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ORIGINAL PAPER

Isolation and characterization of *Vibrio parahaemolyticus* from seafoods along the southwest coast of India

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Abstract The work was aimed to study the microbial quality of the seafood sold in the domestic markets and incidence of *Vibrio parahaemolyticus*. Samples comprising of shellfish, finfish, and cephalopods were collected from various fish markets in and around Cochin. Presumed *V. parahaemolyticus* were identified by standard biochemical tests, and further confirmed by polymerase chain reaction targeting species-specific *tl* gene (450 bp). About 81% of the samples were found to exceed the limits specified for total plate count while total presumptive *V. parahaemolyticus* count was above the limit in 71% of the samples ranging from 5.5×10^5 to 9.7×10^7 and 0.31×10^2 to 7.8×10^6 cfu/g, respectively. Pathogenicity of the identified isolates was confirmed by Kanagawa phenomenon and urease activity. A total of 10% of the isolates exhibited weak haemolysis on Wagatsuma agar, and 1% of the isolates showed urease activity using Christensen's urea agar. Random amplified polymorphic DNA analysis revealed two major clusters based on the species rather than seasonality. The gel pattern revealed 8–10 bands ranging from 0.45 to 3.0 kb. Antibiogram results revealed 85% of the strains sensitive to chloramphenicol and nitrofurantoin. Multiple antibiotic resistance index was found to be 0.4 thus suggesting the risk potential involved in consuming seafoods. The present study has clearly demonstrated the need to adopt seafood safety measures for the products meant for human consumption.

Keywords *Vibrio parahaemolyticus* · PCR · *tl* · Antibiotics · RAPD

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Introduction

Global production of fish and fishery products has more than doubled since 1970, reflecting an increase in capture, and in particular aquaculture production (Delgado et al. 2003). Cochin, the economic capital of Kerala is a major fish-landing center in south west coast of India accounting for 90% of statewide exports. Vibrios of seafood origin have attracted increasing attention from time to time as it is found to be one of the most important causes of human food poisoning. Earlier reports revealed food poisoning due to the consumption of seafood contaminated with *Vibrio* species, particularly *Vibrio parahaemolyticus* (Wong et al. 2000). The incidence of this bacterium increased considerably during recent years in US, Japan and Korea (Lee et al. 2001), and in India it was reported to be doubled in the last 5 years (Chowdhury et al. 2000). The organism has been well recognized as the causative agent of gastroenteritis, wound infections, and septicemia through the consumption of contaminated seafoods (Fujino et al. 1953). Although *V. parahaemolyticus* forms the common cause of gastroenteritis transmitted by contaminated seafood consumption, the true incidence was not known probably due to underreporting of cases and lack of proper study.

Epidemiological studies revealed an association between the Kanagawa phenomenon (KP) positive and

gastroenteritis (Okuda et al. 1997). In the 1980s few gastroenteritis cases were reported from the KP negative *V. parahaemolyticus* isolates, which led to the discovery of the thermostabele direct haemolysin (TDH)-related haemolysin, TRH (Hervio-Heath et al. 2002). Studies revealed the TDH and TRH, encoded by the phenotypic tests viz., KP and urea hydrolysis respectively, were considered as phenotypic markers for pathogenicity (Kaysner et al. 1994). A separate thermolabile hemolysin gene *(tl* gene) was found to be present in all the strains of *V. parahaemolyticus* (Taniguchi et al. 1986) irrespective of their ability to produce TDH or TRH. In the present study *tl* gene, specific to *V. parahaemolyticus* has been applied for definitive identification of *V. parahaemolyticus* isolates.

A DNA-based typing technique that is frequently used to generate strain-specific fingerprinting (Hulton et al. 1991), relies on the polymerase chain reaction (PCR) and primers directed to specific nucleotide sequences. Typing methods viz., pulsed-field gel electrophoresis (Wong et al. 1996), ribotyping (Wong et al. 1999a), and random amplified polymorphic DNA (RAPD) analysis (Wong et al. 1999b) were developed to study the genetic relationships between strains and species of microorganisms (Oakey et al. 1998). Compared with all these methods, RAPD analysis has its merits of being consuming less labor and time. RAPD fingerprinting analysis is a commonly used method in PCR for typing and differentiation of bacteria.

Antibiotics and other chemotherapeutic agents are commonly used in fish farms either as feed additives or immersion baths to achieve either prophylaxis or therapy. As antibiotics were used for feeding animals and for treatment of patients, more drug resistance characteristics were detected in food borne pathogens (Barza 2002). The rapid increase of drug resistant pathogenic bacteria during the recent years has rendered many known antibiotics ineffective. Although the majority of shrimp exported from Kerala are from marine capture, for which antibiotics are not normally an issue, residues have been detected through border checks in the EU even in marine captured seafoods forcing controls on antibiotics onto the agenda of all exporters.

Earlier studies carried out at Cochin were based on the conventional methods used phenotypic assays and biochemical tests to identify virulent strains of *V. parahaemolyticus*, requiring several days for confirmation of the pathogen (Sanjeev and Stephen 1992). In the present study PCR that was found to be rapid and sensitive was used for the detection of this pathogen. The study was aimed to investigate the incidence of *V. parahaemolyticus* in seafoods marketed from this area and to detect the presence of pathogenic isolates, antibiotic resistance, and genetic relatedness among the isolates.

Materials and methods

Sample collection, processing and enrichment

Samples were collected from markets located in and around Cochin for studying the incidence of *V. parahaemolyticus* in various seafoods at regular intervals from October 2005 to September 2006. Finfish samples included *Etroplus suratensis*, *Pampus argentius*, *Megalaspis cordyla, Oreochromis mossambicus*, *Mugil cephalus*, *Sardinella longipes*, and *Labeo rohita*. Shellfishes consisted of *Metapenaeus dobsoni*, *Metapenaeus affinis*, *Macrobrachium rosenbergii*, *Penaeus monodon*, *Fenneropenaeus indicus, Scylla serrata*, *Parapenaeopsis stylifera*, and *Panulirus homarus*. Cephalopod samples included *Sepia pharonis* and *Loligo duvacelli*. All the samples were processed under sterile conditions within 2 h of collection. Bacteriological analysis was performed with three samples separately, and average counts were taken. The samples (25 g) were homogenized with alkaline peptone water (APW) broth (225 ml) in a sterile polythene stomacher bag (Stomacher 400 Seaward medicals, UK) at 230 rpm for 1 min, and enriched in APW broth for 18–24 h.

Isolation and identification of *V. parahaemolyticus*

After 18–24 h of incubation in APW broth (0.5 ml) was aseptically pipetted into thiosulphate citrate bilesalt sucrose agar (TCBS) and tryptone glucose agar (TGA) plates previously preset and dried (56°C, 45 min). The TCBS and TGA plates were incubated at 37°C for 24–30 h and 37°C for 24–48 h, respectively. TGA plates giving colony counts ranging between 30 and 300 were counted and recorded as total plate count (TPC). About 3–4 typical colonies having 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). Sucrose non-fermenting colonies were streaked onto sterile tryptone soy agar slants (TSA) supplemented with NaCl (3% w/v), and maintained at room temperature for further identification. Halophilism tests were performed using tryptone broth with different concentrations of NaCl (0, 3, 6, 8, and 10% w/v). Additional characterization tests for the identification of *V. parahaemolyticus* namely, Gram staining, catalase, cytochrome oxidase, triple sugar iron, lysine iron agar, arginine dehydrolase, lysine and ornithine decarboxylase, and O/129 susceptibility were performed. Tests for glucose oxidation-fermentation were carried out using Hugh-Leifson broth, and arabinose, lactose, mannitol, mannose, salicin, cellobiose, and inositol fermentation tests were also performed following standard procedure (USFDA 2001). All the media were supplemented with NaCl (3% w/v) unless otherwise specified. Colonies that gave typical colony characteristics

were picked up and confirmed by Kanagawa (Wagatsuma 1968) and urease tests (Andrews and Hammack 2001). The presumed positive cultures were confirmed using RAPID Hi-Vibrio^{TM} identification kit (KB007, Himedia, India).

Kaneko and Colwell (1975) suggested that all the colonies growing on TCBS could be regarded as presumptive vibrios (PV) and colonies that appear to be typical (green colonies with dark blue or green centre measuring about 3–5 mm) as *V. parahaemolyticus* were considered as presumptive *V. parahaemolyticus* (PVP). A similar criterion was used in this study for the enumeration of PV and PVP counts in the samples.

Detection of the phenotypic markers for pathogenicity

The phenotypic test for the production of TDH from *V. parahaemolyticus* isolates was studied by testing the KP on Wagatsuma agar (Elliot et al. 1992). Briefly the test strains were inoculated and incubated at 37°C. The production of β-haemolysis zone around the colonies within 24 h was judged as KP positive. The phenotypic marker for the production of TRH was studied by testing urease activity using Christensen's urea agar (Andrews and Hammack 2001).

Antibiotic sensitivity test

Antibiotic sensitivity of the *V. parahaemolyticus* cultures was determined by the standard disk diffusion method (Bauer et al. 1966). Antibiotic discs (Himedia, India) were placed aseptically on the Mueller–Hinton agar plates (three replicates) pre-inoculated with the test culture. The discs were placed at 15 mm away from the edge of the plates, at equal distance, and sufficiently separated from each other to avoid overlapping of the zone of inhibition. The plates were incubated at 37°C for 18–24 h, and the size of inhibition zone was recorded both in test and control isolates. The antibiotics evaluated were polymixin-B, gentamycin, tetracycline, nalidixic acid, chloramphenicols, nitrofurantoin, and trimethoprim. The multiple antibiotic resistance (MAR) index was determined as the ratio between the number of antibiotics for which the organism is resistant and the total number of antibiotics used.

Preparation of bacterial lysate for PCR assay

Bacterial lysate was prepared following established procedure (Karunasagar et al. 1996). The biochemically identified isolate was streaked on TSA plates and the isolated colony was inoculated in the Luria Bertane (LB) broth supplemented with NaCl (2% w/v), and incubated at 37°C under shaking (120 rpm) for 16–18 h. The culture broth was centrifuged (10,000 rpm, 4°C, 1 min; 5804 R, Eppendorf, Germany) to obtain the pellet, which was washed with normal saline (0.8% NaCl w/v), and re-suspended 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 release the DNA. The lysate was clarified by centrifugation (10,000 rpm, 4°C, 5 min), and the supernatant was stored (−20°C) until further use.

The PCR targeting species-specific *tl* gene of *V. parahaemolyticus* was performed (Bej et al. 1999) in a thermocycler (Eppendorf Mastercycler, Germany) using a primer pair (5′ AAA GCG GAT TAT GCA GAA GCA CTG 3′and 5′ GCT ACT TTC TAG CAT TTT CTC TGC 3′) to detect the gene.

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 under 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 (10,000*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 (10,000*g*). Sodium acetate (0.1 volume, 3 M, 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.

PCR conditions for the detection of *tl* gene

PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (13.5 µl), 10× PCR buffer (2.5 µl), primer (1.5 µl), dNTP mix (0.5 µl, 200 mM), template (5 µl), and *Taq* DNA polymerase (0.5 µl). The

components were mixed well and the PCR amplification of the target sequence was arranged in a thermocycler (Eppendorf Mastercycler, Germany), and programmed for 30 cycles of amplification. Each cycle consisted of three step reactions *i.e.*, initial denaturation (94°C, 3 min) followed by 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 1 min) and extension (72°C, 1 min) followed by final extension (72°C, 5 min).

RAPD-PCR conditions

RAPD analysis was carried out using the 10-nucleotide primer, 5′CAG GCG CAC A3′ (Wong 2003). PCR reaction conditions have been optimized for important parameters such as annealing temperature and concentrations of $MgCl_2$, template DNA, *Taq* DNA polymerase, dNTPs and primer. The PCR reaction mixture comprised of 2.0 µl of 10× buffer (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 5 U/µl), primer (30 pM), DNA template (1 µl), and an additional 3.0 µl of $MgCl_2$ (25 mM) adjusted to a reaction volume of 25 µl with an appropriate volume of sterile Milli Q 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 s). The extension was carried out at 72°C (2 min, 30 sec) followed by final extension at 72°C (10 min).

The PCR products were resolved on agarose (1.5% w/v) gel electrophoresis. The gel was stained with ethidium bromide (0.5 mg/ml) and visualized under a UV transilluminator (Alpha Imager, Innotech Corporation, USA). GeneRuler^{TM} 100 bpDNA Ladder (MBI Fermentas, USA) was used as a molecular size marker.

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 market samples based on unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal 1973) using the Gel Compar II software, version 4.0 (Applied-Maths, St-Martens-Latem, Belgium).

Results

About 88% of the shellfish samples have exceeded the limit specified for TPC (USFDA 2001) ranging from 1.1×10^6 to 4.74×10^7 cfu/g, and in the remaining 12% of the samples, TPC values ranged from 1.5×10^5 to 3.96×10^5 cfu/g. Total presumptive vibrio count (TPVC) was found to be in the range of 0.95×10^2 to 5.7×10^5, and total presumptive *V. parahaemolyticus* count (TPVPC) was found to cross the limit (ICMSF 1986) in 64% of the samples (1.21×10^3 to 4.4×10^5 cfu/g). The incidence of *V. parahaemolyticus* was found to be 64%, highest being in summer months (February to May) as evident from Table 1.

About 67% of the finfish samples have exceeded the limit specified for TPC (USFDA 2001) ranging from 5.5×10^5 to 4.5×10^7 cfu/g, and in the remaining samples (33%) TPC was in the range of 2.6×10^4 to 7.2×10^4 cfu/g (Table 1). TPVC was found to be in the range of 2.73×10^2 to 4.2×10^4 and TPVPC was above the limit (ICMSF 1986) in 75% of the samples (1.05×10^3 to 3.6×10^4 cfu/g). Total incidence of *V. parahaemolyticus* in finfish samples was found to be 42%, the highest being found during summer months.

About 80% of the cephalopod samples have exceeded the limit specified for TPC (USFDA 2001) ranging from 1.5×10^6 to 9×10^7 cfu/g and in the remaining 20% of the samples TPC was found to be from 2.0×10^5 to 3.2×10^5 cfu/g. TPVC was found to be in the range of 7.8×10^3 to 9.2×10^6 and TPVPC was recorded above the specified limit (ICMSF 1986) in all the samples (3.6×10^3 to 7.8×10^6 cfu/g), whereas the incidence of *V. parahaemolyticus* was found to be 42%. The results revealed 80% of cephalopod samples collected from Cochin markets were of very poor quality considering the TPC limits (Table 1).

None of the isolates exhibited β-haemolysis while 10% of the isolates exhibited α-haemolysis on Wagatsuma agar, and 1% of the colonies exhibited urease activity on urea agar slants of which one was isolated from the inshore shrimp, *Metapenaeus dobsoni* and the other from *M. affinis*. PCR analysis was carried out by targeting species-specific *tl* gene (450 bp) for all the *V. parahaemolyticus* isolates that included both positive (MTCC 451) and negative controls. All the isolates that were indentified as *V. parahaemolyticus* by biochemical tests were further confirmed by PCR were found to amplify the *tl* gene (Fig. 1).

If more than 70% of the isolates exhibits resistance to a particular antibiotic, then such antibiotic was taken into consideration for the calculation of MAR. In the present study MAR was found to be 0.4. About 100% sensitivity was noticied towards tetracycline, nalidixic acid followed by chloramphenicol, trimethoprim, and nitrofurantoin (Table 2).

V. parahaemolyticus isolates were analyzed by RAPD analysis revealing two major clusters, namely A and B based on the species rather than seasonality of sample collection. Group A included 40% of cultures isolated from shrimps, clustered with group B at $S \leq 20\%$ (S, similarity

Table 1 Microbial quality of seafood samples collected from markets

Months	Shellfish	(Cfu/g)			Samples positive	Finfish	(Cfu/g)			Samples positive	Cephalopod	(Cfu/g)			Samples positive
		TPC[a]	TPVC[b]	TPVPC[c]			TPC[a]	TPVC[b]	TPVPC[c]			TPC[a]	TPVC[b]	TPVPC[c]	
Jan	*F. monodon*	4.18×10^7	3.18×10^4	5.7×10^4	+	*T. mossambica*	5.5×10^5	3.4×10^4	1.2×10^3	–	*S. pharonis*	5.8×10^6	1.78×10^6	1.1×10^6	–
	M. affinis	3.8×10^7	2.17×10^4	1.54×10^4	+										
Feb	*F. indicus*	2.31×10^7	2.61×10^5	1.9×10^5	–	*M. cephalus*	6.2×10^6	2.98×10^3	2.13×10^3	+	*L. duvacelli*	3.4×10^5	8.0×10^3	3.9×10^3	+
	P. homarus	5.5×10^6	5.56×10^5	2.78×10^5	+										
Mar	*M. affinis*	5.85×10^5	4.85×10^5	3.1×10^5	+	*R. kanagurta*	3.4×10^7	7.5×10^3	6.2×10^3	+	*S. pharonis*	9.0×10^6	9.2×10^6	7.8×10^6	+
	P. stylifera	5.8×10^6	4.3×10^5	3.9×10^5	+										
April	*P. homarus*	6.3×10^6	5.2×10^5	4.3×10^5	+	*S. longipes*	4.5×10^7	1.23×10^4	0.78×10^4	–	*S. pharonis*	2.0×10^5	7.8×10^3	3.6×10^3	+
	S. serrata	8.5×10^6	5.7×10^5	4.4×10^5	+										
May	*F. monodon*	4.74×10^7	2.3×10^4	1.7×10^4	+	*E. suratensis*	1.02×10^6	2.54×10^4	1.98×10^4	+	*S. pharonis*	5.8×10^6	1.78×10^6	1.1×10^6	+
	F. indicus	2.3×10^6	1.9×10^4	0.9×10^4	–										
June	*M. affinis*	1.18×10^6	2.6×10^3	1.2×10^3	+	*M.cordyla*	7.2×10^4	6.2×10^3	2.1×10^3	–	*L. duvacelli*	1.5×10^6	2.79×10^6	1.2×10^6	–
	M. dobsoni	1.8×10^6	1.5×10^3	0.75×10^3	–										
July	*F. indicus*	1.5×10^5	1.09×10^3	0.86×10^3	+	*S. longipes*	4.1×10^6	1.25×10^3	0.89×10^3	–	*S. pharonis*	3.3×10^5	7.9×10^3	3.7×10^3	–
	M. dobsoni	3.7×10^7	1.09×10^3	0.9×10^3	+										
Aug	*P. stylifera*	3.3×10^6	1.7×10^4	1.3×10^4	+	*L. rohita*	5.3×10^4	2.73×10^2	0.91×10^2	+	*L. duvacelli*	9.0×10^7	5.79×10^6	4.6×10^5	–
	F. inidicus	1.7×10^6	2.32×10^4	1.7×10^4	–										
Sep	*P. stylifera*	3.7×10^6	2.75×10^4	2.1×10^4	–	*S. longipes*	5.6×10^5	4.2×10^4	3.6×10^4	–	*S. pharonis*	2.0×10^5	7.8×10^3	3.6×10^3	–
	M. dobsoni	2.1×10^5	1.98×10^4	1.73×10^4	+										
Oct	*M. dobsoni*	3.7×10^6	2.2×10^3	0.67×10^3	+	*E. suratansis*	7.02×10^5	2.31×10^4	1.56×10^4	–	*S. pharonis*	2.4×10^5	7.9×10^3	3.8×10^3	–
	M. rosenbergii	5.4×10^6	1.78×10^3	1.21×10^3	+										
Nov	*P. stylifera*	3.9×10^5	2.1×10^4	0.52×10^2	–	*T. mossambica*	2.6×10^4	1.35×10^3	1.05×10^3	–	*S. pharonis*	3.3×10^5	7.9×10^3	3.7×10^3	+
	M. rosenbergii	3.4×10^6	1.25×10^2	0.31×10^2	–										
Dec	*F. monodon*	2.6×10^7	2.31×10^2	1.54×10^2	+	*P. argentius*	5.4×10^4	1.27×10^3	0.85×10^3	+	*L. duvacelli*	3.4×10^5	8.0×10^3	3.9×10^3	–
	F. indicus	2.1×10^6	1.21×10^3	0.73×10^2	–										
	M. rosenbergii	1.1×10^6	0.95×10^2	0.44×10^2	–										

[a]TPC indicates total plate count

[b]TPVC is total presumptive vibrio count

[c]TPVPC implies total presumptive *V. parahaemolyticus* count

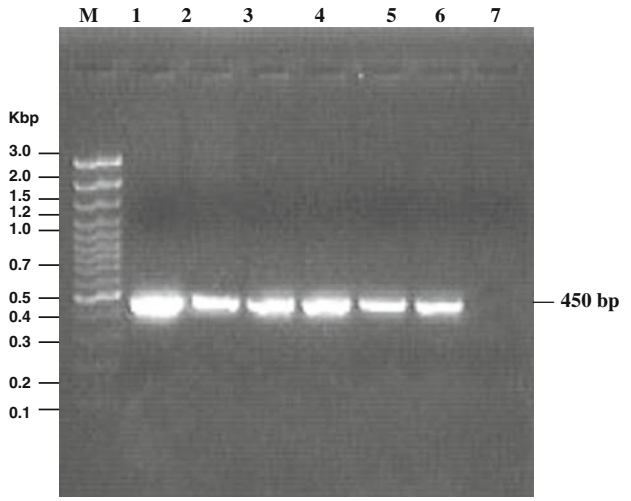

Fig. 1 PCR for the detection of *tl* gene (450 bp). Lane M: DNA ladder (100 bp); Lanes 1: MTCC 451 (type strain); Lanes 1–2: *V. parahaemolyticus* isolates from finfish, lane 3–4 isolates from shrimp, lane 5–6 isolates from cephalopod samples containing *tl* gene, lane 7: PCR negative control

index), which in turn forms the major cluster (60% isolates) having included the isolates from fish and cephalopod samples. Group B was further sub-clustered into two groups, namely B1 (24% isolates) and B2 (36% isolates). B1 clustered with B2 at S $\leq$ 30%. A distinct grouping between clusters was apparent pertaining to the source of isolation, while no such groups were found pertaining to seasonality of samples. *V. parahaemolyticus* positive control (MTCC 451) was found to be grouped with cluster A, which forms the major cluster. Urease positive *V. parahaemolyticus* (NCMB 1902) served as a control for urease activity clustered with the urease positive isolate. This urease positive isolate was recovered from *M. dobsoni* sample and was grouped in the same sub-cluster B1. The gel pattern revealed 8–10 bands ranging from 0.45 to 3.0 kb (Fig. 2). The characteristic fingerprint patterns

Table 2 Antibiotic resistance pattern of *V. parahaemolyticus* isolates

Antibiotics	S%[a]	I%[b]	R%[c]
Ampicillin	9.5	9.5	80.9
Polymixin-B	4.7	4.7	90.4
Streptomycin	14.3	9.5	76.2
Kanamycin	19.1	9.5	71.5
Gentamycin	38.1	52.3	9.5
Neomycin	–	47.6	52.3
Chlorotetracycline	–	14.3	85.7
Oxytetracycline	76.2	23.8	–
Tetracycline	100	–	–
Nalidixic acid	100	–	–
Chloramphenicol	85.7	9.5	4.7
Cephalexin	4.7	19	76.2
Nitrofurantoin	85.7	14.3	
Furazolidone	71.4	23.8	4.7
Trimethoprim	90.5	4.7	4.7

[a] S—sensitivity

[b] I—intermediary sensitivity

[c] R—resistance to the antibiotics

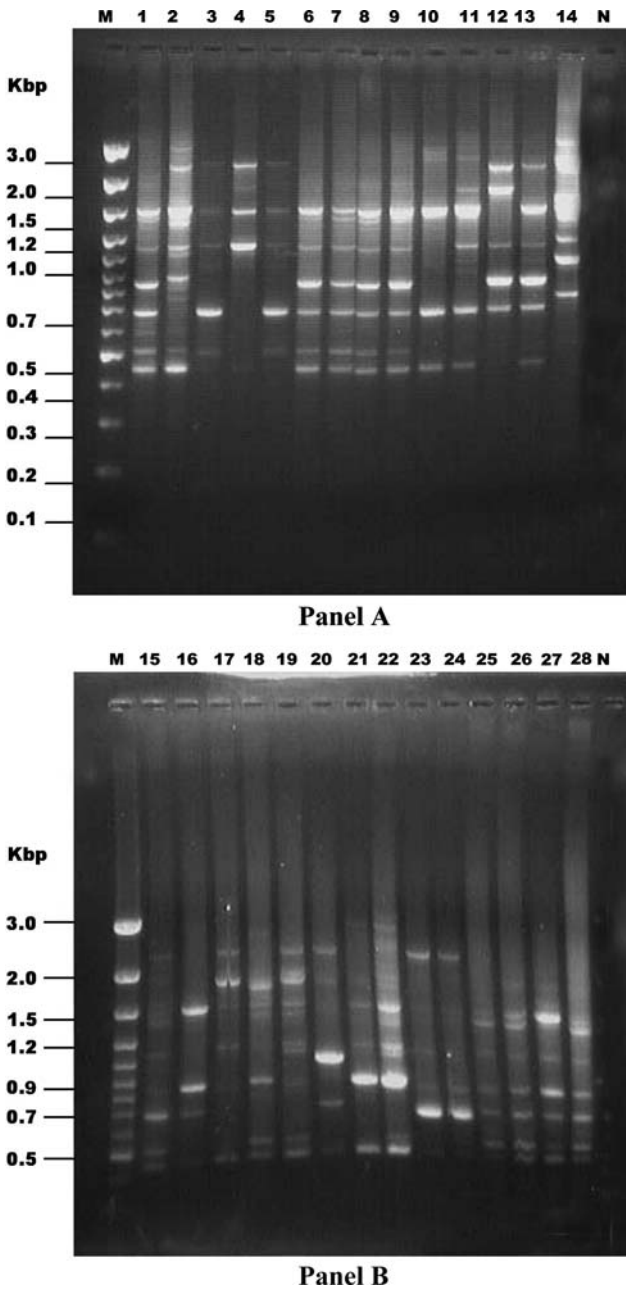

Fig. 2 RAPD-PCR profiles of *V. parahaemolyticus* strains isolated from market samples. Lane M: DNA ladder (100 bp); Lane N: PCR negative control; Panel A: lane 1–9: isolates from finfish samples, lane 10–13 and lane 15–19: isolates from shellfish samples, lane 20–27 in Panel B contain isolates from cephalopod samples, lane 14 contain *V. parhaemolyticus* type strain (NCMB 1902), lane 28 contain *V. parahaemolyticus* type strian (MTCC 451)

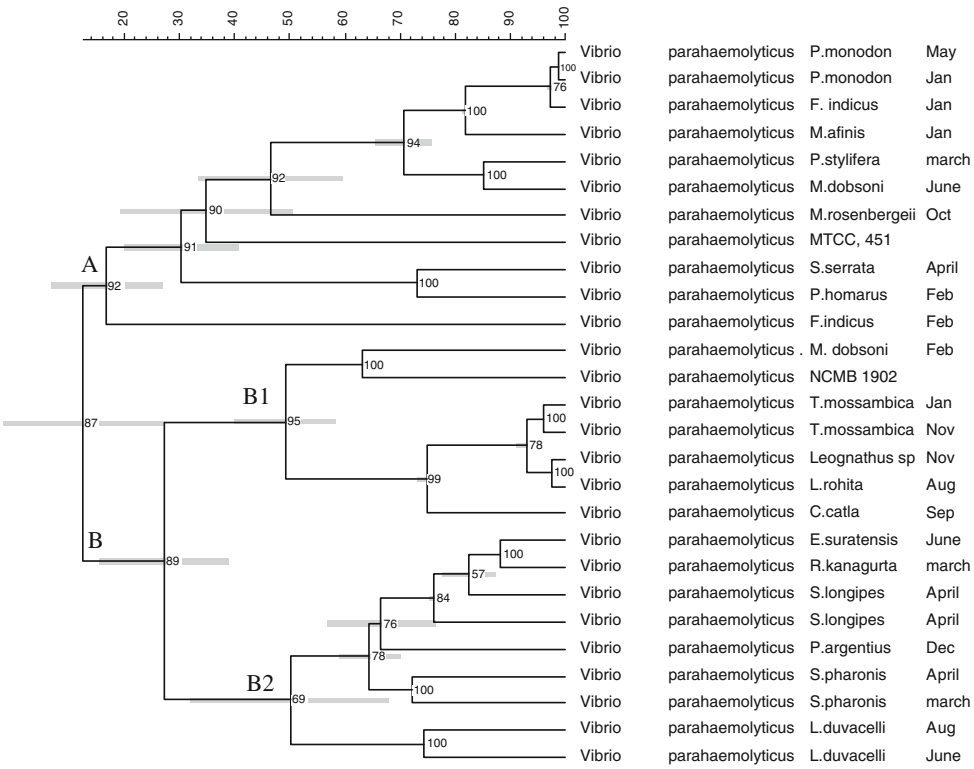

Fig. 3 Dendrogram illustrating the clustering of RAPD profile of *V. parahaemolyticus* isolates from market samples. Bars are shown at each node, corresponding to the standard deviation values in that region of the similarity matrix. The average and the standard deviation of similarity values for the selected nodes and similarity scale are shown above the dendrogram

obtained with primers are shown as dendrogram and gel pictures (Fig. 3).

Discussion

The members of the genus *Vibrio* have been frequently defined as opportunistic and potential pathogenic bacteria of the water bodies especially in tropical waters (Huss 1997), and in India the incidence of *V. parahaemolyticus* has reported to be doubled in the last 5 years (Chowdhury et al. 2000). *V. parahaemolyticus* is an important seafood borne pathogen, and therefore detection of this organism in seafoods is essential.

In this study 81% of the seafood samples analyzed exceeded the limit specified for TPC (USFDA 2001) ranging from 5.5×10^5 to 9.0×10^7 cfu/g, the higher counts were found in the cephalopod samples (Table 1). TPVPC was found to cross the specified limit in 71% of the samples (ICMSF 1986). The possible reason for the higher bacterial and presumptive *V. parahaemolyticus* counts in cephalopod samples can be attributed to the depth zone (150–200 m) from which they were caught. Benthic zone is known to harbor higher microbial communities but lower counts of *V. parahaemolyticus*, which is mainly encountered in the surface seawaters (Tsukamoto et al. 1993). The present study revealed 67% of finfish samples collected from Cochin markets were of poor quality considering the TPC limits set by the USFDA, EU, and Export Inspection Council of India (10^5 cfu/g). It was apparent that quality of fish sold in domestic markets was poor. Nambiar and Iyer (1990) and Nambiar and Surendran (2003) have made a detailed investigation on the microbial quality of seafood samples sold in the retail markets of Cochin, and their results revealed 72% of the samples sold in the retail markets of Cochin were of poor quality based on TPC levels, which exceeded 10^7 cfu/g. These counts were found to be comparatively higher than that reported in the present study. This clearly indicates improvement in the quality of samples marketed from this area over time period due to the implementation of stringent practices viz., Hazard Analysis Critical Control Point (HACCP), Standard Sanitation Operating Procedures (SSOP). Lakshmanan et al. (1993) reported higher bacterial counts in processed squid and cuttlefish as compared to its whole products apparently due to human handling.

The results of this study revealed the incidence of *V. parahaemolyticus* in 55% of the samples. Notably, incidence rates differed with respect to the kind of sample, highest being found in the shellfish samples (64%) followed by finfish (42%), and cephalopods (40%). The higher incidence in shrimps can be correlated with the ability of this bacterium to utilize chitin, which is abundantly available in the crustaceans. Moreover shrimps are rich in free amino acid content, which serves as an excellent growth medium for the proliferation of this bacterium.

Several authors reported the incidence of *V. parahaemolyticus* from various markets located in southwest coast of India (Bandekar et al. 1982; Karunasagar et al. 1984). Sanjeev and Stephen (1993) reported about 72% incidence of *V. parahaemolyticus* from different species of shrimps marketed from this area. Anand et al. (2002) reported higher bacterial load in shrimps (1×10^4–1.21×10^8 cfu/g) from the markets of southeast coast of India. Distribution of vibrios can be attributed to the suspended particulate matter of the water column, and is further influenced by various limiting factors like salinity, pH, depth of water column, tidal cycle, and/or unidentified biological factors. In the present study incidence of *V. parahaemolyticus* was found throughout the sampling period, highest being apparent during the summer months, presumably due to the higher salinities prevailing in those periods.

Incidence of *V. parahaemolyticus* was above 10^3 cfu/g in 42% of the finfish samples. This high count of *V. parahaemolyticus* in particular in finfish samples could be due to the sampling strategy followed in the present study. In brief, meat was picked from different parts of fish sample viz., skin, intestine and gills, and subjected to microbiological analysis as a whole. The intestinal contents of finfish serves as a good reservoir for vibrios apparently due to the higher nutrient content of the intestine, and the capacity of the bacterium to withstand substantially low pH in stomach.

A series of biochemical tests and molecular approach like *tl*-targeted PCR was used (Bej et al. 1999) for the identification of this pathogen. All the biochemically confirmed isolates were found to possess species-specific *tl* gene. The results of this study indicated that the *tl*-targeted PCR method is sensitive and rapid in detecting *V. parahaemolyticus.* However, the biochemical determination and identification of environmental *Vibrio* species has been problematic, time consuming, and often confusing due to their great diversity (Alsina and Blanch 1994). RAPD-PCR generated fingerprinting has been successfully used in this study to discriminate the widely diverse bacterial populations. RAPD analysis revealed two major clusters, namely group A and B. The former included all the cultures isolated from crustaceans, while the latter comprised of fish and cephalopod isolates. The clustering of urease positive control with urease positive isolate into a distinct group B1 reveals their specific identity. In cluster B2 isolates from fish and cephalopods were grouped together thus indicating their genetic similarity. The cultures isolated from cephalopod samples exhibited more similarity to the cultures isolated from finfish rather than crustaceans.

Although 10% of the isolates exhibited weak haemolysis, none of them were found to exhibit β-haemolysis on Wagatsuma agar. This weak haemolysis points towards the presence of virulence factors other than TDH/TRH. The capability of the strains to produce few extracellular enzymes may also lead to the weak haemolysis (Lee et al. 2002). In contrast Sanjeev (1999) and Sudha et al. (2002) reported higher Kanagawa positives from environmental strains of Cochin. Few authors have even correlated the KP with the positive detection of urease by urea hydrolysis (Kaysner et al. 1994; Iida et al. 1998). However, in the present study none of the weak haemolytic strains were found to exhibit urease activity. Athough about 1% of the isolates were found to exhibit urease activity, the potential risk involved in consuming such seafoods cannot be ignored due to its short generation time.

Drug resistance in environmental and clinical isolates of *V. cholerae* is well known (Vijayalakshmi et al. 1997). In 2001 residues of antibiotics emerged as a major concern for fish and fishery products exported to EU and United States. Antibiotics such as chloramphenicol and nitrofurans have been set at the limit of determination. In recent years, there has been an increase in the clinical importance of non-cholerae vibrios, but little is known about their antibiotic susceptibility. The occurrence of MAR among the bacterial species could be a problem associated with transfer or resistance to other organisms of human/veterinary significance (Kasper et al. 1990). In the present study MAR index was found to be 0.4 indicating the higher resistance acquired by the isolates. This study presents the sensitivity of the *V. parahaemolyticus* isolates from this area towards life saving drugs, viz., tetracycline, nalidixic acid followed by chloramphenicol, trimethoprim, nitrofurantoin thus indicating the safety of seafood products. However, few number of isolates exhibited resistance towards gentamycin. The significance of MAR in different ecosystem was also studied by Kaspar et al. (1990), and the results of the present study are in agreement with their study.

In conclusion, the study revealed detection of this pathogen by PCR using species specific, *tl* gene was found to be rapid and sensitive. This study demonstrated the incidence of *V. parahaemolyticus* in more than 50% of the samples marketed with a value of 0.4 as MAR index suggesting environmental significance. RAPD-PCR generated fingerprints has been successfully used to discriminate diverse bacterial populations. Although the incidence of urease positive and haemolytic strains was meager the risk involved in consuming such seafoods cannot be ignored due to its short generation time. However, the incidence of *V. parahaemolyticus* isolates sensitive to life saving drugs indicates the safety of the seafood products marketed from Cochin.

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