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
maritime 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 septicemia 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 coastal 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. Approximately 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>
<thead>
<tr>
<th>Sl. No.</th>
<th>Target Genes</th>
<th>Primer sequence (5’→3’)</th>
<th>Product Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>toxR</td>
<td>F: GTC TTC TGA CGC AAT CGT TG&lt;br&gt;R: ATA CGA GTG GTT GCT GTC ATG</td>
<td>368 bp</td>
<td>Kim et al. (1999)</td>
</tr>
<tr>
<td>2</td>
<td>tdh</td>
<td>F: GAA AAG GTC TCT GAC TTT TGG AC&lt;br&gt;R: TGG AAT AGA ACC TTC ATC TTC ACC</td>
<td>269 bp</td>
<td>Bej et al. (1999)</td>
</tr>
<tr>
<td>3</td>
<td>trh</td>
<td>F: TTG GCT TCG ATA TTT TCA GTA TCT&lt;br&gt;R: CAT AAC AAA CAT ATG CCC ATT TCC G</td>
<td>500 bp</td>
<td>Bej et al. (1999)</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

Systematic bacteriological examination of total 150 shrimp and prawn samples resulted in the recovery of 5 (3.33%) V. parahaemolyticus 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.
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 amplify 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 thr 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 thr 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* (Miyamoto et al., 1969).

<table>
<thead>
<tr>
<th>Sl. no.</th>
<th>Type of the Sample</th>
<th>No. of samples examined</th>
<th>No. of samples positive</th>
<th>Per cent value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shrimp Sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fresh water shrimp/prawn</td>
<td>80</td>
<td>2</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>Marine water shrimp/prawn</td>
<td>70</td>
<td>3</td>
<td>4.28</td>
</tr>
<tr>
<td>2</td>
<td>Human Samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hand swab from fish handlers</td>
<td>27</td>
<td>2</td>
<td>7.40</td>
</tr>
<tr>
<td></td>
<td>Human stool sample</td>
<td>23</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
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

REFERENCES


