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ORIGINAL ARTICLE

# Detection of *ctx* gene positive non-O1/non-O139 *V. cholerae* in shrimp aquaculture environments

**Rao B. Madhusudana · P. K. Surendran**

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**Abstract** Water and post-larvae samples from black tiger (*Penaeus monodon*) shrimp hatcheries; pond water, pond sediment and shrimp from aquaculture farms were screened for the presence of *V. cholerae*. A *V. cholerae*-duplex PCR method was developed by utilizing *V. cholerae* species specific *sodB* primers and *ctxAB* genes specific primers. Incidence of *V. cholerae* was not observed in shrimp hatchery samples but was noticed in aquaculture samples. The incidence of *V. cholerae* was higher in pond water (7.6%) than in pond sediment (5.2%). Shrimp head (3.6%) portion had relatively higher incidence than shrimp muscle (1.6%). All the *V. cholerae* isolates ($n$=42) belonged to non-O1/non-O139 serogroup, of which 7% of the *V. cholerae* isolates were potentially cholera-toxigenic (*ctx* positive). All the *ctx* positive *V. cholerae* ($n$=3) were isolated from the pond water. Since, cholera toxin (CT) is the major contributing factor for c*holera gravis*, it is proposed that the mere presence of non-O1/non-O139 *V. cholerae* need not be the biohazard criterion in cultured black tiger shrimp but only the presence of *ctx* carrying non-O1/non-O139 *V. cholerae* may be considered as potential public health risk.

**Keywords** *Vibrio cholerae* · Choleratoxigenic · *ctx* · Non-O1 and non-O139 · Duplex PCR · Black tiger shrimp · *Penaeus monodon*

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## Introduction

The genus Vibrio is a member of the family *Vibrionaeceae* and consists of 44 recognized species of which 12 species occur in human clinical specimens (Bergey's Manual of Systematic Bacteriology 2005). *V. cholerae* O1 is the causative agent of pandemic cholera. *V cholerae* O139 (the Bengal strain) was reported as another cause of cholera (Ramamurthy et al. 1993). The epidemic causing strains of *V. cholerae* (O1 or O139 serogroups) produce cholera toxin (CT) which is the major contributing factor for profuse diarrohea (c*holera gravis*) characterized by severe diarrohea with rice water stools, devastating dehydration and electrolyte imbalance. *V. cholerae* non-O1 serogroups are widely distributed in the aquatic environment and are free living in nature. Strains of non-O1 *V. cholerae* greatly outnumber O1 strains in the environment and majority of these isolates lack the classical virulence factors such as cholera toxin and toxin co-regulated pilus.

Cholera Toxin (CT) encoded by *ctxAB* is responsible for the severe diarroheal symptoms elicited by *V. cholerae* (Kaper et al. 1995). CT is a potent A-B type exotoxin composed of 5 B subunits (ctxB) which binds holotoxin to the cell receptor and one A subunit (ctxA) which provides intracellularly toxigenic activity. *V. cholerae* O1 strains and O139 strains produce CT (Nair and Takeda 1993) but the expression of CT is rare in other serogroups of *V. cholerae* non-O1 (Said et al. 1994). However, there are atypical environmental strains that possess the *ctx* gene (Nair et al. 1988; Rivera et al. 2001). The toxigenic O139 serogroups

having arisen from recombination with toxigenic O1 strains (Faruque et al. 2000b) and it has been hypothesized that *V. cholerae* non-O1/non-O139 strains can acquire genes for toxin production by transduction and might be the source of new epidemics.

PCR methods that target a single gene/sequence, multiple genes within *V. cholerae,* or two or more pathogens in a single PCR assay have been used by several researchers (Brasher et al. 1998; Bacteriological Analytical Manual 2001; Singh et al. 2002; Jing et al. 2003; Karunasagar et al. 2003; Panicker et al. 2004a; Fraga et al. 2007; Tarr et al. 2007; Khuntia et al. 2008). Tarr et al. (2007) developed a multiplex PCR for *V. vulnificus*, *V. parahaemolyticus*, *V. mimicus and V. cholerae* wherein the intra-specific variation in the conserved housekeeping gene *viz*., *sodB* was used as a source of marker for *V. cholerae* and as the house keeping genes are invariably present in all isolates this PCR method helps in the detection of all *V. cholerae* isolates irrespective of their toxigenic status. An effective PCR method for detecting enterotoxigenic *Vibrio cholerae* in food samples was described in Bacteriological Analytical Manual (2001). This PCR selectively amplifies a specific DNA fragment within the *ctxAB* operon of *V. cholerae* and detects only cholera toxin producing *V. cholerae* but does not provide information on non-cholera-toxigenic *V. cholerae*. A duplex-PCR that detects all *V. cholerae* isolates and simultaneously provides information on the choleratoxigenic potential is essential for risk analysis of food.

Cultured shrimp, mainly the black tiger shrimp (*P. monodon*) constitutes a major portion of Indian fishery export. During the year 2007–08, a total of 1,06,165 MT of shrimp was produced from a culture area of 1,22,078.80 ha (MPEDA 2008). Cultured shrimp processing waste is also a rich source for important by-products like chitin and carotenoids (Raghu et al. 2008). The importance of *V. cholerae* in the *P. monodon* aquaculture is mainly as a biological hazard that compromises post-harvest quality and leads to rejections from the importing countries. Rapid Alert System for Food and Feed (RASFF) of the European Union has issued alert notifications for the presence of either *V. cholerae* or *V. cholerae* non O1/non-O139 in frozen black tiger shrimp (*P. monodon*) imported by the European Union (EU) countries from shrimp exporting countries including India (http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm).

The present study was taken up to screen samples from shrimp aquaculture system for potential cholera-toxigenic *V. cholerae*, develop a duplex-PCR method for the simultaneous detection of *V. cholerae* and differentiation of cholera toxin producing *V. cholerae* and determine the effect of fish and crustacean meats on the performance of the duplex-PCR.

## Materials and methods

*Samples from shrimp culture system* Water ($n=7$) and post-larvae ($n=7$) samples from seven *Penaeus monodon* (black tiger) shrimp hatcheries; pond water ($n=5$), pond sediment ($n=5$) and shrimp samples ($n=5$) from five *P. monodon* aquaculture farms on the East Coast of India were screened.

*Isolation and identification of V. cholerae* Thiosulfate Citrate Bile Sucrose Agar (TCBS Agar) was used for isolating vibrios. Serial ten fold dilutions of shrimp muscle, shrimp head, pond sediment and pond water were spread plated on TCBS agar, incubated at 37 °C for 24 h. The sucrose fermenting colonies on the TCBS plates were purified by streak dilution and identified to the species level by using the identification scheme of Noguerola and Blanch (2008). Isolates that were arginine dihydrolase negative, lysine decarboxylase positive, ornithine decarboxylase positive, indole positive, ONPG test positive, showed growth at 0%NaCl positive, growth at 8%NaCl negative, produced acid from sucrose were identified as *V. cholerae*. The *V. cholerae* isolates identified by the above mentioned schemes were confirmed by performing the tests described for *V. cholerae* species identification (Bergey's Manual of Systematic Bacteriology 2005).

*E.coli* Tergitol seven agar was used to determine *E.coli* counts and final counts were arrived after confirmation on Eosin Methylene Blue agar and by performing indole, methyl red, Voges-Proskauer and citrate (IMVC) tests.

*Slide agglutination test for V. cholerae O1 and V. cholerae O139 and V. cholerae non-O1/non-O139* *V. cholerae* isolates were initially tested with polyvalent somatic O antiserum (Difco *Vibrio cholerae* antiserum Poly [Hikojima, Inaba, Ogawa], Becton, Dickinson and Company, Franklin Lanes, NJ, USA) and the isolates that gave positive agglutination reaction were classified as *V. cholerae* O1. Cultures that gave negative reaction with polyvalent somatic O antiserum were further tested for agglutination using *V. cholerae* O139 antiserum (*V. cholerae* antiserum O139 Bengal, Denka Seiken Co Ltd, Tokyo, Japan) and those *V. cholerae* cultures that gave positive agglutination reaction were classified as *V. cholerae* O139. *V. cholerae* cultures that gave negative reaction both with polyvalent somatic O antiserum and O139 antiserum were classified as *V. cholerae* non-O1/non-O139.

*PCR* PCR for the detection of *V. cholerae* using species specific primers (Tarr et al. 2007) was performed using 1 µl of each *sodB* (Table 1) primer (10 µM stock) in a final PCR reaction volume of 20 µl. *V. cholerae* yield an amplicon of 248 bp. PCR for the detection of enterotoxigenic *V.*

**Table 1** Details of the *V. cholerae* species specific and ctx specific primers

| Organism | Primer | Sequence | Target | Amplicon size | Reference |
|---|---|---|---|---|---|
| *V. cholerae* | *Vc.sodB*-F | 5′-AAG ACC TCA ACT GGC GGT A – 3′ | *sodB* | 248 bp | Tarr et al. (2007) |
| | *Vc.sodB*-R | 5′-GAA GTG TTA GTG ATC GCC AGA GT – 3′ | | | |
| *V. cholerae* cholera-toxigenic | *ctx*F | 5′-TGA AAT AAA GCA GTC AGG TG – 3′ | *ctxAB* | 777 bp | Bacteriological Analytical Manual (2001) |
| | *ctx*R | 5′-GGT ATT CTG CAC ACA AAT CAG– 3′ | | | |
| *V. cholerae* O1 | O1F2-1 | 5′-GTT TCA CTG AAC AGA TGG G-3′ | *V. cholerae* O1-rfb specific primers | 192 bp | Hoshino et al. (1998) |
| | O1R2-2 | 5′-GGT CAT CTG TAA GTA CAA C- 3′ | | | |
| *V. cholerae* O 139 | O139F2 | 5′- AGC CTC TTT ATT ACG GGT GG-3′ | O139-rfb specific primers | 449 bp | Hoshino et al. (1998) |
| | O139R2 | 5′-GTC AAA CCC GAT CGT AAA GG-3′ | | | |

*cholerae* was performed (Bacteriological Analytical Manual 2001) using *ctx* primers (Table 1) at 0.5 µM concentration of each primer and 3% (v/v) APW lysate as template. *ctxAB* positive *V. cholerae* cultures yield an amplicon of 777 bp. PCR for *V. cholerae* O1 and *V. cholerae* O139 was performed as per Hoshino et al. (1998) using O1F2-1 and O1R2-2 primers (*V. cholerae* O1-rfb specific primers) and O139F2 and O139R2 primers (O139-rfb specific primers) (Table 1). *V. cholerae* O1 cultures yield an amplicon 192 bp whereas *V. cholerae* O139 cultures yield an amplicon 449 bp. *V. cholerae* non-O1/non-O139 cultures do not yield any of these amplicons. *V. cholerae* (MTCC 3906), *V. vulnificus* (MTCC 1145), *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802) cultures were used for standardizing the PCR reactions.

*Duplex PCR for the simultaneous detection of V. cholerae and differentiation of cholera toxin producing V. cholerae isolates (V. cholerae-duplex PCR)* A *V. cholerae*-duplex PCR method was developed by utilizing *V. cholerae* species specific *sodB* primers (Tarr et al. 2007) and *ctxAB* genes specific primers (Bacteriological Analytical Manual 2001). This method was designed to detect *V. cholerae* isolates and differentiate *ctx* toxin producing strains.

*V. cholerae-duplex PCR reaction conditions* One ml of *V. cholerae* culture (24 h at 37 °C) grown in $T_1N_1$ (Tryptone 1%, NaCl 1%) was centrifuged at 10,000 rpm for 10 min. The cell pellet was resuspended in 100 µl of Tris-EDTA (TE) buffer, placed in a dry bath for 5 min at 95 °C and the crude lysate was used as template. The PCR amplification reactions were performed in a final volume of 20 µl. Each reaction mixture contained 0.5 µM each of PCR species specific primers, 0.5 µM each of *ctxAB* primers, 18 µl of master reaction mix containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM MgCl2, 200 µM each dATP, dCTP, dGTP and dTTP, 1 U of *Taq* polymerase and 1.2 µl of crude lysate. PCR reaction was carried out in Minicycler (Minicycler PTC-150, MJ Research, MA, USA) programmed to perform a denaturation step at 93 °C for 15 min, followed by 35 cycles consisting of 40 s at 92 °C, 1 min at 57 °C and 1.5 min at 72 °C. The last extension step was extended to 7 min longer. Agarose gel analysis of *V. cholerae*-duplex PCR products (Sambrook and Russell 2001) was carried out by loading 10 µl of the PCR product in a 2% agarose gel containing 1 µg/ml Ethidium bromide in TAE buffer and electrophoresed. After appropriate migration with constant voltage of 5–10 V/cm (Electrophoresis Powerpack, Bangalore Genei, India) the agarose gel was scanned using a gel documentation system (Alpha Imager, Alpha Innotech Corporation, USA). *V. cholerae* cultures yield 248 bp amplicon; *ctxAB* positive *V. cholerae* cultures yield two amplicons viz., 248 bp and 777 bp.

*End point dilution of V. cholerae-duplex PCR* The sensitivity of *V. cholerae*-duplex PCR was determined by making serial 10 fold dilutions of *V. cholerae* culture (grown in $T_1N_1$, incubated for 24 h at 37 °C) in normal saline ranging from undiluted to $10^{-6}$ viz., $10^6$ *V. cholerae* cells, $10^5$ *V. cholerae* cells, $10^4$ *V. cholerae* cells, $10^3$ *V. cholerae* cells, 100 *V. cholerae* cells, 10 *V. cholerae* cells and 1 *V. cholerae* cell. From each tube, 1 ml was withdrawn for template preparation and subjected to *V. cholerae*-duplex PCR.

*Suitability of V. cholerae-duplex PCR in detecting V. cholerae in fish and crustaceans samples* Freshwater fish (carp, *Labeo rohita*), marine fish (tuna, *Euthynnus affinis*),

black tiger shrimp (*Penaeus monodon*) and three spot crab (*Portunus sangulnolentus*) meats were used. All the food matrices were initially tested negative for the presence of *V. cholerae*. Fish, shrimp or crab meat (25 g) was homogenized with sterile alkaline peptone water (225 ml) and spiked with known number of *V. cholerae* (*ctx* positive) cells ($10^6$ cells) and incubated at 37 °C. The samples were drawn after 18 h of incubation for PCR analysis.

## Results and discussion

*Vibrio loads in shrimp hatchery and farm samples* Vibrio loads were higher in *P. monodon* hatchery samples than in aquaculture farm samples (Table 2). Shrimp post-larvae had maximum loads of Vibrios ($2.1\times10^5$ CFU/g). Shrimp head portion had relatively higher counts of Vibrios ($3.5\times10^4$ CFU /g) than shrimp muscle portion ($1.4\times10^4$ CFU /g). Hatchery waters had higher Vibrio loads (2400 CFU /ml) than farm waters (150 CFU /ml). Sucrose non-fermenting Vibrios were higher in shrimp head portion (59%) and hatchery waters (51%) whereas more than 90% of the Vibrios in post-larvae, pond water, pond sediment and shrimp muscle portions were sucrose fermenters. Gomez-Gil et al. (1998) reported that Vibrio spp. isolated from the digestive tract of a population of healthy juvenile *Litopenaeus vannamei* consisted of both sucrose and non-sucrose fermenters whereas the haemolymph contained only non-sucrose fermenters.

*Incidence of V. cholerae in shrimp hatchery and farm samples* Two hundred ten Vibrio cultures isolated from water ($n$=105) and post-larvae ($n$=105) samples from shrimp hatcheries; 250 Vibrio cultures isolated and purified from pond water ($n$=75), pond sediment ($n$=75) and shrimp ($n$=100) from aquaculture farms were screened for the presence of Vibrio species. Incidence of *V. cholerae* was not observed in shrimp hatchery samples as none of the 210 Vibrio cultures obtained from hatchery water or post-larvae were identified as *V. cholerae*. However, a total of 42 vibrio isolates out of the 250 vibrio cultures obtained from shrimp aquaculture samples were identified as *V. cholerae* by biochemical tests. The incidence of *V. cholerae* was higher in pond water (7.6%) than in pond sediment (5.2%). Shrimp head (3.6%) portion had relatively higher incidence of *V. cholerae* than shrimp muscle (1.6%). The incidence of *V. cholerae* in pond water, pond sediment, shrimp head portion and shrimp muscle as a percentage of the total *V. cholerae* isolates ($n$=42) was 45.2%, 31%, 14.3% and 9.5%, respectively (Table 2). All the *V. cholerae* isolates obtained from shrimp aquaculture system were grouped as *V. cholerae* non-O1/non-O139 as they yielded negative agglutination results with polyvalent somatic O antiserum and O139 antiserum. All these *V. cholerae* isolates yielded the specific amplicon (248 bp) in the PCR employing *sodB* primers thereby confirming their identity as *V. cholerae* (Fig. 1). Three *V. cholerae* isolates were positive in the PCR employing *ctxAB* primers and yielded a single specific amplicon of 777 bp size. *V. cholerae* was absent but non-01 serotype *V. cholerae* was reported from *Penaeus monodon* ponds in India (Otta et al. 1999).

*V. cholerae-duplex PCR method for the simultaneous detection and differentiation of choleratoxigenic V. cholerae* In this study a *V. cholerae*-duplex PCR method was developed by utilizing *V. cholerae* species specific (Tarr et al. 2007) and *ctxAB* genes specific primers (Bacteriological Analytical Manual 2001). The reason for selecting the *ctxAB* and *sodB* primers for developing *V. cholerae*-duplex PCR was that the annealing temperatures for the *ctxAB* primers (55 °C) and *sodB* primers (57 °C) were nearer. This method was designed to detect *V. cholerae* isolates and simultaneously differentiate *ctx* toxin producing strains.

**Table 2** Incidence of *V. cholerae* in black tiger shrimp culture system

| Shrimp culture sample | Total Vibrio load CFU/g or CFU/ml[a] | Incidence of *V. cholerae*[b] | Incidence of *V. cholerae* O1 | Incidence of *V. cholerae* O139 | Incidence of non-O1/ non-O139 *V. cholerae* | Incidence of *ctx* positive *V. cholerae* |
|---|---|---|---|---|---|---|
| Shrimp hatchery water | $2.4 \times 10^3 \pm 2.2 \times 10^3$ | 0 | Negative | Negative | Negative | Negative |
| Shrimp post-larvae | $2.1 \times 10^5 \pm 1.1 \times 10^5$ | 0 | Negative | Negative | Negative | Negative |
| Shrimp pond water | $1.5 \times 10^2 \pm 42$ | 7.6% (45.2%)[c] | Negative | Negative | Positive | Positive (7%)[c] |
| Shrimp pond sediment | $1.5 \times 10^3 \pm 6.4 \times 10^2$ | 5.2% (31%)[c] | Negative | Negative | Positive | Negative |
| Shrimp head | $3.5 \times 10^4 \pm 2.2 \times 10^4$ | 3.6% (14.3%)[c] | Negative | Negative | Positive | Negative |
| Shrimp muscle | $1.4 \times 10^4 \pm 1.42 \times 10^4$ | 1.6% (9.5%)[c] | Negative | Negative | Positive | Negative |

[a] Mean ± SD

[b] As percentage of the total Vibrios ($n$=250) isolated from shrimp aquaculture ponds

[c] As percentage of *V. cholerae* ($n$=42) isolates from shrimp aquaculture ponds

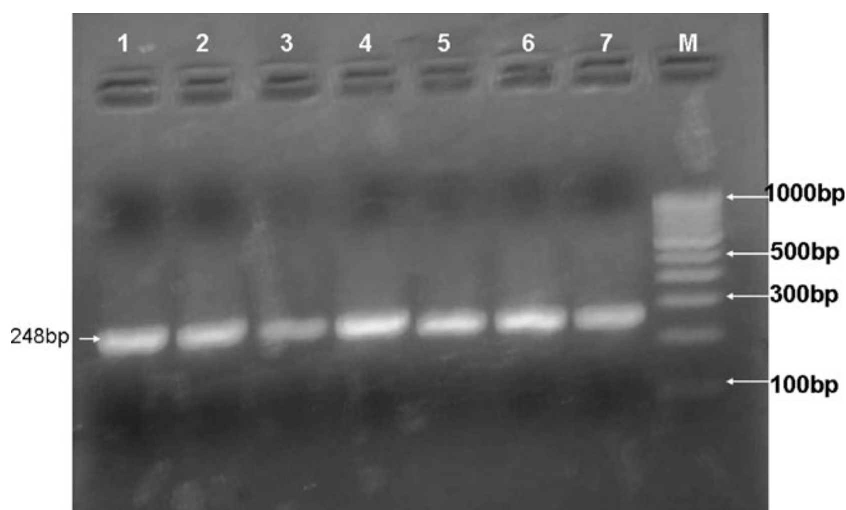


**Fig. 1** PCR for the detection of *V. cholerae* using species specific primers. Lane 1–3, *V. cholerae* isolates from shrimp pond water; Lane 4–5, *V. cholerae* isolates from shrimp pond sediment; Lane 6, *V. cholerae* isolates from shrimp muscle; Lane 7, *V. cholerae* (MTCC 3906); All *V. cholerae* yielded the species specific amplicon (248 bp) with *sodB* primers; Lane8, 100 bp DNA ladder (GeNei)

PCR amplification cycle conditions for the *ctxAB* primers was 34 cycles consisting of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The thermal cycling profile for the *sodB* primers was 35 cycles consisting of 40 s at 92 °C, 1 min at 57 °C and 1.5 min at 72 °C. The primer annealing condition of 1 min at 57 °C was used in this *V. cholerae*-duplex PCR. A simple template preparation procedure as mentioned in material and methods was used for the *V. cholerae*-duplex PCR. The crude lysate of *V. cholerae* culture was found sufficient for obtaining specific result in the *V. cholerae*-duplex PCR method. The *V. cholerae*-duplex PCR was initially standardized using *V. cholerae* (MTCC 3906), *V. vulnificus* (MTCC 1145), *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802) cultures and *ctx* negative *V. cholerae* (laboratory culture collection) cultures. *V. cholerae* (*ctx* negative) cultures yielded a single amplicon (248 bp) whereas *ctxAB* positive *V. cholerae* cultures yielded two amplicons (248 bp and 777 bp). Other Vibrio species did not yield the *V. cholerae* specific amplicon. The results indicate that the *V. cholerae*-duplex PCR was specific to *V. cholerae* and the PCR cycle conditions were adequate for obtaining the desired result.

All the 42 *V. cholerae* isolates obtained from shrimp aquaculture system were subjected to *V. cholerae*-duplex PCR. Thirty nine *V. cholerae* cultures yielded a single amplicon of 248 bp indicating that they were *V. cholerae* but non-choleratoxigenic. Three *V. cholerae* cultures yielded two amplicons viz., species specific 248 bp and cholera toxin specific 777 bp, thereby indicating that they were cholera toxin producing strains of *V. cholerae* (Fig. 2). The specificity of the *V. cholerae*-duplex PCR was confirmed by screening the remaining 208 Vibrios cultures isolated from aquaculture ponds. None of the samples

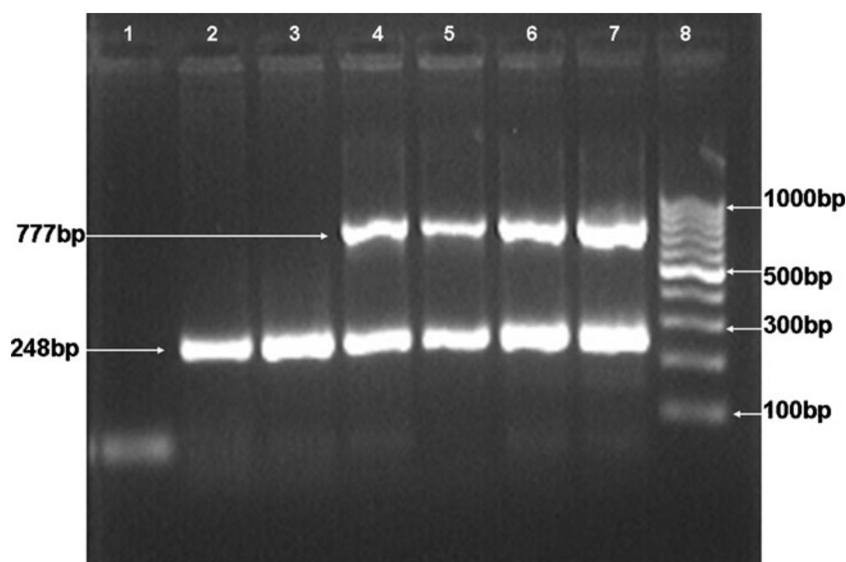


**Fig. 2** Duplex PCR for the simultaneous detection of *V. cholerae* and differentiation of cholera toxin producing *V. cholerae* isolates. Lane 1, Negative control; Lane 2–3, Cholera toxin negative *V. cholerae* isolates from shrimp pond water; Lane 4–6, Cholera toxin positive *V. cholerae* isolates from shrimp pond water; Lane 7, *V. cholerae* (MTCC 3906); Lane 8, 100 bp DNA ladder (Gene Ruler™, Fermentas)

yielded the specific amplicons in *V. cholerae*-duplex PCR indicating the specificity of *V. cholerae*-duplex PCR. The *V. cholerae*-duplex PCR and the dichotomous scheme of Noguerola and Blanch (2008) yielded the same result; in both the cases the same cultures were identified as *V. cholerae*.

*V. cholerae* was not detected in shrimp hatchery water and post-larvae. The results indicate that, even though *V. cholerae* was detected in shrimp pond sediment, shrimp head and shrimp muscle samples majority of the isolates were found to be non-choleratoxigenic as the isolates did not yield the *ctxAB* specific amplicon in *V. cholerae*-duplex PCR. Only 7% of the *V. cholerae* isolates (three isolates) were potentially cholera-toxigenic (Table 2). These three *V. cholerae* isolates were obtained from pond water and they agglutinated neither with polyclonal somatic O antiserum nor with O139 antiserum; thereby grouping them as *V. cholerae* non-O1/non-O139 serogroup. These three isolates failed to yield O1 specific or O139 specific amplicon when tested in PCR (Fig. 3) reconfirming that the isolates were indeed *V. cholerae* non-O1/non-O139. The faecal indicator, *E. coli* was detected in aquaculture farms but not in shrimp hatchery samples and the mean *E. coli* levels were high in pond water (123±87 CFU/g) than in shrimp head (16±16.7 CFU/g) and shrimp muscle (12±11 CFU/g). This may suggest that the *ctx* positive *V. cholerae* in pond water might have entered through faecal pollution either through the source water or through feed. Farm made feeds were reported positive for *V. cholerae* (Raghavan 2003). However, pond sediment samples had higher mean *E. coli* levels (204±133 CFU/g) than pond water (123±87 CFU/g) but *ctx* positive *V. cholerae* could not be isolated from these sources which suggests that the relationship between faecal bacteria and presence of *ctx* positive *V. cholerae* is non-existent. A negative correlation was observed between total vibrio counts and *E. coli* ($r=-0.54$) in the shrimp culture system. The incidence of *V. cholerae* observed in aquaculture ponds might also have been due to natural inhabitation. The autochthonous existence of *V. cholerae* in environment has been previously reported by several researchers (Colwell et al. 1981; Lee et al. 1982). Moreover, the presence of *V. cholerae* could not be correlated with faecal indicators (Hood and Ness 1982; Filetici et al. 1997).

The structural genes for the *ctx* element reside on a filamentous phage ctxΦ (Waldor and Mekalanos 1996). CTXΦ is found in all epidemic *V. cholerae* isolates but is rarely recovered from the non-O1/non-O139 *V. cholerae* environmental isolates. A DNA probe study showed that a small percentage of environmental strains of *V. cholerae* non-O1 have the *ctx* gene (Nair et al. 1988). Virulence genes including *ctxAB* were found among environmental strains from Calcutta, India (Chakraborty et al. 2000). Occurrence of *ctxA* was found among 10% on non-O1/non-O139 environmental isolates from Brazil (Rivera et al. 2001). Clinical toxigenic *V. cholerae* isolates are closely related to non-toxigenic environmental strains (Jiang et al. 2000) and CT genes are highly mobile among environmental isolates. The spread of CT genes in the environment can be facilitated by the exposure of CTXΦ positive strains to sunlight (Faruque et al. 2000a). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a *V. cholerae* O1 strain by horizontal gene transfer (Bik et al. 1995; Comstock et al. 1996; Waldor and Mekalanos 1994; Faruque et al. 2000b). Similarly, *V. cholerae* non-O1/non-O139 strains can also acquire toxigenic genes for toxin production by transduction and therefore might be the

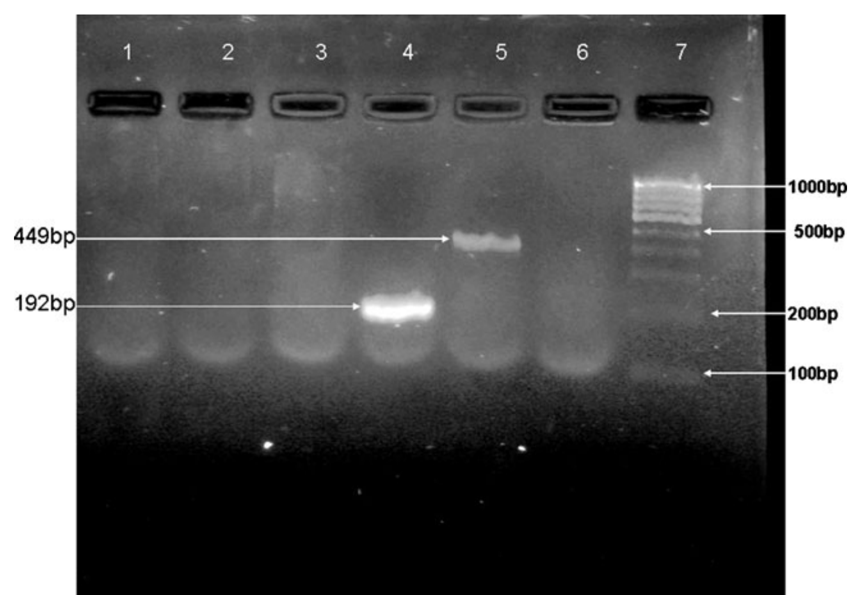


**Fig. 3** PCR for testing the O1 and O139 status of *ctx* bearing *V. cholerae* isolates from shrimp pond water. Lanes 1–3, *ctx* positive *V. cholerae* isolates from shrimp aquaculture system failed to yield O1 specific or O139 specific amplicon indicating that the isolates were *V. cholerae* non-O1/non-O139; lane 4, Known *V. cholerae* O1 isolate from laboratory culture collection; lane 5, *V. cholerae* (MTCC 3906) – *V. cholerae* O139; lane 6, Known *V. cholerae* non-O1/non-O139 from laboratory culture collection; lane 7, 100 bp DNA ladder (GeNei)

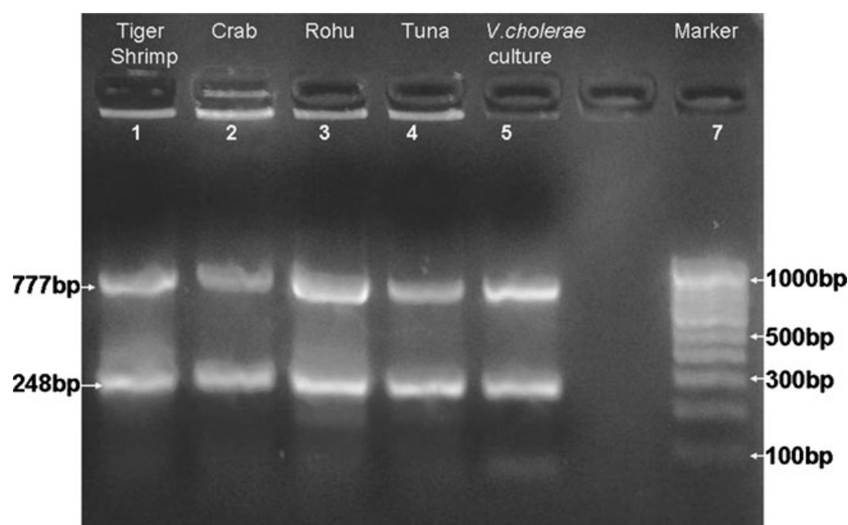


**Fig. 4** Performance of *V. cholerae* duplex-PCR in different food matrices. Lane 1, Tiger shrimp, (*Penaeus monodon*); Lane 2, 3 spot crab (*Portunus sangulnolentus*); Lane 3, Freshwater fish (carp, *Labeo rohita*); Lane 4, marine fish (tuna, *Euthynnus affinis*) meats spiked with *V. cholerae* (*ctx* positive) cells yielded positive PCR result for the presence of *Vibrio cholerae* species specific amplicon (248 bp) and cholera toxin specific amplicon (777 bp amplicon); Lane 8, 100 bp DNA ladder (GeNei)

source of new epidemics. Hypothesis of the existence of a TCP-independent mechanism for infection by CTXΦ was proposed by Jiang et al. (2003). The choleratoxigenic *V. cholerae* (non-O1/non-O139) isolated from shrimp aquaculture system showed greater genetic similarity with *ctx* negative *V. cholerae* than amongst *ctx* positive *V. cholerae* (Rao and Surendran 2010).

Keeping these in view, the screening of environmental and food samples for the presence of *ctx* positive *V. cholerae,* especially within the non-O1/ non-O139 serogroup, is of paramount importance. The *V. cholerae* duplex-PCR method developed in this study would be a useful tool to screen for *ctx* positive *V. cholerae*. Even though the existence of *ctx* carrying non-O1/non-O139 *V. cholerae* isolates was very low in shrimp culture system, the ecological significance of *ctx* genes among these *V. cholerae* non-O1 and non- O139 isolates in the shrimp aquaculture environment needs to be further investigated. Study on the expression of *ctx* gene by the *ctx* positive non-O1/non-O139 *V. cholerae* was not performed. Additional study on the expression of *ctx* genes would unravel the ecological significance of *ctx* genes in non-O1/non-O139 *V. cholerae*

The autochthonous existence of non-O1/non-O139 *V. cholerae* in aquatic environment has been reported from several areas world over. Non-O1/non-O139 *V. cholerae* appears to constitute part of the microflora of prawns (Nair et al. 1991) and oysters (McIntyre et al. 1979; Salamaso et al. 1980). While, non-O1/O139 *V. cholerae* may be occasionally found in shrimp, there is no known risk of cholera associated with these serotypes in shrimp. Risk assessments by FAO/WHO presented a very small risk of acquiring cholera through consumption of imported warm-water shrimp (FAO/WHO 2005). However, RASFF alert notifications (numbers: 2005.778, 2008.AXE, 2008.BDH) were issued for the presence of non-O1/O139 *V. cholerae* in black tiger shrimps exported from India. (http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm). The biohazard status of non-O1/non-O139 *V. cholerae* which appears to be autochthonous bacterial flora in the black tiger shrimp culture system needs to be reassessed. It is proposed that the mere presence of *V. cholerae* non-O1/non-O139 in cultured black tiger shrimp need not be considered as the criterion for biohazard leading to rejection of shrimp exports but *ctx* carrying *V. cholerae* non-O1/non-O139 may be considered potential public health risk.

**Table 3** Effect of different fish/shrimp matrices on the performance of *V.cholerae*-duplex PCR

| Fish/shrimp sample | Total vibrio load (before spiking with *V.cholerae*) | Inhibitory effect of the food matrix on *V.cholerae*-duplex PCR result | Result of *V.cholerae*-duplex PCR (spiked sample) |
|---|---|---|---|
| Black tiger shrimp (*Penaeus monodon*) | $4.6\times10^{4}$ CFU/g | No inhibitory effect | Positive |
| Marine fish, tuna (*Euthynnus affinis*) | $1.6\times10^{3}$ CFU/g | No inhibitory effect | Positive |
| Freshwater fish, Carp *(Labeo rohita)* | 60 CFU/g | No inhibitory effect | Positive |
| Crab meat, 3 spot crab (*Portunus sangulnolentus*) | $4.8\times10^{3}$ CFU/g | No inhibitory effect | Positive |

However, further studies are needed to establish *V. cholerae* non-O1/non-O139 as native flora of black tiger shrimp culture system. Other virulence factors associated with *V. cholerae* are *tcpA, hlyA, zot*, *ace, rtxA*, etc. Fraga et al. (2007) reported that *V. cholerae* isolates from the environment lacked *ctxA* and *tcpA* but contained *hlyA* (98·7%), *rtxA* (99·0%), *toxR* (98·7%) and *stn-sto* (1·9%) The present study did not search for other virulence genes and further studies on the prevalence of these genes in *ctx* positive non-O1/non-O139 would aid in risk assessment.

The sensitivity of *V. cholerae*-duplex PCR was determined using templates prepared from different *V. cholerae* cell concentrations. The *ctxAB* specific primers yielded an amplicon only when the concentration of *V. cholerae* cells was 1000 *V. cholerae* cells/ml and above whereas the species specific *sodB* primers yielded an amplicon at a concentration as low as 100 *V. cholerae* cells/ml. The intensity and thickness of both the amplicon bands decreased with decrease in *V. cholerae* cell numbers. The sensitivity attained was similar that of reported sensitivities for multiplex PCR. The sensitivity of the multiplex PCR for the detection of *V. cholerae* targeting *ctxA, ace, zot, tcpA* and *toxR* was $10^2$ CFU/ml (Jing et al. 2003). The sensitivity of multiplex PCR for the detection of toxigenic and pathogenic *V. cholerae* from direct water sources using specific primers targeting *ompW*, *ctxB*, *rfbG*, *zot* and *tcpB* was $5\times10^4$ *V. cholerae* cells per reaction (Goel et al. 2007). A duplex PCR targeting the genes *gyrB* and *tl* for specific identification of *V. parahaemolyticus* could detect as few as 250 CFU/ml in pure cultures (Vongxay et al. 2006). In the multiplex PCR (*vvh* fragment and *viuB*) for *V. vulnificus*, the sensitivity of detection for both targeted genes was 10 pg of purified DNA, which correlated with $10^3$ CFU/ml of pure culture (Panicker et al. 2004b)

The suitability of *V. cholerae*-duplex PCR in detecting *V. cholerae* in fish (rohu, tuna) and crustaceans (black tiger shrimp, three spot crab) samples was studied to know whether inhibitory substances present in these foods might adversely affect the performance of PCR. All the above mentioned food matrices did not show any inhibitory effect on *V. cholera* duplex-PCR as all the spiked samples yielded the *V. cholerae* species specific amplicon and *ctx* specific amplicon (Fig. 4). The specificity of the *V. cholerae* duplex-PCR is further strengthened by the fact that the primers yielded the specific amplicon even in the presence of initial resident Vibrio populations ranging between 60 CFU /g and $4.6\times10^4$ CFU/g in these food matrices (Table 3). The *V. cholerae* duplex-PCR can be of use in the post-harvest quality analysis of fish and crustaceans.

In this study, we studied the incidence of *V. cholerae* in black tiger shrimp aquaculture system and detected non-O1/non-O139 *V. cholerae* in aquaculture samples but not in hatchery samples. Seven percent of the non-O1/non-O139 *V. cholerae* isolates were potentially cholera-toxigenic. This work reports a new method viz., *V. cholerae*-duplex PCR method by utilizing *V. cholerae* species specific *sodB* primers and *ctxAB* genes specific primers. *V. cholerae* duplex-PCR may be particularly usefulness in the risk assessment of fish and shrimp as *V. cholerae* non-O1/non-O139 strains can acquire toxigenic genes for toxin production and therefore might be the source of new epidemics.

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