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Distribution of Potentially Pathogenic *Vibrio parahaemolyticus* in Seafood and the Aquatic Environment of Mumbai, India

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Abstract

The occurrence of total and pathogenic *Vibrio parahaemolyticus* in fresh seafood and the coastal environment of Mumbai, India was examined in this study. Samples comprising of fish, shellfish, coastal sediment and coastal waters were analyzed for *V. parahaemolyticus* by selective enrichment and isolation. Biochemically identified isolates were tested for the presence of *tlh* (thermolabile hemolysin), *tdh* (thermostable direct hemolysin) and *trh* (*tdh*-related hemolysin) by polymerase chain reaction (PCR). While Chromogenic Vibrio (CV) agar yielded *V. parhaemolyticus* from all samples (100%), TCBS agar yielded *V. parahaemolyticus* from 57.1% of the samples. The incidence of trh^+ *V. parahaemolyticus* was high, being isolated from 16.4% of the samples analyzed, while the tdh^+ *V. parahaemolyticus* were isolated from 1.4% of samples. Two tdh^-, trh^+ isolates from the coastal water were positive by a in pandemic group-specific (GS) PCR. The study suggests that seafood and coastal environment may harbor pathogenic *V. parahaemolyticus* with characteristics of pandemic clones, although their incidence is very low. Further, sodium taurocholate (ST) broth and chromogenic *Vibrio* (CV) agar combination is highly suitable for the isolation of total and pathogenic *V. parahaemolyticus* from seafood and environmental samples.

Keywords: *Vibrio parahaemolyticus*, *tdh*, *trh*, seafood, pandemic clone

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Introduction

Pathogenic vibrios are a major concern in seafood, particularly in raw or minimally cooked seafood (Bonnin-Jusserand et al., 2017). Among various pathogenic vibrios encountered in seafood, *Vibrio parahaemolyticus* has emerged as one of the leading causes of seafood-borne infections globally (Su & Liu, 2007). This bacterium is a common inhabitant of the coastal-marine environment and is often associated with plankton, fish and shellfish. Infections with *V. parahaemolyticus* are associated with the consumption of raw or partially cooked seafood (Okuda et al., 1997; DePaola et al., 2000; Su & Liu, 2007). Infections caused by *V. parahaemolyticus* can range from mild, self-limiting diarrhea, to more severe wound infection and life threatening septicemia (Yeung & Boor, 2004). Both pathogenic and non-pathogenic strains of *V. parahaemolyticus* exist. Pathogenic strains of *V. parahaemolyticus* produce either or both of a thermostable direct hemolysin *(tdh)* and *tdh*-related hemolysin (*trh*) (Joseph et al., 1982; Nishibuchi & Kaper, 1995).

The detection of pathogenic strains in seafood is important in considering relatively low prevalence of pathogenic strains among the total *V. parahaemolyticus* (Deepanjali et al., 2005; Parvathi et al., 2006). No dominant serovars of *V. parahaemolyticus* were associated with seafood-borne infections until the appearance of O3:K6 pandemic serotype in India in 1996 (Matsumoto et al., 2000). Pandemic clones of *V. parahaemolyticus* possess *tdh*, but not the *trh* gene, and do not produce urease (Matsumoto et al., 2000). The new O3:K6 strain and its variants are recognized as the pandemic clones of *V. parahaemolyticus* and have been involved in several outbreaks across the world (Nair et al., 2007). The occurrence of *V. parahaemolyticus* with pandemic characteristics has

been reported from seafood in India (Raghunath et al., 2008; Pal & Das, 2010; Parthasarathy et al., 2016). Similar studies on the incidence of pathogenic *V. parahaemolyticus* in seafood and the coastal environment of Mumbai, India are scanty. Therefore, the present study was initiated with the following objectives; i) to study the incidence of pathogenic *V. parahaemolyticus* in fish shellfish and coastal environment of Mumbai, India and ii) to identify *V. parahaemolyticus* with features of pandemic clones among pathogenic *V. parahaemolyticus*.

Materials and Methods

A total of 140 samples comprising of fish (40), shellfish (32), coastal sediment (23) and coastal water (45) were collected and analyzed for the presence of *V. parahaemolyticus*. The samples were collected from different fish markets and landing centers located in and around North Western Mumbai, India. *V. parahaemolyticus* strains AQ4037 (tdh^-, trh^+) and O3:K6 (tdh^+, trh^-) were used as reference strains.

For selective enrichment of *V. parahaemolyticus*, two enrichment broths namely alkaline peptone water (APW) and sodium taurocholate broth (ST) were used. Twenty-five grams of fish, shellfish or sediment (25 ml in case of water samples) was homogenized in 225ml of enrichment broth and incubated statically for 18-24 h at 37°C. A loopful each from the enrichment broth was streaked separately on thiosulfate citrate bile salt sucrose agar (TCBS) and HiCrome *Vibrio* agar (CV) (HiCrome, Hi-Media, Mumbai, India) and incubated for 24 h. Typical colonies of *V. parahaemolyticus*, green on TCBS and bluish-green on CV agar, were subcultured on Luria Bertani (LB) agar containing 3% salt and subjected to further identification using a series of biochemical tests (FDA, 2004).

The colonies identified as *V. parahaemolyticus* by biochemical tests were confirmed by species-specific PCR assays targeting *toxR* and *tlh* genes (Bej et al., 1999; Kim et al., 1999). Pathogenic *V. parahaemolyticus* were identified by PCR detection of either or both of *tdh* and *trh* genes (Bej et al., 1999). For the detection of pandemic strains of *V. parahaemolyticus*, a pandemic group-specific PCR for the *toxRS* gene was used as described previously (Matsumoto et al., 2000; Okura et al., 2004).

The *tdh* sequences in PCR products of varying sizes obtained with some isolates were blotted onto a nylon membrane and Southern hybridized using biotin-labeled *tdh* PCR product as the probe. The *tdh* PCR product from the reference strain O3:K6 was labeled with biotin-dUTP using a biotin labeling kit (Thermo Scientific, USA). The PCR products were separated on 1% agarose gel and Southern blotted onto positively charged nylon membrane in an alkaline condition (Brown, 2001). Hybridization was done overnight at 42°C in a hybridization oven (UVP, USA). Following hybridization, the membrane was subjected to three washing steps: twice with 5X SSC (sodium saline citrate) buffer, 0.5% [W/V] SDS at 50°C, 5 min each; twice with 0.1X SSC, 1% [W/V] SDS at 42°C for 15 min and once with 2X SSC for 5 min at room temperature. The membrane was subjected to a blocking step by incubating in bovine serum albumin for 1 h at 60°C. Streptavidin-alkaline phosphatase (SA: AP; 1:5000 diluted) (Thermo Scientific, USA) was added and the membrane was incubated for 10 min at room temperature. For color development, the membrane was incubated with BCIP-NBT with gentle shaking at 37°C until the bands were clearly visible.

Results and Discussion

In the present study, *V. parahaemolyticus* was isolated from all (100%) of the 140 samples analyzed irrespective of the source or the season (Table 1). A total of 688 isolates were confirmed as *V. parahaemolyticus* by biochemical tests followed by a species-specific PCR assay (Table 1 & Fig. 1). Several studies from India have reported the incidence of *V. parahaemolyticus* in seafood ranging from 40-75.9% (Chakraborty et al., 2008; Pal & Das, 2010; Sudha et al., 2012). *V. parahaemolyticus* is a natural inhabitant of coastal-marine environment and is usually present in numbers ranging from 4 to 1000 cells per 100 ml of seawater (De Paola et al., 1990). Studies have shown that in temperate waters, the density of *V. parahaemolyticus* positively correlates with the water temperature (Duan & Su, 2005). In contrast, water temperature and salinity have least effect on the abundance of *V. parhaemolyticus* in oysters in tropical waters (Deepanjali et al., 2005).

The APW-TCBS combination is the most commonly used selective enrichment-isolation method for *V. parahaemolyticus* from seafood (FDA, 2004). In this study, an additional selective enrichment broth (ST) and a selective agar (CV) were used and the recovery efficiency of *V. parahaemolyticus* in four different combinations namely as APW-CV, ST-CV,

Table 1. Isolation of *V. parahaemolyticus* (VP) from seafood, coastal water and sediment samples using a combination of two selective enrichment broths and selective plating media

Samples	No. analyzed	No. (%) positive for VP on CV		No. (%) positive for VP on TCBS		No. of VP isolated on CV		No. of VP isolated on TCBS	
		APW	ST	APW	ST	APW	ST	APW	ST
Fish	40	35 (87.5)	40(100)	10 (25)	16 (40)	48	57	15	23
Shellfish	32	30 (93.7)	32(100)	20 (62.5)	21(65.6)	63	75	30	55
Water	45	42 (93.3)	45 (100)	25 (55.5)	29 (64.4)	64	72	22	32
Sediment	23	18 (78.2)	23 (100)	9 (39.1)	14 (60.8)	34	49	18	31
Total	140	125 (89.2)	140(100)	66 (47.1)	80 (57.1)	209	253	85	141

Table 2. Incidence of pathogenic (*tdh*$^+$ and/*trh*$^+$) *V. parahaemolyticus* (VP) in different samples analyzed in this study

Samples	No. analyzed	No. (%) positive for pathogenic VP	No. (%) of *trh*$^+$ samples	No. (%) of *tdh*$^+$ samples
Fish	40	0	0	0
Shellfish	32	2 (6.25)	2 (6.2)	0
Water	45	18 (40)	16 (35.5)	2 (4.4)
Sediment	23	5 (21.76)	5 (21.7)	0
Total	140	25 (17.85)	23 (16.4)	2 (1.4)

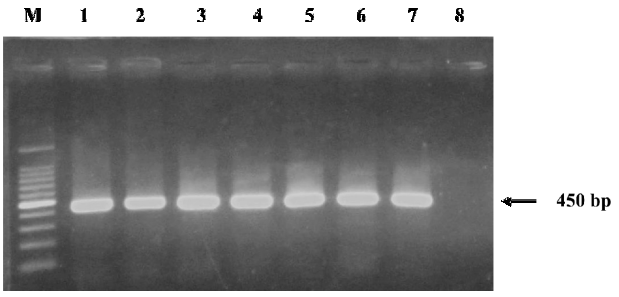

Fig. 1. Detection of *tlh* gene of *V. parahaemolyticus*
Lane M: GeneRuler 100 bp (Fermentas, USA); Lane1: positive control (AQ4037), Lane 2-6: *V. Parahaemolyticus* isolates from different samples

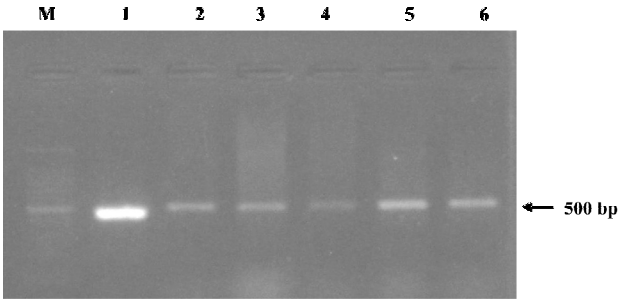

Fig. 2. Detection of *trh* gene of *V. parahaemolyticus*
Lane M: GeneRuler 100 bp (Fermentas, USA), Lane1: positive control (AQ4037), Lane2-6: *V. Parahaemolyticus* isolates containing *trh* gene from water samples

APW-TCBS and ST-TCBS were compared. It was observed that, 67.15% of the total confirmed isolates of *V. parahaemolyticus* were from Chromogenic *Vibrio* agar, while only 32.84% of the isolates were from TCBS (Table 1). Chromogenic medium contains a chromogenic substrate instead of sucrose used in conventional isolation media such as the TCBS agar (Hara-Kudo et al., 2003). *V. parahaemolyticus* produce bluish green colonies on CV agar, while *V. mimicus* appear as creamy colonies and *V. vulnificus* as pale green colonies.

Higher efficiency of *V. parahaemolyticus* isolation has been reported using a chromogenic isolation medium (CHROMagar *Vibrio,* Paris, France) following a two-step enrichment in APW and salt polymixin broth (Blanco-Abad et al., 2009).

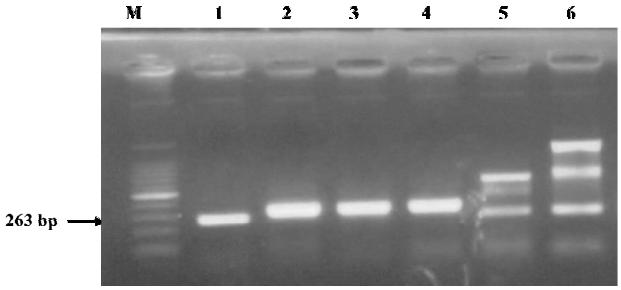

Fig. 3. PCR amplification of *tdh* gene of *V. parahaemolyticus* Lane M, GeneRuler 100bp (Fermentas, USA); lane 1, positive control (O3:K6); lanes 2-6: *V. Parahaemolyticus* isolated from water samples. The amplification products were subjected to Southern blotting and hybridization using a biotin-labeled polynucleotide probe (Fig. 4).

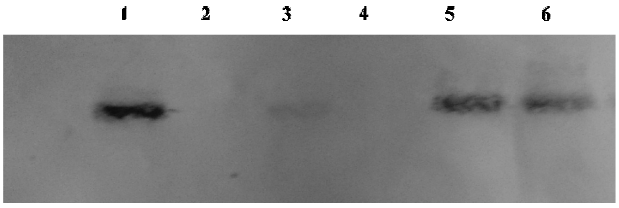

Fig. 4. Southern blotting and hybridization of *tdh*-gene products using biotin-labeled polynucleotide probe

In the present study, all 140 samples tested on CV agar were positive for the presence of *V. parahaemolyticus*. The isolation rate on TCBS agar was 57.1% (Table 1). A previous study, reported *V. parahaemolyticus* isolation rates of 16 and 27% on TCBS and CV agars respectively, from retail seafoods in the Netherlands (Hassan et al., 2012). The low recovery from TCBS plate might be due to the overgrowth of other *Vibrio* species especially yellow colored colonies which mask the detectability of *V. parahaemolyticus*. Other sucrose non-fermenting bacteria such as *V. mimicus* and *V. vulnificus* also produce green colonies on TCBS agar which are difficult to differentiate from *V. parahaemolyticus*. The higher recovery rate of *V. parahaemolyticus* on CV agar could be attributed to their easily distinguishable bluish-green colonies of lower background colonies compared to the TCBS agar. The result of the present study suggests that CV is the most suitable medium for the isolation of *V. parahaemolyticus* from seafood and environmental samples. This observation is supported by several previous studies which have reported better isolation rates of *V. parahaemolyticus* on chromogenic agar medium (Bilung et al., 2005; Blanco-Abad et al., 2009). Chromogenic media such as the ChromID Vibrio are superior to TCBS agar in yielding *V. cholerae* and *V. parahaemolyticus* from clinical samples (Eddabra et al., 2011).

The study tried to further ascertain the performance of two selective enrichment broths, ST and APW, for their efficiencies in yielding *V. parahaemolyticus* on selective agars. The results suggest that ST broth was superior compared to APW broth and allowed better isolation of *V. parahaemolyticus* (Table 1). All the samples analyzed in this study yielded *V. parahaemolyticus* on CV agar following enrichment in ST broth. Thus, ST broth-CV agar combination is recommended for the isolation of *V. parahaemolyticus* from environmental samples. In comparison, the APW-TCBS combination yielded *V. parahaemolyticus* from only 47.14% of the samples (Table 1). A previous study from India reported better isolation of *V. parahaemolyticus* from ST broth compared to APW (Raghunath et al., 2008). The

Table 3. Comparative efficiencies of two selective enrichment broths and two selective media in yielding pathogenic *V. parahaemolyticus* (VP) from different samples

Samples	No. positive for pathogenic VP	No. of *trh*$^+$ samples on CV		No. of *trh*$^+$ samples on TCBS		No. of *tdh*$^+$ samples on CV		No. of *tdh*$^+$ samples on TCBS	
		APW	ST	APW	ST	APW	ST	APW	ST
Fish	-	-	-	-	-	-	-	-	-
Shellfish	2 (6.25%)	-	2	-	-	-	-	-	-
Water	18 (40%)	12	16		4	-	2		1
Sediment	5 (21.76%)	-	5	-	-	-	-	-	-
Total	25 (17.85%)	12	23	-	4	-	2	-	1

high recovery rate on ST broth might be due to the presence of sodium taurocholate and $MgCl_2$ which may have a role in selecting *V. parahaemolyticus*.

All the confirmed (*tlh*-positive) isolates of *V. parahaemolyticus* were tested for the presence of thermostable direct hemolysin (*tdh)* and thermostable direct hemolysin-related hemolysin (*trh*), the marker genes for pathogenic strains of *V. parahaemolyticus* (Fig. 2 & 3). The *tdh* gene specific PCR did not yield the expected amplification product of 263 bp with any of the isolates. However, five isolates yielded strong amplicons of higher size with *tdh*-specific primers (Fig. 3). These products were subjected to Southern blotting and hybridization using a biotin-labeled polynucleotide probe. Positive hybridization signals were obtained with three products confirming that these products were indeed from the *tdh* gene (Fig. 4). Based on the PCR and hybridization results, the incidence of *tdh*$^+$ *V. parahaemolyticus* was 1.42% (Table 2). Studies have shown that the incidence of pathogenic *V. parahaemolyticus* can vary from 0% to 59.3% in seafood and marine samples (Deepanjali et al., 2005; Raghunath et al., 2009; Hassan et al., 2012; Xu et al., 2016). However, in general, the incidence of *tdh*$^+$ *V. parahaemolyticus* is very low (<1%) in environmental and seafood samples (García et al., 2009; Di Pinto et al., 2012).

The incidence of *trh*$^+$ *V. parahaemolyticus* was relatively higher in the samples analyzed in this study (Table 2 & Fig. 2). Of the 140 samples analyzed, *trh*$^+$ *V. parahaemolyticus* were isolated from 16.42% of the samples (Table 2). Previous studies from India have reported high incidence of *trh*$^+$ *V. parahaemolyticus* compared to *tdh*$^+$ isolates. The incidence of *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus* in oysters in Mangalore, India was reported to be 6.1% and 59%, respectively (Deepanjali et al., 2005). A slightly better detection rate (8.4%) of *tdh*$^+$ *V. parahaemolyticus* was achieved by performing PCR directly on the enrichment broths (Raghunath et al., 2009). Similar trends in the prevalence of *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus* have been reported from other geographical regions. Bilung et al. (2005) isolated 62 *V. parahaemolyticus* from 100 samples of cockles in Malaysia and detected *tdh* gene in only two isolates and *trh* gene in 11 isolates. A relatively higher incidence (12.8%) of *tdh*$^+$ *V. parahaemolyticus* has been reported in Alabama oysters (DePaola et al., 2003) and in fish harvested from coastal waters of Spain (12%) (Rodriguez-Castro et al., 2010).

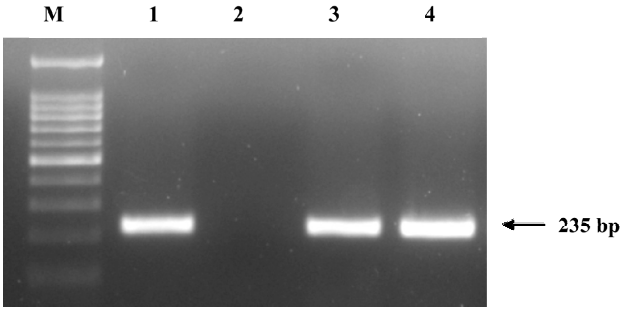

Fig. 5. Pandemic group-specific PCR assay showing positive amplification signals with two *trh*$^+$ isolates of this study.

Lane 1, *V. parahaemolyticus* reference strain O3:K6; lane 2, negative control; lanes 3 & 4, *trh*-positive *V. parahaemolyticus* isolated from the water samples.

In the present study, pathogenic *V. parahaemolyticus* were isolated largely from the water samples (12.85%) (Table 2). Twenty-three *trh*$^+$ *V. parahaemolyticus* were isolated of which 16 were from the water samples, five were from the sediment and two were from the shellfish (Table 3). The *tdh*$^+$ *V. parahaemolyticus* were isolated from only the water samples (Table 3). Water samples accounted for 27.61% of the total confirmed isolates of *V. parahaemolyticus* (Table 1), second only to shellfish samples, which contributed 32.41% of the total *V. parahaemolyticus*. Higher levels of total pathogenic *V. parahaemolyticus* generally indicate the presence of pathogenic *V. parahaemolyticus*, although there is no direct correlation between the two (Deepanjali et al., 2005). It may be speculated that although the density of *V. parahaemolyticus* in filter feeding shellfish is several fold higher than that in the surrounding water, the isolation of pathogenic strains may be hindered by the presence of large background flora in highly contaminated water bodies. However, in the present study, pathogenic *V. parahaemolyticus* were not detected in any of the fish samples.

Two selective enrichment broths compared in this study varied in yielding pathogenic *V. parahaemolyticus* from the samples. ST broth performed better in terms of yielding *tdh*$^+$ and *trh*$^+$ *V. parahaemolyticus*, while the APW broth yielded only *trh*$^+$ *V. parahaemolyticus* (Table 3). The isolation rate was better on CV agar compared to TCBS agar (Table 3). All 25 samples positive for pathogenic *V. parahaemolyticus* yielded *tdh*$^+$/*trh*$^+$ isolates with ST broth-CV agar combination, 12 with APW broth-CV

agar combination, five with ST broth-CV agar combination and none with APW-TCBS combination (Table 3). These results clearly suggest higher efficiency of ST broth-CV agar combination in selecting pathogenic *V. parahaemolyticus* from seafood and coastal water samples.

All pathogenic *V. parahaemolyticus* isolates of this study were subjected to pandemic group-specific PCR (GS-PCR). Two *trh*$^+$ isolates from water samples yielded specific amplicons in a GS PCR described by Okura et al. (2004) suggesting that these isolates may be carrying genetic markers of pandemic clones of *V. parahaemolyticus*. None of the isolates were tested positive by the GS-PCR described by Matsumoto et al. (2000). Pandemic *V. parahaemolyticus* are known to harbor only *tdh* gene and are urease negative (Okuda et al., 1997; Matsumoto et al., 2000). The *trh* and GS-PCR positive isolates from this study will be of interest to further investigate their virulence gene composition and pathogenic potential.

In the present study, low incidence of *tdh*$^+$, but a relatively higher incidence of *trh*$^+$ *V. parahaemolyticus* in seafood and the coastal environments of Mumbai was observed. Using Southern hybridization, possible sequence variants of *tdh* gene were detected although the human health significance of such isolates from the environment remains to be investigated. Further, the study suggests that ST broth-CV agar combination results in better recovery of total and pathogenic *V. parahaemolyticus* from seafood and the environmental samples.

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