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Incidence and Molecular Typing of *Vibrio parahaemolyticus* from Tiger Shrimp Culture Environments along the Southwest Coast of India

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Vibrio parahaemolyticus is one of the most prevalent food-borne pathogens along the southwest coast of India, where marine foods are frequently consumed. Shrimp (*Penaeus monodon*) and environmental samples were collected from aquaculture farms located in and around Cochin. Confirmation of the biochemically identified strains with species-specific *tox*R gene and detection of virulent genes viz., *tdh* and *trh* was performed by polymerase chain reaction (PCR). The phenotypic markers for the presence of *tdh* and *trh* genes were assayed by Kanagawa phenomenon and urease activity, respectively. Protease activity was examined to identify other potential virulence factors. After phenotypic characterization of bacterial strains fingerprinting of genomic DNA was carried by various typing methods, *viz*., random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus sequence (ERIC), repetitive extragenic palindromic sequence (REP), and ribosomal gene spacer sequence (RS) PCR methods to assess the genetic diversity within the isolates. Eighteen percent of the samples were found positive for the incidence of *V. parahaemolyticus* by biochemical protocols and *toxR* (368 bp) targeted PCR. PCR analyses revealed 1% of the samples positive for *tdh* (269 bp) and *trh* (500 bp) gene. RAPD analysis revealed clustering of toxigenic strains into a single group. Cluster analysis revealed the conglomeration of isolates into two, five, and seven major groups using RS, ERIC, and REP PCR methods, respectively. RS PCR generated fewer amplified bands compared to REP and ERIC PCR methods, thus giving scope for higher discrimination. Moreover, RS PCR patterns were more discernible visually from other patterns, suggesting RS PCR as a considerably practical method for routine use.

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Key Words: PCR; *Penaeus monodon*; tdh; trh; typing; *Vibrio parahaemolyticus*

INTRODUCTION

Aquaculture production in India has increased considerably during recent years, contributing more than 4% to the world aquaculture (Ayyappan and Biradar, 2002). *Penaeus monodon* (Fabricius, 1798) is one of the most popular and commercially important brackish water tiger shrimp in India. Cochin accounts for more than 90% of statewide exports dominated by shrimp. Reports show that food poisoning due to the consumption of seafood contaminated with *V. parahaemolyticus* has increased considerably during recent years in the United States, Japan, and Korea (Daniels et al., 2000a; Lee et al., 1997), and in India it has almost doubled (Chowdhury et al., 2000). *V. parahaemolyticus* is a Gram-negative, halophilic bacterium ubiquitous in marine and estuarine environments. It is one of the leading causes of seafood-borne gastroenteritis, especially in countries where seafood consumption is high (Trison and Kelly, 1984). Acute enteritis caused by *V. parahaemolyticus* is characterized by a self-limiting watery diarrhoea, often accompanied by nausea, vomiting, and/or abdominal cramps; occasionally bloody diarrhoea can also occur (Potasman et al., 2002). Almost all *V. parahaemolyticus* strains isolated from clinical specimens exhibit a β-type haemolysis on Wagatsuma agar, called the Kanagawa phenomenon (KP), whereas only 1–2% of environmental strains were found to exhibit this phenomenon (Cook et al., 2002). These pathogenic strains were found to produce virulence proteins *viz*., thermostable direct haemolysin (TDH), TDH-related haemolysin (TRH), or both (Nishibuchi and Kaper, 1995). The phenotypic markers for the presence of *tdh* and *trh* genes were assayed by Kanagawa phenomenon and urease activity, respectively (Suthienkul et al., 1995; Osawa et al., 1996; Okuda et al., 1997), while the species-specific gene *toxR* (toxin regulatory) was reported to be present in all the *V. parahaemolyticus* strains (Tada et al., 1992; Kim et al., 1999; Chakraborty and Surendran, 2008) irrespective of their pathogenicity. In recent studies, extracellular proteases produced by pathogenic vibrios (Lee et al., 1997) were also directly correlated to the pathogenicity and virulence of the organism.

Subspecies typing of *V. parahaemolyticus* isolates may be useful for tracking the organisms causing disease and as well as the ecology of the strains. A number of molecular methods for typing of this bacterium have been described. Di Pinto et al. (2005) used collagenase gene sequences as a genetic marker for species identification of *V. alginolyticus, V. cholerae,* and *V. parahaemolyticus*. For epidemiological purposes, different molecular typing methods, such as pulsed-field gel electrophoresis (PFGE) or ribotyping, have been developed for this pathogen; however, these methods are mostly labor-intensive and time-consuming (Wong and Lin, 2001). RAPD analysis has the merits of being less labor-intensive and faster to complete (Wong et al., 1999;

Chakraborty et al., 2008). Analysis of the enterobacterial repetitive intergenic consensus sequence (ERIC) PCR has been previously used for the subtyping of *Vibrio cholerae* (Rivera et al., 1995). It was found to be a useful tool for evaluating genetic and epidemiological relationships among *V. parahaemolyticus* strains (Marshall et al., 1999). Besides ERIC-PCR, methods based on the highly conserved ribosomal gene spacer sequence (RS) and the 38-bp repetitive extragenic palindromic sequence (REP) in *Enterobacteriaceae* and other bacteria have been used for typing pathogenic bacteria (Stubbs et al., 1999) such as *V. parahaemolyticus*. The discriminative abilities of these PCR methods closely approached or even exceeded those of PFGE and ribotyping (Wong and Lin, 2001).

In this study, we report the incidence, isolation, characterization, and comparison of typing methods used for evaluating genetic relationships in the strains of *V. parahaemolyticus* isolated from *Penaeus monodon* and its environmental samples collected from farms located in and around Cochin on the southwest coast of India.

MATERIALS AND METHODS

Sample Collection, Processing, and Enrichment

Samples were collected fortnightly over a period of one year from four brackish water farms located in and around Cochin for studying the incidence of *V. parahaemolyticus* in tiger shrimp (*Penaeus monodon*), water, and sediment samples. Standard statistical methods were adopted in collection of samples and processed under sterile conditions within 2 h of collection. Bacteriological analysis was performed with the three water samples separately and average counts were taken. Water sample (25 ml) was added to sterile alkaline peptone water (APW, 225 ml) containing 3% NaCl (w/v) for enrichment. Four random samples of sediment from each culture farm were collected aseptically in sterile polythene bags. The samples were centrifuged ($8500 \times g$, 5 min) and 25 g of each sediment sample was added to APW (225 ml). Similarly, shrimp samples (25 g) were homogenized with APW (225 ml) in a sterile polythene stomacher bag and blended in a homogenizer (Stomacher 400 Seaward medicals, UK) at 230 rpm for 1 min. The samples were incubated at 37°C for 18–24 h for enrichment. The total number of samples collected from each farm along with their codes and time of sampling is given in Table 1.

Isolation and Identification of *V. parahaemolyticus*

After 18–24 h of incubation, 0.5 ml of broth was aseptically pipetted into preset dried (56°C, 45 min) thiosulphate citrate bilesalt sucrose (TCBS) agar plates and incubated for 24–30 h. About three to four typical colonies having

Table 1: Presumed *Vibrio parahaemolyticus* isolates from various farms isolated at regular intervals from different sources.

		Farms			
Month	**source**	**Pngd**	**Vypn**	**Chertl**	**Challanm**
Jan	Water	PngWj	VypWj	ChrWj	ChallWj
	Shrimp	PngSj	VypSj	ChrSj	ChallSj
	Sediment	PngDj	VypDj	ChrDj	ChallDj
Feb	Water	PngWf	VypWf	ChrWf	ChallWf
	Shrimp	PngSf	VypSf	ChrSf	ChallSf
	Sediment	PngDf	VypDf	ChrDf	ChallDf
Mar	Water	PngWr	VypWr	ChrWr	ChallWr
	Shrimp	PngSr	VypSr	ChrSr	ChallSr
	Sediment	PngDr	VypDr	ChrDr	ChallDr
April	Water	PngWa	VypWa	ChrWa	ChallWa
	Shrimp	PngSa	VypSa	ChrSa	ChallSa
	Sediment	PngDa	VypDa	ChrDa	ChallDa
May	Water	PngWm	VypWm	ChrWm	ChallWm
	Shrimp	PngSm	VypSm	ChrSm	ChallSm
	Sediment	PngDm	VypDm	ChrDm	ChallDm
June	Water	PngWj	VypWj	ChrWj	ChallWj
	Shrimp	PngSj	VypSj	ChrSj	ChallSj
	Sediment	PngDj	VypDj	ChrDj	ChallDj
July	Water	PngWl	VypWl	ChrWl	ChallWl
	Shrimp	PngSl	VypSl	ChrSl	ChallSl
	Sediment	PngDl	VypDl	ChrDl	ChallDl
Aug	Water	PngWg	VypWg	ChrWg	ChallWg
	Shrimp	PngSg	VypSg	ChrSg	ChallSg
	Sediment	PngDg	VypDg	ChrDg	ChallDg
Sep	Water	PngWs	VypWs	ChrWs	ChallWs
	Shrimp	PngSs	VypSs	ChrSs	ChallSs
	Sediment	PngDs	VypDs	ChrDs	ChallDs
Oct	Water	PngWo	VypWo	ChrWo	ChallWo
	Shrimp	PngSo	VypSo	ChrSo	ChallSo
	Sediment	PngDo	VypDo	ChrDo	ChallDo
Nov	Water	PngWn	VypWn	ChrWn	ChallWn
	Shrimp	PngSn	VypSn	ChrSn	ChallSn
	Sediment	PngDn	VypDn	ChrDn	ChallDn
Dec	Water	PngWd	VypWd	ChrWd	ChallWd
	Shrimp	PngSd	VypSd	ChrSd	ChallSd
	Sediment	PngDd	VypDd	ChrDd	ChallDd

Pngd, Vypn, Chertl, Challanm indicates different farms with their codes; W, S, D codes for water, shrimp and sediment respectively, and the time of sampling has been coded with Jan (j), Feb (f), Mar (r), April (a), May (m), June (j), July (l), Aug (g), Sep (s), Oct (o), Nov (n), Dec (d).

green or bluish green color with dark blue or green centre measuring about 3–5 mm were picked from TCBS plate, and each one was inoculated into sterile sucrose broth supplemented with NaCl (3% w/v). The colonies that were randomly picked were checked for purity on the nonselective medium to confirm the purity of the isolates. Only sucrose nonfermenting colonies were streaked onto sterile tryptone soy agar (TSA) slants supplemented with NaCl (3% w/v) and maintained at room temperature for further identification. Growth sensitivity to NaCl (0, 3, 6, 8, and 10% w/v) test was performed using

tryptone broth. Additional characterization tests for the identification of *V. parahaemolyticus* namely, Gram staining, catalase, cytochrome oxidase tests, triple sugar iron tests, lysine iron agar tests, arginine dehydrolase tests, lysine and ornithine decarboxylase test, and O/129 susceptibility tests, were performed (USFDA, 2001). Tests for glucose oxidation-fermentation were carried out using Hugh-Leifson broth, and arabinose, lactose, mannitol, mannose, salicin, and inositol fermentation tests were also performed. The presumed positive cultures were further confirmed with a RAPID Hi-Vibrio™ identification kit (KB007, Himedia, India).

Detection of Phenotypic Markers for *tdh* and *trh*

To detect the production of thermostable direct haemolysin (*tdh*) from *V. parahaemolyticus* isolates, the KP was studied on Wagatsuma agar (Elliot et al., 1992). Briefly, the test strains were inoculated and incubated at 37°C. β-haemolysis around colonies within 24 h was judged as positive Kanagawa reaction (KP+). As phenotypic marker of *trh* gene from *V. parahaemolyticus* isolates, the urease activity (Ure) was assayed in the Christensen's urea agar (Andrews and Hammack, 2001).

Detection of Protease Activity

Protease activity of *V. parahaemolyticus* isolates was examined using single diffusion method on nutrient agar containing beef extract (3g/L), peptone (10g/L), supplemented with NaCl (3% w/v) and agar (1.5% w/v). This medium was supplemented with skimmed milk (5% w/v) for carrying out the test (Iyer et al., 2000). Briefly, the molten agar (18-20 ml) was poured into the sterilized Petri plates, allowed to solidify, and the test cultures were seeded on the agar by surface inoculation methods followed by incubation at 37°C for 20–24 h. The test was considered positive when an opaque halo was observed around the growth.

Bacterial Lysate for PCR Assay

Bacterial lysate was prepared following established procedure (Ausubel et al., 1987). The biochemically identified isolate was streaked on TSA plates. The colonies were inoculated in the Luria Bertani (LB) broth supplemented with NaCl (2% w/v) and incubated at 37°C with shaking (120 rpm) for 16–18 h. The broth cultures were centrifuged (12,000 x g, 4°C, 1 min; 5804 R, Eppendorf, Germany) to obtain the pellet, which was washed with normal saline (0.8% NaCl w/v) and resuspended with DNA-free sterile distilled water (200 μL). The resulting suspension was heated at 98 ± 2°C for 15–20 min in a water bath to lyse the cells and to release the DNA. The lysate was clarified by centrifugation (12,000 × g, 4°C, 5 min), and the supernatant was stored (–20°C) until further use.

The PCR targeting *toxR* gene of *V. parahaemolyticus* was performed (*21*) using primer pairs (5' GTC TTC TGA CGC AAT CGT TG 3' and 5' ATA CGA GTG GTT GCT GTC ATG 3') in a thermocycler (Eppendorf Mastercycler, Germany). The PCR products were resolved on agarose (1.5% w/v) gel electrophoresis.

Extraction and Purification of Chromosomal DNA

Genomic DNA from *V. parahaemolyticus* isolates was extracted following the method of Ausubel et al. (1987). Briefly, a colony with typical characteristics was picked from the TCBS plate and was grown overnight in LB broth containing NaCl (3% w/v) and incubated at 37°C with shaking (120 rpm) for 16–18 h. DNA was extracted from culture in exponential phase of growth of the organism by giving a wash with normal saline (NaCl 0.8% w/v), followed by resuspending in TE buffer (10 mM Tris–Cl, 1 mM Na_2EDTA, pH 8.0). Alkaline lysis was performed with sodium dodecyl sulfate (20% w/v) (SDS, SRL India) and proteinase K (20 mg/ml, Sigma, Aldrich Chemical, USA). After 1 h of incubation at 37°C, NaCl (5 M) was added along with cetyl trimethyl ammonium bromide/NaCl solution to complex with the polysaccharides. DNA was purified from proteins and other cellular constituents using an equal volume of chloroform-isoamyl alcohol (24:1 v/v) followed by centrifugation (12,000 × g) for 5 min. Further purification of the supernatant was achieved by adding an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1 v/v) to the supernatant, followed by centrifugation (12,000 × g). Sodium acetate (0.1 volume, 3M, pH 5.2) was added to the supernatant to chelate the salts followed by chilled absolute alcohol (2 volume) to precipitate the DNA. The sample (0.2 ml) was centrifuged (12,000 × g) for 10 min and the DNA pellet was washed once with cold ethanol (70% v/v) before being dried under vacuum. The purified DNA was resuspended in TE buffer and stored at –20°C until further use.

Multiplex PCR Amplification

Multiplex PCR (m-PCR) amplification was performed following established procedure (Bej et al., 1999) with some modifications. m-PCR amplification was optimized in a total reaction volume of 50 μL consisting of sterile Milli Q water (29.9 μL), 2.5 μL of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl and 15 mM $MgCl_2$), oligonucleotide primers (30 pm) for *tdh, trh* (2.5 μL) each and *toxR* (1.5 μL) gene, 0.5 μL dNTP mix (200 mM, Finnzymes, Finland), template (2 μL, pure genomic DNA), additional 1.5 μL, $MgCl_2$ (2.5 mM), and 0.6 μL, *Taq* DNA polymerase (3units/μl, Bangalore Genei, India). The primer sequences were listed in Table 2. The components were mixed well, and the PCR amplification of the target sequence was arranged in a thermocycler (Eppendorf Mastercycler, Germany) and

Table 2: Primers used in the study.

Typing method	Primers	Nucleotide sequence	Reference
toxR	Forward	5′ GTC TTC TGA CGC AAT CGT TG 3′	Kim et al. (1999)
	Reverse	5′ ATA CGA GTG GTT GCT GTC ATG 3′	
trh	Forward	5′ TTG GCT TCG ATA TTT TCA GTA TCT 3′	Bej et al. (1999)
	Reverse	5′ CAT AAC AAA CAT ATG CCC ATT TCC G 3′	
tdh	Forward	5′GTA AAG GTC TCT GAC TTT TGG AC3′	Bej et al. (1999)
	Reverse	5′TGG AAT AGA ACC TTC ATC TTC ACC 3′	
RS PCR	Forward	5′CAA GGC ATC CAC CGT3′	Wong and Lin (2001)
	Reverse	5′GAA GTC GTA ACA AGG3′	
ERIC PCR	Forward	5′ATG TAA GCT CCT GGG GAT TCA C3′	Wong and Lin (2001)
	Reverse	5′AAG TAA GTG ACT GGG GTG AGC G3′	
REP PCR	Forward	5′NNN RCG YCG NCA TCM GGC3′	Wong and Lin (*45*)
	Reverse	5′RCG YCT TAT CMG GCC TAC3′	

programmed for 30 cycles of amplification. Each cycle consisted of three-step reactions, that is, initial denaturation (94°C, 3 min) followed by 30 cycles of denaturation (94°C, 1 min) and annealing (58°C, 1 min). The primer extension was carried out at 72°C (1 min). Following the amplification cycles, samples were kept at 72°C for 10 min to allow final extension of an incompletely synthesized DNA. PCR-amplified products were then separated in an agarose (1.8% w/v) gel electrophoresis. The amplicon sizes were determined by comparison with standard 100-bp DNA molecular weight marker (Gene Ruler, Fermentas International, Germany). The gel was stained with ethidium bromide (0.5 mg/ml) and visualized under a UV transilluminator (Alpha Imager, Innotech Corporation, USA).

DNA Sequencing

Primers based on the *toxR, trh,* and *tdh* gene fragments were used to amplify and to sequence DNA of these amplicons using Beckman coulter CEQ 8000 genetic analysis system (Bioserve Biotechnologies, India). *V. parahaemolyticus* isolates, which generated amplicons of the expected size, that is, 368 bp for *toxR,* 500 bp for *trh*, and 269 bp for *tdh,* were used for sequencing. Sequence assembly, analysis, and alignments were performed with DNA using Chromas LITE software (Version 2.01, Technelysium Pvt. Ltd., Australia). The

nucleotide sequence data was analyzed for homology with the published sequence in the GenBank database using the Basic Local Alignment Search Tool (BLAST), and percent homology was noted. The nucleotide sequences were submitted to the GenBank to obtain the accession numbers.

Rapid Amplified Polymorphic DNA (RAPD) PCR Assay

RAPD was carried out using an oligonucleotide primer, 5'CAG GCG CAC A3' (Wong, 2003). PCR reaction conditions have been optimized for important parameters such as primer annealing temperature and concentrations of $MgCl_2$, template DNA, *Taq* DNA polymerase, dNTPs, and primer. The PCR reaction mixture consisted of reaction buffer (2.0 μL, 10 × *taq*) (100 mM Tris–HCl pH 8.3, 500 mM KCl, 20 mM $MgCl_2$ and 0.001% gelatin), dNTP mix (1.0 μL, 200 mM), 0.5 μL of *Taq* DNA polymerase (2.5 units, Fermentas 5U/μL), primer (30 pm), DNA template (1 μL), and an additional 3.0 μL of $MgCl_2$ (2.5 mM) adjusted to a reaction volume of 25 μL with an appropriate volume of sterile MilliQ water. Amplifications were performed on a thermocycler (Eppendorf Mastercycler, Germany), which was programmed for an initial denaturation (94°C, 5 min) followed by 45 cycles of denaturation (94°C, 1 min) and primer annealing (38°C, 1 min, 30 sec). The extension was carried out at 72°C (2 min, 30 sec) followed by final extension at 72°C (10 min).

ERIC, RS, and REP PCR Assay

Vibrio parahaemolyticus isolates, positive (*V. parahaemolyticus*, NCMB 1902, MTCC 451) and negative controls (*V. alginolyticus*, MTCC 4439) were grown overnight at 37°C on a tryptic soy agar plate. Total DNA was purified from overnight culture broths following the standard procedure (Ausubel et al., 1987). The primers used for all PCRs (ERIC, RS, and REP) are listed in Table 2. PCR amplification was optimized in a total reaction volume of 25 μL consisting of sterile Milli Q water (11.9 μL), 10X PCR buffer (2.5 μL), each primer (2.5 μL), dNTP mix (1.0 μL, 200 μM), template (1 μL), and *Taq* DNA polymerase (0.6 μl, 3U/μL) and $MgCl_2$ (3.0 μL, 2.0 mM) was mixed well. All amplifications were performed on a thermocycler (Eppendorf Mastercycler, Germany) comprising 30 cycles. Each cycle consisted of initial denaturation (95°C, 7 min), annealing (52°C, 1 min), and extension (72°C, 5 min) for ERIC PCR; initial denaturation (95°C, 7 min), annealing (45°C, 1 min), and extension (65°C, 5 min) for REP PCR; and initial denaturation (95°C, 7 min), annealing (55°C, 1 min), and extension (70°C, 5 min) for RS PCR followed by a final extension (70°C, 5 min) remaining the same for all the three PCRs. Amplified products were resolved on agarose gels (2.0% w/v) stained with ethidium bromide (Sigma, 0.5 μg/mL). Electrophoresis was carried out at 7–10 V cm^{-1} for 90 min in tris acetate (TAE, pH 8.0) buffer. A 100-bp DNA ladder was used as a molecular

size marker (Gene RulerTM, Fermentas, Germany). The resolved bands were visualized on UV-transilluminator at a wavelength of 360 nm and photographed using an UV gel documentation system (Alpha Imager, Innotech Corporation, USA).

Cluster Analysis

Similarity matrix was built for *V. parahaemolyticus* isolates using Pearson's correlation co-efficient. Cluster analysis was performed and dendrogram was constructed using the data matrix of all the strains isolated from farm samples based on unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973) using Gel Compar II software, version 4.0 (Applied-Maths, St-Martens-Latem, Belgium).

RESULTS

A total of 144 samples were collected during the study, of which 18% were found to be positive for the incidence of *V. parahaemolyticus*. The highest incidence was found in shrimp (46%) followed by water (35%) and sediment (19%). The presumed positive cultures were tested with a RAPID Hi-Vibrio^{TM} identification kit (KB007, Himedia, India) for additional confirmation. Multiplex-PCR revealed the presence of *tox*R gene in all biochemically confirmed isolates. Virulent genes viz., *trh* and *tdh* were amplified in 1% of the strains isolated from water and shrimp samples (Fig. 1). Ten percent of the isolates exhibited weak haemolysis, while only 1% of the isolates exhibited β-haemolysis and urease activity on Wagatsuma and Christensen's agar, respectively. However, protease activity was observed in 80% of the isolates. Enrichment of samples revealed higher counts (cfu per mL/g) in sediment samples (2.5×10^2 to 2.7×10^9) followed by shrimp (0.5×10^2 to 3.2×10^4) and water (0.4×10^1 to 3.3×10^3) particularly in the summer months (Table 3). The nucleotide sequences of the amplified products were analyzed for homology with the published sequence in the GenBank database using the Basic Local Alignment Search Tool (BLAST) and percent homology was noted. The *toxR, trh,* and *tdh* gene fragments exhibited 95–99%, 93–97%, 93–95% similarity, respectively, with the published sequences. The accession numbers obtained from the GenBank for the selected isolates were bankit841705 EF016489, bankit914939 EF640380, and bankit916366 EF640372 for *tox*R, *trh,* and tdh genes, respectively. All the positive confirmed isolates along with their sample codes and typing codes used for carrying out various PCR reactions are given in Table 4.

RAPD-PCR Analysis of *V. parahaemolyticus* Isolates

Gel electrophoresis pattern of RAPD-PCR products revealed 6–8 bands ranging approximately from 0.25–2.5 Kb (Fig. 2). The resemblance of typing

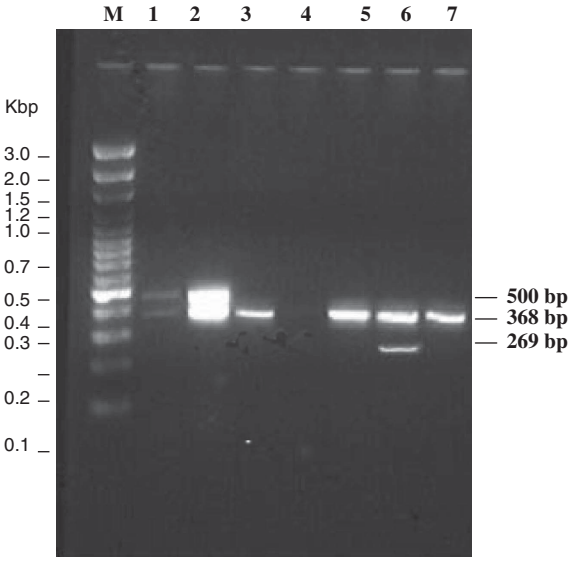

Figure 1: Agarose gel electrophoresis of PCR-amplified products using multiplex PCR for *trh* (500 bp), *toxR* (368 bp), *tdh* (269bp) genes; Lane M: DNA ladder (100-bp); Lanes 1: *V. parahaemolyticus* type strain (NCMB 1902) with *trh* and *toxR* genes, Lane 2: *V. parahaemolyticus* isolate with *trh* and *toxR* genes from shrimp (sample code: Pngsa), Lane 4: negative control, Lane 3, 5 and 7: strain with *toxR* gene from sediment samples (sample codes: VypDj, PngDr, VypDm), Lane 6: strain containing *toxR* and *tdh* genes from water sample (code: ChallWm).

Table 3: *Vibrio parahaemolyticus* counts (cfu per g/ml) from various sources.

Month	Shrimp	Water	Sediment
Jan	1.8×10^4	0.5×10^1	1.1×10^5
Feb	0.86×10^4	1.7×10^2	0.5×10^5
Mar	3.2×10^4	1.1×10^3	2.4×10^7
April	2.5×10^4	2.7×10^3	3.9×10^7
May	1.9×10^4	3.3×10^3	2.1×10^9
June	0.9×10^3	1.7×10^3	2.7×10^9
July	1.1×10^2	0.4×10^1	1.4×10^3
Aug	0.5×10^2	1.9×10^1	1.6×10^3
Sep	0.75×10^3	1.5×10^1	1.3×10^3
Oct	0.8×10^3	1.2×10^1	1.0×10^3
Nov	1.1×10^3	2.0×10^1	2.5×10^2
Dec	1.05×10^3	2.4×10^1	1.5×10^3

patterns among different isolates of *V. parahaemolyticus* was determined by cluster analysis, which is represented as dendrogram. Cluster analysis revealed two major groups A and B based on the similarity index (S). Cluster A was grouped into two subgroups *viz*., A1a and A1b. Cluster A1a included 26% of the strains isolated during September to November, clustered with subgroup

Table 4: *Vibrio parahaemolyticus* positive isolates from different farms indicating their source, typing code, time of sampling.

source	Typing code				source	Typing code				source	Typing code			
Shrimp	**RAPD**	**ERIC**	**REP**	**RS**	**Water**	**RAPD**	**ERIC**	**REP**	**RS**	**sediment**	**RAPD**	**ERIC**	**REP**	**RS**
PngSj	R1	E1	P2	S1	VypWj	R10	E11	P10	S10	VypDj	R19	E22	P19	S21
ChrSj	R2	E2	P3	S2	PngWf	R11	E12	P11	S11	PngDr	R20	E23	P20	S22
PngSf	R3	E3	P4	S3	ChallWf	R12	E13	P12	S12	VypDm	R21	E24	P21	S23
VypSf	R4	E4	P5	S4	PngWr	R13	E14	P13	S13	VypDn	R22	E25	P22	S24
ChrSr	R5	E5	P6	S5	VypWr	R14	E15	P14	S16	ChallDd	R23	E26	P23	S25
ChallSr	R6	E6	P7	S6	ChrWa	R15	E16	P15	S17					
PngSa	R7	E7	P8	S7	ChallWm	R16	E17	P16	S18					
ChrSa	R8	E8	P9	S8	PngWg	R17	E18	P17	S19					
VypSm	R9	E9	P24	S9	VypWo	R18	E19	P18	S20					
ChrSm	R24	E20	P25	S26										
PngSn	R25	E27	P26	S27										
ChrSd	R26	E28	P27	S28										

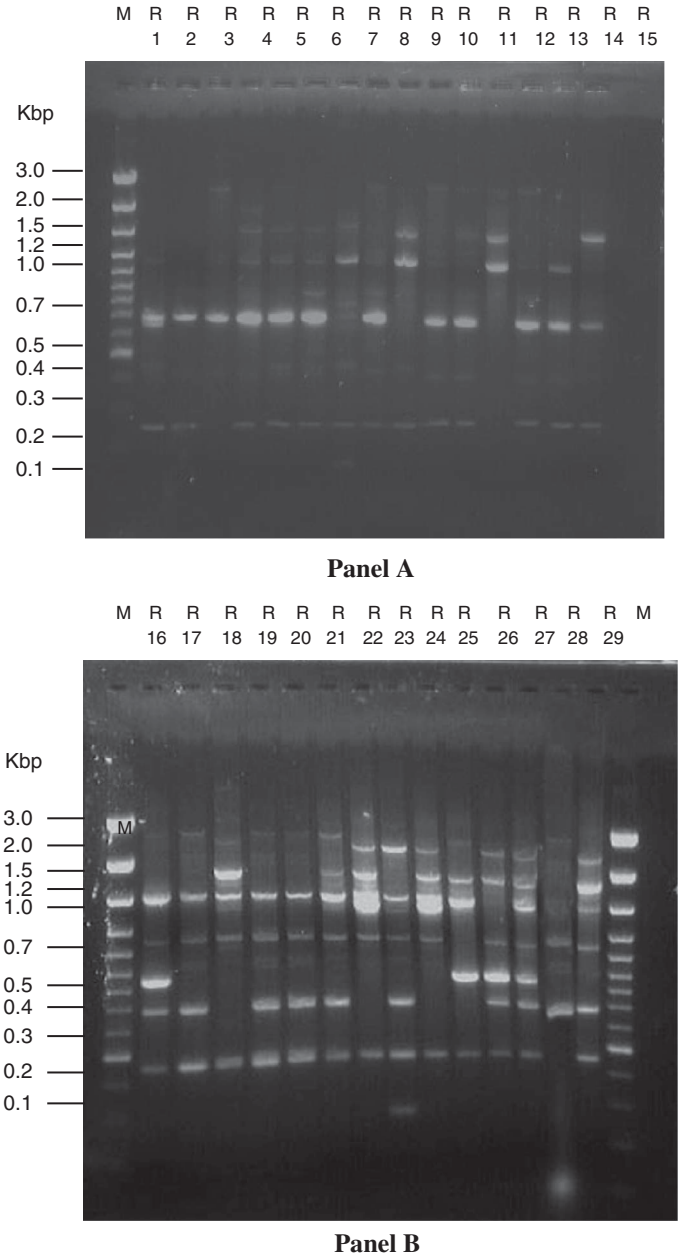

Figure 2: RAPD-PCR profiles of *Vibrio parahaemolyticus* strains isolated from farm samples. Lane M: 100-bp DNA ladder, Panel A: Lane 1–9 and 24–26 (sample code: R1 to R9 and R24 to R26) contain *V. parahaemolyticus* strains from shrimps isolates moreover Lane 9: *tdh* positive, Lanes 10–15 (sample code: R10 to R15) and Lanes 16–18 (sample code: R16 to R18) in Panel B contain *V. parahaemolyticus* strains isolated from water samples and Lane 18: *trh* positive and Lanes 19–23 (sample code: R19 to R23) strains isolated from sediment samples, Lane 27: *V. alginolyticus* (MTCC 4439) Lane 28: strain from *Escherichia coli,* Lane 29: *V. parhaemolyticus* type strain (NCMB 1902).

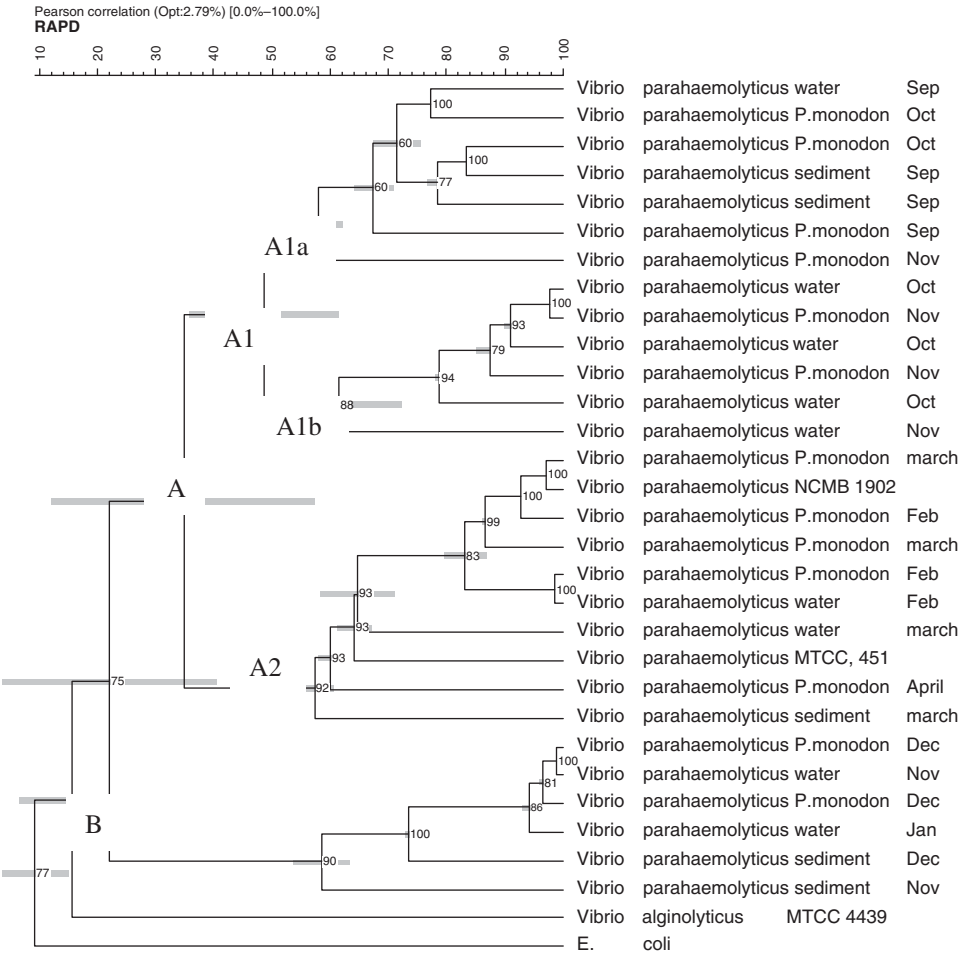

Figure 3: Phylogenetic analysis of RAPD profile of *Vibrio parahaemolyticus* isolates from farm samples. Bars are shown at each node, corresponding to the standard deviation of values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes are shown above the dendrogram. The similarity scale is shown above this dendrogram.

A1b at S >48%. A1b included the strains isolated during October to November clustered with subgroup A2 at S >35%. A2 cluster included strains (30%) isolated during February to April. Group A clustered with group B at S >20% had strains (22%) isolated during November to January. The positive controls formed cluster within the main cluster. *V. alginolyticus* type strain (MTCC 4439) which served as negative control (species wise) clustered at S >15% while *E. coli* (MTCC 729) clustered with the main cluster at S <10% (Fig. 3) with the main group.

ERIC-PCR Analysis of *V. parahaemolyticus* Isolates

The gel pattern of ERIC-PCR revealed 15–20 amplified bands with sizes ranging between 130 and 1,200 bp in the *V. parahaemolyticus* isolates. Several bands with molecular sizes of 330, 400, 520, 560, 660, 700, 800, 900, and 1,200 bp were common in most isolates, while 330, 400, 660, 700, and 900-bp bands were discernable in all the *V. parahaemolyticus* isolates (Fig. 4).

Dendrogram of ERIC-PCR analysis of *V. parahaemolyticus* cultures isolated from farm samples revealed five major clusters *viz*., A, B, C, D, and E based on seasonality of isolation rather than source of isolation. Cluster A subgrouped into A1, included strains (23.1%) isolated from water and shrimp samples during September and October. A1 clustered with subgroup A2 at S $\geq$54% placed cultures (19.2%) isolated from water, sediment, and shrimp during March to May along with the positive control. A2 clustered with group B at S $\geq$50% had a single culture isolated from water sample during May. Group B clustered with group C at S $\geq$45% subgrouped into C1 placed strains (19.2%) isolated during November and December clustered with C2 at S $\geq$60% included strains (7.7%) isolated during November to January. Group C clustered with group D at S $\geq$35% had 15.4% cultures isolated during March and April. Group D clustered with E at S $\geq$30% placed cultures isolated during January and February. *V. alginolyticus* formed a separate cluster at S $\geq$5% with the main cluster (Fig. 5).

RS-PCR Analysis of *V. parahaemolylticus* Isolates

The gel pattern of RS-PCR analysis of the farm samples produced six amplified bands with molecular sizes varying between 350 and 1500 bp, while three amplified bands (350, 650, and 720 bp) occurred in all the *V. parahaemolyt,icus* isolates (Fig. 6).

The dendrogram of RS-PCR analysis of isolates revealed two major clusters *viz*., A and B based on the seasonality rather than source of samples. Subgroup A1a placed strains (11.1%) isolated during November, clustered with A1b at S $\geq$50% included cultures (22.2%) isolated during September to November. Group A1 clustered with A2 at S $\geq$30% placed strains (18.5%) isolated during October and November. Thus group A includes the strains isolated during September to November clustered with group B at S $\geq$25% included the strains isolated during December to April. Subgroup B1 included isolates (22.2%) obtained during January to April including the *V. parahaemolyticus* type strain (NCMB 1902). B1 clustered with B2 at S $\geq$35% included isolates (26%) procured during December to March. Negative control formed a separate cluster distinctly away from the main cluster (Fig. 7).

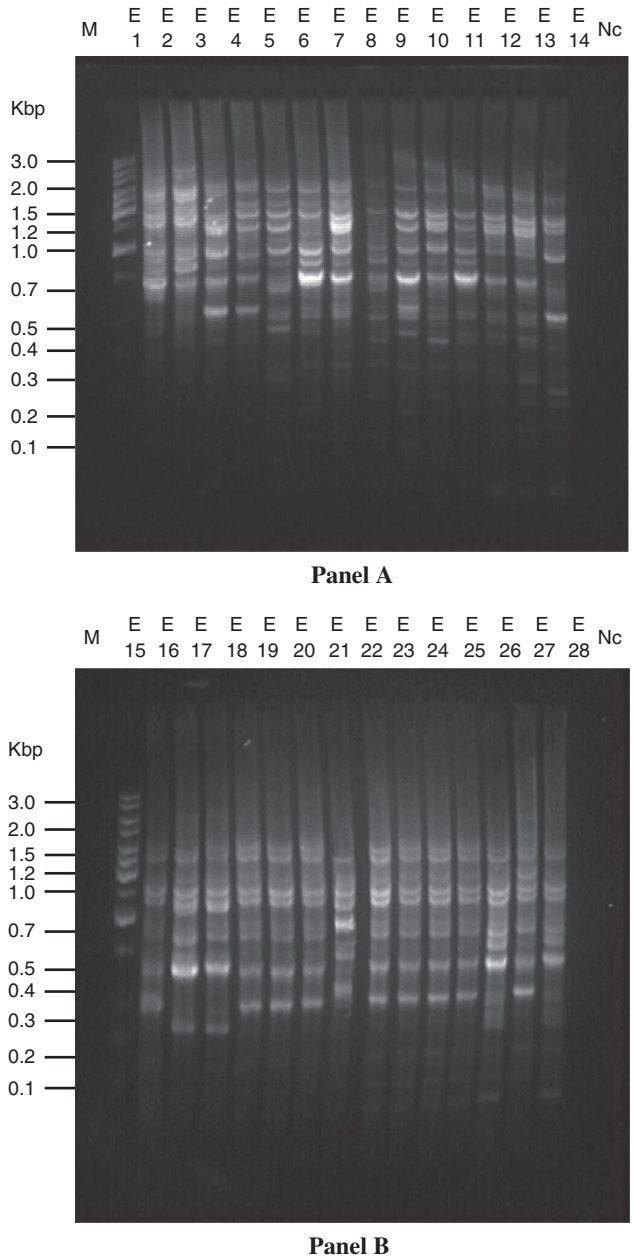

Figure 4: ERIC-PCR profiles of *V. parahaemolyticus* strains isolated from farm samples. Lane M: 100-bp DNA ladder, Lane Nc: PCR negative control, Panel A: Lanes 1–9 and 20, 27, 28 (sample code: E1 to E9 and E20, E27, E28) contain *V. parahaemolyticus* strains from shrimps isolates, Lane 10: contain *V. parahaemolyticus* type strain (NCMB 1902), Lanes 11–14 (sample code: E11 to e14) and Lanes 15–19 (sample code: E15 to E19) in Panel B contain *V. parahaemolyticus* strains isolated from water samples and Lane 21: contain *V. alginolyticus* type strian (MTCC 4439), Lanes 22–26 (sample code: E22 to E26) contain strains isolated from sediment samples.

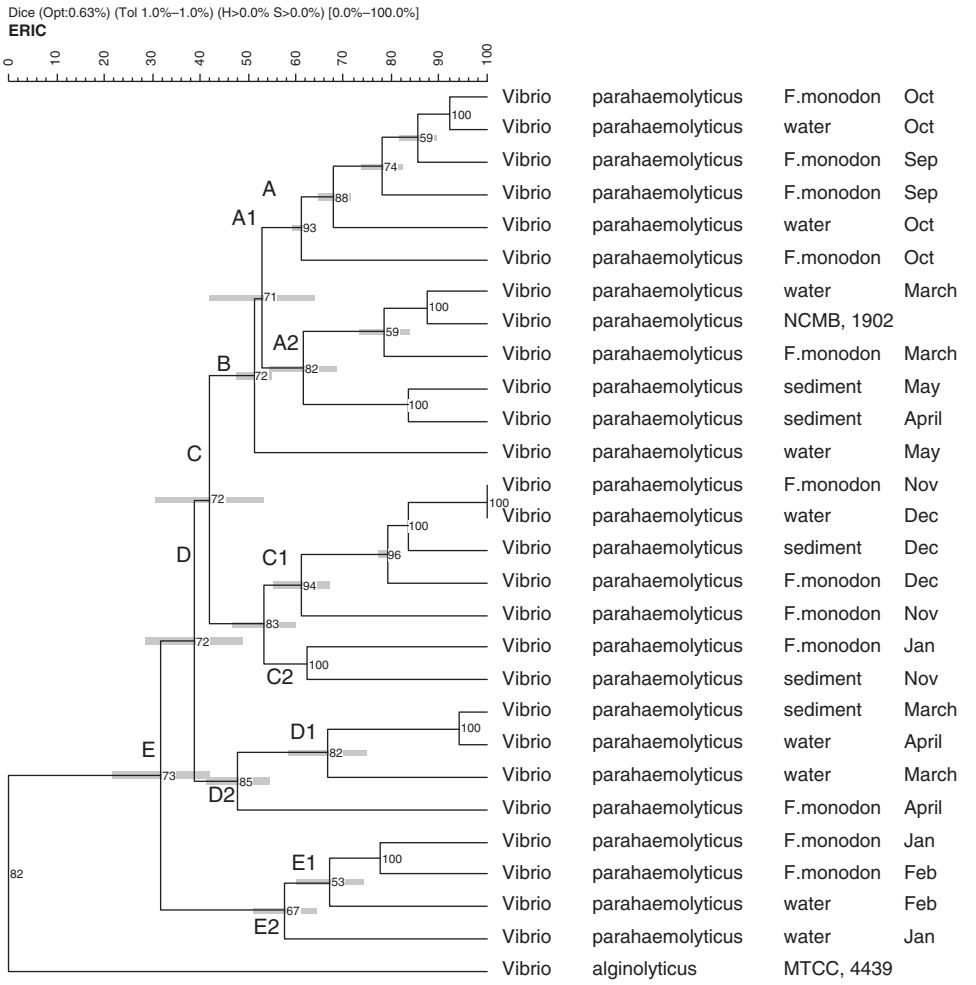

Figure 5: Phylogenetic analysis of ERIC-PCR profile of *V. parahaemolyticus* isolates from farms samples. Bars are shown at each node, corresponding to the standard deviation of values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes are shown above the dendrogram. The similarity scale is shown above this dendrogram.

REP PCR Analysis of *V. parahaemolyticus* Isolates

The gel pattern of REP-PCR analysis of cultures isolated from farm samples revealed 10 amplified bands with molecular sizes varying between 150 bp and 2500 bp. The bands with molecular sizes *viz*., 950 bp, and four amplified bands having 150, 480, 570, 600, and 950 bp molecular sizes were clearly visible in all the *V. parahaemolyticus* isolates (Fig. 8).

The dendrogram analysis revealed seven groups *viz*., A, B, C, D, E, F, and G based on seasonality of isolation rather than the source of samples. Group A

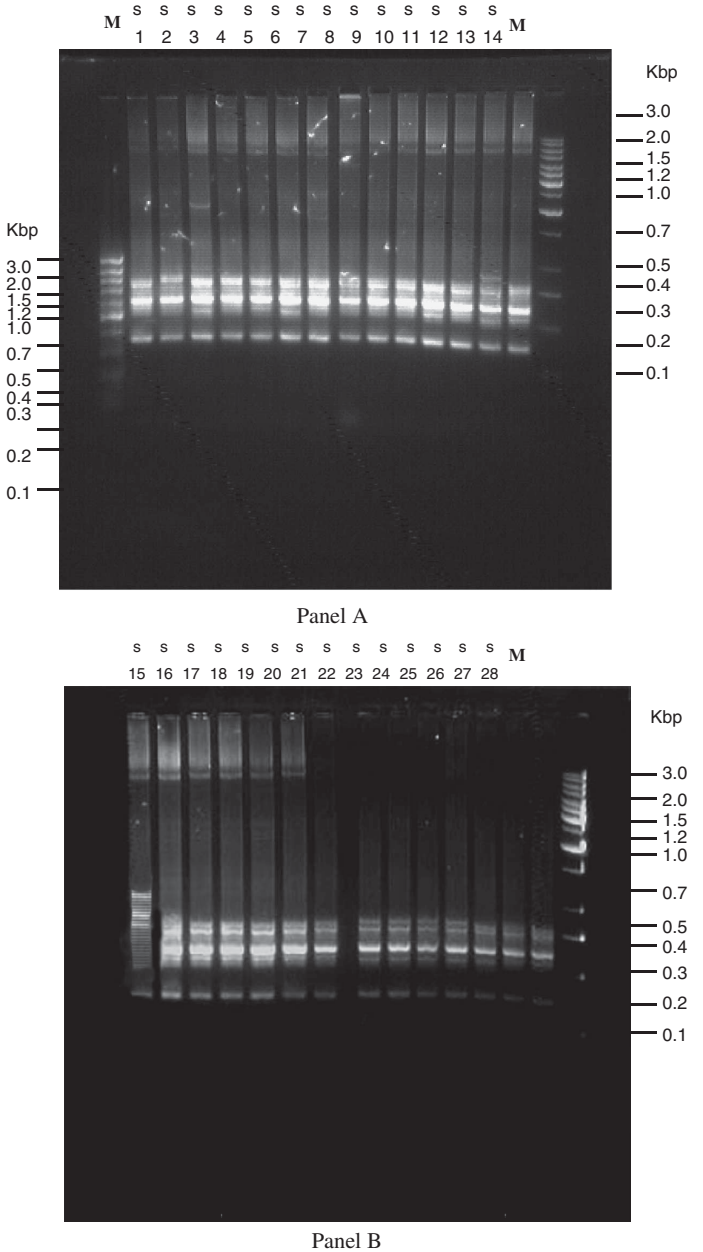

Figure 6: RS-PCR profiles of *V. parahaemolyticus* strains isolated from farm samples. Lane M: 100-bp DNA ladder, Lane 14: Type strain (NCMB 1902) Panel A: Lanes 1–9 and 26–28 (sample code: S1 to S9 and S26 to S28) contain *V. parahaemolyticus* strains from shrimps isolates, Lanes 10–13 (sample code: S10 to S13) and Lanes 16–20 (sample code: S16 to S20) in Panel B contain *V. parahaemolyticus* strains isolated from water samples and Lanes 21–25 (sample code: S21 to S25) contain strains isolated from sediment samples, Lane 15 contain strain from *V. alginolyticus* type strain (MTCC 4439).

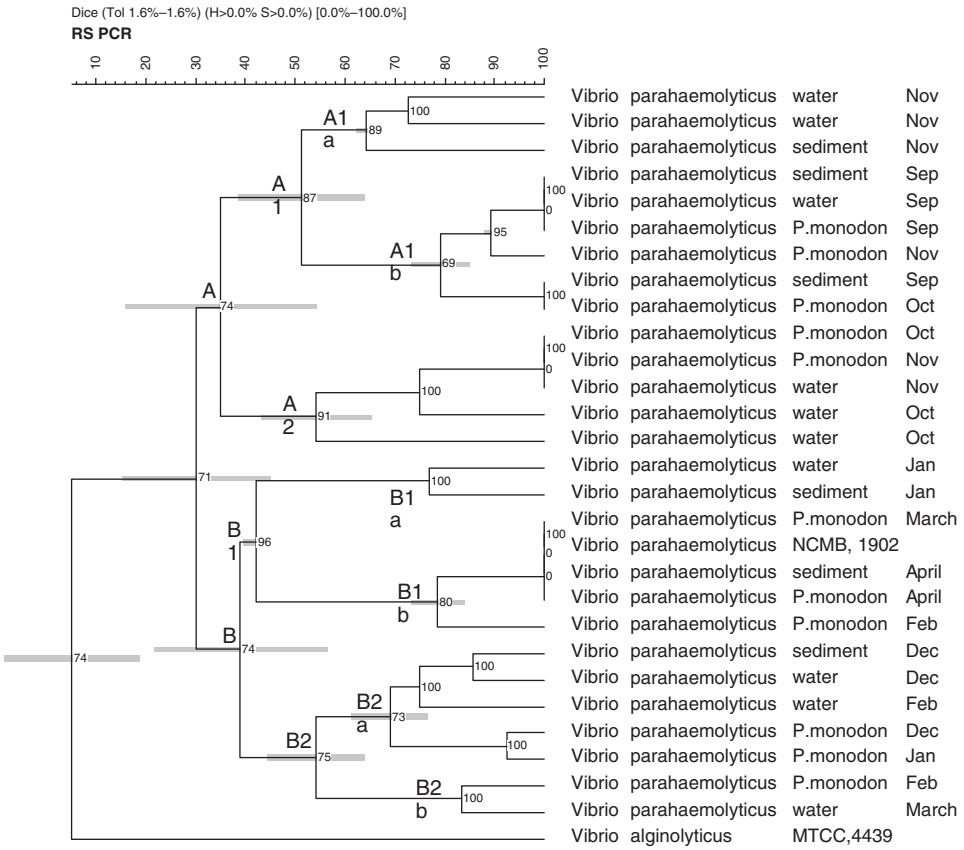

Figure 7: Phylogenetic analysis of RS profile of *V. parahaemolyticus* isolates from farm samples. Bars are shown at each node corresponding to the standard deviation of values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes are shown above the dendrogram. The similarity scale is shown above this dendrogram.

included 36% of the isolates of which A1 included the cultures isolated during March and April clustered with A2 at S ≥90%, which in turn placed cultures isolated during February to April. Group A clustered with group B at S ≥88% included a culture isolated from sediment during January. Group D clustered with group C at S ≥85% placed strains (12%) isolated during December to February. Group C clustered with group D at S ≥83% included strains obtained during December and February clustered with group E at S ≥75%. Group E clustered with group F at S ≥65% placed cultures (20%) isolated during September to November, which also included two positive controls. Group F clustered with group G that in turn included cultures (16%) isolated during September and October. Negative control formed a separate cluster distinctly away from the main cluster (Fig. 9). All isolates were assigned with a profile

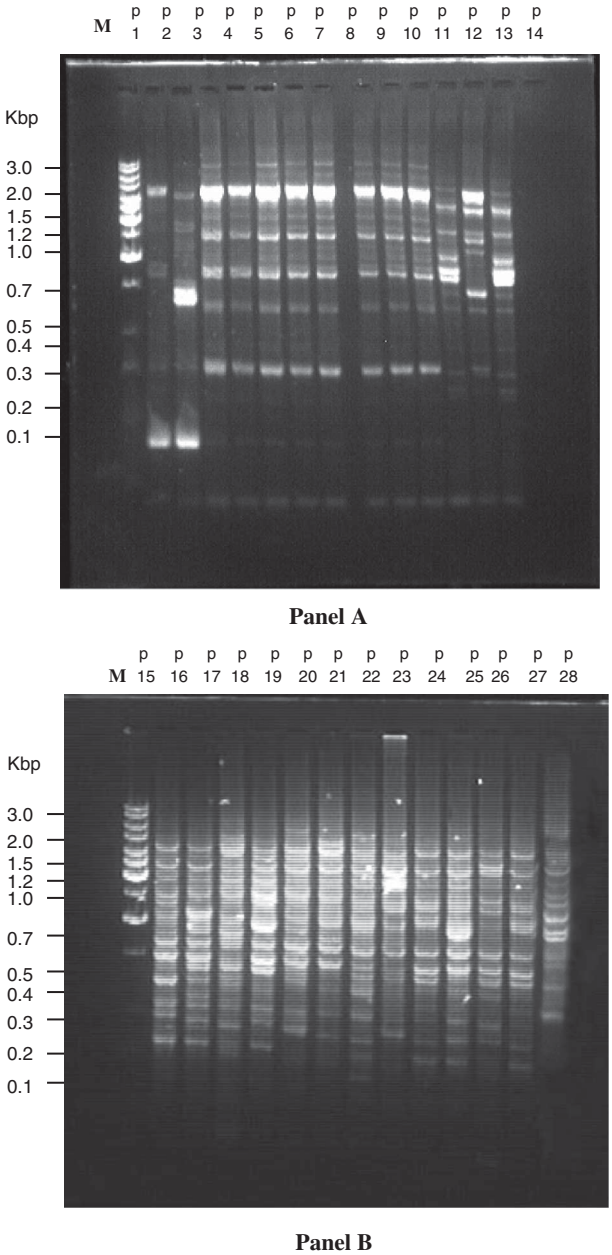

Figure 8: REP-PCR profiles of *V. parahaemolyticus* strains isolated from farm samples. Lane M: 100-bp DNA ladder, Panel A, Lane 1: *V. parahaemolyticus* type strain (NCMB 1902), Lane 2–9 (sample code: P2 to P9) and Lanes 24–27 (sample code: P24 to 27) contain *V. parahaemolyticus* strains from shrimps isolates, Lanes 10–14 (sample code: P10 to P14) and Lanes 15–18 (sample code: P15 to P18) in Panel B contain *V. parahaemolyticus* strains isolated from water samples and Lanes 19–23 (sample code: P19 to P23) contain strains isolated from sediment samples, Lane 27 *V. alginolyticus* type strain (MTCC 4439), Lane 28 PCR negative control.

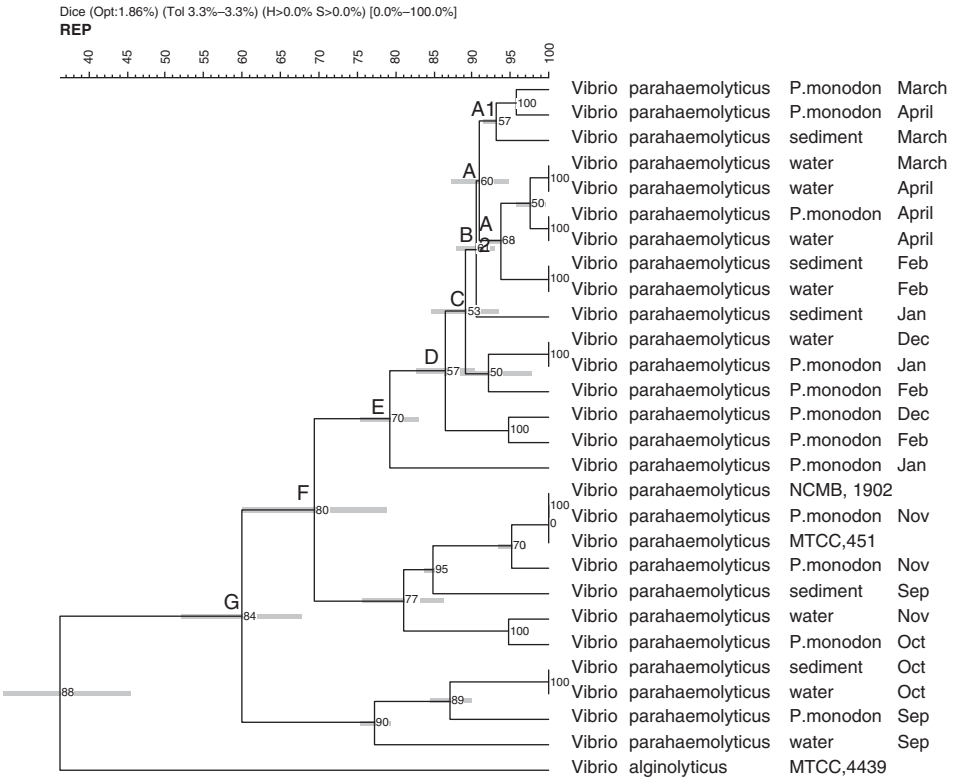

Figure 9: Phylogenetic analysis of REP profile of *V. parahaemolyticus* isolates from farm samples. Bars are shown at each node corresponding to the standard deviation of values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes are shown above the dendrogram. The similarity scale is shown above this dendrogram.

code, and a combined dendrogram constructed based on the combined profile data revealed three major clusters and subclusters within it (Fig. 10) based on the seasonality rather than source of sample.

DISCUSSION

Vibrio parahaemolyticus is a halophilic organism that has received considerable attention for its high recovery in seafood. It is the major cause of gastroenteritis, where raw, semi-processed seafood or cross contamination of temperature-abused cooked seafood by raw seafood are consumed as part of the daily diet (Honda and Iida, 1993). Although *V. parahaemolyticus* forms the common cause of gastroenteritis transmitted by seafood consumption (Daniels et al., 2000b), the true incidence was not known probably due to underreporting of cases and lack of proper study. Conventionally, seafood-borne microbial

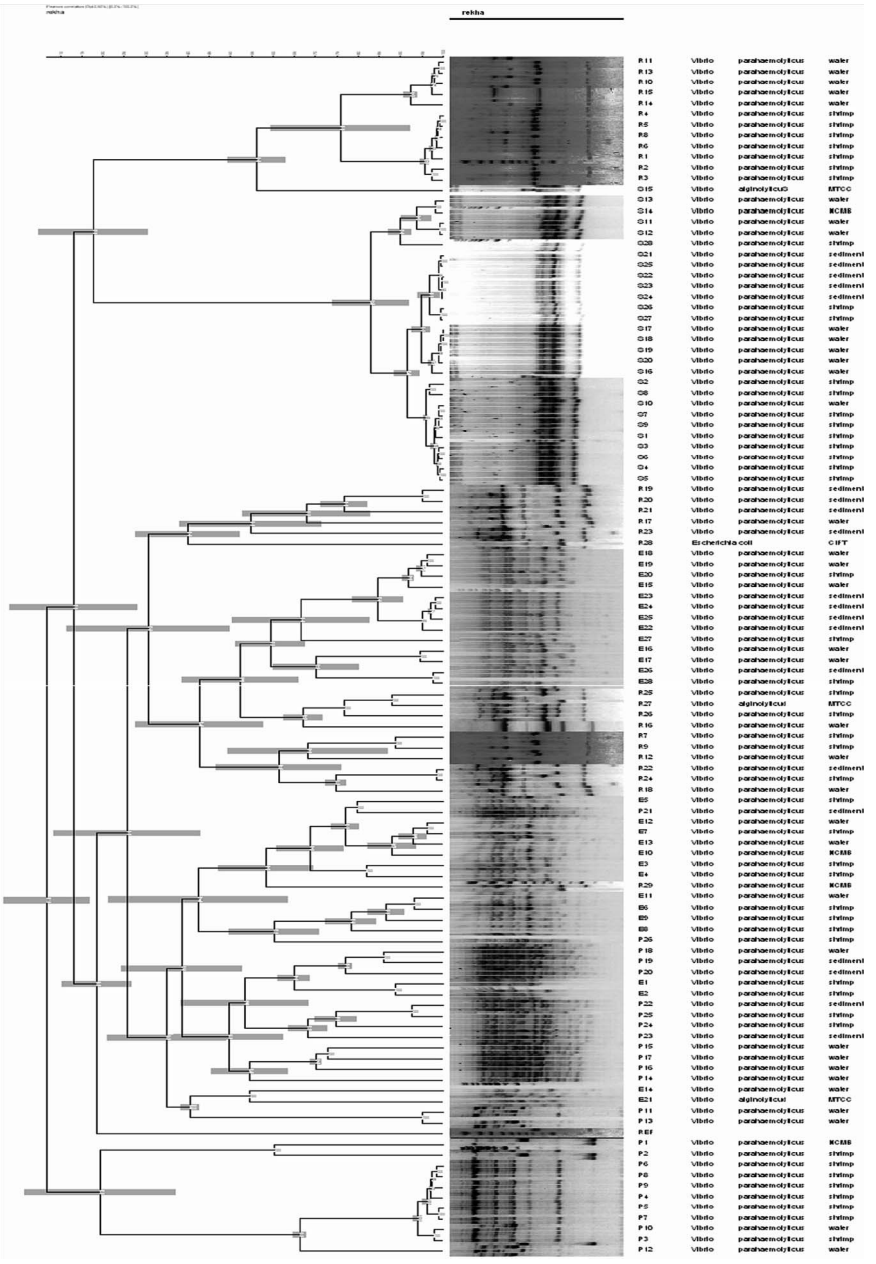

Figure 10: Phylogenetic analysis of RAPD, ERIC, RS, and REP PCR profiles of *V. parahaemolyticus* isolates from farm samples. Bars are shown at each node corresponding to the standard deviation of values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes are shown above the dendrogram. The similarity scale is shown above this dendrogram.

pathogens are identified by culture-based methods and biochemical tests, which take five to seven days to complete. This pathogen has the ability to exist in viable but nonculturable (VBNC) state in the environment (Wong and Wang, 2004) where the conventional biochemical protocols fail to detect its presence. Molecular methods such as polymerase chain reaction (PCR) has the advantage over the existing biochemical methods in detecting few number of stressed cells in a relatively short period of time. Thus the applications of PCR-based DNA amplification methods have greatly increased the speed at which these pathogens are detected (Gonzalez-Rodriguez et al., 2002). In addition, simultaneous amplification of targeted DNA from samples having multiple virulence genes in a single PCR reaction has further improved the efficiency of the detection process.

In the present study, all biochemically confirmed *V. parahaemolyticus* isolates from shrimp, water, and sediment samples gave amplification for *toxR* gene, which is found to be specific for *V. parahaemolyticus.* About 1% of the isolates from shrimp and water samples exhibited amplification for *trh* and *tdh* genes, but the isolate that amplified the *tdh* gene did not give amplification for the *trh* gene. Also, the isolate with *trh* gene did not give amplification for *tdh*, thus suggesting both *trh* and *tdh* genes may not be present in the same sample. In our study, no correlation was apparent between the possession of *tdh* gene and urease production. Moreover, none of the urease-positive isolates were found to contain the *tdh* gene as evident from PCR analysis. However, few workers have tried to correlate the presence of *tdh* gene and urease activity in *V. parahaemolyticus* (Kaysner et al., 1987). So a PCR reaction targeting both genes (*tdh* and *trh*) simultaneously is necessary to detect all haemolysin-producing strains in this pathogen. In the present study m-PCR approach was successfully used to detect multiple genes *viz*., *toxR, trh,* and *tdh* in various strains of *V. parahaemolyticus* isolates collected from different samples. Earlier Bej et al. (1999) reported the simultaneous amplification of *tl, trh,* and *tdh* genes using m-PCR. The present study provides the first direct evidence for the incidence of pathogenic *tdh* and *trh* positive *V. parahaemolyticus* strains from the southwest coast of India.

Many extracellular proteases are suggested to play an important role in virulence of *Vibrio* spp. In the present study, protease activity was observed in 80% of the *V. parahamolyticus* isolates and its reference strains independently of the presence of *tdh* and *trh* genes. This confirms the presence of other virulence factors besides TDH and TRH toxins in *V. parahaemolyticus*, contributing to enteropathogenisis. In a recent study (Lee et al., 2002), a protease was identified as the major virulence factor in a clinical *tdh* and *trh* negative *V. parahaemolyticus* isolate. Although 10% of the isolates signaled weak haemolysis, only 1% was found to exhibit β-haemolysis on Wagatsuma agar, which was later confirmed to be *tdh* positive by PCR. The results in this study exhibited a direct correlation between the presence of *tdh* gene and

Kanagawa reaction suggesting both tests are mandatory for the confirmation of *tdh* gene. The weak haemolysis points toward the presence of virulence factors other than *tdh* in this pathogen. The capability of strains to produce few extracellular enzymes may also lead to weak haemolysis (Lee et al., 2002). In this study only 1% of the isolates, which gave urease activity on Christensen's urea agar, were found to be positive for *trh* gene. The results indicated phenotypic tests; that is, Kanagawa phenomenon and urease activity could be considered convenient and useful markers for predicting *tdh* and *trh* genes. Similar results were observed by Suthienkul et al. (1995).

The DNA-based typing of isolates is of great importance for tracking epidemiological and causal relationships. RAPD-PCR analysis pattern revealed two major clusters A and B with 6–8 bands ranging from 0.25 to 2.5 Kb. The virulent strains that gave amplification for *tdh, trh,* and its type strain (NCMB 1902) were found lying in the same subcluster A2. These toxigenic strains have been isolated in the summer, and their occurrence can be correlated with the higher salinities prevailing in those months, as variation in salinity was found comparatively higher than temperature during the study period. Clustering of a specific isolate, which was later confirmed to be *tdh* positive by PCR along with *trh* positive isolate and its reference strain, indicates that the RAPD method was useful in clustering toxigenic strains into a single group. The possible explanation for the clustering of *tdh* with *trh* strains could be supported by the studies of Nishibuchi et al. (1989), who stated that the *trh* gene isolated from a *V. parahaemolyticus* strain A24037'was found to show 69% similarity with the *tdh* gene. Similarly Mahmud et al. (2006) could differentiate the toxigenic strains by the RAPD method, revealing 10–12 bands ranging from 0.25 to 2.4 Kb. It is evident from the dendrogram that all the *V. parahaemolyticus* isolates formed two major clusters. Although *Vibrio algninolyticus* formed a separate cluster but placed within the main cluster, which can be explained by the fact that *V. alginolyticus* and *V. parahaemolyticus* belong to the same genus *Vibrio* that were earlier considered as two biotypes of *V. parahaemolyticus* (Shewan and Veron, 1974). Investigations using 16S rRNA sequence analysis revealed 99.7% homology between *V. parahaemolyticus* and *V. alginolyticus* (Ruimy et al., 1994), while *E. coli* formed a cluster quite distinctly separate from the main cluster indicating less similarity with *Vibrio parahaemolyticus*. Sudheesh et al. (2002) used RAPD technique for clustering *V. parahaemolyticus* and *V. alginolyticus* into two groups, while Bhowmick et al. (2006) grouped *V. cholerae* into genetically homogeneous or heterogeneous strains. Thus the RAPD method can be efficiently used in molecular subspecies typing of *V. parahaemolyticus* to differentiate toxigenic strains from nontoxigenic ones.

The PCR typing methods using specific primers designed on the basis of the repeated and conserved sequences in bacteria, and more stringent annealing conditions display more promising fingerprints. Marshall et al. (1999)

found that ERIC-PCR using a 22-mer primer specific for the enterobacterial repetitive intergenic consensus sequence (ERIC) is useful for evaluating genetic and epidemiological relationships among *V. parahaemolyticus* strains. Besides ERIC-PCR, methods based on the highly conserved ribosomal gene spacer sequence (RS) and the 38-bp repetitive extragenic palindromic sequence (REP) in *Enterobacteriaceae* and other bacteria have been used for typing of pathogenic bacteria (Stubbs et al., 1999). As all samples were obtained from the same area, clusters formed based on the seasonality rather than the source of sample. Sanjeev (1999) reported higher incidence of this bacterium during summer than winter, presumably due to lower salinities during those periods. However, in temperate regions the higher incidence of this organism was correlated with the rise in temperature (Kaneko and Colwel, 1978). In the present study comparatively higher isolations were obtained during winter than summer. This could be due to the absence of prominent winters in this area.

ERIC-PCR is the most widely adopted method of the above three PCR typing methods, and has been applied for typing of many species, including *V. cholerae* (Shangkuan et al., 1997) and *V. parahaemolyticu*s (Wong and Lin, 2001). ERIC-PCR method was used to specifically identify O3:K6 *V. parahaemolyticus* isolates in less than 6 h in the United States (Khan et al., 2001). Marshall et al. (1999) compared 38 clinical strains of *V. parahaemolyticus* isolates from the 1997 Canadian outbreak using ERIC-PCR. Using a single primer for the amplification ERIC-PCR was useful for evaluating genetic and epidemiological relationships among *V. parahaemolyticus* strains (Marshall et al., 1999). Both REP-PCR and ERIC-PCR are based on the presence of repetitive conserved sequences in bacteria. The REP-PCR method is based on the presence of 38 bp REPs in *Enterobacteriaceae* and other bacteria, and has been applied for many species (Stubbs et al., 1999) to differentiate toxigenic *V. cholerae* O1 strains from nontoxigenic and non-O1 strains based on the fingerprint profiles.

All PCR typing methods described in this study could differentiate *V. parahaemolyticus* from other species (negative control) and effectively project the intraspecific diversity in the strains. Although ERIC-PCR is rapid, relatively easy to perform, and can be used as a single method for typing, REP-PCR could be the better alternative between the two owing to its higher rate of reproducible fingerprints. In ERIC-PCR, some of the minor light amplification bands were found to be inconsistent, thus complicating pattern differentiation. However, since the RS-PCR patterns were more easily discernible visually than the REP-PCR or ERIC-PCR patterns, the former can be suggested as a practical method for routine use. Although the discriminative efficiency of these PCR typing methods differed from each other, these methods are effective for typing strains. Wong and Lin (2001) reported that the results obtained by these PCR methods mirrored those of the PFGE

method for some outbreaks, although they differed slightly for other outbreaks. This finding implies that these methods measure genetic diversity that persist in the strains and it makes interpretation of the relationships between strains more complex revealing the genetic heterogeneity among the isolates. There have been some old reports on the similarities at molecular genetic level among some strains of *V. parahaemolyticus* (Anderson and Ordall, 1972). Recently, Zanetti et al. (1999) observed intraspecies variation in *V. alginolyticus* strains using DNA amplification.

CONCLUSION

This study shows that m-PCR technique is a very useful tool in the detection of pathogenic strains of *V. parahaemolyticus* from aquaculture systems. The results revealed the first direct evidence of the incidence of pathogenic, *tdh* and *trh* positive *V. parahaemolyticus* isolates in shrimp culture systems. Although the incidence of *tdh*/*trh* positive virulent strains was found to be low but the probable public health risk involved in consuming seafood cannot be neglected because of its short generation time. DNA-based typing of isolates is of great importance for tracking epidemiological and causal relationships. In this study RAPD, ERIC, REP, and RS PCR were performed to understand the genetic diversity among the isolates. These methods can be used in molecular subspecies typing of *V. parahaemolyticus* independently or as a supplement to other typing methods when sensitive typing is required. All methods have high discriminative ability, but RS-PCR seems to be more practical with fewer amplification bands and patterns, simplifying the interpretation of data and exhibiting high discriminative ability.

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