ISSN 0975 - 6299 Vol 2 / Issue 4 / Oct - Dec 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE MICROBIOLOGY

DETECTION OF *VIBRIO PARAHAEMOLYTICUS* IN SHELL FISH BY CULTURAL AND POLYMERASE CHAIN REACTION

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ABSTRACT

A total of 105 shell fish samples (35 each of prawn, shrimp and crab) collected from local markets were subjected to cultural methods and PCR for the presence of *V. parahaemolyticus* and their toxins using primers for *toxR*, *tdh* and *trh* genes respectively. Out of total 105 samples, positive isolates of *V. parahaemolyticus* were found in 17 prawn samples, 25 shrimp and 19 crab samples by cultural method where as by PCR assay the organism was found in 24 samples of prawn, 33 shrimp and 27 crab samples. Out of 84 positive isolates of *V. parahaemolyticus* by PCR method, 11 showed presence of *tdh*, 37 had *trh* and 6 had both *tdh* and *trh*. Selective broths (Alkaline PeptoneWater and Salt Polymyxin Broth) were more efficient than non-selective broths (Brain Heart Infusion and Tryptic Soy Broth) and Salt Polymyxin Broth (SPB) was superior over Alkaline Peptone Water (APW) in selective broths for isolation of this organism by both cultural and PCR methods. The sensitivity of PCR method for this organism was 2.5cfu.

KEY WORDS

Vibrio parahaemolyticus, PCR, Cultural method, Prevalence, Shell fish.

INTRODUCTION

Vibrio parahaemolyticus is a gram-negative bacterium that occurs naturally in the marine environment. This human pathogen is frequently found in shellfish and can cause acute gastroenteritis or traveler's diarrhea characterized by diarrhea, vomiting and abdominal cramps through consumption of contaminated raw or partially cooked fish or shellfish[23]. This organism was first identified as a causative agent of food-borne gastroenteritis after a large outbreak (272 illnesses and 20 deaths) associated with consumption of sardines in Japan[9] in 1951. In India, *V. parahaemolyticus* was first isolated from a case of gastroenteritis[2] and about 10% of the cases of gastroenteritis in patients admitted to the Infectious Disease Hospital in Kolkata are due to *V. parahaemolyticus*[4].

V. parahaemolyticus accounts for about 70% of the gastroenteritis associated with seafood in Japan[12] and this food poisoning is most common in Japan and Southeast Asia, although they occurred occasionally in other parts of the world[20]. *V. parahaemolyticus* accounts for 35% to more than 50% of the bacterial food borne illness outbreaks occurring annually in Taiwan[3,25]. The incidence of *V. parahaemolyticus* infection in recent years has been increasing in many parts of the world, due to the emergence of O_3:K_6 serotype carrying only the *tdh* gene that is responsible for most out breaks worldwide[17] since, 1996.

Enumeration of this organism from seafood is important in the context of current FDA guidelines, which stipulate that seafood should contain less than 10,000 cells per gram[18], but inadequacy of this is indicated by the outbreaks that occurred in the united states, despite the *V. parahaemolyticus* number being lower than the permissible limit[19].

Involvement of *V. parahaemolyticus* in gastroenteritis among some staff members at the Christian Medical College and Hospital, Vellore following a social get-together has further emphasized the public health hazard caused by this organism in India[15]. Shell fish have been reported to concentrate vibrio species 100 fold compared with the amount in the surrounding water through filtration[6].

Detection of pathogenic *V. parahaemolyticus* is traditionally done by the Wagutsuma agar test for the Kanagawa reaction, which requires fresh human or rabbit RBC. This hemolysis is due to the production of a thermostable direct hemolysin (TDH)[22]. Both TDH and TRH are considered as major virulence factors[23].

The conventional isolation procedure includes growth in enrichment broth like APW[8] (Alkaline PeptoneWater) or SPB[10] (Salt Polymyxin Broth) incubated at 37^0C for 18-24 hours to increase the ratio of *V. parahaemolyticus* to other organisms. The broth cultures are then plated onto Thiosulphate Citrate Bile salt Sucrose agar incubated at 37^0C for 18-24 hours. *V. parahaemolyticus* is unable to ferment sucrose resulting in green color colonies with blue centres[8].

The present investigation was carried out to isolate *V. parahaemolyticus* from prawn, shrimp and crab samples to investigate the presence of toxins (*tdh* and *trh* genes).

MATERIALS AND METHODS

Thirty five samples of 50g each of prawn, shrimp and crabs were collected

aseptically from local fish markets. Samples (10gm) were enriched in 90 ml of APW and 90 ml of SPB (Himedia) at 37^{0}C for 24 hours. The enriched cultures were streaked on selective media i.e Thiosilphate Citrate Bile salt Sucrose agar and plates were incubated at 37^{0}C for 24 hours. The green color colonies with blue centres were taken for further confirmation by biochemical tests like indole, methyl red, VP, TSI, urease and nitrate tests.

All the samples were subjected to PCR analysis for the presence of *V. parahaemolyticus* by PCR method using primers specific to *toxR*[14] with an amplification size of 368bp. The samples positive for *V. parahaemolyticus* by PCR method were further examined for the presence of toxins, *tdh*[13] and *trh*[24] using specific primers with an amplification size of 623bp and 460bp respectively (Fig-1).

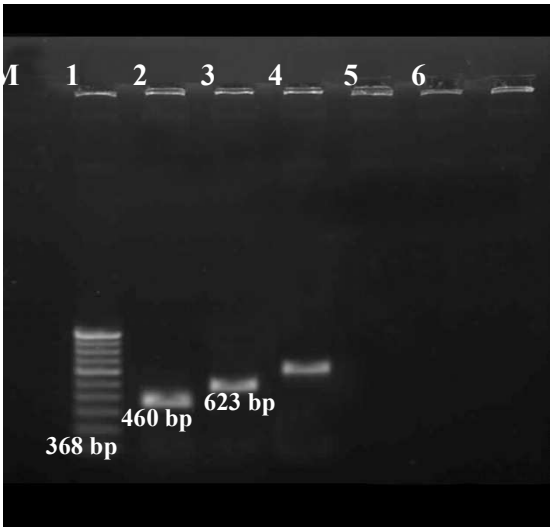

Figure 1
Comparison between amplicon products obtained from genes toxR, tdh and trh of Vibrio parahaemolyticus

Lane M: 100bp DNA Ladder
Lane 1: Amplicon product obtained by using primers from *toxR*
Lane 2: Amplicon product obtained by using primers from *tdh*
Lane 3: Amplicon product obtained by using primers from *trh*

Pure culture of *V. parahaemolyticus* obtained from MTCC, Chandigarh was used as known positive strain in PCR analysis. 1.5 ml of enriched broths were taken into centrifuge tubes and bacteria were pelleted by centrifugation at 8000rpm for 10 min To the pellet 50µl of molecular grade water was added and incubated at 100^{0}C for 10 min and snap chilled to release DNA. Then centrifuged at 10,000rpm for 5 min and the supernatants were used as DNA templates for PCR amplification.

Bacterial DNA amplification was done in 20µl reaction mixture containing 2µl of 10X Taq DNA polymerase buffer (containing 100mM Tris with P^H 9.0, 500mM KCl, 15mM $MgCl_2$ and 1% Triton X-100), 2µl of 10mM of dNTP mixture, 0.9U of Taq DNA polymerase (Genei, Bangalore), 2µl of each of 4 pmoles/µl of forward and reverse primers

(Genei, Bangalore) and 5µl of crude bacterial cell lysate. Make this mixture to 20µl using molecular grade water. Amplification was done in a Thermal Cycler (Eppendorf) following the conditions shown in Table.1. The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5µg/ml) and visualized with an UV transilluminator (Fig 1, 2).

Table.1
Cycling conditions used for three sets of primers

S.No.	Step	*toxR*	*tdh*	*Trh*
1.	Initial denaturation	94^0C/5min	94^0C/10min	94^0C/5min
2.	Final denaturation	94^0C/1min	94^0C/1min	94^0C/1min
3.	Annealing	63^0C/2min	5^0C/1.5min	48^0C/1min
4.	Initial extension	72^0C/1.5min	72^0C/1.5min	72^0C/1min
	Final extension	72^0C/10min	72^0C/10min	72^0C/5min

To know the sensitivity of *V. parahaemolyticus*, homogenized shell fish was inoculated at the rate of 250 cfu, 25 cfu, 2.5 cfu and 0.25 cfu per 10 g of shell fish and transferred to two different enrichment media i.e. SPB and APW. Both the media were incubated at 37^0C for 8h and18h and subjected to PCR assay and conventional culture testing for *V. parahaemolyticus* and only PCR assay for toxins.

Different enrichment broths like SPB, APW (selective), BHI and TSB (non-selective) were evaluated for their efficiency.

RESULTS AND DISCUSSION

The PCR represents a rapid procedure with both high sensitivity and specificity for the immediate detection and identification of specific pathogenic bacteria from different food materials[11,16]. The advantage of PCR over conventional isolation is its ability to distinguish virulent and avirulent strains, saves time and hence molecular methods are valuable in such cases[5].

In the present study, the sensitivity of PCR was found to be 25cfu and 2.5cfu for APW after 8h and 18h incubation respectively where as it was 2.5cfu for SPB at both 8h and 18h of incubation. But it was 250cfu and 25cfu for APW and 25cfu and 2.5cfu for SPB after 8h and18h of incubation respectively by cultural method. With a step of overnight enrichment, very high level of sensitivity (1cfu) can be obtained[26]. Almost similar level of sensitivity (25CFU/10g) was reported by some published scientific studies[5] using colony hybridization method.

In the present study, growth of *V. parahaemolyticus* was good in selective broths (APW and SPB) compared to non-selective broths (BHI and TSB) and in selective broths SPB was superior to APW both by cultural and PCR methods (Fig-2). The recovery of *V. parahaemolyticus* was reported to be maximum in SPB (P^H 8) at 37^0C[1].

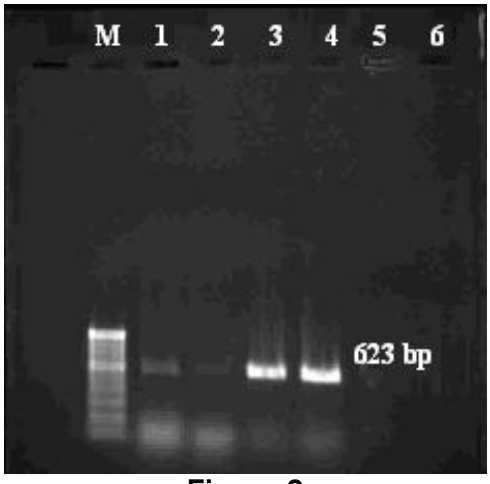

Figure 2
Evaluation of PCR compatibility of enrichment broths for the detection of *tdh*
Lane M: 100bp DNA ladder
Lane 1 & 2: APW
Lane 3 & 4: SPB

Results for the presence of *V. parahaemolyticus* in various samples are presented in table.2. In this study, *V. parahaemolyticus* was isolated from 68.6% (24 out of 35) of the prawn samples by PCR method and 48.6% (17 out of 35) by cultural methods. *V. parahaemolyticus* was isolated from 94.3% (33out of 35) of shrimp by PCR method where as by cultural method, the isolation rate was 71.4% (25 out of 35). The incidence of *V. parahaemolyticus* in fresh water prawns (68.6%) was low compared to marine shrimp (94.3%), which might be due to the halophilic nature of this organism[7,21]. *V. parahaemolyticus* was isolated from 77.1% (27out of 35) of crab by PCR method where as 54.3% (19 out of 35) by cultural method.

Table.2
Occurrence of *V. parahaemolyticus* in different samples

S.No.	Sample	No. of samples	Results for *V. parahaemolyticus*		*tdh*	*trh*	Both (tdh&trh)
			Cultural	PCR			
1.	Prawn	35	17	24	3	9	1
2.	Shrimp	35	25	33	4	13	3
3.	Crab	35	19	27	4	15	2

For a total of 105 samples (prawn, shrimp and crab), cultural method could detect only 58.1%, where as PCR assay gave 80% of the samples positive for *V. parahaemolyticus*. This study shows that PCR method was more accurate than cultural

method for the isolation of *V. parahaemolyticus* and many of the published studies reveal the same opinion[7].

Of the 84 samples positive for *V. parahaemolyticus* by PCR method, 11 (13.09%) showed presence of *tdh* (prawn-3, shrimp-4, crab-4), 37 (44.04%) showed *trh* (prawn-9, shrimp-13, crab-15) and 6 (7.14%) showed presence of both *tdh* and *trh* (prawn-1, shrimp-3, crab-2). According to this study, the production of *trh* was high compared to *tdh.*

Since *Vibrio parahaemolyticus* strains carrying the *tdh* and *trh* genes are virulent and responsible for diarrhea in humans, PCR is a very specific and rapid method for detecting the virulent *Vibrio parahaemolyticus* strains in food samples and human stools.

ACKNOWLEDGEMENT

The authors are thankful to the Sri Venkateswara Veterinary University for providing necessary facilities and financial support to carry out this work.

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