



Reverse transcription-PCR assay for the rapid detection of viable *Vibrio cholerae* from fresh and processed shrimp

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ABSTRACT

Viable *Vibrio cholerae* in seafood was rapidly detected by the Reverse transcription-PCR (RT-PCR) assay developed by targeting mRNA *ctxA* gene without any pre-enrichment. The PCR product size of the selected gene was 308 bp, which aided in the identification of *V. cholerae*. This RT-PCR assay was rapid, as it detected *V. cholerae* from fresh shrimp tissue within 5 min on bio-inoculation when the cell count was 1,000,000 cells. The pathogen was detected in 5 h when the load was 110 cells. The assay even detected the pathogen in shrimp that has been cooked for 10 min, frozen for 30 days and even in dried shrimp. Other food-borne pathogens like *Salmonella*, *Staphylococcus aureus* and *Listeria monocytogenes* were not detected by this assay. The developed RT-PCR assay is thus specific and rapid in detecting the viable *Vibrio cholerae* in seafood without any pre-enrichment.

Keywords: RT-PCR, Seafood, Shrimp, *Vibrio cholerae*

Introduction

The epidemic cholera, caused by *Vibrio cholerae*, is the watery diarrhea resulting in dehydration and sometimes death. The important reservoir for *V. cholerae* is the coastal waters and it is normally transmitted to human beings through contaminated water and seafood (Colwell *et al.*, 1981). Two important serogroups O1 and O139 of *V. cholerae* among 200 known serogroups are associated with epidemic cholera (Cholera Working Group, 1993). The Inaba, Ogawa and Hikojima are the three serotypes under the serogroup O1 and the serotype is divided into two biotypes *viz.*, classical and El Tor (Kaper *et al.*, 1995). The non-O1 and non-O139 serogroups of *V. cholerae* are not associated with epidemics, but with the occasional outbreaks of cholera-like diseases. Though the pathogenesis of cholera is complex, the major virulence factor of *V. cholerae* is the cholera toxin (CT) encoded by the *ctxA* gene (Kaper *et al.*, 1995). CT causes increased secretion of electrolytes and water into the lumen of the intestine. The *ctxA* gene located in genome of the CTX filamentous prophage (Lee *et al.*, 1999) is responsible for massive and watery diarrhea of cholera.

The culture-based method for the detection of *V. cholerae* in fishery products is laborious, time consuming and often not specific. Though few alternative analytical strategies are proposed, PCR based method is found to be the highly specific molecular diagnostic tool (Wang *et al.*, 1997; Jeyasekaran *et al.*, 2011a). The sensitivity of the PCR

based technique may be reduced dramatically when applied to complex biological samples (Lantz *et al.*, 2000), even though this can be extremely effective on pure solutions of nucleic acids. However, PCR does not provide information related to cell viability, as the DNA molecules from live and dead cells cannot be distinguished by the PCR (Josephson *et al.*, 1993; Masters *et al.*, 1994). A pre-enrichment step is essentially performed to allow viable cells to grow and multiply that improve the discrimination power of PCR (Wang *et al.*, 1997). Pre-enrichment takes 6-48 h to complete, which is a major drawback in PCR (Call *et al.*, 2001). Since only viable bacteria produce toxins that cause human disease, the distinction between viable and dead bacteria is important (Law, 2000).

It would be of enormous significance for food, industrial, environmental and medical applications, if a nucleic acid-based method is developed and applied directly to samples without pre-enrichment indicating the viability of microbes. A method for the detection of specific mRNA in individual bacterial cells was described by Tolkar-Neilsen *et al.* (1997). Most mRNA in living bacterial cells has a half-life of only a few minutes (Alifano *et al.*, 1994). Hence, the detection of mRNA shall be a good indicator of viability of cells (Hill, 1996; Sheridan *et al.*, 1998). Investigations on the relationship between microbial mRNA and viability in *V. cholerae* and *Listeria monocytogenes* using RT-PCR were specifically studied by Bej *et al.* (1996) and Klein and

Juneja (1997). The present study was undertaken to detect viable *V. cholerae* from shrimp tissue using mRNA based RT-PCR assay without pre-enrichment.

Materials and methods

Type cultures of bacteria and other materials

Vibrio cholerae type cultures used are *Vibrio cholerae* O139 (SG 24), *V. cholerae* El Tor (N 16961) and *V. cholerae* Classical (V 154). Type cultures of other bacterial pathogens like *Salmonella enterica* serovar Typhi (ATCC 122235), *S. enterica* serovar Enteritidis (ATCC 13065), *Staphylococcus aureus* (ATCC 12598), *Escherichia coli* (ATCC 9637), *Aeromonas hydrophila* (ATCC 1457) and *Listeria monocytogenes* (ATCC 12232) were used to check specificity of the assay. Trypticase soy broth (TSB) was used for growing type cultures and incubation was done at 37°C for 18 h prior to the extraction of total RNA. Media and ingredients supplied by Hi-Media Laboratories Pvt. Ltd, Mumbai, India were used in the experiment. Nuclease free water obtained from molecular grade water purification system (Sartorius Stedim Biotech, Gottingen, Germany) was used for development of assay.

Extraction of total RNA from bacterial type cultures and shrimp tissue

The total RNA was extracted from type cultures using a commercial RNA extraction kit [Real Biotech Corporation (RBC), Ohio, USA]. One milliliter of broth culture (18 h old) was used to obtain purified 50 µl of RNA following the manufacturer's protocol. In the case of shrimp tissue, about 30 mg tissue homogenate was used to obtain 50 µl of RNA as per the protocol of the manufacturer. The RNA was treated with DNase I (Fermentas, Whatman, USA) for removing any contaminating DNA. The DNase treated RNA was immediately frozen at -70°C until used as a template for cDNA synthesis. Biophotometer (Eppendorf AG, Germany) was used to measure the concentration and purity of the extracted RNA.

cDNA synthesis

The RNA was converted into cDNA by performing reverse transcription as per the standard protocol (Fermentas, Waltham, U.S.A). Each reaction tube contained extracted RNA (1-5 µg), random hexamer primer (0.2 µg µl⁻¹) and the volume was made up to 11 µl with nuclease free water. After incubation at 65°C for 5 min, the tubes were placed on ice and the components provided in the kit *viz.*, 5X reaction buffer, ribolock RNase inhibitor (20 U µl⁻¹), 10 mM dNTP mix and M-MuLV transcriptase buffer (20 U µl⁻¹) were added to the final volume of 20 µl. The tubes were again incubated at 37°C for 60 min and then heated at 70°C for 5 min for terminating the reaction. The cDNA obtained was stored at -20°C until used as template in PCR.

Primers

Forward and reverse primer pair (*ctx A* F 5' ACA GAG TGA GTA CTT TGA CC 3' and *ctx A* R 5' ATA CCA TCC ATA TAT TTG GGA G 3') used was based on mRNA of *ctx A* gene (amplified a product size of 308 bp) for *Vibrio cholerae* (Hoshino *et al.*, 1998). The primer (desalted) supplied by Ocimum Biosolutions Inc., Indianapolis, USA was used.

Reverse transcription-Polymerase chain reaction (RT-PCR)

A gradient master cycler (Eppendorf AG, Hamburg, Germany) was used for performing the RT-PCR. A biosafety cabinet Type II (Clean Air Systems, Chennai, India) was used for handling all PCR mixtures. The amplification was performed in 25 µl of reaction mixture containing 1 µl of template cDNA of *V. cholerae*, 10X PCR buffer 100 mM Tris, pH 9.0, 500 mM KCl, 15 mM MgCl and 0.1% gelatin, 100 mM of each dNTP, 30 pmols of each primer and 1.25 U of Taq DNA polymerase. The optimised RT-PCR conditions were initial denaturation at 94°C for 3 min, followed by denaturation at 94°C for 60 s; annealing at 55°C for 60 s; extension at 72°C for 90 s, and final extension at 72°C for 3 min. The resulting RT-PCR product was run on 2% agarose gel containing ethidium bromide (5 mg ml⁻¹) in Tris acetate EDTA buffer (TAE, pH 8.5) along with 100 bp DNA ladder.

Specificity of RT-PCR assay

The specificity of the developed RT-PCR for *Vibrio cholerae* was tested by performing the RT-PCR assay with other food-borne bacterial pathogens such as *Salmonella enterica* serovar Typhi (ATCC 122235), *S. enterica* serovar Enteritidis (ATCC 13065), *Staphylococcus aureus* (ATCC 12598), *Escherichia coli* (ATCC 9637), *Aeromonas hydrophila* (ATCC 1457) and *Listeria monocytogenes* (ATCC 12232).

Sensitivity of RT-PCR assay in shrimp tissue

Bio-inoculation studies of type culture of *Vibrio cholerae* in shrimp tissue was performed for testing the sensitivity of the developed RT-PCR assay. A tissue homogeniser was used for homogenising the shrimp tissue. Approximately 1g of tissue homogenate was taken in sterile eppendorf tubes. Overnight grown type culture (1.00 x 10⁷ CFU ml⁻¹) of *V. cholerae* O139 (SG 24) was serially diluted up to 10⁻⁹ and an aliquot of 50 µl was inoculated into the tissue. The bacterial count in each serial dilution was ascertained by plating onto thiosulphate citrate bile salt sucrose (TCBS) agar plates and incubated at 37°C for 48 h (Shikongo-Nambabi *et al.*, 2012). The bio-inoculated tissue homogenates were incubated for 5, 10, 20 min (for 10⁻¹ and 10⁻² dilutions) and 3, 4, 5 h (for 10⁻⁷, 10⁻⁸ and 10⁻⁹ dilutions) at 37°C. Different dilutions of bio-inoculated samples were prepared for various inoculation periods to get a range of bacterial cells from 10¹ to 10⁶ as per earlier reports of Sheridan *et al.* (1998).

Uninoculated tissue homogenate was used as a control. After incubation, 30 mg of tissue homogenate was used for the extraction of total RNA, and for performing RT-PCR assay as described earlier. The experiment was independently repeated thrice for concurrent results.

Validation of RT-PCR assay

The shrimp tissue samples were divided into 4 lots to validate the developed RT-PCR assay in detecting viable *V. cholerae* from the shrimp samples that had been subjected to different processing treatments *viz.*, freezing, cooking and drying. The first lot was the uninoculated shrimp tissue and designated as 'Control or C. The remaining three lots were subjected to different processing treatments. About 30 g of tissue was divided into three lots of 10 g and 1 ml each of tenfold serial dilution (10^{-1} and 10^{-2} , equivalent to 10^6 and 10^5 cells, respectively) of an overnight grown culture of *V. cholerae* O139 (SG 24) containing 1×10^7 CFU ml $^{-1}$, was inoculated into 10 g of shrimp tissue. All the experiments were carried out in triplicates. The second lot was subjected to slow freezing (SF) in a deep freezer at -20°C as per the protocol given by Jeyasekaran *et al.* (2002) and subdivided into two lots. They were designated as SF15 and SF30 with respect to the storage periods *i.e.* 15 days and 30 days respectively. The third lot was cooked at 100°C in boiling water for 5, 10 and 20 min and designated as C5, C10 and C20 respectively. The fourth lot was dried under direct sunlight for a period of 15 days and subdivided into two sub-lots. Samples were taken for analysis after storage of 15 and 30 days and designated as D15 and D30, respectively.

Results and discussion

Reverse transcription-Polymerase chain reaction (RT-PCR)

RT-PCR performed for the *ctxA* gene yielded a product of 308 bp only in case of *V. cholerae* (Fig. 1). The selected target gene and also the presence of inhibitors in the food matrix that may prevent sufficient extraction of template for amplification or may inhibit the amplification process are the important factors affecting the detection of pathogenic microorganisms in food by RT-PCR. Bickely *et al.* (1996) have reported the effect of inhibitors in a variety of products, including blood, faeces and food. However, many of the obstacles can be overcome by enrichment procedures that increase the cell numbers (McIngvale *et al.*, 2002).

Klein and Juneja (1997) developed a reverse transcription-PCR (RT-PCR) for reliably detecting viable *L. monocytogenes* by targeting mRNA of *iap*, *hlyA* and *prfA* genes. Yaron and Matthews (2002) detected viable *E. coli* using RT-PCR assay by targeting *rfbE*, *fliC* and *stx1* mRNA genes. The detection of viable *Salmonella* by targeting *invA* mRNA from the artificial contaminated spinach, tomatoes and pepper without any false positive results from dead cells

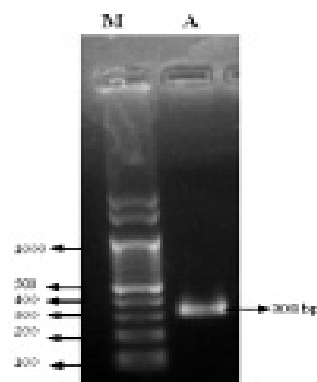


Fig. 1. RT-PCR assay for *Vibrio cholerae*. Lane A - *ctxA*; Lane M - 100bp DNA ladder

was reported by Gonzalez-Escalona *et al.* (2009). All the above studies employed the pre-enrichment procedure. But, in the present study, viable *V. cholerae* from shrimp without pre-enrichment targeting mRNA *ctxA* gene was detected by the RT-PCR assay developed. Since the present assay did not involve any pre-enrichment, it was rapid when compared to the RT-PCR assays developed by earlier workers.

Specificity and sensitivity of RT-PCR assay in shrimp tissue

The assay showed 100% specificity of the selected gene, as it was specific only for *Vibrio cholerae* and did not get amplified in case of bacterial pathogens like *Salmonella* sp., *L. monocytogenes*, *S. aureus* and *E. coli* (Table 1 and Fig. 2).

Morin *et al.* (2004) also reported that the presence of other bacteria did not interfere in RT-PCR detection of the bacterial pathogens like *Salmonella* and *E. coli* in clinical isolates. RT-PCR assay showed amplification of *V. cholerae* specific gene in 5, 10 and 20 min incubated shrimp tissue in the dilutions of 10^{-1} and 10^{-2} which was equivalent to above 10^6 cells (Fig. 3).

There was no amplification of *V. cholerae* specific gene in the 3 and 4 h incubated shrimp tissue in the dilutions of 10^{-7} , 10^{-8} and 10^{-9} equivalent to 20, 67, 11, 36, 2 and 6 cells,

Table 1. Bacterial strains tested with the presence/absence of tested gene

Bacterial strains	<i>ctxA</i> gene
<i>Vibrio cholerae</i> O139 (SG 24)	+
<i>Vibrio cholerae</i> El Tor (N 16961)	+
<i>Vibrio cholerae</i> Classical (V 154)	+
<i>Salmonella enterica</i> serovar Typhi (ATCC 122235)	-
<i>S. enterica</i> serovar Enteritidis (ATCC 13065)	-
<i>Staphylococcus aureus</i> (ATCC 12598)	-
<i>Escherichia coli</i> (ATCC 9637)	-
<i>Aeromonas hydrophila</i> (ATCC 1457)	-
<i>Listeria monocytogenes</i> (ATCC 12232)	-

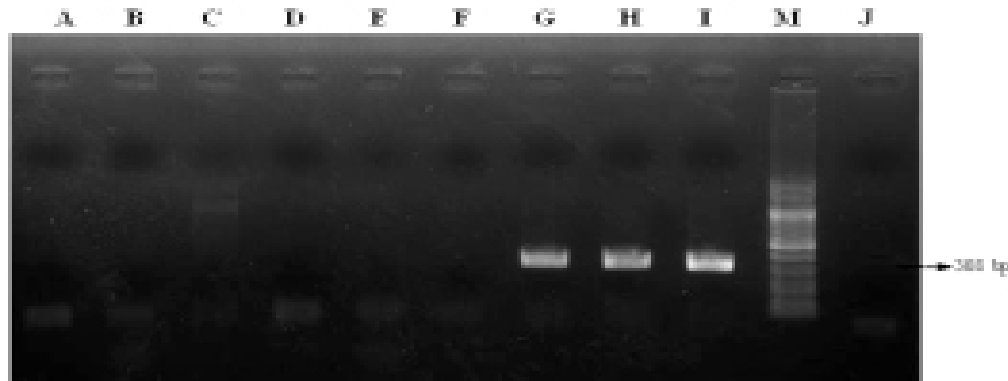


Fig. 2. Specificity of RT-PCR assay developed for *Vibrio cholerae*.

Lane A - *Salmonella enterica* serovar Typhi (ATCC 122235); Lane B - *S. enterica* serovar Enteritidis (ATCC 13065); Lane C - *Staphylococcus aureus* (ATCC 12598); Lane D - *Escherichia coli* (ATCC 9637); Lane E - *Aeromonas hydrophila* (ATCC 1457); Lane F - *Listeria monocytogenes* (ATCC 12232); Lane G - *Vibrio cholerae* O139 (SG 24), Lane H - *V. cholerae* El Tor (N 16961), Lane I - *V. cholerae* Classical (V 154); Lane M - 100 bp DNA ladder; Lane J - Control (Uninoculated)

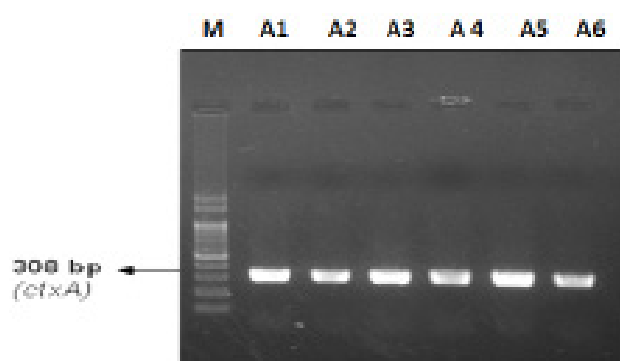


Fig. 3. Sensitivity of RT-PCR assay developed for *Vibrio cholerae* targeting mRNA *ctxA* gene

Lanes A1, A3, A5 - 10^{-1} diluted sample incubated for 5, 10 and 20 min; Lanes A2, A4, A6 - 10^{-2} diluted sample incubated for 5, 10 and 20 min; Lane M - 100 bp DNA ladder

respectively and the amplification was observed in 5 h incubated shrimp tissue in the dilutions of 10^{-7} and 10^{-8} equivalent to 143 and 110 cells. Though 91 cells were present in 10^{-9} diluted shrimp tissue in 5 h, no amplification of specific gene of *V. cholerae* was found (Fig. 4). Theron *et al.* (2000) evaluated the performance of a semi-nested *ctxAB* gene specific PCR for the detection of pathogenic *V. cholerae* in environmental water and drinking water sources within 10 h. The sensitivity obtained in this work for RT-PCR assay is much better when compared to the earlier works reported on bacterial pathogens (Klein and Juneja, 1997; Yaron and Matthews, 2002). Klein and Juneja (1997) found that 10^4 cells of *L. monocytogenes* is required for its detection targeting mRNA of *hlyA* gene by RT-PCR assay. Yaron and Matthews (2002) also observed that 10^7 cells of *E. coli* is needed for its detection targeting mRNA of *fliC* gene by RT-PCR assay. Since the estimated infective dose of

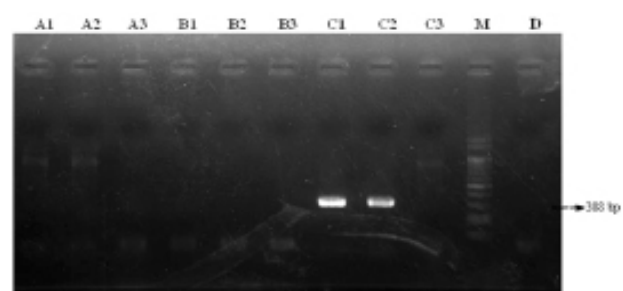


Fig. 4. Sensitivity of RT-PCR assay developed for *Vibrio cholerae* targeting mRNA *ctxA* gene

Lanes A1, A2 and A3 - 10^{-7} , 10^{-8} and 10^{-9} diluted samples incubated for 3 h; Lanes B1, B2 and B3 - 10^{-7} , 10^{-8} and 10^{-9} diluted samples incubated for 4 h; Lanes C1, C2 and C3 - 10^{-7} , 10^{-8} and 10^{-9} diluted samples incubated for 5 h; Lane M - 100 bp DNA ladder; Lane D - Control (Uninoculated)

V. cholerae is about 10^6 cells (FDA, 2012), the detection limit of the developed RT-PCR assay is sufficient for screening the pathogen below the infective dose.

Validation of RT-PCR assay

The stability of mRNA based RT-PCR assay for the detection of viable *V. cholerae* was studied by carrying out bio-inoculation experiments. The RT-PCR assay detected viable *V. cholerae* in frozen shrimp tissue even on the 30th day of storage (Fig. 5).

The RT-PCR assay developed in the present study detected viable *V. cholerae* even after cooking for 20 min (Fig. 6). *V. cholerae* was also detected by RT-PCR assay even on the 30th day in dried shrimp tissue (Fig. 7). Sheridan *et al.* (1998) have also reported that mRNA was detected in heat (100°C for 5 min) treated cells of *E. coli* even after incubation at room temperature for 16 h. They have also observed

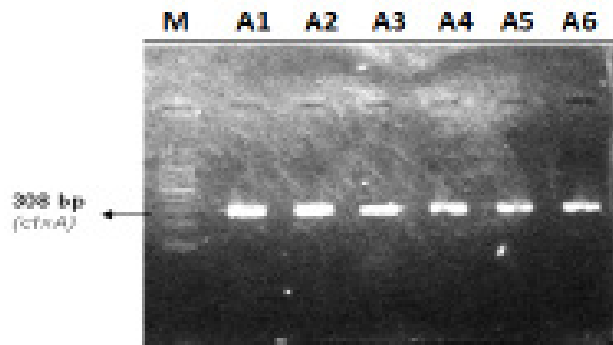


Fig. 5. Validation of RT-PCR assay for the detection of viable *Vibrio cholerae* using specific mRNA *ctxA* gene in shrimp tissue on frozen storage

Lanes A1, A3, A5 - 10^{-1} diluted sample frozen stored for 1, 15 and 30 days; Lanes A2, A4, A6 - 10^{-2} diluted sample frozen stored for 1, 15 and 30 days; Lane M - 100 bp DNA ladder

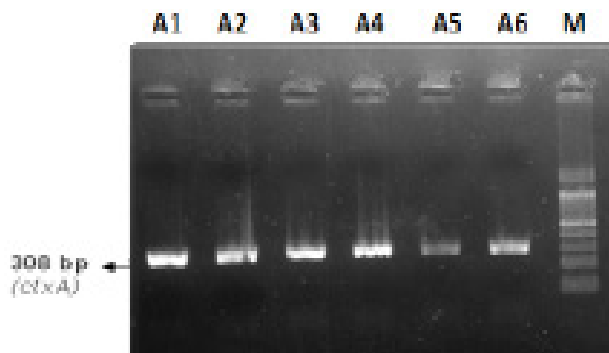


Fig. 6. Validation of RT-PCR assay for the detection of viable *Vibrio cholerae* using specific mRNA *ctxA* gene in cooked shrimp tissue

Lanes A1, A3, A5 - 10^{-1} diluted sample cooked for 5, 10 and 20 min; Lanes A2, A4, A6 - 10^{-2} diluted sample cooked for 5, 10, 20 min; Lane M - 100 bp DNA ladder

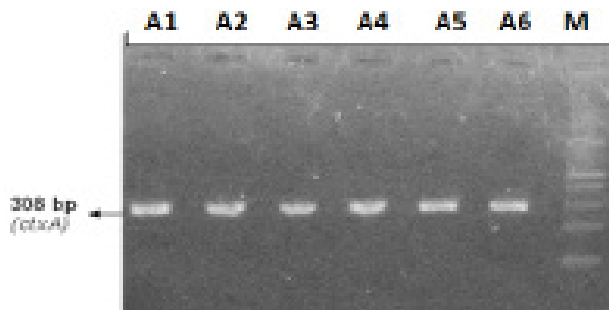


Fig. 7. Validation of RT-PCR assay for the detection of viable *Vibrio cholerae* using specific mRNA *ctxA* gene (308 bp) in shrimp tissue on drying

Lanes A1, A3, A5 - 10^{-1} diluted sample dried and stored for 1, 15 and 30 days; Lanes A2, A4, A6 - 10^{-2} diluted sample dried and stored for 1, 15 and 30 min days. Lane M - 100 bp DNA ladder

that detection of mRNA after heat treatment varied with the stability of different mRNA sequences targeted by the oligonucleotide primers designed as well as with the relative abundance of each mRNA type in a given cell.

Expansion of the international food trade resulted in the emergence of members of vibrios as the most important emerging food and waterborne pathogens both in developing and developed countries (Jeyasekaran *et al.*, 2011b). These human pathogenic vibrios are transmitted either by direct contact or indirectly through contaminated seafood products (Kumar, 2008; Jeyasekaran *et al.*, 2011b). Since some of the environmental strains are lacking the genes, the identification of cholera toxin is important (Rivera *et al.*, 2001). Development of a suitable methodology for rapid, viable, sensitive and specific monitoring of these bacteria in food products is always necessary and attract more interest, as it would greatly facilitate the sanitary control of food products and lower the incidence of associated food-borne infections. It is well known that mRNA detection by RT-PCR is the most promising technique for investigating the viability of bacteria (Call *et al.*, 2001). RT-PCR for the detection of *Vibrio* spp. from clinical stool samples were attempted by Call *et al.* (2001) and Morin *et al.* (2004). Detection of *ctxA* mRNA by RT-PCR in the present study indicated that the loss of viability was well correlated with the loss of the RT-PCR signal in the cooked samples.

It can be concluded that the reverse transcriptionPCR (RT-PCR) assay developed in this study was rapid, reliable, specific and sensitive in the detection of viable *V. cholerae* in seafood targeting mRNA of *ctxA* gene without pre-enrichment. This RT-PCR assay detected only the viable *V. cholerae* but not the other pathogens (*Salmonella*, *Escherichia coli*, *Staphylococcus aureus*, *Aeromonas hydrophila* and *Listeria monocytogenes*) tested.

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