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Isolation, Identification and Molecular Characterization of *Vibrio parahaemolyticus* from Shrimp Samples from South Gujarat of Navsari District

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ABSTRACT

Shrimp cultivation faced serious problems with diseases caused by viruses and bacteria causing severe economic losses. Of the bacterial infections, *Vibrio parahaemolyticus,* have been frequently associated with fatalities both, in hatcheries and grow out ponds. *V. parahaemolyticus* is pathogenic to human besides fish and other aquatic lives. For systematic bacteriological examination of aseptically collected all samples were brought to the laboratory in Ice box and they were further processed for isolation, identification and characterization of *V. parahaemolyticus* isolates on the basis of their morphological, cultural and biochemical characteristics. Out of total 150 samples of shrimp 5 (3.33%) isolates of *V. parahaemolyticus* were obtained which included 3 (4.28%, 3/70) from marine shrimp samples and 2 (2.5%, 2/80) from freshwater shrimp samples. Out of 27 samples of the hand swabs of fish handlers, 2 (7.40 %) were positive for *V. parahaemolyticus*. However, none of 23 human stool samples was positive for the pathogen. The pathogenicity of 7 isolates of *V. parahaemolyticus* was tested on Wagatsuma agar contained human red blood cells. Only one *V. parahaemolyticus* isolate (33.33%, 1/3) cultured from marine shrimp sample was Kanagawa Phenomenon positive, expressing β- haemolysin on Wagatsuma agar. Rest of all the isolates were KP negative. all 7 *V. parahaemolyticus* isolates amplified the species specific *toxR* (368 bp) gene. While ruling out pathogenic nature of the isolates by PCR, 1 out of 7 (14.28 %) isolates exhibited amplification of virulent *tdh* (269 bp) gene. However, not a single *V. parahaemolyticus* isolate contained *trh* (500bp) gene.

Keywords: Shrimp, *Vibrio parahaemolyticus*, Diarrhoea, Acute gastroenteritis

Shrimp cultivation is an economically important agricultural activity worldwide. In international trade, the most prominent product from aquaculture is marine shrimp, with approximately 26 per cent of the total product comes from pond-reared *Penaeid* species i.e. *Litopenaeus vannamei* and *Penaeus monodon* (Shanmugasundaram *et al.*, 2015).

With its vast brackish water resources and congenial climatic conditions, India has good scope for shrimp production (Ponnusamy and Pillai, 2014). Gujarat is having a vast brackish water area of 3.76 lakh hectares throughout 1,600 km long coastline which is ideal for shrimp culture. Large number of shrimp farms have been constructed and major activities related to shrimp culture have been concentrated on coastal belt of South Gujarat (Vadher and Kapila, 2014).

Shrimps and Prawns of various kinds have certainly been a source of protein for human consumptions from very early times. The most common *Vibrio* species found in farming phases of black tiger shrimp in India were *V. alginolyticus, V. parahaemolyticus* and *V. vulnificus* (Bhaskar and Setty, 1994). Bacteria of the genus *Vibrio* are ubiquitous in

marine and estuarine aquatic ecosystems where shrimp dwells naturally and are farmed. Several *Vibrio spp*. form part of the natural biota of fish and shellfish with association of *V. harveyi and V. parahaemolyticus* in bacterial infections in shrimp. Among more than 20 *Vibrio* species known to be associated with human disease, *V. cholerae, V. parahaemolyticus* and *V. vulnificus* are most important. Depending on the species involved, the clinical manifestations are different, ranging from gastroenteritis to septicaemia and wound infection (Gopal *et al*., 2005).

Reports showed that food borne illness 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.*, 2000; Lee *et al.*, 1997), and in India it has almost doubled (Chowdhury *et al.*, 2000).

The occurrence of *V. parahaemolyticus* in marine and estuarine environments is of special interest from the public health point of view too, since most outbreaks of gastroenteritis caused may result in more severe infections than those caused by sewage-borne viral and bacterial pathogens (Rippey, 1994).

Vibrio parahaemolyticus is a Gram-negative, halophilic, non spore forming rod, either straight or has a single, rigid curve. When grown in liquid medium, motility is exhibited by a single polar flagellum. *V. parahaemolyticus* is inhibiting temperate and tropical estuarine, marine and costal environment worldwide (Baumann and Schubert, 1984).

Consumption of raw or undercooked seafood, particularly shellfish, contaminated with *V. parahaemolyticus* lead to development of acute gastroenteritis characterized by diarrhoea, headache, vomiting, nausea, abdominal cramps and low fever. It is recognized as the leading cause of human gastroenteritis associated with seafood consumption in the world (Kaysner and DePaola, 2001; Su and Liu, 2007).

Clinical isolates of *V. parahaemolyticus* most often produce either the thermostable direct hemolysin (*TDH*) or *TDH*-related hemolysin (*TRH*) encoded by *tdh* and *trh* genes, respectively. However, only bacteria producing virulence factors, i.e. *TDH* and/or *TRH*, are considered to be pathogenic and can cause acute gastroenteritis or invasive septicaemia (Bisha *et al.,* 2012).

Thermostable direct Hemolysin is capable of producing β haemolysis on Wagatsuma agar which is called Kanagawa phenomenon (KP). Most of the strains (90 %) isolated from clinical cases show this type of haemolysis, while only 1–2 % of the strains of environmental origin are KP positive (Drake *et al.*, 2007).

MATERIALS AND METHODS

Samples

A total aseptically collected 150 shrimp samples, 27 samples of hand swabs from fish handlers were collected from retail fish outlets, and 23 stool samples from patients suffered with digestive disturbances after consumption of seafood reported to private clinics, all from Navsari city were investigated.

Shrimp samples

Altogether 150 shrimp samples from different ecosystems viz. marine and freshwater comprising of shrimp and prawns like white leg shrimp, black tiger shrimp and brown shrimp (*Metapenaeus dobsoni*) sold in and around Navsari city were collected aseptically in sterile polythene bags. Each sample bag was labelled indicating code number, type of the sample, date of collection etc. Samples were placed in the insulated box containing ice and brought to the departmental P.G. laboratory for further investigation.

The Human samples

From the human subjects, stool samples and hand swabs collected aseptically directly in APW enrichment broth and brought to the departmental laboratory and incubated at 35-37°C for 16-18 hrs.

Isolation and identification of *V. parahemolyticus*

Samples of freshwater and marine shrimps and prawns were subjected to obtain surface tissues, gills, and guts and hepato-pancreas. About 25g of each type of sample (Gills, Guts and Hepato-pancreas) was thoroughly triturated in a sterile mortar and pestle with use of 225 ml PBS (0.85% NaCl), pH 7.2-7.5, than inoculate 3-tube, multiple dilution, alkaline peptone water (APW) MPN series (i.e., add 1 ml portions of each 1:10 and higher dilution to sets

of 3 tubes containing 10 ml APW). Incubate tubes 16-18 h at 35-37°C. Inoculations of MPN tubes completed within 15-20 min of dilution preparation. From the human subjects, stool samples and hand swabs collected aseptically directly in APW enrichment broth and brought to the departmental laboratory and incubated at 35-37°C for 16-18 hrs. Subsequently they were processed in similar procedures follow for shrimp samples. A loopful of culture from APW after 18-24h enrichment was streaked onto Thiosulfate citrate bile salt sucrose agar (TCBS) and Vibrio parahaemolyticus sucrose agar (VPSA) incubated at 37 °C for 24 h. The characteristic large colonies (3-4mm) with light blue or green centers on TCBS and VPSA were regarded as presumptive *V. parahaemolyticus* and further subjected to morphological, cultural and biochemical characterization. A series of biochemical tests as per BAM, USFDA method (Kaysner and DePaola, 2004) was used for the identification of *Vibrio* isolates.

Detection of Pathogenicity by Kanagawa test

The pathogenicity of *V. parahaemolyticus* has been related to its ability to cause β-haemolysis on a special high salt medium called wagatsuma agar known as Kanagawa Phenomenon or reaction.

The Kanagawa reaction was carried on Wagatsuma agar using 2% human RBCs. Loopfuls of overnight grown culture of *V. parahaemolyticus* isolates were spot inoculated onto Wagatsuma agar plates and incubated at 37 °C for 24 h. The β-haemolysis of human RBCs after 24 h incubation was interpreted as positive for Kanagawa reaction (Beauchat, 1982).

PCR assay

PCR was performed separately for *toxR, tdh* and *trh* genes for the biochemically characterized isolates. The DNA of isolates of *V. parahaemolyticus* was prepared by bacterial lysis by heat application method. Approxemately loopful of culture was taken in microcentrifuge in 100ul of sterilized DNAse and RNAse free milliQ water (milipore, USA). Then vortexed and samples were heated at 95°C for 10 min, cell debris was removed by centrifugation and 3ul of supernatant was used as a DNA template in PCR reaction mixture. PCR was performed with three sets of primer pairs specific for the *toxR, tdh* and *trh* gene shown in Table 1.

Table 1: Primer pairs used for virulence characterization of *Vibrio parahaemolyticus*

Sl. No.	Target Genes	Primer sequence (5'→3')	Product Size	Reference
1	*toxR*	**F:** GTC TTC TGA CGC AAT CGT TG **R:** ATA CGA GTG GTT GCT GTC ATG	368 bp	Kim *et al.* (1999)
2	*tdh*	**F:** GTA AAG GTC TCT GAC TTT TGG AC **R:** TGG AAT AGA ACC TTC ATC TTC ACC	269 bp	Bej *et al.* (1999)
3	*trh*	**F:** TTG GCT TCG ATA TTT TCA GTA TCT **R:** CAT AAC AAA CAT ATG CCC ATT TCC G	500 bp	Bej *et al.* (1999)

RESULTS AND DISCUSSION

Systematic bacteriological examination of total 150 shrimp and prawn samples resulted in the recovery of 5 (3.33%) *V. parahemolyticus* isolates as shown in Table 2. The findings of the present study were in approximation to 5.5 per cent as reported by Khamesipour (2014). However, lower incidence of 0.5 and 1.8 per cent was recorded by Hosseini *et al*. (2004) and Raissy *et al*. (2015), respectively. In contrast to the findings of present work, earlier studies conducted by Kshirsagar *et al.* (2013); Sperling *et al.* (2015) and Shanmugasundaram *et al.* (2015) reported higher incidence of 11.66, 80.80 and 83.40 per cent, respectively. This could be due to variation in the sample size, different geographical conditions etc.

The incidence of *V. parahaemolyticus* in marine prawns in the present study was 4.28 per cent, which is lower than 15.00 per cent (6/40) recorded by Kshirsagar *et al*. (2013).

The incidence of *V. parahaemolyticus* in fresh water shrimp in the present study was 2.5 per cent which is in approximation to 5 per cent reported by Kshirsagar *et al.* (2013). However, quite higher incidence of 78.8 and 83.4 per cent reported by Anjay *et al.* (2014) and Shanmugasundaram *et al.* (2015), respectively, indicating quite high level of contamination. The findings of the present study indicated relatively lower threat to the public health. By advising for proper kitchen hygiene measures to consumers, their health could be safeguarded.

Table 2: Isolation of *V. parahaemolyticus* from Shrimp and human samples

Sl. No.	Type of the Sample		No. of samples examined	No. of samples positive	Per cent value	
1	Shrimp Sample	Fresh water shrimp/prawn	80	2	2.50	
		Marine water shrimp/prawn	70	3	4.28	3.33
2	Human Samples	Hand swab from fish handlers	27	2	7.40	
		Human stool sample	23	0	0.00	4.00

In the present study, *V. parahaemolyticus* was found 7.4 per cent (2/27) samples of the hand swabs of fish handlers as shown in table no. 2. This finding is lower than that of Mohammed (2012) who reported 13.2 per cent (7/53) incidence in the hand swabs of fish handlers.

In the present study, none of 23 stool samples from patients suffered with digestive disturbances after consumption of seafood found positive for *V.parahaemolyticus*. Hernández-Díaz *et al.* (2015), Velazquez-Roman *et al.* (2012) and Mohammed (2012) reported 2.4 per cent, 5.09 per cent and 7.7 per cent incidence of *V. parahaemolyticus* in human stool samples, respectively

In the present study, all the seven isolates of *V. parahaemolyticus* were found to amlify the species specific *toxR* (368bp) gene and all the seven isolates of *V. parahaemolyticus* (5 from shrimp and 2 from human samples) were subjected to PCR for detection of the *tdh* gene and one isolated obtained from marine shrimp (14.28%) yielded desired amplified product of 269 bp which is positive for Kanagawa phenomenon. The observation of the present study were in close proximity to 11.11 per cent reported in the literature reviewed so far (Kshirsagar *et al*., 2013). While, none of the seven isolates (all of shrimp and human samples) of *V. parahaemolyticus* subjected to PCR exhibited amplification of *trh* gene (500 bp). The findings of the present study were similar to that of Kshirsagar *et al*. (2013) and Zulkifli *et al.* (2009) who also failed to amplify *trh* gene in any of *V. parahaemolyticus* isolates they studied.

Detection of Pathogenicity by Kanagawa Reaction

Haemolytic activity with 7 isolates (5 from shrimp and 2 from human sample) was tested on Wagatsuma agar using human red blood cells. one *V. parahaemolyticus* isolate (33.33%, 1/3) cultured from marine shrimp sample was Kanagawa Phenomenon positive, expressing β-haemolysin on Wagatsuma agar shown in Fig. 1. All the samples of human subject (2) and fresh water shrimp (2) were KP negative.

One of seven (14.28 %) *V. parahaemolyticus* isolates was Kanagawa Phenomenon positive. Looking to the type of the samples, not a single isolate cultures from fresh water shrimp and human subject was Kanagawa Phenomenon positive. From three marine isolates of *V. parahaemolyticus*, single (33.33%) was Kanagawa Phenomenon positive.

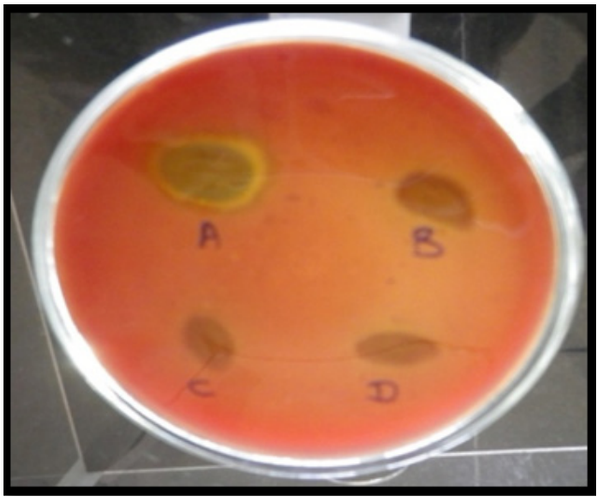

Fig. 1: Kanagawa Phenomenon (KP) on Wagatsuma agar A: Kanagawa positive reaction and B, C and D: Kanagawa negative reaction

Kanagawa positive strains contain a thermostable direct haemolysin (TDH), which might be responsible for gastroenteritis syndrome by *V. parahaemolyticus* (Miyamato *et al.,* 1969).

The incidence of Kanagawa positive strains of *V. parahaemolyticus* marine ecosystems stresses the need for hygienic handling of sea foods at every stage. Honda *et al.,* (1988) identified a TDH- related haemolysin (TRH) from Kanagawa negative strains of *V. parahaemolyticus* and this TRH was immunologically similar but not identical to TDH. Therefore, it appeared to be evident that the Kanagawa negative strains of *V. parahaemolyticus* also produce some toxic materials which may play some role in the pathogenicity.

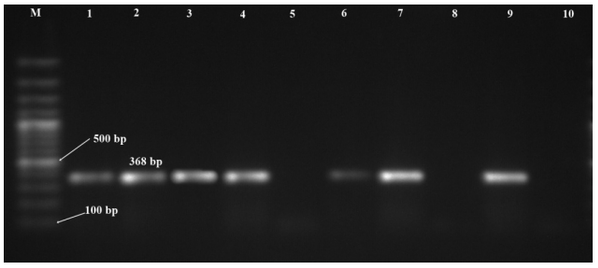

Fig. 2: Agarose gel showing amplification product of toxR gene of *V. parahaemolyticus* M1 and M2: 100 bp DNA marker Lane 1-4 and 6,7,9 : Amplification product of *toxR* gene (368 bp) Lane 5 and 8 : Negative sample Lane 10: Negative control

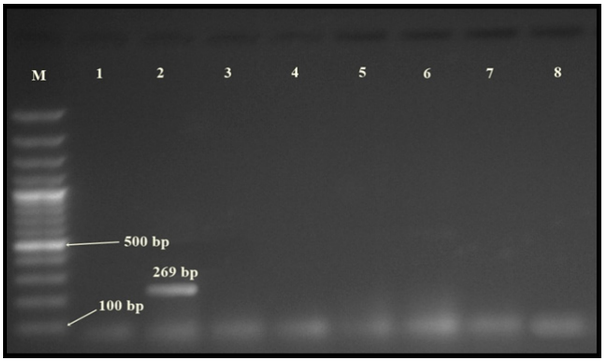

Fig. 3: Agarose gel showing amplification product of *tdh* gene of *V. parahaemolyticus* M: 100 bp DNA marker Lane 2: Amplification product of *tdh* gene (269 bp) Lane 1 and 3-7: Negative sample Lane 8: Negative control

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