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Prevalence and Genomic Characterization of *Vibrio parahaemolyticus* isolated from Molluscan Shellfish and their Inhabiting Water of Coastal Karnataka, India

Vilasini Udyavara*, Sangeetha Madambithara Sivadasan, Dechamma Mundanda Muthappa, Santhosh Kogaluru Shivakumaraswamy and Moleyur Nagarajappa Venugopal

Department of Fisheries Microbiology, College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Mangalore, 575002, Karnataka, India
**Corresponding author*

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A B S T R A C T

Vibrio parahaemolyticus is a halophilic bacterium that commonly inhabits the marine and estuarine environments. This organism is known as one of the major leading causative agent for gastroenteritis, often related to consumption of raw or undercooked seafood. In this study, molluscan shellfish collected from various estuarine environments of Dakshina Kannada and Udupi districts of Karnataka were tested and analyzed for the presence of *V. parahaemolyticus*. These isolates were confirmed both by phenotypic, genotypic methods and checked for presence of virulence factors too. A total of 203 isolates of *Vibrio parahaemolyticus* were isolated of which, 20 isolates showed positive for *tdh* gene. The 20 isolates were further studied for Kanagawa phenomenon, production of thermostable direct haemolysin, antibiotic susceptibility and the presence of antibiotic resistant gene, which are related to pathogenicity in humans.

Introduction

Vibrio parahaemolyticus is a seafood-borne pathogen, a causative agent of gastroenteritis in humans (Bhuiyan *et al.*, 2002). It is a Gram-negative, halophilic bacterium that occurs naturally in estuarine environments worldwide and found frequently in seawater, sediments, plankton, finfish and shellfishes (Pavia *et al.*, 1989). The occurrence of *V. parahaemolyticus* in estuarine water is greatly influenced by a combination of temperature, salinity, and pH of water (Hayat Mahmud *et al.*, 2006). Illness is most frequently associated with the consumption of raw or under cooked seafood and seafood contaminated with the bacterium after cooking (Rippey, 1994). This bacterium can cause gastroenteritis in humans only when it propagates in the harvested seafood to the number exceeding the infectious dose when consumed by humans without proper cooking (Okuda *et al*., 1997a). The density of the organism in the environment and seafood vary greatly and is influenced by season, location, sample type and fecal pollution (Depaola *et al*., 2003; Oliver and Kaper, 2001).

V. parahaemolyticus is commonly isolated from samples on thiosulfate citrate bile salt

sucrose (TCBS) agar (Kobayashi *et al.*, 1963) plates on which it grows as large, sticky, bluish-green colonies. The thermolabile hemolysin gene (*tlh*) was reported to be the signature molecular marker of *V. parahaemolyticus* (Gutierrez West *et al.*, 2013). Not all the strains of *V. parahaemolyticus* are considered as pathogenic. Members of this species that produce virulence factors, (Nishibuchi and Kaper, 1995) *tdh* and /or the *trh*, are considered to be pathogenic that code for two well-characterized hemolysin proteins, thermostable direct hemolysin (*tdh*), and *tdh*-related hemolysin (*trh*) (Firdausi *et al.*, 2005) and can cause acute gastroenteritis. The hemolytic activity of this pathogen on high salt blood agar (Wagatsuma agar) is known as Kanagawa phenomenon (KP), which is associated with the presence of *tdh* gene (Honda and Iida, 1993). The early epidemiological investigations revealed a very strong association between the KP and gastroenteritis. It has been demonstrated that the KP, a beta-hemolysis in Wagatsuma agar, is associated with most clinical strains but with very few environmental strains (Zen *et al.*, 1971). Therefore, *tdh* has been considered a major virulence factor of *V. parahaemolyticus*. In this study, the prevalence and characterization of *V.parahemolyticus* in water and molluscan shellfish samples collected from coastal Karnataka is presented.

Materials and Methods

Sampling sites

The water samples were collected from Netravathi-Gurupura, Mulki estuary of Dakshina Kannada District and Sasthan, Gangolli estuary of Udupi District, Karnataka once in a month for the period of one year from October 2013 to September 2014.

Collection of samples

The water samples and available molluscan shellfish samples such as clams (*Meretrix* spp), oysters (*Crassostrea* spp) and green mussels (*Perna viridis*) were collected aseptically and were brought to the laboratory for further analysis.

Enrichment, isolation and characterization of *V. parahaemolyticus*

Molluscan shellfish samples (10 to 12 sample) collected from each site were scrubbed, shucked and the meat was blended separately. To 225 ml of the alkaline peptone water (APW), 25g of blended meat sample or 25ml of water sample was added and incubated at 37^0C for overnight. A loopful of APW enriched sample was taken and streaked onto thiosulfate-citrate-bile salts-sucrose (TCBS,) agar (HiMedia, Mumbai) plates and were incubated at 37^0C for overnight. Typical green (sucrose negative) colonies appearing on TCBS were considered as *V. parahaemolyticus* and selected for further identification. Phenotypic characterization of the *V. parahaemolyticus* was preferred by subjecting to a battery of biochemical tests following FDA bacteriological analytical manual (FDA, 2004).

Kanagawa phenomenon

The biochemically confirmed *V. Parahemolyticus* cultures were spotted aseptically on Wagatsuma blood agar (Wagatsuma, 1968) grown overnight at 37^0C. The Kanagawa phenomenon was considered positive for those *V. parahaemolyticus* strains which showed a characteristic halo surrounding the growth due to β-hemolysis.

Genotypic characterization

DNA extraction

Total genomic DNA was extracted from all pure cultures of *V. Parahemolyticus* strains using CTAB method (Ausubel *et al*., 1995). About 1.5 ml of an overnight grown culture from each strain of *V. parahaemolyticus* was concentrated by centrifugation at 10,000 rpm for 5 min. The bacterial pellet was resuspended in 567 µl of 1 x TE buffer, then 30µl of 10% SDS and 3 µl of 20mg/ml proteinase K were added and mixed. The samples were treated with 100µl of 5M NaCl and 80µl of cetrimide hexadecyltrimethyl ammonium bromide (CTAB)/NaCl solution and incubated for 10 min at 65^0C in water bath. The genomic DNA was extracted by using chloroform/isoamyl alcohol (24:1, v/v) and phenol–chloroform–isoamyl alcohol (25:24:1, v/v). The DNA was precipitated with 0.6 volume of isopropanol. The concentration and purity of the extracted DNA was analyzed spectrophotometrically by measuring optical density at 260 and 280 nm wavelengths in Nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

Polymerase Chain Reaction (PCR) for the detection of total and pathogenic *V. parahaemolyticus*

PCR was performed on extracted DNA for the detection of total and pathogenic *V. parahaemolyticus*. For the species identification of *V. parahemolyticus tlh* and *toxR* gene was used and for the detection of virulence determinants, *tdh* and *trh* genes were targeted using specific primers (Table 2). The PCR was carried out in a 30 µl mixture consisting of 3 µl of 10X buffer (Genei, Bangalore), 50µM each of the four deoxy nucleotide triphosphates (dNTPs), 10pmol of each primer, and 1.0U of Taq DNA polymerase (Genei, Bangalore). The extracted DNA was used as template. The PCR assays were performed in a programmable thermocycler (MJ Research, USA).

Antibiogram assay: Antibiotic sensitivity tests were performed for *tdh* positive *V. parahaemolyticus* strains by the standard disk diffusion method (Bauer *et.al.,* 1966) on Muller-Hinton agar as recommended by Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines. The standard antibiotic discs (HiMedia, Mumbai) were placed on the bacterial lawn cultures on Muller Hinton agar plates. The plates were incubated at 37^0C for 24 hours. The antibiotics used were nalidixic acid (30mcg), tetracycline (30mcg), co-trimoxazole (25mcg), ciprofloxacin (5mcg), chloramphenicol (30mcg), ampicillin (10mcg), nitrofurantoin (300mcg), imipenem (10mcg), meropenem (10mcg), cefotaxime (30mcg) and piperacillin-tazobactam (100/10mcg).

Detection of antibiotic resistance gene by polymerase chain reaction (PCR)

The isolates showed resistance to antibiotics was tested by various genes. The genes responsible for the resistance to antibiotics were detected by PCR by using specific primers (Table 3).

Results and Discussion

Characterization of *V. parahaemolyticus*

A total of 203 isolates of *V. parahaemolyticus* were isolated from 116 samples analyzed. The isolates were identified to be *V. parahaemolyticus* based upon their ability to give typical biochemical reactions as listed in the USFDA (2001).

Genotypic analysis revealed that out of 203 isolates, all the isolates tested were positive for *toxR* and *tlh* and negative for *trh* (Fig.1). Only 20 isolates out of 203 isolates showed positive reaction for *tdh* gene (Table 2). These 20 isolates were used for further antibiogram assay.

Kanagawa phenomenon

Twenty *tdh* positive *V. parahaemolyticus* isolates were inoculated on Wagatsuma agar medium which contained 5% washed human erythrocytes. The plate was incubated at 37^0C for 24 hours. All 20 inoculated isolates tested showed a clear hemolytic zone around the colonies indicating their positive reaction for Kanagawa phenomenon.

Antibiotic sensitivity of *V. parahaemolyticus* strains

Among 20 *tdh* positive isolates tested for antibiotic susceptibility, all isolates were susceptible to one or more antibiotics. Out of 20 isolates, all isolates shown resistance to ampicillin (95%), meropenem (15%), cefatoxin (55%), tetracycline, chloramphenicol and piperacillin (5%), ciprofloxacin (20%). None of the isolates were resistant to antibiotics like imipenem, gentamycin, cotrimoxazole and nalidixic acid. The isolate shown tetracycline resistance was checked for the presence of gene responsible for the resistance. One among 20 isolates showed resistance (SO 107) to *tetA, tetB, tetC* and *tet E* gene tested by amplifying gene at particular base pair (Fig 2). None of the isolates showed the resistant genes for chloramphenicol gene *Cat 1, Cat 2, Cat 3, Cml A, Cml B, floR* tested.

The genome of *V. parahaemolyticus* is highly versatile and presence of virulent and pathogenic strains is prominent in marine environments (Gennari *et al.,* 2012).*V. parahaemolyticus* can be considered ubiquitous in the marine environment (Jones *et al*., 2012). In the present study we isolated a total of 203 isolates of *V. parahaemolyticus* from both water and molluscan shellfish samples. Seafood such as fish, shellfish, crustaceans and plankton may harbor *V. parahaemolyticus*. Presence of this organism in seawater is highly influenced by the seasonal variation. Isolates from sea water were reported to be pathogenic to humans (Nelapathi *et al.,* 2011) but *V. parahaemolyticus* outbreaks are invariably related to seafood consumption and pathogenic strains are rarely isolated from seafood. The *tlh* gene has been used as species specific marker for the *V. parahemolyticus* strain (Gutierrez West *et al.,* 2013). The difficulty to isolate virulent strains of *V. parahaemolyticus* from environmental samples such as seafood coastal water and sediments was reported (Hara *et al*., 2001). This difficulty in isolating *tdh* producing colonies on agar plates may be due to the fact that the relative proportion of *tdh* producing *V. parahaemolyticus* to total *V. parahemolyticus* is extremely low in some samples (Yukiko *et al.,* 2003).

V. parahaemolyticus strains producing *tdh* and *trh* are considered pathogenic (Kaufman *et al*., 2003; Depaola *et al*., 2003). A small proportion of the *V. parahaemolyticus* isolates in the environment are known to be virulent and carry virulence genes *tdh* and/or *trh* (Janda *et al*., 1988). Major pathogenicity of *V. parahaemolyticus* has been associated with Kanagawa positive (KP+) isolates capable of producing β-hemolysis on Wagatsuma agar. It is known that the Kanagawa phenomenon is due the production of *tdh*, which is highly heat stable at 100^0C up to10 mins (Nishibuchi and Kaper, 1995).

Table.1 List of *V. parahaemolyticus* virulence strain isolated

Serial number	Strain number	Month/year of isolation	Site of collection	Source	*tdh*	*trh*
1	SBC5	October'13	Sasthan	Clams	+	-
2	SBC13	October'13	Sasthan	Clams	+	-
3	SBC25	October'13	Sasthan	Clams	+	-
4	GO6	October'13	Gangolli	Oysters	+	-
5	SC5	December'13	Sasthan	Oysters	+	-
6	MC25	December'13	Mulki	Clams	+	-
7	GW58	December'13	Gangolli	Water	+	-
8	SO107	December'13	Sasthan	Oyster	+	-
9	SC125	December'13	Netravathi	Clams	+	-
10	SC 130	February '14	Sasthan	Clams	+	-
11	SW 20	February '14	Sasthan	Oysters	+	-
12	SW 39	February '14	Sasthan	Water	+	-
13	NGW17	March '14	Netravathi-Gurupura	Water	+	-
14	SW28	March '14	Sasthan	Water	+	-
15	SW32	March '14	Sasthan	Water	+	-
16	SW34	March '14	Sasthan	Water	+	-
17	SC35	March '14	Sasthan	Clams	+	-
18	SO37	March '14	Sasthan	Oysters	+	-
19	SO41	March '14	Sasthan	Oysters	+	-
20	GO47	March '14	Gangolli	Oysters	+	-

Table.2 Primers used for the confirmation of *Vibrio parahemolyticus* isolates and their virulence determinants.

Gene	Sequence 5'- 3'	Size (bp)	Tm (°C)	Reference
tlh	F:AAAGCGGATTATGCAGAAGCACTG	450	65	Bej *et. al.*, (1999)
	R:GCTACTTTCTAGCATTTTCTCTGC			
toxR	F: GTCTTCTGACGCAATCGTTG	368	63	Kim *et al.*, (1999)
	R:ATACGAGTGGTTGCTGTCATG			
tdh	F: CCACTACCACTCTCATATGC	251	55	Tada *et al.*,(1992)
	R:GGTACTAAATGGCTGACATC			
trh	F:GGCTCAAAATGGTTAAGCG	250	55	
	R:CATTTCCGCTCTCATATGC			

Table.3 Primers used for the detection of different antibiotic resistant gene

Antimicrobials	Resistant genes	Sequence 5'- 3'	Size (bp)	Reference
Tetracycline	*tet A*	**F:** TTGGCATTCTGCATTCACTC **R**: GTATAGCTTGCCGGAAGTCG	494	Menggen *et al*., 2007
	tet B	**F**: CAGTGCTGTTGTTGTCATTAA **R**: GCTTGGAATACTGAGTGTTAA	571	
	tet C	**F**: CTTGAGAGCCTTCAACCCAG **R**: ATGGTCGTCATCTACCTGCC	418	
	tet D	**F**: GCAAACCATTACGGCATTCT **R**: GATAAGCTGCGCGGTAAAAA	546	
	tet E	**F**: TATTAACGGGCTGGCATTTC **R**: AGCTGTCAGGTGGGTCAAAC	544	
	tet G	**F**: TATTAACGGGCTGGCATTTC **R**: AGCTGTCAGGTGGGTCAAAC	550	
Chloramphenicol	*Cat 1*	**F**: AACCAGACCGTTCAGCTGGAT **R**: CCTGCCACTCATCGCAGTAC	549	
	Cat 2	**F**: AACGGCATGATGAACCTGAA **R**: ATCCCAATGGCATCGTAAAG	547	
	Cat3	**F**: ATCGGCATCGGTTACCATGT **R**: ATCCCCTTCTTGCTGATATT	531	
	Cml A	**F**:GGCCTCGCTCTACGTCATC **R**:GCGACACCAATACCCACTAGC	662	
	Cml B	**F**:ACTCGGCATGGACATGTACT **R**:ACGGACTGCGGAATCCATAG	840	
	floR	**F**:ATGACCACCACACGCCCCG **R**:AGACGACTGGCGACTTCTCG	1213	

Fig.1 Gel-electrophoresis of PCR amplifiesd products of *V.parahaemolyticus* for A:tlh gene with 450 bp; B: toxR gene with 368 bp; C:tdh gene with 251 bp.

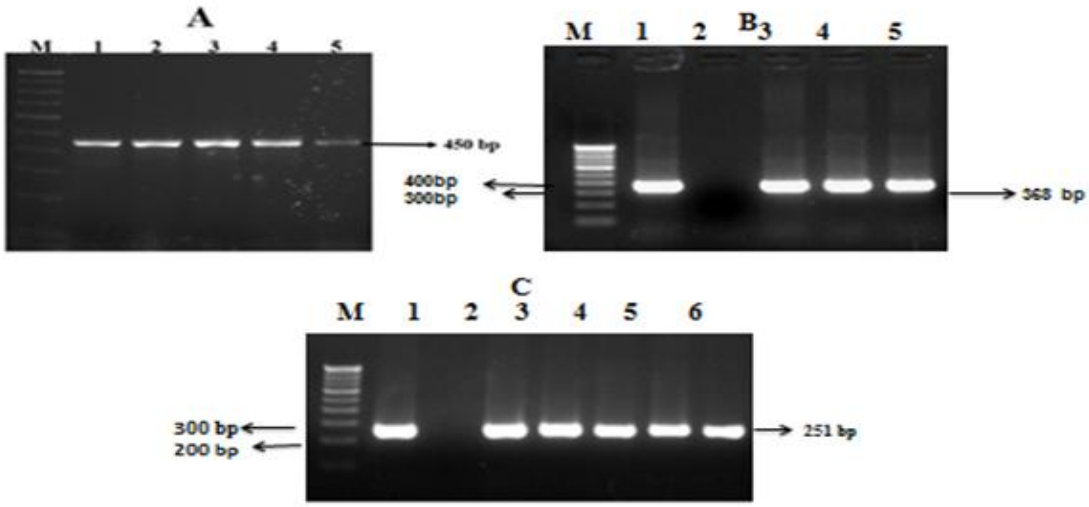
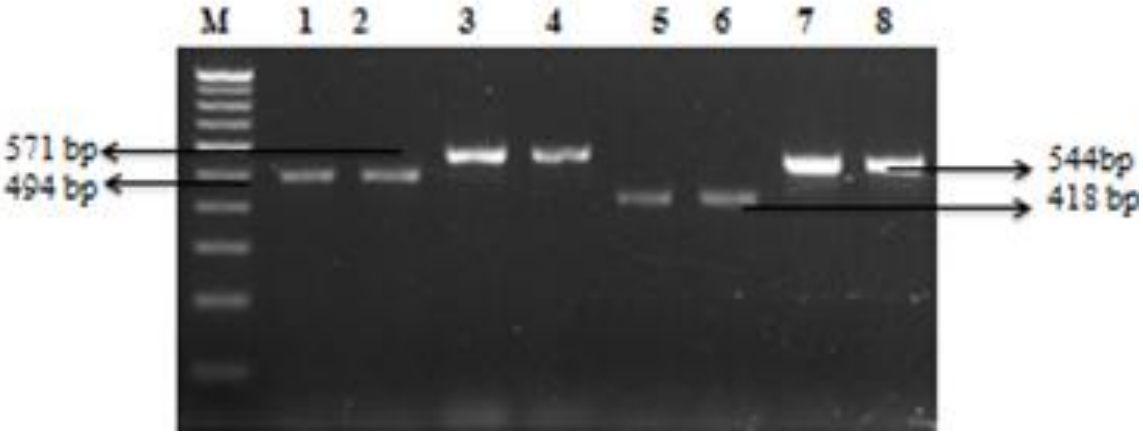


Fig.2 Gel-electrophoresis of PCR amplifiesd products of tetracycline resistant genes. Lane M: 100bp maraker; lane 1&2: tetA (571 bp); lane 3&4: tetB (494 bp); lane 5&6: tet C (418 bp); lane 7&8: tetE (544 bp)



Generally 0.2- 10% of environmental *V. parahaemolyticus* isolates are potentially pathogenic based on presence of tdh/trh genes (Su and Liu, 2007; Martinez Urtaza *et al*., 2008; Miyamoto *et al.*, 1969). Present study assessed 20 pathogenic strains of *tdh+* (9.85%) on the Wagatsuma blood agar that showed positive with β-hemolysis, correlating positive amplification with the PCR analysis for *tdh* gene. *V. parahaemolyticus* exhibited a direct correlation with thermo stable hemolysin genes and positive reaction for Kanagawa phenomenon on Wagatsuma blood agar.

Food contaminated with antibiotic resistance bacteria could be a major threat to public health as there is distinct possibility that gene encoding antibiotic resistant determinants carried on mobile genetic elements may be transferred to other bacteria of human clinical significance (Heinitz *et al*., 2000). Traditionally *Vibrios* are considered to be susceptible to antimicrobials (Oliver, 2006a). In the present study, 95% of the isolates showed ampicillin resistance, Joseph *et al.*, (1978) reported that 90% of the *V. parahaemolyticus* out of 160 isolates was ampicillin resistant and showed β- lactamase activity. Multiple studies conducted in other countries has also reported ampicillin-resistant The high percentage resistance of ampicillin among *V. parahaemolyticus* isolates suggests that ampicillin has a potentially low efficiency in the treatment of *V. parahaemolyticus* infections. It was also found that the drugs like tetracycline, cefotaxime, ceftazidime, and fluoroquinolones, remained highly effective against both the *Vibrio* spp. The results from our study shows that the *Vibrios* were susceptible to the majority of the antibiotics tested and in most of the cases, gastroenteritis caused by this organisms can be even treated with oral rehydration itself (Daniels *et al*., 2000).

In conclusion, the ecology of *Vibrio* spp. is intimately linked to estuarine and marine environments. The concentrations of *V. parahaemolyticus* pathogenic strains in shellfish samples are low, but they may propagate to infectious doses before consumption. The presence of *V. parahaemolyticus* in shellfishes is inevitable, though not all the *V. parahaemolyticus* detected in our shellfish

samples were pathogenic, few samples found to contain high levels of virulent strains of *V. parahaemolyticus* addressing the concern towards public health. Therefore, proper handling of these molluscs should be channeled not only to the retailers, but also to the consumers. For instance, the consumers should be educated on the risk involved and preventive measures, such as proper storage and cooking practices. If the filter feeders are not properly handled, they may serve as a vehicle of food poisoning by *V. parahaemolyticus* through consumption or as cross-contaminants.

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