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Effect of Season on the Prevalence of Virulence Genes of *V. parahaemolyticus* in Molluscan Shellfish along the Thoothukudi Coast, India

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A B S T R A C T

Commercially available molluscan shellfish such as oysters and clams were collected monthly along the Rochepark, Thoothukudi and brought to the laboratory in moist condition immediately after harvesting and analysed for the prevalence of virulence genes of *V. parahaemolyticus.* The meat was carefully shucked (meat and fluid) from a pool of 6 – 8 oysters/clams and used. Prevalence of tdh, trh genes of *V. parahaemolyticus* was confirmed by PCR as well as Direct plating method (TCBS). The present study revealed that the effect of season on the presence of virulence genes of *V. parahaemolyticus* was insignificant compared to other reports.The result of this study shows less prevalence of pathogenic *V. parahaemolyticus* and moderate to high prevalence of non-pathogenic *V. parahaemolyticus* in both oysters and clams during summer season followed by winter season. It could also be observed that 2% *Crassostrea* sp. showed positive for pathogenic (*tdh*) *V. parahaemolyticus* and 61% *Crassostrea* sp. and 51.2% of *Meretrix* sp. from HB showed positive for non-pathogenic *V. parahaemolyticus.*

Introduction

Vibrios are widely distributed in sea water and estuarine environment worldwide and are part of the natural flora of zooplankton, coastal fish and shellfish. Their number depends on water salinity and temperature and cannot be usually found in water with temperatures below 150 C (Thompson *et al.,* 2006). However, climate changes taking place in the world can promote their survival and geographical spread, resulting in a potential increase of exposure and the possibility that the number of infections will increase in the world. Not all strains isolated from the environment or food are considered pathogenic, but only those that produce virulence factors such as haemolysin (Zhang and Austin 2005). *Vibrio parahaemolyticus* strains possessing tdh, trh or both genes can produce thermostable direct haemolysin (TDH) and thermostable direct-related haemolysin (TRH) respectively. For this reason it is very important to monitor the presence and spread of these micro-organisms in the natural environment with particular reference to haemolysin producing strains.

Currently, most of the studies present in the literature regard the isolation of pathogenic Vibrio in sea water, molluscs, crustaceans and sediments (Robert-Pillot *et al.,* 2004; Normanno *et al.,* 2006; CovazziHarriague *et al.,* 2008; Vezzulli *et al.,* 2009). The greatest number of seafood associated illnesses is from consumption of molluscan shellfish such as oysters, clams and mussels as consequences of the filter feeding habit of these organisms that concentrate environmentally derived human enteropathogens in their tissues without affecting their infectivity. Therefore, such shellfish can cause human food borne illnesses if consumed raw or after inadequate processing. Information derived from epidemiologic investigations and surveillance systems indicates an upward trend in food borne illnesses in some areas linked with consumption of molluscan shellfish. Worldwide, the majority of outbreaks have been linked to oysters followed by clams and mussels, and most of the reports originate from the United States, followed by Europe, Asia and Australia (Potasman *et al.,* 2003). *Vibrio parahaemolyticus* is recognized as one of the most important agents for seafood-associated gastroenteritis and stands for 10% of the Indian outbreaks (Deepanjali *et al.,* 2005).

Human development along Thoothukudi coast greatly accelerated environmental pressure on downstream estuarine and coastal ecosystems and resulted overall decline in the livability of the coastal zone. Survivability of pathogenic bacteria in marine environment depend on certain biotic and abiotic factors. The genus *Vibrio* is widely distributed in aquatic environment especially in coastal water. Of the 98 species, 11 species are recognized as human pathogens and most of the infections with *V.parahaemolyticus* are known to be associated with either consumption of seafood or exposure to marine environments. Hence, this study is conducted to understand the prevalence of virulence genes of *Vibrio parahaemolyticus* in molluscan shellfish along thoothukudi coast.

Materials and Methods

Study Area and Raw material

Commercially available molluscan shellfish such as oysters and clams were collected monthly along the Rochepark, Thoothukudi and brought to the laboratory in moist condition immediately after harvesting and used for the analysis. The collected oyster and clam samples were washed well using water to remove dirt and mud and measured for length and weight, which ranged from 13.0 to 21.0 cm and 87.42 g to 429.43 g and 3.1 to 4.7 cm and 35.46 to 41.90 g respectively.

Identification of Species

Species of oysters identified taxonomically as *Crossostrea madrasensis* and clams as *Meretrix meretrix* by following the keys as per FAO species catalogue (Heemstra and Randall., 1993).

Isolation, Identification and detection of *V. parahaemolyticus* using PCR

The prevalence of *V. parahaemolyticus* in oysters was studied by using species specific marker *tlh*gene (Themolabilehemolysin gene) and the virulence genes such as *tdh* gene (Thermostable direct hemolysin) (Fig.3) and *trh* gene (Thermostable direct hemolysin related hemolysin).The samples were subjected for the isolation of *V. parahaemolyticus* as per FDA Bacteriological Analytical Manual (USFDA, 1998). The meat was carefully shucked (meat and fluid) from a pool of 6 – 8 oysters/clams without contamination into a sterile blender jar, homogenized and transferred to the Alkaline peptone water and incubated at 37^0C for 24 h

for primary enrichment. After this, 1.5 microliter of pre enriched molluscan samples in APW was taken and pelletized using Refrigerated Centrifuge at 12000 rpm for 3 min (Eppendorf, Germany). Then the supernatant was discarded and the pellet obtained was used for the extraction of DNA using DNA extraction kit (Hi-media, Bombay). Further, the extracted DNA was used for the identification and confirmation of *V. parahaemolyticus* and its virulence genes using PCR. The *tlh* gene, a species specific signature marker of *V.parahaemolyticus* was detected by using primers directed to the *tlh*gene detection. The PCR was carried out in a 25 µl mixture consisting of 10 µl of PCR master mix (Hi-Media Laboratories Pvt. Ltd., Bombay), 1 µl of 10 pmol of each primer, and 11 µl of water. Two microliters of eluted DNA was used as DNA template. The PCR assays were performed in a programmable thermocycler (Applied Biosystems, USA). In all the reactions, an initial denaturation of DNA template at 94°C for 5 min, annealing at 63°C and a final elongation at 72°C for 5 min were followed. Positive bands in the agarose gel confirmed the presence of *V. parahaemolyticus* in both oyster and clam samples.

On the other hand, detection and confirmation of *V. parahaemolyticus* was also done conventionally by using TCBS agar which is a selective medium for *V. parahaemolyticus*. A loopful of pre - enriched APW culture was streaked on thiosulfate citrate-bile salts-sucrose (TCBS) agar and confirmed that the colonial morphology was typical of *V.parahaemolyticus*.

Detection of pathogenic *V. parahaemolyticus* using PCR

PCR assay was performed to detect virulencegenes of *V.parahaemolyticus* viz., *tdh* with a size of 250 bp (Thermostable direct hemolysin) (Fig.3) and *trh* with a size of 251bp (Thermostable direct hemolysin related hemolysin) in oysters and clams used in this study. For each assay, 1.5µl of each isolates grown in LB broth was used for DNA extraction (Hi Media, Bombay). The eluted DNA was used as a template for the PCR assay. PCR was carried out in a 25 µl mixture consisting of 10 µl of PCR master mix (Hi Media, Bombay), 1 µL of 10 pmol of each primer and 11 µl of water. Two microliters of eluted DNAwas used as DNA template. The PCR assays were performed in a programmable thermocycler (Applied Biosysytems, US). In all the reactions, an initial denaturation of DNA template at 94°C for 5 min, annealing at 55°C and a final elongation at 72°C for 5 min were followed. Electrophoresis was done using 2% gel to detect the virulence genes of *V. parahaemolyticus*. Positive bands with a size of 250 bp in agarose gel confirmed the presence of virulence gene *tdh* of *V. parahaemolyticus*in both oyster and clam samples.

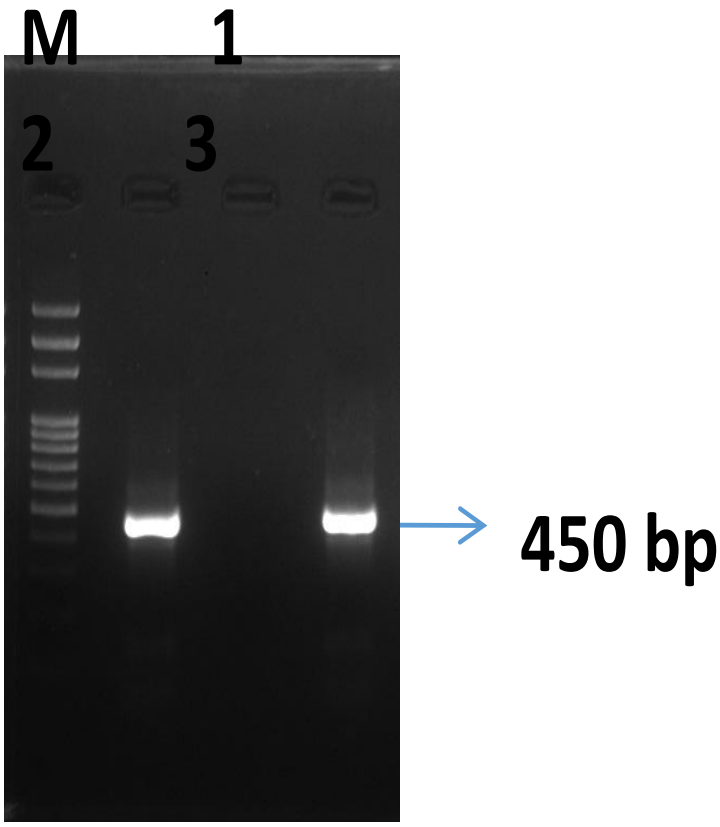
Results and Discussion

Molluscan shellfish samples such as oysters (*Crossostrea madrasensis*) and clams (*Meretrix meretrix*) (Fig.1A & 1B) were collected from Harbour Beach of Thoothukudi (monthly). The effect of season on the prevalence of pathogenic *V. parahaemolyticus* was analysed using species specific primer *tlh* (Thermolabilehemolysin gene) (Fig.2) followed by the primers encoding for their virulence genes viz., *tdh* (Thermostable direct hemolysin) (Fig.3) and *trh* (Thermostable direct hemolysin related hemolysin). Virulence gene, *tdh* of *V. parahaemolyticus* was detected only from 2% of isolates (Fig. 6) that are obtained from oysters during summer season and it might be due to influence of season on prevalence of pathogenic *V. parahaemolyticus.*

Fig.1A *Crosstrea madrasensis*

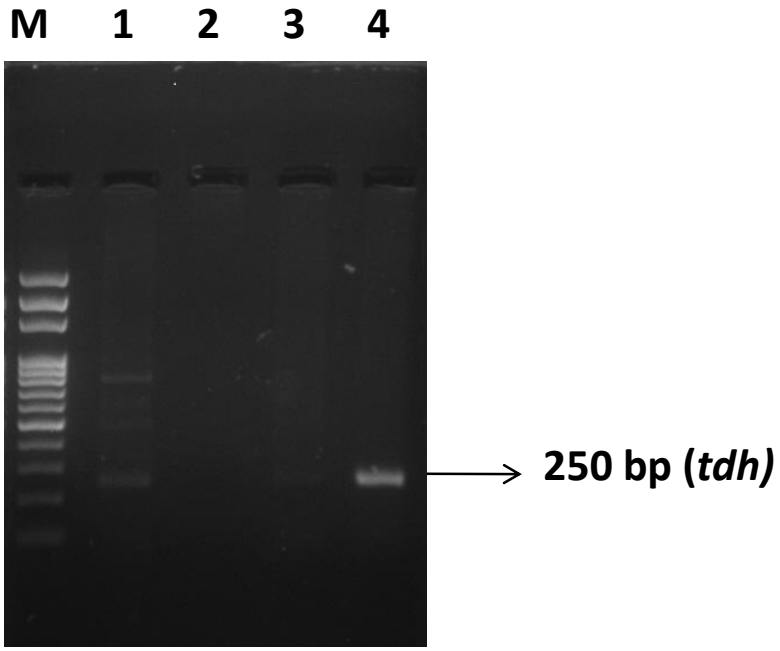
Fig.1b *Meretrix meretrix*

Fig.2 Detection of *V. parahaemolyticus* using species specific *tlh* gene



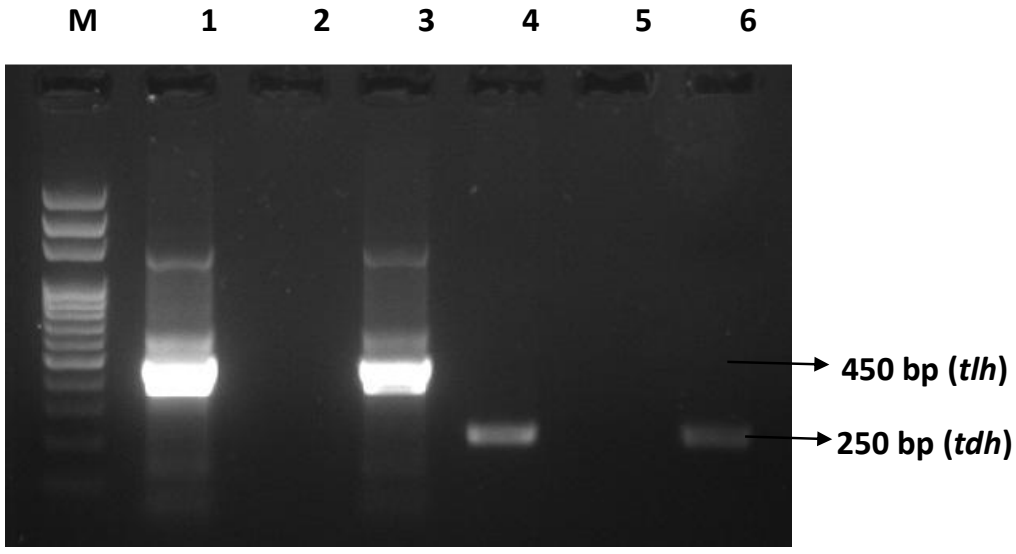
Lane M- 100 bp DNA Marker; Lane 1– Positiive control for *tlh* gene of *V. parahaemolyticus;* Lane 2–Negative control for *tlh* gene of *V. parahaemolyticus* Lane 3 & 4 -Samples positive for *tlh* gene of *V. parahaemolyticus*

Fig.3 Detection of virulence gene of *V. parahaemolyticus* using *tdh* gene



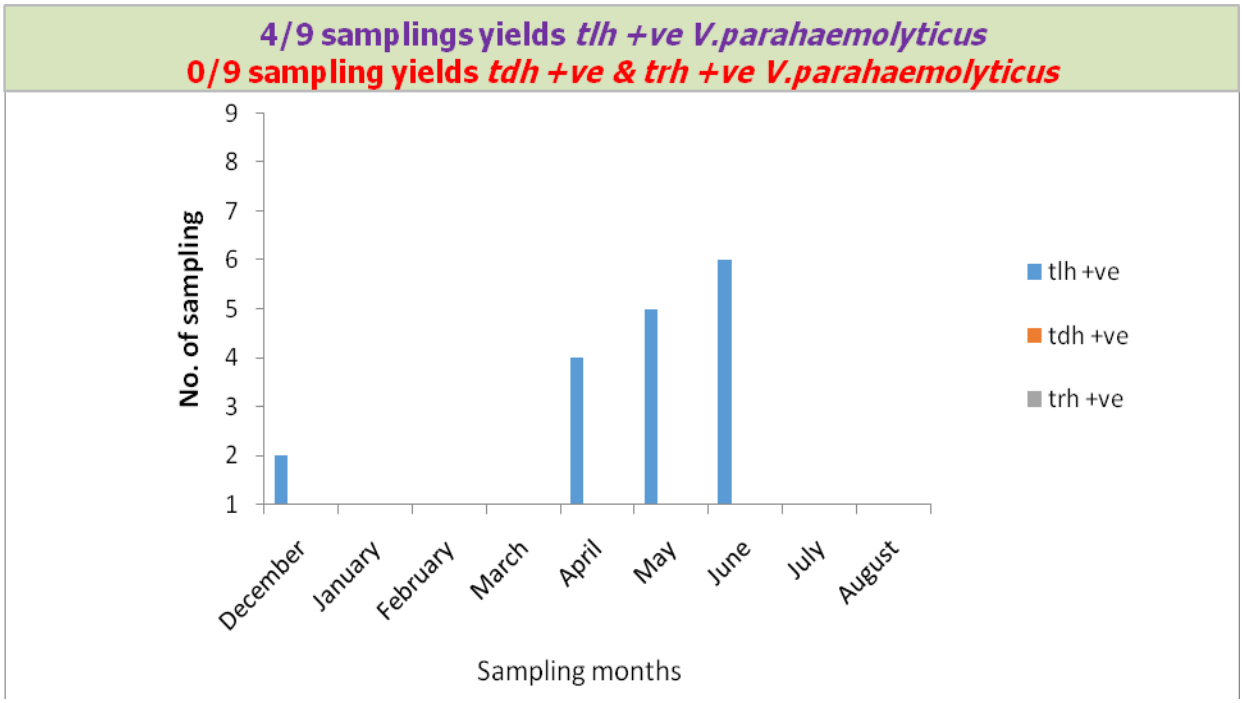
Lane M- 100 bp DNA Marker; Lane 1– Positiive control for *tdh* gene of *V. parahaemolyticus;* Lane 2–Negative control for *tdh* gene of *V. parahaemolyticus* Lane 3 & 4 -Samples positive for *tdh* gene of *V. parahaemolyticus*

Fig.4 Detection of *V. parahaemolyticus* using species specific primer targeting *tlh* gene and virulence gene of *V. parahaemolyticus* targeting *tdh* gene



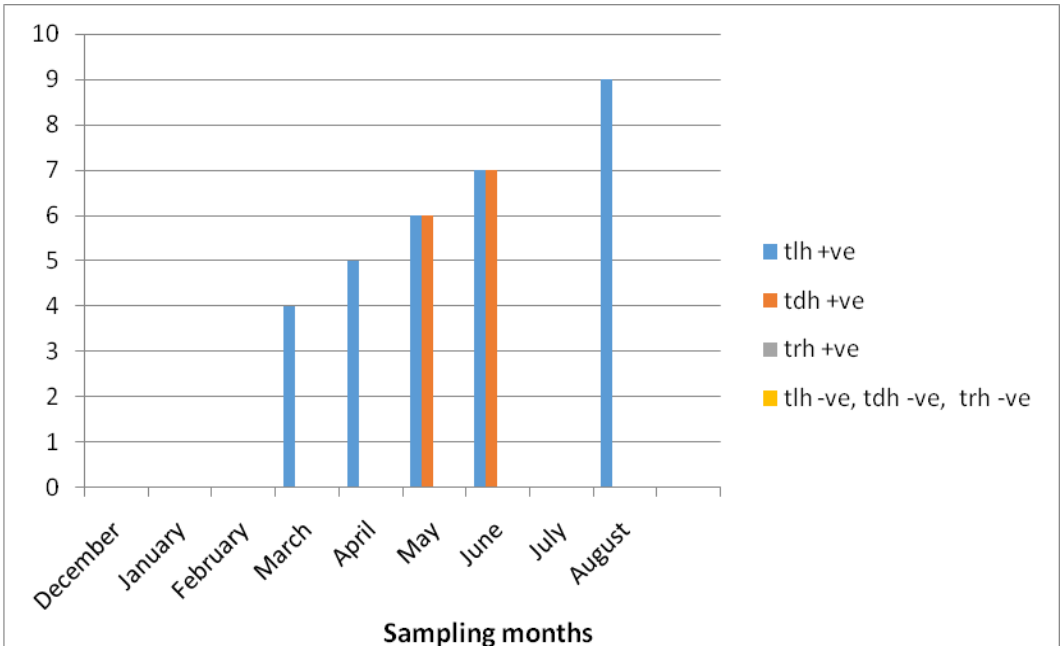
Lane M- 100 bp DNA Marker; Lane 1– Positive control for *tlh* gene of *V. parahaemolyticus;* Lane 2–Negative control for *tlh* gene of *V. parahaemolyticus;* Lane 3 -Sample positive for *tlh* gene of *V. parahaemolyticus;* Lane 4 – Positiive control for *tdh* gene of *V. parahaemolyticus;* Lane 5 – Negative control for *tdh* gene of *V. parahaemolyticus;* Lane 6 -Sample positive for *tdh* gene of *V. parahaemolyticus*

Fig.5 Prevalence of pathogenic and non-pathogenic *V. parahaemolyticus* in Clam



5/9 samplings yields *tlh* +ve *V.parahaemolyticus*
2/9 samplings yields *tdh* +ve & *trh* +ve *V.parahaemolyticus*

Fig.6 Prevalence of pathogenic and non-pathogenic *V. parahaemolyticus* in Clams



The present study revealed that the influence of season on the prevalence of pathogenic *V. parahaemolyticus* in mollucan shellfish along Thoothukudi coast was significant while not significant in prevalence of non-pathogenic *V. parahaemolyticus* along Thoothukudi coast.

Further, the remaining isolates obtained from *Crassostrea* sp. and *Meretrix* sp. did not contain any of these said virulence genes (*tdh and trh*) of *V. parahaemolyticus* during summer and also in winter season. Similarly, the growth and survival of *V.parahaemolyticus* increased with increasing storage temperature and reached a peak of 7.5 log10 CFU/g at 30°C in oysters (Parveen *et al.,* 2012). Likewise, the growth of *V.parahaemolyticus* increased with increasing temperature up to a level of 7.1 log10 CFU/g at 30.6°C after 29 h (Piquer *et al.,* 2011). The observed growth at 300C showed higher variation than the other storage temperatures like 20 and 100C. This could indicate that 300C is close to the maximum growth rate for *V.vulnificus* (Dasilva *et al.,* 2012). The study also shows that there was no trh gene detected from both *Crassostrea* sp. and *Meretrix* sp and it agrees with the earlier reports as it is less prevalent than *tdh*.

The result of this study shows less prevalence of pathogenic *V. parahaemolyticus* and moderate to high prevalence of non-pathogenic *V.parahaemolyticus* in both oysters and clams during summer season followed by winter season. This supports the evidence that the growth condition of 37^0C is considered to be the optimal growth condition with highest growth rate and mean population density than any other storage temperature (Liu *et al.,* 2016). It could also be observed that 2% Crassostrea sp. showed positive for pathogenic (*tdh*) *V. parahaemolyticus* and 61% *Crassostrea* sp. and 51.2% of *Meretrix* sp. from HB showed positive for non-pathogenic (Fig.5 & 6) *V. parahaemolyticus* (Fig.4). Since the study area is one of the hottest places in Tamil Nadu, effect of season on the prevalence of *V. parahaemolyticus* might be low. Several studies indicate that *V. parahaemolyticus* in mollusks are significantly correlated with seawater temperature; where, reported temperature ranges varied from: 10 to 33°C (DePaola *et al.,* 2003); 9.9 to 32.7°C (Phillips *et al.,* 2007); 14.4 to 29.2°C (Sobrinho *et al.,* 2010); 7.7 to 29.7°C (Haley *et al.,* 2014); and 7.9 to 25.5°C (Cruz *et al.,* 2015).However, presence of virulence gene of *V. parahaemolyticus* during summer season could be due to the influence of season. The frequency of detection of pathogenic *V. parahaemolyticus* was significantly related to water temperature. In our model, the temperature was not significantly associated with total *V. parahaemolyticus* presence (Table 4), when the model included location. These results agree with those reported by Deepanjali *et al.,* (2005), who observed no statistically significant correlation with tropical seawater temperature from 34 to 24°C, and, with Zimmerman *et al.,* (2007) who did not find any correlation with temperature ranging from 22.4 to 33.8°C either. However, temperature was significantly negatively associated with total *V. parahaemolyticus* presence in our study. Since, failure to detect virulence genes of *V. parahaemolyticus* in shellfish was more frequently attributed to the low numbers and uneven distribution of the organism, further studies on prevalence of pathogenic *V. parahaemolyticus* involving molluscan shellfish need to be studied. It can be concluded that the influence of season on the prevalence of virulence genes of *V.parahaemolyticus* was not much significant.

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