

Identification of *Vibrio campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an *Artemia* model

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Shrimp diseases are frequently reported to be caused by closely related vibrios, and in many cases they are tentatively but inaccurately identified as *Vibrio harveyi* and related vibrios. In the present study, 28 biochemically identified *V. harveyi*-related strains isolated from diseased shrimps were randomly selected for further characterization by molecular tools. Twenty-six strains were identified as *Vibrio campbellii* and two as *V. harveyi* by sequence analysis of 16S rRNA and uridylate kinase genes. Haemolysin-gene-based species-specific multiplex PCR also confirmed these results. Experimental challenge studies using *Artemia* as a model showed that eight isolates were highly pathogenic, three were moderately pathogenic and the remaining 17 were non-pathogenic. Ribotyping with *Bgl*I clearly distinguished *V. campbellii* from *V. harveyi*, but it failed to separate pathogenic and non-pathogenic clusters. *Artemia* nauplii challenged with a fluorescently labelled highly pathogenic strain (IPEY54) showed patches in the digestive tract. However, no patches were observed for a non-pathogenic strain (IPEY41). Direct bacterial counts also supported colonization potential for the highly pathogenic strain. To our knowledge, this is the first report on the isolation and accurate identification of large numbers of *V. campbellii* associated with shrimp disease in aquacultural farms. *V. campbellii* has long been considered to be non-pathogenic and classified with *V. harveyi*-related bacteria. However, we show that this species may be an emerging aquaculture pathogen. This study will help to formulate suitable strategies to combat this newly identified pathogen.

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INTRODUCTION

Halophilic vibrios such as *Vibrio harveyi* are ubiquitous in the marine environment and are implicated as the causes of several diseases in wild and cultured aquatic organisms. Due to the plasticity of *Vibrio* genomes, with frequent

horizontal gene transfer events, species boundaries are very narrow in the marine environment (Fraser *et al.*, 2007). Hence, the identification of *V. harveyi* and related species isolated from the marine environment is sometimes difficult. Luminous vibrios including *V. harveyi* have been implicated principally with disease outbreaks in shrimp larval culture facilities and grow-out ponds worldwide. Due to the high level of phylogenetic similarity among marine vibrios, bacteria associated with disease outbreaks have often been misidentified. For example, although strain LMG 19703^T (or AK1^T) showed 99.4% sequence similarity in 16S rRNA with *Vibrio mediterranei* (ATCC 43341^T), Kushmaro *et al.* (2001) initially classified it as a new species, *Vibrio shiloi*, due to large differences in phenotypic properties. Later, Thompson *et al.* (2001), on the basis of genotypic features such as fluorescent amplified fragment length polymorphism and DNA–DNA hybridization, as

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Abbreviations: ASW, artificial seawater; DOC, days of culture; DTAF, 5-(4,6-dichlorotriazin-2-yl)aminofluorescein; NJ, neighbour joining.

The GenBank/EMBL/DDBJ accession numbers for the sequence data for the experimental strains in this study are AB469289–AB469316 (16S rRNA), and AB469317–AB469324, AB469417–AB469436 (*pyrH*). The accession numbers for the *V. parahaemolyticus* reference strain (Vp61) are AB561143 (16S rRNA) and AB561144 (*pyrH*).

Supplementary material is available with the online version of this paper.

well as phenotypic analyses, proposed that strain LMG 19703^T should be considered as a subspecies of *V. mediterranei*. Similarly, two recently isolated crustacean pathogens were close to *Vibrio campbellii*, *V. harveyi* and *V. rotiferianus*, but DNA–DNA hybridization later proved them to be a new species, *Vibrio owensii* (Cano-Gómez *et al.*, 2010). Misidentification of bacterial pathogens in fish/shrimp farm settings may create problems in selecting appropriate prophylactic measures. In recent years, sequencing and comparison of the 16S rRNA gene has become an important tool for identification of bacterial species (Greenwood *et al.*, 2005). However, *V. harveyi* and *V. campbellii* share more than 97% similarity in their 16S rRNA gene sequences and show a 69% match in DNA–DNA hybridization (Gomez-Gil *et al.*, 2004). Further, the well-studied *V. harveyi* strain BB120 was recently proved to be *V. campbellii* by microarray-based comparative genomic hybridization (Lin *et al.*, 2010). However, based on comparison of a series of housekeeping genes (*pyrH*, *topA*, *ftsZ* and *mreB*) a few researchers have successfully identified and differentiated *V. campbellii* from *V. harveyi* (Thompson *et al.*, 2007).

In addition to problems of accurate identification, a second challenge is to understand the virulence potential of marine vibrios, which is still difficult due to the lack of simple and reliable animal models. Nauplii of the brine shrimp *Artemia* have been used in many studies because this shrimp can be cultured easily in gnotobiotic conditions (Austin *et al.*, 2005). Bacterial interaction or colonization with challenged organisms is a complex process. To study colonization potential, several techniques have been employed such as the use of cell lines (Olsson *et al.*, 1992) and direct observation by scanning electron microscopy (Lavilla-Pitogo *et al.*, 1990). These processes involve fixation and preparation of samples for microscopy, but do not permit *in vivo* observations of recently killed organisms or guarantee that the observed bacteria are those inoculated or of interest. Thus, the use of a pathogenic strain labelled with fluorescent dye was considered to be an appropriate method to study the *in vivo* colonization potential.

Vibriosis was reported during the months of October to December 2004 in three shrimp farms near Chennai, south India. In this study, we aimed to confirm the identification and to characterize bacterial strains isolated from the diseased shrimps and farm environments using molecular tools in addition to conventional culture techniques. We demonstrated the pathogenic potential and colonization abilities of *V. campbellii* strains using an *Artemia* model. Our studies indicate that *V. campbellii* may be a potential and emerging shrimp pathogen.

METHODS

Isolation place, sample collection and bacterial isolation. An incidence of disease was observed in the shrimp *Penaeus monodon* in three farms (here designated A, B, and C) with five, six and eight

ponds, respectively, in north coastal Chennai, south India (see Supplementary Fig. S1, available with the online version of this paper). Average pond size was 0.5–1.0 ha (1 ha=10 000 m²), with a stocking density of 8–12 shrimps m⁻² (semi-intensive culture). All three farms were located within a 9 km radius. Water supply to all ponds was from the Bay of Bengal through a common estuary. The water used was treated with 2% chlorine, followed by sedimentation for 24 h in a reservoir pond, and was finally supplied to the culture ponds through inlet canals. Two to four aerators (1 hp each) were used in each pond after 60 days of culture (DOC), depending upon the size of the pond. Salinity and pH were measured using a refractometer (Erma) and a pH pen (Eutech Instruments), respectively, twice daily starting from DOC60 (APHA, 1998), and the water temperature was measured once a day. All diseased and moribund shrimps were immediately stored in ice after collection and were analysed in the laboratory within 3–4 h. Samples were washed thoroughly with sterile distilled water. The hepatopancreas, muscles and gills of the diseased shrimps were dissected with sterile scissors and homogenized in physiological saline under aseptic conditions. Water samples were collected in duplicate at approximately 10 cm depth, using 100 ml sterile plastic flasks. Sediments from the affected ponds were sampled with a bottom collector (Van Veen type) and two portions of the top 1 cm layer were transferred aseptically to UV-sterilized plastic bags. Pond water and bottom soil samples were also included in this study to establish any possible epidemiological relationship with the experimental strains. Appropriate dilutions of homogenized samples from shrimp body parts (samples were collected from many shrimps) – hepatopancreas, muscle, gills, etc. – as well as pond water and bottom soil were plated on ZoBell's marine agar (Himedia) by spreading (Buck & Cleverdon, 1960). Isolated bacteria from the body parts of shrimps were considered as shrimp disease isolates. A random selection of colonies was screened with an array of biochemical tests and stored in LB broth containing 3% sodium chloride and 25% glycerol at –80 °C.

Biochemical tests. Phenotypic characterization of all bacterial isolates was done following a scheme described elsewhere (Alsina & Blanch, 1994; Holt *et al.*, 1994). Details of the biochemical tests are described in the Supplementary Material.

Sequence analysis of 16S rRNA and *pyrH* genes. Genomic DNA was prepared according to the method of Ausubel *et al.* (2002). Partial sequencing of the 16S rRNA and uridylate kinase (*pyrH*) genes was done for all 28 strains as described previously (Haldar *et al.*, 2007; Thompson *et al.*, 2007). The sequence of the *pyrH* gene from all experimental strains was compared with published sequence data in GenBank using BLAST (<http://www.ncbi.nih.gov/BLAST>). Alignment score and expect value (*E* value) were also evaluated. Highest scoring hit and lowest *E* value were taken as possible matches (Ausubel *et al.*, 2002).

For each experimental strain, the sequences for both 16S rRNA and *pyrH* genes were aligned and a phylogenetic tree was constructed with concatenated sequences by neighbour-joining (NJ) analysis using MEGA4 software (Kumar *et al.*, 2008). Reference strains of *V. campbellii* (ATCC 25920^T) and *V. harveyi* (ATCC 14126^T) were used in the construction of the phylogenetic tree. Both 16S rRNA and *pyrH* sequences of the *Vibrio* core group members *V. rotiferianus* (CAIM573 and CAIM574) and *V. parahaemolyticus* (Vp61) were used as references and outgroup, respectively (Dorsch *et al.*, 1992). The robustness of each topology was checked by 500 bootstrap replications.

The sequence data for the experimental strains have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AB469289–AB469316 (16S rRNA), and AB469317–AB469324, AB469417–AB469436 (*pyrH*). The accession numbers for the *V.*

parahaemolyticus reference strain (Vp61) are AB561143 (16S rRNA) and AB561144 (*pyrH*).

PCR-based identification. A recently developed species-specific multiplex PCR targeting the haemolysin (*hly*) gene was also used to reconfirm the identification of *V. campbellii* and *V. harveyi*. Details of primer sequences and PCR conditions were as described by Haldar *et al.* (2010a).

Ribotyping. Genomic DNA of all the test strains was digested with the restriction enzyme *Bgl*I (Takara Bio) overnight at 37 °C and electrophoresed in 1% agarose gel with a Chef Mapper (Bio-Rad), using the Auto Algorithm program (separation limit of 1–25 kb). The DNA was then transferred to nylon membranes by Southern blotting. The membranes were hybridized under high-stringency conditions (Lin *et al.*, 1993) with an [α -³²P]dCTP (111 TBq mmol⁻¹; Perkin Elmer)-labelled 7.5 kb *Bam*HI fragment of pKK3535 (Brosius *et al.*, 1981; Faruque *et al.*, 1994), and were then exposed to BAS film and analysed by FLA-3000 (Fuji Film).

Gnotobiotic *Artemia* culture. *Artemia* culture and challenge experiments were performed in autoclaved artificial seawater (ASW) prepared with seawater salt according to the instructions of the manufacturer (Matsuda). *Artemia* cysts were purchased from Tetra (Tokyo, Japan). All the experiments were performed with the same batch of *Artemia* nauplii. Bacteria-free nauplii were obtained by chlorination according to the procedure described by Defoirdt *et al.* (2005), with a slight modification (see the Supplementary Material for the decapsulation method). After hatching, 50 nauplii were randomly collected, ground and mixed with 100 μ l physiological saline in aseptic conditions, and bacterial counts were checked on LB agar. Healthy nauplii were transferred to ASW (500 nauplii in 100 ml) and maintained aseptically for 1 day.

Preparation of bacterial inocula. Several doses of bacteria were prepared according to the method of Gomez-Gil *et al.* (1998), with some modifications. Briefly, a single colony was inoculated into 3 ml LB broth (Becton Dickinson) with 3% sodium chloride and the culture was incubated at 37 °C in an incubator shaker (Innova 4000, New Brunswick Scientific) at 180 r.p.m. for 15 h. One millilitre of freshly prepared bacterial culture was centrifuged at 9100 g for 5 min and bacterial inocula were prepared by adding an appropriate volume of ASW to the cell pellet to obtain an OD₆₁₀ of 1.5 in a UV/vis spectrophotometer (Beckman Coulter).

Infection study. Thirty *Artemia* nauplii were transferred to a 15 ml test tube with 6 ml ASW 24 h after the chlorination treatment. The nauplii were kept for 6 h for acclimatization, and appropriate bacterial doses were added to obtain three concentrations, 10², 10⁵ and 10⁸ c.f.u. ml⁻¹, in each tube for the challenge experiment. Co-culture was done under sterile conditions in an incubator shaker (Innova 4300) at 26 °C with constant shaking (80 r.p.m.). Dead nauplii were collected and bacterial strains were isolated after 24 h. Subsequently, 16S rRNA gene sequence was performed. After 48 h, the numbers of live *Artemia* nauplii in each tube were counted and the mean percentage mortality (M_{obs}) with standard deviation (SD) was calculated. Nauplii were not fed throughout the experimental period to keep the experimental set-up contamination free as described by Marques *et al.* (2005). Generally *Artemia* can survive in a healthy condition for up to 72 h without feeding (Orozco-Medina *et al.*, 2002). All experiments were performed with three replicates per treatment.

Control experiments. *V. harveyi* strain BB120 (also known as BAA-1116), recently identified as *V. campbellii* and reported to be pathogenic for *Artemia* nauplii, was used as a positive control (Bassler *et al.*, 1997). A bacterium producing green colonies on

thiosulphate-citrate-bile-sucrose agar (Nissui Pharmaceutical Co.) was isolated from an *Artemia* cyst. It was non-pathogenic and identified as *Vibrio* sp. by 16S rRNA gene sequencing. This strain was used as a negative control. Another set of tubes containing only 30 *Artemia* nauplii in ASW without any bacterial inoculation was used as experimental control. Percentage mortality in this experimental control (M_{control}) was used for calculating corrected percentage mortality as described below. One hundred microlitres of water from the experimental control was plated on LB agar every day during the experimental period. Presence of a bacterial colony on either of these plates was considered as a failure of the experiment and the experiment was repeated.

Determination of the LC₅₀ (50% lethal concentration). LC₅₀ was determined using the probit method (Wardlaw, 1985) by plotting the log value of bacterial concentration (x -axis) against the probit value of corrected percentage mortality (y -axis). Corrected mortality was calculated by the following formula: Corrected mortality (%) = $[(M_{\text{obs}} - M_{\text{control}}) / (100 - M_{\text{control}})] \times 100$.

There is no probit value for 0% or 100% corrected mortality. As a result, it was not possible to calculate LC₅₀ values for strains which caused either 100% or 0% corrected mortality at any of the concentrations used.

Fluorescent labelling. The methodology described by Soto-Rodriguez *et al.* (2003a) for labelling bacteria with fluorescent dye was followed, with slight modification. A highly pathogenic (IPEY54) and a non-pathogenic (IPEY41) strain were labelled with the fluorescent dye 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) (see the Supplementary Material for details of the methodology).

Strains IPEY54 and IPEY41 were each inoculated at approximately 10⁵ c.f.u. ml⁻¹ in a similar way as in the bacterial challenge study, and after 24 h, accumulation of fluorescently labelled bacteria in the digestive tract was observed by fluorescence microscopy using a BP 470–490 nm excitation filter, DM 505 nm dichroic filter and BA 512–542 nm barrier filter with 100 \times magnification (Leica DFC350 FX, Leica Microsystems).

Colonization assay. The highly pathogenic strain IPEY54 and the non-pathogenic strain IPEY41 were selected to compare the colonization potentials. Hatching of *Artemia* nauplii in gnotobiotic conditions is described in the Supplementary Material. Bacterial doses were prepared as described above. A total of 375 nauplii were cultured in 75 ml ASW with approximately 10⁵ c.f.u. ml⁻¹ of bacteria for 24 h at 26 °C with shaking at 80 r.p.m. to allow possible bacterial colonization of the *Artemia* nauplii. The bacterial dose was selected on the basis of a previous study (Soto-Rodriguez *et al.*, 2006), where 10⁴–10⁶ c.f.u. ml⁻¹ of a luminous *Vibrio* were used to study the colonization potential against *Litopenaeus vannamei* larvae. Then 50 live *Artemia* were taken out and washed thoroughly with ASW without damaging any body parts. After this, all the nauplii were treated with 10 ml ASW containing 50 μ g chloramphenicol ml⁻¹, quickly transferred to fresh ASW without antibiotics and washed twice to remove all the bacteria loosely attached to the outer surface. This step was performed very rapidly to prevent engulfment of any antibiotics by the *Artemia* nauplii. Ten *Artemia* were crushed and plated with appropriate dilution on LB agar. The bacterial load in the surrounding water was also determined. Thirty *Artemia* were recultured in 6 ml ASW for another 24 h to check bacterial accumulation and possible colonization inside the bodies of the nauplii. At the end of the assay, 10 crushed nauplii and 100 μ l surrounding water were plated again with appropriate dilution on LB agar. Colonization potentials were calculated by the following formula: Colonization potential = $[(\text{c.f.u. ml}^{-1} \text{ per nauplius} - \text{c.f.u. ml}^{-1} \text{ in surrounding water}) \text{ after } 48 \text{ h}] / [(\text{c.f.u. ml}^{-1} \text{ per nauplius} - \text{c.f.u. ml}^{-1} \text{ in surrounding water}) \text{ after } 24 \text{ h}]$.

RESULTS

Epidemic information

During 60 days of shrimp culture, the disease symptoms such as reduction of average daily growth and increase in food conversion ratio were first observed in the ponds of farm A, closest to the mouth of the estuary. In the following 2 weeks, similar symptoms were recorded in the other two farms (B and C). No viral disease such as white spot syndrome was reported from any of the ponds throughout the culture period. During culture the water temperature varied from 28 to 32 °C and the pH of the water was slightly alkaline (7.8–8.2).

Biochemical characters

Initially, 88 bacterial strains were biochemically tested (data not shown). All these strains were Gram-negative, oxidase-positive and negative for vibriostatic agent O/129 (10 µg); all showed no growth in 0% sodium chloride, and grew well in medium supplemented with 3–6% sodium chloride. The results of the biochemical tests indicated that all the strains were halophilic *Vibrio* species. All the strains were negative for arginine dihydrolase reaction, and positive for indole production and gelatin production, establishing close identity with *V. harveyi*-related organisms. However, variable results were recorded for the majority of strains in the ornithine decarboxylase test, which is considered as the key test for differentiating *V. campbellii* from *V. harveyi* (Alsina & Blanch, 1994).

Identification by 16S rRNA and *pyrH* genes

Among the 88 biochemically identified bacterial strains, 28 from different origins (Table 1) were randomly selected for further molecular characterization. When the *pyrH* gene sequences were compared with a non-redundant database at NCBI, 26 strains showed highest similarity (100%) with published *V. campbellii* sequences and the remaining two with published *V. harveyi* sequences (data not shown). The lowest *E* value was considered for all comparisons. Construction of a phylogenetic tree with concatenated sequences of 16S rRNA (496 bp) and *pyrH* genes (467 bp) with MEGA4 software using NJ analysis revealed a similar identification pattern. The sequences of *V. campbellii* (ATCC 25920^T) and *V. harveyi* (ATCC 14126^T) were used as references. *V. rotiferianus* and *V. parahaemolyticus* formed separate clusters (Fig. 1). Similar phylogenetic trees were formed when constructed with minimum-evolution (ME), maximum-parsimony (MP) and UPGMA analysis using the same MEGA4 software (data not shown). The *pyrH* gene-based phylogenetic tree using NJ analysis also successfully differentiated *V. campbellii* groups from *V. harveyi* (Supplementary Fig. S2).

PCR-based identification

Species-specific multiplex PCR targeting the *V. campbellii* and *V. harveyi hly* gene also clearly identified 26 strains as

V. campbellii and the remaining two as *V. harveyi* (data not shown).

Ribotyping

Ribotyping with *Bgl*I restriction enzyme revealed three distinct clusters among the 28 strains. Two *V. harveyi* strains (IPEY37 and IPEY52) formed cluster I. Clusters II and III each contained 13 strains. Cluster I differed from clusters II and III by four and five DNA bands, respectively (Fig. 2). In this study, *Bgl*I-based ribotyping was able to discriminate *V. campbellii* from *V. harveyi* successfully, but the intra-specific discrimination efficiency of *V. campbellii* was not very high. The strains of cluster I were non-pathogenic (Table 1). When an attempt was made to relate ribotype clusters to the origin of the strains, it was found that both strains of cluster I had originated from pond water, whereas the majority of strains in clusters II and III originated from diseased shrimps. An interesting correlation between time of isolation and ribotype clusters was found. Both strains of cluster I were isolated during DOC60 and all strains of cluster II were isolated throughout the culture period (DOC60 to DOC135). Strains of cluster III were isolated from the samples during the initial (DOC60 to DOC90) and later (DOC135) stages of culture, but not during DOC105 and DOC120.

Pathogenic potential

The pathogenic potential varied among the strains. The pathogenicity of each strain against *Artemia* nauplii was determined on the basis of LC₅₀. Strains having an LC₅₀ of <10³ c.f.u. ml⁻¹ were considered as highly pathogenic, those with an LC₅₀ of 10³ to 10⁷ c.f.u. ml⁻¹ as moderately pathogenic, and those with an LC₅₀ of >10⁷ c.f.u. ml⁻¹ as non-pathogenic. LC₅₀ was not determined for 11 non-pathogenic strains, which showed 0% corrected mortality at some of the bacterial doses (10², 10⁵ and 10⁸ c.f.u. ml⁻¹). Of the 28 strains, 8 were highly pathogenic, 3 were moderately pathogenic and 17 were non-pathogenic in the *Artemia* experimental model (Table 1). The positive control strain BB120 showed moderate pathogenicity as expected (Bassler *et al.*, 1997). Negligible mortality was observed in the negative control (*Vibrio* sp.). In the bacteria-free control, 6% average mortality was observed, which was used to calculate corrected mortality. Detailed results of the challenge experiment are shown in Supplementary Table S1. Among five environmental strains (pond and estuarine water, bottom soil), four (IPEY37, IPEY52, IPEY63 and IPEY77) were non-pathogenic and only one (IPEY60) was moderately pathogenic to *Artemia* nauplii.

Colonization assay

To examine the colonization potential of highly pathogenic and non-pathogenic *V. campbellii* strains, *V. campbellii* was labelled with fluorescent dye and used for challenge in the

Table 1. Strain details and results of molecular identification, ribotype and pathogenic potential against *Artemia*

Strain	Source	DOC*	16S rRNA	Ribotype cluster	<i>Artemia</i> challenge	
					LC ₅₀	Pathogenicity
IPEY37	Pond water	60	<i>V. harveyi</i>	I	$>1.7 \times 10^8$	None
IPEY41	Diseased shrimp	60	<i>V. campbellii</i>	II	$>2.4 \times 10^8$	None
IPEY45	Diseased shrimp	60	<i>V. campbellii</i>	III	$>3.9 \times 10^8$	None
IPEY46	Diseased shrimp	60	<i>V. campbellii</i>	III	1.2×10^2	High
IPEY50	Diseased shrimp	60	<i>V. campbellii</i>	III	$>3.8 \times 10^8$	None
IPEY52	Pond water	60	<i>V. harveyi</i>	I	$>3.1 \times 10^8$	None
IPEY53	Diseased shrimp	60	<i>V. campbellii</i>	III	2.7×10^6	Moderate
IPEY54	Diseased shrimp	75	<i>V. campbellii</i>	III	3.4×10^1	High
IPEY55	Diseased shrimp	75	<i>V. campbellii</i>	II	5.8×10^1	High
IPEY60	Estuary water	75	<i>V. campbellii</i>	II	7.9×10^5	Moderate
IPEY63	Bottom soil	75	<i>V. campbellii</i>	III	4.4×10^7	None
IPEY64	Diseased shrimp	90	<i>V. campbellii</i>	III	$>3.6 \times 10^8$	None
IPEY65	Diseased shrimp	90	<i>V. campbellii</i>	III	$>3.4 \times 10^8$	None
IPEY67	Diseased shrimp	90	<i>V. campbellii</i>	II	0.7×10^1	High
IPEY70	Diseased shrimp	90	<i>V. campbellii</i>	II	2.1×10^2	High
IPEY71	Diseased shrimp	90	<i>V. campbellii</i>	III	0.5×10^1	High
IPEY72	Diseased shrimp	90	<i>V. campbellii</i>	II	$>2.9 \times 10^8$	None
IPEY74	Diseased shrimp	105	<i>V. campbellii</i>	II	1.2×10^2	High
IPEY76	Diseased shrimp	120	<i>V. campbellii</i>	II	6.7×10^4	Moderate
IPEY77	Bottom soil	120	<i>V. campbellii</i>	II	$>2.4 \times 10^8$	None
IPEY78	Diseased shrimp	120	<i>V. campbellii</i>	II	$>7.0 \times 10^8$	None
IPEY79	Diseased shrimp	135	<i>V. campbellii</i>	III	$>4.8 \times 10^8$	None
IPEY80	Diseased shrimp	135	<i>V. campbellii</i>	III	$>3.4 \times 10^8$	None
IPEY82	Diseased shrimp	135	<i>V. campbellii</i>	II	7.8×10^2	High
IPEY83	Diseased shrimp	135	<i>V. campbellii</i>	III	$>3.0 \times 10^8$	None
IPEY84	Diseased shrimp	135	<i>V. campbellii</i>	II	$>3.9 \times 10^8$	None
IPEY87	Diseased shrimp	135	<i>V. campbellii</i>	II	$>1.5 \times 10^8$	None
IPEY88	Diseased shrimp	135	<i>V. campbellii</i>	III	$>3.4 \times 10^8$	None

*DOC, days of culture.

Artemia model. After fluorescent labelling (Fig. 3a), bacterial viability and growth (IPEY54) were not changed. Equal haemolytic zones were also observed for both fluorescently labelled and non-labelled strain IPEY54 (data not shown). These results indicated that the fluorescent dye did not have any adverse effect on the survival, growth and pathogenic expression of the experimental strains. When fluorescently labelled strain IPEY54 was observed by fluorescence microscopy, active motility was observed. *Artemia* challenged with fluorescently labelled pathogenic strain IPEY54 showed obvious patches of fluorescently labelled bacteria in the intestine of the nauplii (Fig. 3b), whereas no such clumps were observed in the case of fluorescently labelled non-pathogenic strain IPEY41 (Fig. 3c). In a control experiment, when *Artemia* was cultured with only fluorescent dye, the dye was observed in the whole of the digestive tract (Fig. 3d), due to its filter-feeding nature. After 24 h infection of *Artemia* nauplii with *V. campbellii* strains IPEY54 and IPEY41, the total bacterial counts were $13 \pm 0.3 \times 10^6$ c.f.u. ml⁻¹ per nauplius and $82 \pm 7 \times 10^5$ c.f.u. ml⁻¹ per nauplius, respectively. A sharp increase of 84-fold in bacterial counts for the highly

pathogenic strain (IPEY54) and a slight decrease of 10-fold (0.1-fold increase) for the non-pathogenic strain (IPEY41) were found after 24 h when washed *Artemia* were kept in ASW without any bacterial inoculation (Table 2). This result indicates that the highly pathogenic strain IPEY54 can colonize and multiply inside the *Artemia* nauplii.

DISCUSSION

The *Vibrio* infection in the farmed shrimps was reported during the post-monsoon season, when the water temperature was increasing. Elevation in water temperature and constant alkaline pH may help the spread of *Vibrio* in the farm setting.

Identification of the causative agent is the first step towards understanding the nature of a disease in any setting. Hence, initially phenotype-based identification was carried out. However, phenotype-based identification of marine bacteria involves time-consuming techniques with low discriminating power (Alsina & Blanch, 1994). Numerous studies have already been done to identify many *Vibrio*

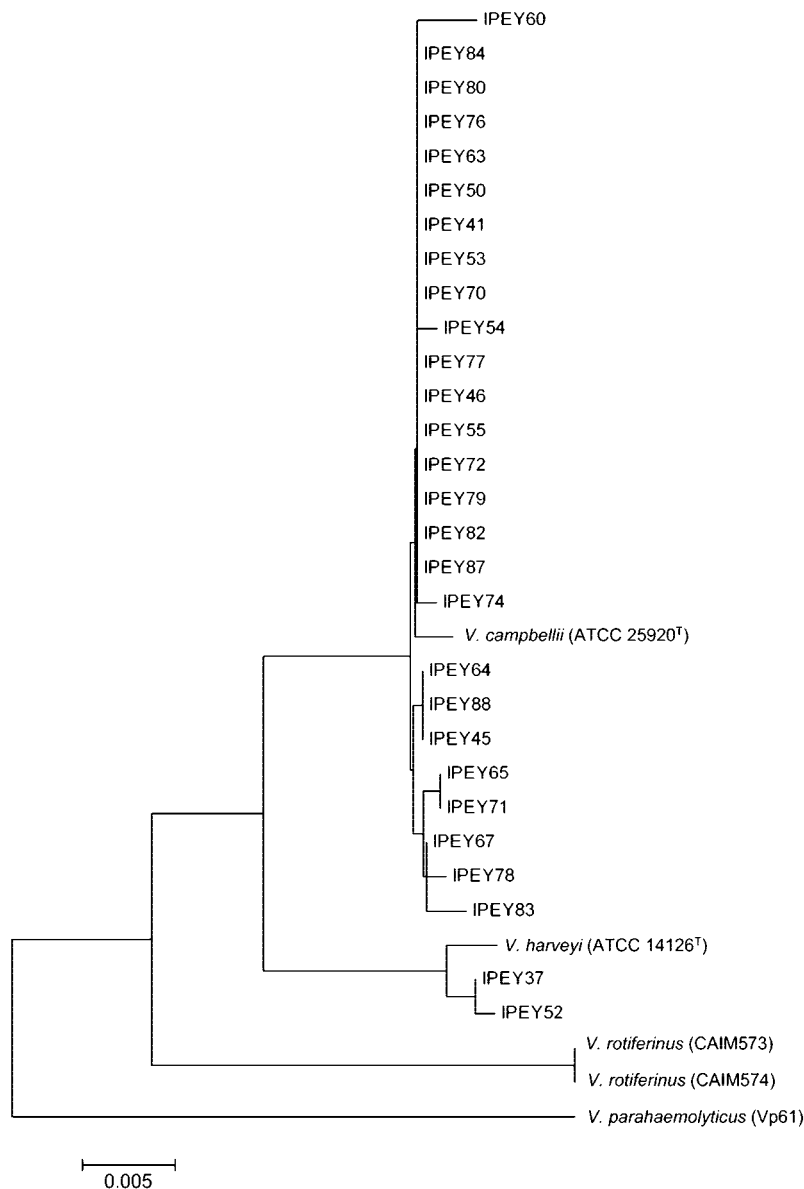


Fig. 1. Phylogenetic tree based on concatenated sequences of partial 16S rRNA *pyrH* genes using NJ analysis in the MEGA4 software. The *V. campbellii* type strain (ATCC 25920^T) clustered with a group of 26 experimental isolates, and the *V. harveyi* type strain (ATCC 14126^T) clustered with the other two experimental isolates. Sequences of the respective genes from two *V. rotiferianus* (CAIM573, CAIM574) strains and a *V. parahaemolyticus* (Vp61) strain were also included, and they were out-grouped.

species on the basis of biochemical tests, Biolog GN techniques, etc. However, these methods have failed to distinguish closely related vibrios like *V. harveyi* and *V. campbellii* (Thompson *et al.*, 2004a, b; Fraser *et al.*, 2007). In the present study, the biochemical tests gave preliminary indications that all 88 strains isolated from diseased shrimps and farm environments were presumptive *Vibrio* with considerable similarity to *V. harveyi*-related isolates, especially *V. campbellii*. Thus, a systematic attempt was made to correctly identify the disease-causing bacteria in the present study. To this end, 28 isolates of diverse origins within the shrimp farm ponds were randomly selected and characterized by different molecular tools. Thompson *et al.* (2005, 2007) analysed a series of housekeeping genes and concluded that there is no single gene which can clearly differentiate all species of vibrios. Further, it was reported

that *V. harveyi* and *V. campbellii* have less than 97% *pyrH* gene sequence similarity and it is possible to differentiate a series of *V. campbellii* and *V. harveyi* species on the basis of this gene sequence (Thompson *et al.*, 2007). The *pyrH* gene was therefore sequenced in this study to identify *V. campbellii* and *V. harveyi* correctly (Supplementary Table S1). A phylogenetic tree with concatenated sequences of the 16S rRNA and *pyrH* genes using NJ analysis also supported the above identification (Fig. 1). To strengthen the precise identification, all identified strains were reconfirmed by a recently developed simple multiplex PCR method, which can easily and accurately identify *V. campbellii* and *V. harveyi* on the basis of the *hly* gene (Haldar *et al.*, 2010a). Recently similar strategies were followed to successfully identify a number of disease-associated *V. harveyi* strains in a fish hatchery in Malta (Haldar *et al.*, 2010b).

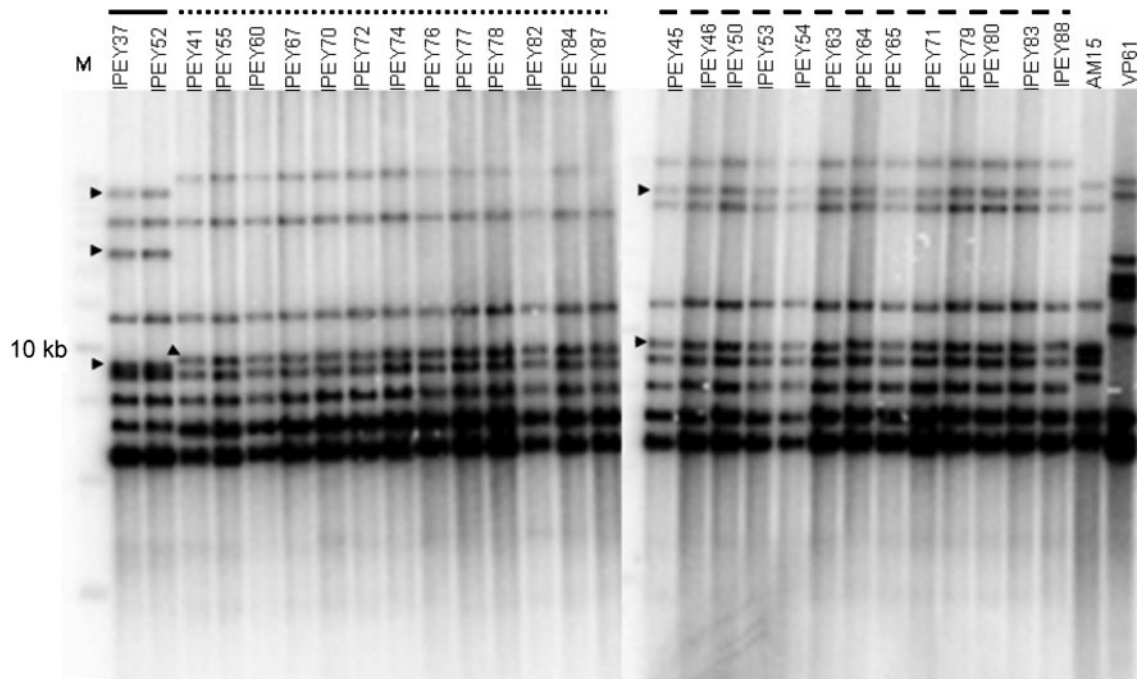


Fig. 2. Ribotype pattern of the test strains along with *V. harveyi* (AM15) and *V. parahaemolyticus* (Vp61) standard strains after digestion with *Bgl*I. Differences in DNA bands are indicated by arrowheads. The three clusters are marked by a solid line (cluster I), a dotted line (cluster II) and a dashed line (cluster III) above the figure.

After identification of disease-associated strains, the next major task was to evaluate the virulence potential of each strain. *Artemia* is an excellent model to study the mode of action of probiotics and pathogenic bacteria under gnotobiotic conditions (Marques *et al.*, 2005). Austin *et al.* (2005) evaluated the pathogenic potential of several

vibrios using rainbow trout and *Artemia* as test organisms. Their study revealed that some of the *Vibrio* species, such as *V. brasiliensis*, *V. coralliilyticus*, *V. fortis*, and *V. neptunius*, were highly pathogenic against *Artemia* nauplii, but *V. campbellii* was considered as a low- or non-pathogenic species. However, in the present investigation,

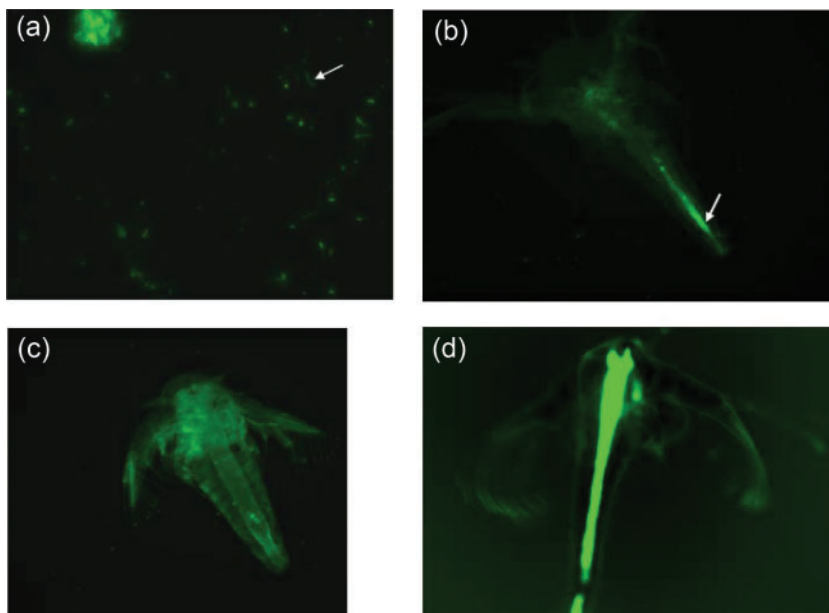


Fig. 3. Epifluorescent photomicrographs of fluorescently labelled highly pathogenic and non-pathogenic *V. campbellii* strains (strains IPEY54 and IPEY41, respectively). (a) Fresh mono-dispersed strain IPEY54 labelled with the fluorescent dye DTAF. Live fluorescently labelled bacteria are indicated by the arrow. (b) Clusters of fluorescently labelled bacteria in the midgut of an *Artemia* nauplius exposed to strain IPEY54 for 24 h at a density of 10^5 c.f.u. ml^{-1} . A patch of bacteria in the digestive tract is indicated by an arrow. (c) No fluorescent patch was observed in the midgut of an *Artemia* nauplius exposed to strain IPEY41 for 24 h at density of 10^5 c.f.u. ml^{-1} . (d) *Artemia* nauplius incubated with DTAF (0.5 mg ml^{-1}) alone (control). All figures are magnified $\times 100$.

Table 2. Colonization potentials of the highly pathogenic *V. campbellii* strain IPEY54 and the non-pathogenic strain IPEY41

	Counts of IPEY54 (c.f.u. ml ⁻¹)			Counts of IPEY41 (c.f.u. ml ⁻¹)			C.f.u. ml ⁻¹ in control <i>Artemia</i>
	Per nauplius	In surrounding water	Fold increase*	Per nauplius	In surrounding water	Fold increase*	
Initial load	0	0		0	0		0
After 24 h	13 ± 0.3 × 10 ⁶	1.2 ± 0.1 × 10 ²		82 ± 7 × 10 ⁵	1.2 ± 0.1 × 10 ²		0
After 48 h	11 ± 0.4 × 10 ⁸	11 ± 1.1 × 10 ²	84	10 ± 0.5 × 10 ⁵	13 ± 1.2 × 10 ²	0.1	0

*Fold increase in counts per nauplius between 24 and 48 h.

half of the *V. campbellii* strains isolated from diseased shrimps were moderately to highly pathogenic against *Artemia* nauplii. There are a few reports in which the pathogenic potentials of selected *V. campbellii* strains have been evaluated (Lavilla-Pitogo *et al.*, 1990; Phuoc *et al.*, 2008), but our study seems to be the first description of widespread damage caused by *V. campbellii* in a shrimp aquaculture setting. It would be interesting to challenge shrimp larvae with this set of strains, but a reproducible challenge protocol for penaeid larvae is not yet available (Soto-Rodriguez *et al.*, 2003a).

It is well known that marine and estuarine environments provide a habitat where vibrios can be exposed to high levels of gene transfer by transduction (Jiang & Paul, 1998), and consequently transfer of virulence factors may occur between marine bacteria (Hacker *et al.*, 2003). The present study also indicated that some unknown factor(s) might have transformed half of the *V. campbellii* strains to be pathogenic against *Artemia* nauplii. Bacterial adherence to the epithelial cells is an important virulence determinant for many mucosal pathogens and is a prerequisite for the pathogenesis (Finlay & Falkow, 1997). Using a paraffin-carving technique Lavilla-Pitogo *et al.* (1990) observed that luminescent bacteria adhered to the oral region of *P. monodon* larvae. However, this technique is time consuming, and does not enable the pinpointing of the exact location of the bacteria. Moreover, when a particular strain is used, it does not guarantee its differentiation from the naturally occurring bacteria. Compared to the paraffin-carving technique, use of a fluorescent stain has several advantages for specific marking of bacteria for pathological studies. It allows selective observation of bacteria inside and outside the host, ensuring that the inoculated bacteria alone are targeted. Using this technique, our challenge experiment showed that the highly pathogenic strain (IPEY54) colonized the gut after 24 h. In the non-pathogenic strain (IPEY41), the number of fluorescently labelled bacteria was very low and no patches were observed in the gut. This result may indicate the association of the pathogenic strain with the mortality of *Artemia* nauplii. Another study suggested that *Artemia franciscana* nauplii have a maximum capacity for ingestion of bacterial cells ranging from 10² to 10⁴ c.f.u. ml⁻¹ per

nauplius, independent of the bacterial density to which they are exposed but dependent on the bacterial strain employed (Soto-Rodriguez *et al.*, 2003b). In the present study when the highly pathogenic (IPEY54) and the non-pathogenic (IPEY41) strains were used for challenge at the concentration of 10⁵ c.f.u. ml⁻¹ with *Artemia* nauplii, the bacterial load of IPEY54 increased from 10⁶ to 10⁸ c.f.u. ml⁻¹ over a 24 h period; however, the mechanism of pathogenicity is not clear. We assume that *V. campbellii* must have had a high growth rate in the gut of *Artemia*, and cell invasiveness might be involved as previously reported in *V. proteolyticus* (Verschuere *et al.*, 2000).

Conclusions

In this study, 88 strains were initially identified as *V. harveyi*-related bacteria by biochemical tests. In subsequent more detailed studies of 28 strains, the majority of the strains were found to be *V. campbellii*, establishing mono-specific dominance during the period of shrimp culture. Given the absence of symptoms of viral infection such as white spot disease throughout the shrimp culture, and favourable environmental conditions, the disease seen in the shrimp farm ponds seems to have been caused by *V. campbellii* only. In addition the source water was the same for all the farms, which will have helped to spread the disease in a short time. We have demonstrated the pathogenic potential of *V. campbellii* in a crustacean model to support this view. This appears to be the first report regarding the isolation of large numbers of *V. campbellii* from diseased shrimp farms and demonstrating their pathogenicity in an *Artemia* experimental model. It will be worthwhile to study the mechanisms of pathogenicity to help develop prophylactic measures against this newly emerging *Vibrio* sp. to reduce its spread.

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