y.-H. and Weng, S.-F. (2005) Isolation and characterization of novel giant *Stenotrophomonas maltophilia* phage SMA5. *Appl Environ Microbiol* **71**, 1387–1393.

Duckworth, D.H., Glenn, J. and McCorquodale, D.J. (1981) Inhibition of bacteriophage replication by extrachromosomal genetic element. *Microbiol Rev* **45**, 52–71.

Hantke, K. (1978) Major outer membrane proteins of *E. coli* K12 serve as receptors for the phages T2 (protein Ia) and 434 (protein Ib). *Mol Gen Genet* **164**, 131–135.

Harris, L.J. and Owens, L. (1999) Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. *Dis Aquat Organ* **38**, 11–22.

d'Herelle, F. (1917) An invisible microbe that is antagonistic to the dysentery bacillus. *Comptes rendus Acad Sci Paris* **165**, 373–375.

Johansson, M.L., Quednau, M., Molin, G. and Ahrne, S. (1995) Randomly amplified polymorphic DNA (RAPD) for rapid typing of *Lactobacillus plantarum* strains. *Lett Appl Microbiol* **21**, 155–159.

Karunasagar, I., Shivu, M.M., Girisha, S.K., Krohne, G. and Karunasagar, I. (2007) Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture* **268**, 288–292.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Munro, J., Oakey, J., Bromage, E. and Owens, L. (2003) Experimental bacteriophage-mediated virulence in strains of *Vibrio harveyi*. *Dis Aquat Organ* **54**, 187–194.

Nasu, H., Iida, T., Sugahara, T., Yamaichi, Y., Park, K.S., Yokoyama, K., Makino, K., Shinagawa, H. *et al.* (2000) A filamentous phage associated with recent pandemic *Vibrio parahaemolyticus* O3:K6 strains. *J Clin Microbiol* **38**, 2156–2161.

Oakey, H.J. and Owens, L. (2000) A new bacteriophage, VHML, isolated from a toxin-producing strain of *Vibrio harveyi* in tropical Australia. *J Appl Microbiol* **89**, 702–709.

O'Flaherty, S., Ross, R.P., Flynn, J., Meaney, W.J., Fitzgerald, G.F. and Coffey, A. (2005) Isolation and characterization of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections. *Lett Appl Microbiol* **41**, 482–486.

Pasharawipas, T., Thaikua, S., Sriurairatana, S., Ruangpan, L., Direkbusarakum, S., Manopvisetcharean, J. and Flegel, T.W. (2005) Partial characterization of a novel bacteriophage of *Vibrio harveyi* isolated from shrimp culture ponds in Thailand. *Virus Res* **114**, 63–69.

Price, C. and Rooyen, J.V. (2001) Characterisation and identification of isolated bacteriophage from a polluted water source. *J Mol Cell Biol* **177**, 144–151.

Ruangpan, L., Danayadol, Y., Direkbusarakom, S., Siurairatana, S. and Flegel, T. (1999) Lethal toxicity of *Vibrio harveyi* to cultivated *Penaeus monodon* induced by a bacteriophage. *Dis Aquat Organ* **35**, 195–201.

Shivu, M.M., Rajeeva, B.C., Girisha, S.K., Karunasagar, I., Krohne, G. and Karunasagar, I. (2007) Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Environ Microbiol* **9**, 322–331.

Shukla, J., Khan, M., Tiwari, M., Sannarangaiah, S., Sharma, S., Rao, P.V.L. and Parida, M. (2009) Development and evaluation of antigen capture ELISA for early clinical diagnosis of chikungunya. *Diagn Microbiol Infec Dis* **65**, 142–149.

Smith, H.W. and Huggins, M.B. (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *J Gen Microbiol* **128**, 307–318.

Solorzano, L. (1969) Determination of ammonia in natural waters by the phenolhypochlorite method. *Limnol Oceanogr* **14**, 799–801.

Strickland, J.D.H. and Parson, T.R. (1968) *A Practical Hand Book of Water Analysis*. Canada: Fisheries Research Board.

Su, M.T., Venkatesh, T.V. and Bodmer, R. (1998) Large- and small-scale preparation of bacteriophage λ lysate and DNA. *Biotechniques* **25**, 44–45.

Twort, F.W. (1915) An investigation on the nature of ultra-microscopic viruses. *Lancet* **186**(4814), 1241–1243.

Vinod, M., Shivu, M., Umesha, K., Rajeeva, B., Krohne, G. and Karunasagar, I. (2006) Isolation of *Vibrio harveyi* bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. *Aquaculture* **255**, 117–124.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Influence of *Vibrio harveyi* phage cocktail on (a) NO_2-N and (b)NO_3-N production (–▲–) Control, (–□–) Test. The cocktail phages added to the eighth day stabilized test consortium.

Figure S2. Spatial distribution of sampling locations for *Vibrio harveyi* phage isolation.

Figure S3. RAPD profile of *Vibrio harveyi* phage DNA with primer P-292. Lane 1, 1 kb DNA ladder; lane 3, *Vi ha* 15; lane 4, *Vi ha* 19; lane 5, *Vi ha* 21; lane 6, *Vi ha* 32; lane 7, *Vi ha* 68; lane 8, 100 bp DNA ladder.

Figure S4. RAPD profile of *Vibrio harveyi* phage, *Vi ha* 6 DNA with primer P-292. Lane 1, 100 bp DNA ladder; lane 2, *Vi ha* 6.

Figure S5. RAPD profile of *Vibrio harveyi* phage DNA with primer P-293. Lane 1, 1 kb DNA ladder; lane 2, *Vi ha* 6; lane 3, *Vi ha* 15; lane 4, *Vi ha* 19; lane 5, *Vi ha* 21; lane 6, *Vi ha* 32; lane 7, *Vi ha* 68; lane 8, 100 bp DNA ladder.

Table S1. The details of sampling and isolation of *Vibrio harveyi* phages.

Table S2. *Vibrio harveyi* phages and their percentage of lysis (Percentage is calculated from infection with 87 *V. harveyi* strains).

Table S3. Cross-infectivity of segregated phages to species of *Vibrio, Aeromonas* and probiotics. (Plaques denoted as +++ clear, ++ moderately clear and + turbid).

Table S4. *Vibrio harveyi* isolates used in the study and their source.