ISOLATION AND CHARACTERIZATION OF *Vibrio* spp. FROM SEA FOOD AND ENVIRONMENTAL SAMPLES IN AND AROUND CHENNAI CITY

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ABSTRACT

Vibrio is a major sea food pathogen which needs to be monitored regularly for devising appropriate control strategy to ensure food safety among sea food consumers and handlers. The present study aims to ascertain the occurrence of Vibrio spp. in seafood viz fishes, prawn/shrimps, crabs and water samples collected from various fish markets in and around Chennai city, Tamil Nadu, India. A total of 175 samples were screened, of which 106 (60.57%) samples were found positive by PCR targeting the 16s rRNA of Vibrio genus and all the positive isolates were subjected to toxR gene identification by Multiplex PCR for species level identification. At the species level 28(26.41%) were positive for Vibrio parahemolyticus (Vp), 6 (5.67%) positive for Vibrio Cholerae (Vc), 2 (1.89%) positive for Vibrio vulnificus (Vv). The presence of mixed vibrio species were also observed of which 56 samples (43.40%) revealed the presence of Vp and Vc, 7 (6.60%) with Vp and Vv, 3 (2.84%) with Vv and Vc and 14 samples with (13.20%) Vp, Vv and Vv. The present study identified the presence along with mixed Vibrio spp. in most of the samples tested from the study area.

Key words: Crabs, Fish, Seafood, Vibrio cholera, Vibrio parahemolyticus, Vibrio vulnificus

INTRODUCTION

Vibrio is the genus of Gram-negative rod-shaped bacteria which are catalase and oxidase positive, motile with bipolar flagella, sensitive to the vibriostatic agent (O/129), require sodium chloride for growth and widespread in the coastal and estuarine ecosystems (Baumann and Schubert, 1984; Farmer III *et al.,* 2005).

This bacterial group has the capacity to cause serious alimentary toxico-infection and watery diarrhoea and is implicated to the consumption of raw or undercooked contaminated fish or shellfish. Pathogenic *Vibrio* species pose a considerable public

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health threat as they result in sporadic and epidemic human infections, and hence represents an important microbial group in the field of food safety and quality.

Major vibrios that cause sea foodborne icthyozoonoses are *V. cholerae, V. mimicus, V. parahaemolyticus* and *V. vulnificus* (Oliver, 2006). *Vibrio cholerae* is an important pathogenic *Vibrio* with the serogroups O1 and O139 to be specifically considered. The major virulence factor of *V.cholera* the CTX cholera toxin. *Vibrio parahaemolyticus* is a halophilic bacterium which causes acute gastroenteritis on consumption of raw contaminated sea foods and naturally inhabits marine waters. *V. parahaemolyticus* has two major species-specific pathogenic markers the tdh that encodes the thermostable direct hemolysin (TDH) which can lyse red blood cells and the *trh* encoding *tdh* related hemolysin (TRH). *Vibrio vulnificus* is a pathogenic species that inhabits in seawater and causes gastroenteritis or septicemia and can results in severe infections and high mortality in immune compromised patients with chronic liver disease.

The phenotypic identification of *Vibrio* species is often problematic as there are only a few common characteristics and owing to the intra-species variation in biochemical characteristics. The application of molecular methods such as PCR may enable easy and rapid identification of the species. The *tox*R gene that encodes the trans-membrane proteins involved in the regulation of virulence-associated genes is an ideal target for a simple multiplex PCR, because it appears to be well conserved among the bacteria within *Vibrionaceae,* but sufficiently divergent to distinguish between different *Vibrio* spp (Kim *et al.,* 1999; Osorio and Klose, 2000). With this background the present study was envisaged to study the occurrence of *Vibrio* spp.in different sea food and environmental samples collected in and around Chennai city, Tamil Nadu.

MATERIAL AND METHODS

Sample collection

A total of 175 sea food and environmental samples were collected from various fish markets in and around Chennai city, Tamil Nadu, India. The sample includes 50 fishes, 60 prawn/shrimps, 30 crabs, 5 shells and 30 water samples.

Bacterial culture, isolation and identification

The samples were enriched in alkaline peptone water (APW; Himedia, India) at pH 8.5 at 37˚Cfor 6 hours. The enriched samples were plated on selective media thiosulfate citrate bile salts sucrose agar (TCBS; Himedia, India) containing 1 per cent sodium chloride (NaCl), and incubated at 37˚C for 18 to 24 hour. The presumptive *V. parahaemolyticus* strains (green with blue tinge on TCBS Agar), presumptive *V. cholera* strains (yellow on TCBS Agar), and presumptive *V. vulnificus* strains (dark green/yellow on TCBS Agar), were purified on Nutrient agar (NA; Himedia, India) containing 3 per cent NaCl (BAM Protocol, 2014). The presumptive colonies were confirmed by gram staining and biochemical tests.

DNA preparation

A few presumptive colonies from nutrient agar were transferred to 100 µL of sterile water. The bacterial cells was lysed by boiling at 100°C for 5 min, followed by immediate chilling for 2-3 min at -20°C and then centrifuged at 5000 x g for 15 min at 4°C. The recovered the supernatant was as template in the PCR assay.

Polymerase chain reaction

The polymerase chain reaction was performed with primers targeting the 16s rRNA for identification of at genus level. Further, multiplex-PCR was performed with primers targeting the *toxR* gene for the identification of *V. parahaemolyticus, V. cholera* and *V. vulnificus*. The primer sequences used and the thermal cyclic conditions are provided in Table 1. The PCR amplicons were electrophoresed in a 1.5 percent agarose gel, stained using ethidium bromide and the results were visualized the Gel documentation system (Bio Rad, India).

RESULTS

Isolation and identification of *Vibrio* spp. based on cultural characteristics and PCR

The enrichment with alkaline peptone water for sea food samples was necessary for rejuvenation and enrichment of the *Vibrio* spp. The thiosulphate citrate bile salt sucrose agar (TCBS) is the most widely used media to isolate *Vibrio* spp. from sea food and environmental samples. The colony morphology of the *Vibrio spp.* is as follows: green with blue tinge colony for *Vibrio parahemolyticus,* yellow color colony for *Vibrio cholera* and dark green/ yellow for *Vibrio vulnificus.*

Table 1. List of primers used in the present study

Name	Sequence	Product length (bp)	Annealing temperature	Reference
V16S rRNA-F	CGG TGA AAT GCG TAG AGA T	663	57^0C	Sudha *et al.,* 2014
V16S rRNA-R	TTA CTA GCG ATT CCG AGT TC			
U-toxR-F	GAS TTT GTT TGG CGY GAR CAA GGT T	Vp-297 Vc-640 vv-435	55^0C	Bauer and Rørvik, 2007
vp-toxR-R	GGT TCA ACG ATT GCG TCA GAA G			
vc-toxR-R	GGT TAG CAA CGA TGC GTA AG			
vv-toxR-R	AAC GGA ACT TAG ACT CCG AC			

Out of 175 samples screened in the present study we found that 106 samples were positive for the 16s rRNA specific simplex PCR confirming the genus level identification of *Vibrio* (Fig. 1). The multiplex PCR for the *toxR* gene enabled differentiation of *Vibrio* species as *Vibrio parahemolyticus (297bp), Vibrio cholera (640bp)* and *Vibrio vulnificus (435bp)*. Among the 106 *Vibrio* positive samples we found that 28 (26.41%) positive for *Vibrio parahemolyticus* (Vp), 6 (5.67%)

positive for *Vibrio cholera* (Vc) and 2 (1.89%) positive for *Vibrio vulnificus* (Vv). The mixed infections were observed in 56 samples (43.40%) with Vp and Vc, 7 (6.60%) with Vp and Vv, 3 (2.84%) with Vv and Vc and 14 (13.20%) with Vp, Vv and Vc (Fig. 2 and Table 2).

DISCUSSION

The presence of *Vibrio* spp. in sea food is highly variable based on different seafood samples, time and place of sample collection. From the present study, it is evident that the use of salt at 1-3 per cent is beneficial in enriching the growth of *Vibrio* in laboratory cultural techniques. The prevalence of *Vibrio* spp. in sea food in present study as assessed by the cultural technique was found to be 60.57 per cent (106/175) and confirmation by PCR targeting the 16s rRNA gene, concurred with the report of Azwai *et al.,* (2016), which reported an incidence of *Vibrio* spp.

Table 2. Prevalence of *Vibrio* spp. indifferent seafood samples

Organism	Water	Shrimp	Fish	Crab	Total
Vibrio parahemolyticus	4	8	8	8	**28**
Vibrio cholerae	0	2	2	2	**6**
Vibrio vulnificus	0	2	0	0	**2**
Vp+Vc	8	12	22	14	**56**
Vp+Vv	0	5	1	1	**7**
Vv+Vc	0	3	0	0	**3**
Vp+Vv+Vp	7	7	0	0	**14**

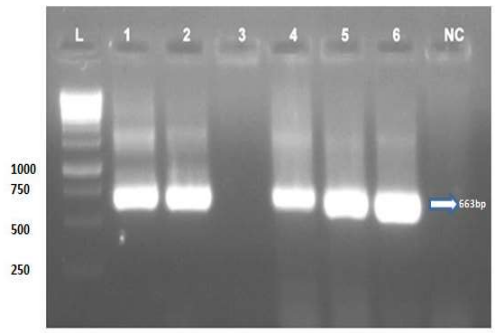

Fig. 1. PCR gel image depicting the amplification 16s rRNA gene (663bp)

[L: DNA Ladder (Genederix 1kb), NC: Negative control, 1-6: sea food samples]

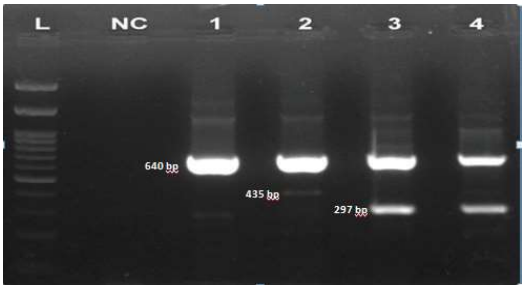

Fig. 2. PCR gel image depicting the amplification of toxR gene by Multiplex PCR

[L: DNA Ladder (Genederix 100bp), NC: Negative control, 1,3,4: Vp-297bp+Vc-640bp, 2:Vv-435bp+Vc-640bp amplified from sea food samples]

of 51.6 per cent in seafood, meat and meat products in Libya. Similarly, Alaboudi *et al.,* (2016) detected 66% positivity of typical *Vibrio* isolates from 200 sea food samples. In Indian sub-continent, a study by Sudha *et al.,* (2014), found that 65.5 per cent of the 110 samples screened were reported to be positive for *Vibrio* spp. The *Vibrio* spp. was the major pathogen from seafood to cause diseases in humans and, for this reason, it may be considered as an agent of zoonoses (Austin, 2010).

In the present study, out of 106 positive samples screened for species level identification of *Vibrio* spp. using toxR gene by multiplex PCR was applied by Bauer and Rørvik, (2007) and reported its utility in the reliable identification of *V. parahaemolyticus, V. vulnificus* and *V. cholerae*. In the present study, there was a higher frequency of *Vibrio parahemolyticus* which concurred with results of Yu *et al.,* 2016 who have also reported that *V. parahaemolyticus* in shellfish to be 34.3% (48/140). Xu *et al.,* (2016) also reported that 36.2 % of the samples to be positive in sea food. In India, a study by Sudha *et al.,* (2014), 68.1 per cent samples were found to be positive for *V. parahaemolyticus* from seafoods. The occurrence of *Vibrio cholera* from seafoods was earlier documented by Kumar and Lalitha, (2013) who reported that 39per cent positivity.A study by Hill *et al.,* (2011) and Senderovich *et al.,* (2010) concluded that fish species harvested from Indian coastal water bodies had higher number of *Vibrio* spp. and indicated the risk of this pathogen to consumers.

CONCLUSION

The occurrence of *Vibrio* spp. in seafood samples may be due to environmental stressors, improper handling and storage and cross contamination of sea food. Hence there is a need for strict hygienic measuresto be adopted at various levels of production till it reaches the consumers to ensure safety among sea food consumers and handlers.

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