Detection of total and pathogenic *Vibrio parahaemolyticus* by Polymerase chain reaction using toxR, tdh and trh genes

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Abstract

The levels of total and pathogenic *Vibrio parahaemolyticus* were estimated in 105 samples (each 35 of fresh water fish, sea fish and mutton) using cultural and polymerase chain reaction (PCR) methods by amplification of toxR gene for total *V. parahaemolyticus*, tdh and trh genes for pathogenic *V. parahaemolyticus*. Out of 105 samples, 30 (28.6%), 39 (37.1%) samples gave positive results for total V. parahaemolyticus by cultural and PCR methods respectively. Out of 39 samples positive for PCR 6 (5.7%), 28 (26.6%) and 3 (8.6%) were positive for tdh, trh and both tdh and trh respectively.

Key Words: *Vibrio parahaemolyticus,* PCR,. Gene, Laboratory Technique, Pathogenic Organism.

Introduction

Vibrio parahaemolyticus is an important enteric pathogen that causes acute gastroenteritis and traveler'diarrhoea in humans (Depaola et. al., 1990) occurs in estuarine environment world wide. This organism was first discovered in Japan in 1950 in association with a food poisoning case (Fujino et. al., 1953). V. parahaemolyticus illness is most frequently associated with the consumption of raw or undercooked seafood and food recontaminated with the bacterium after cooking (Rippey, 1994).

97% of the strains isolated from diarrhoeal patients were positive for hemolytic activity on wagutsuma's agar, while only 1% of environmental isolates showed the hemolysis on the agar (Sakazaki et. al., 1968). This hemolysis is known as kanagawa phenomenon and considered as useful marker of the virulent V. parahaemolyticus. The hemolysis that causes the phenomenon is called Thermostable Direct Hemolysin (TDH) (Honda and Iida, 1993). Clinical isolates of kanagawa phenomenon negative V. parahaemolyticus that produces a toxin named TDH-related hemolysin (TRH) have also been reported to cause gastroenteritis similar to that of TDH producing isolates (Honda et. al., 1988; Shirai et. al., 1990).

TDH and TRH, encoded by the tdh and trh genes respectively are considered to be important virulence factors in the pathogenesis of V. parahaemolyticus.

70%of the seafood borne gastroenteritis cases in Japan are due to V. parahaemolyticus (Depaola et. al., 1990) and in India this organism accounts for about 10% of gastroenteritis cases admitted to the Infectious Disease Hospital in Kolkata (Deb et. al., 1975).

V. parahaemolyticus strains possess a regulatory gene, *toxR*, which is present in all the strains irrespective of their ability to produce TDH or TRH. Thus the PCR targeted to the *toxR* gene can be used as a method for identification at the species level (Kim *et. al.,* 1999).

Materials and Methods:

Thirty five samples of 50g each fresh water fish and sea fish were collected aseptically from local fish markets and 35 mutton samples were collected from modern slaughter house, Chengicherla, Hyderabad. Samples (10gm) were enriched in 90 ml of APW 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 colour 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*. The samples positive for *V. parahaemolyticus* by PCR method were further examined for the presence of toxins (*tdh* and *trh*) using specific primers listed in the table.1.

Table-1. Oligonucleotide primers used in the study

Primer	Target gene	Primer sequence (5`-3`)	Fragment size (bp)	Reference
toxR-F	toxR	GTC TTC TGA CGC AAT CGT TG	368	Kim et. al., 1999
toxR-R	toxR	ATA CGA GTG GTT GCT GTC ATG	368	Kim et. al., 1999
M-454	tdh	CGT TGA TTA TTC TTT TAC GA	623	Karunasagar et. al., 1996
M-441	tdh	TTT GTT GGA TAT ACA CAT	623	Karunasagar et. al., 1996
TRH-F	trh	CTC TAC TTT GCT TTC AGT	460	Suthienkul et. al., 1995
TRH-R	trh	AAT ATT CTG GAG TTT CAT	460	Suthienkul et. al., 1995

Pure culture of *V. parahaemolyticus* were obtained from MTCC, Chandigarh was used as known positive strain in PCR analysis. 1.5 ml of enriched broths were taken into eppendorf 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 in PCR for DNA 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), 2µl of each of 4 pmoles/µl of forward and reverse primers and 5µl of crude bacterial cell lysate. Make this mixture to 20µl using molecular grade water. Amplification was done following the conditions shown in Table.2.

The amplified DNA fragments were resolved by agarose gel electrophoresis, stained with ethidium bromide (0.5µg/ml) and visualized with a UV transilluminator shown in Fig.1.

Results and Discussion:

Results for the presence of *V. parahaemolyticus* in various samples are presented in table.3.

Out of 35 samples of each fresh water fish and sea fish samples, PCR gave positive results for 17 (48.6%) and 20 (57.1%), where as cultural method gave 12 (34.3%) and 17 (48.6%) positive results for total *V. parahaemolyticus* respectively. The percent of positive results by cultural method compared to PCR assay method was 70.6 and 85 for fresh and sea fish samples respectively.

Raghunath *et. al.* (2008) reported high incidence of *V. parahaemolyticus* in marine fish both by cultural (55.5%) and PCR (66.6%) methods, compared to present study results of 48.6% and 57.1% respectively. High level of incidence (71.4%) in fish samples was reported by Vitela *et. al.* (1993). Low level of incidence of *V. parahaemolyticus* in marine fish was reported by several scientists i.e 21.74% (Das *et. al.,* 2009), 25% (Nithya Quintiol *et. al.,* 2007), 29.3% (Wong *et. al.,* 1999) and 17.5% (Wong *et. al.,* 1995), whereas Baross and Liston (1970) reported almost similar findings (52%) of this study.

Compared to 48.6% incidence of *V. parahaemolyticus* in fresh water fish in the present study, low incidence was reported by many scientists i.e zero (Gjerde and Boe, 1981), 2.6% (Chan *et. al.,* 1989), 10% (Jaksic *et. al.,* 2002), 12% (Nithya Quintiol *et. al.,* 2007) and 13.83% (Das *et. al.,* 2009). An incidence of 16-20%of *V. parahaemolyticus* was reported in brakish water fish by Nithya Quintiol *et. al.* (2007) and Das *et. al.* (2009).

Two (5.7%) and one (2.8%) positive results for *V. parahaemolyticus* were obtained by PCR and cultural methods respectively out of 35 mutton samples. The percent of positive results by cultural method compared to PCR assay was 50. The incidence of *V. parahaemolyticus* (5.7%) in mutton samples in the present study was almost similar to the findings (6.6%) of Wong *et. al.* (2000), whereas low incidence (1.3%) was reported by He-Lianhua *et. al.* (2005).

For a total of 105 samples (fresh water fish, sea fish and mutton), cultural method could detect only 28.6%, where as PCR assay gave 37.1% of the samples positive for *V. parahaemolyticus*. In this study, PCR method was more accurate than cultural method for the isolation of *V. parahaemolyticus*. Similar opinion was given by Dileep *et. al.* (2003).

Of the 39 samples positive for *V. parahaemolyticus* by PCR method, 9 showed presence of *tdh (fresh water*

Table-2: Cycling conditions used for three sets of primers

S.No.	Step	toxR	tdh	trh
1.	Initial denaturation	94^{o}C/5min	94^{o}C/10min	94^{o}C/5min
2.	Final denaturation	94^{o}C/1min	94^{o}C/1min	94^{o}C/1min
3.	Annealing	63^{o}C/2min	55^{o}C/1.5min	48^{o}C/1min
4.	Initial extension	72^{o}C/1.5min	72^{o}C/1.5min	72^{o}C/1min
5.	Final extension	72^{o}C/10min	72^{o}C/10min	72^{o}C/5min

Table-3. Occurrence of *V. parahaemolyticus* in different samples

S.No.	Sample	No. of Samples	Results for *V. parahaemolyticus*		tdh	trh	Both
			Cultural	PCR			
1.	Fresh water fish	35	12	17	2	12	1
2.	Sea fish	35	17	20	4	16	2
3.	Mutton	35	1	2	0	0	0

fish-2, sea fish-4 and mutton-nil), 28 showed *trh (fresh water fish-12, sea fish-16 and mutton-nil)* and 3 showed presence of both *tdh* and *trh (fresh water fish-1, sea fish-2 and mutton-nil)*.

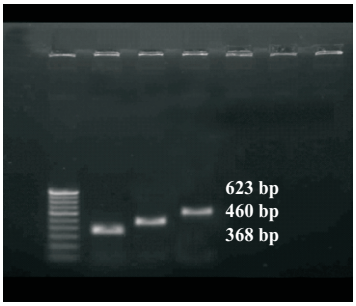

Fig.1: Comparison between amplicon products obtained from genes *toxR, tdh* and *trh* of Vibrio parahaemolyticus
Lane M: 100bp DNA Ladder
Lane 1: Amplicon product obtained by using primers from *toxR*
Lane 2: Amplicon product obtained by using primers from *tdh*
Lane 3: Amplicon product obtained by using primers from *trh*

Out of 17 and 20 fresh water fish and sea fish samples (positive for *V. parahaemolyticus*), PCR gave 2 (5.7%) and 4 (11.4%) positive results for *tdh*, 12 (34.3%) and 16 (45.7%) for *trh* and 1(2.8%) and 2 (5.7%) for both *tdh* and *trh* respectively. Raghunath *et. al.* (2008) reported 11.1% incidence of *tdh* in sea fish, which is almost coinciding with the findings (11.4%) of the present study, whereas the incidence of *trh* is more (45.7%) in this study compared to 11.1% (Raghunath *et. al.,* 2008). Baffone *et. al.* (2000) reported higher incidence (20%) of *tdh* in marine water compared to present study. The incidence of *tdh* in fresh water fish in the present study (5.7%) is less than 20.56% reported by Sanjeev and Stephen (1995).

Out of 2 mutton samples positive for *V. parahaemolyticus*, none were positive for *tdh or trh in the present study*.

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