TRIPLEX REVERSE TRANSCRIPTION-PCR FOR DETECTING VIABLE TOXIGENIC *VIBRIO CHOLERAE* IN WATER SAMPLES IN THAILAND

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Abstract. Detection of toxigenic *Vibrio cholerae* O1/O139 in aquatic environment is difficult to achieve using the culture method. For direct detection of viable toxigenic *V. cholerae* in aquatic environment, we developed a triplex reverse transcription (RT)-PCR, targeting genes for the outer membrane protein (*omp*W), cholera toxin A (*ctx*A) and toxin-coregulated pilli (*tcp*A) and compared the assay with the culture method. After enrichment of the bacteria-containing filters in alkaline peptone water for 6 hours, the sensitivity of triplex RT-PCR for detecting *V. cholerae* was 7 cfu/ml. Of the 80 environmental water samples collected from various regions in Northeast Thailand, triplex RT-PCR detected 15 toxigenic and 20 non-toxigenic *V. cholerae*, whereas the culture method detected only 3 toxigenic *V. cholerae* - containing water samples. These results show that this triplex RT-PCR method could be used as an alternative tool for rapid and sensitive identification of viable toxigenic *V. cholerae* in environmental water samples.

Keywords: *Vibrio cholerae*, triplex reverse transcription-PCR, environmental water samples, Thailand

INTRODUCTION

Vibrio cholerae, the major cause of water-borne cholera, continues to be a major public health problem in many countries (Shears, 2001; Swaddiwudhipong *et al*, 2008; Wiwanitkit, 2008). Of more than 200 known serogroups of *V. cholerae*, O1 and O139 are the two most common serogroups associated with epidemic cholera (Shears, 2001; Alam *et al*, 2007).

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Detection of *V. cholerae* in aquatic water samples using the conventional culture method has been unsuccessful because certain *V. cholerae* strains can achieve a viable but non-culturable (VBNC) resting state, which allows them to survive in aquatic environments (Colwell, 2000; Alam *et al*, 2007). In this state, cells are still metabolically active and maintain their pathogenicity (Colwell *et al*, 1996). Thus VBNC *V. cholerae* can escape detection by conventional testing methods, but can later recur as endemic cholera (Binsztein *et al*, 2004; Alam *et al*, 2007). Besides not being able to detect VBNC *V. cholerae* the culture method is also time-consuming and laborious.

PCR with its demonstrated sensitivity and specificity has been modified as multiplex PCR that can simultaneously detect *Vibrio* species and virulence genes (Goel *et al*, 2005, 2007). PCR cannot, however, distinguish between DNA of live and dead cells, whereas reverse transcription (RT)-PCR can be used to identify both viable and VBNC bacteria (Sheridan *et al*, 1998; Lleo *et al*, 2000). Although multiplex RT-PCR (mRT-PCR) has been applied for detecting *E. coli* O157:H7, *V. cholerae* O1 and *Salmonella* Typhi in clinical isolates (Morin *et al*, 2004), and *Vibrio cholerae*/*mimicus*, *V. parahaemolyticus*/*alginolyticus* and *Campylobacter jejuni*/*coli* spiked in human stool (Kurakawa *et al*, 2012), mRT-PCR has not been applied for the simultaneous detection of toxigenic and various virulence genes of *V. cholerae* in environmental water.

The aim of the current study was, therefore, to develop triplex RT-PCR for detection of both *V. cholerae* and virulence-associated genes (*omp*W, *ct*xA and *tcp*A) of viable toxigenic *V. cholerae* in environmental water samples and to compare this method with the conventional culture method.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains were obtained from the American Type Culture Collection (ATCC), the Department of Medical Sciences Thailand Culture Collection (DMST), the Maryland Pathogen Research Institute, University of Maryland, College Park, Maryland, USA, clinical and environmental sources at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, Thailand, and the Department of Microbiology Laboratory, Faculty of Medicine, Khon Kaen University.

V. cholerae O1 (clinical strains), *V. cholerae* O139 (MO10; GenBank Taxonomy No. 345072), *V. cholerae* non-O1/non-O139 (O5, O6, O8 and O14), *V. mimicus* ATCC 33653, *V. vulnificus* ATCC 27562, *V. fluvialis* DMST 19347, *V. alginolyticus* DMST 14800, *V. parahaemolyticus* ATCC 17802, *Salmonella* spp (clinical and environmental strains), *Shigella dysenteriae* DMST 15111, *Shigella flexneri* DMST 4423, *Shigella boydii* DMST 28180, *Aeromonas hydrophila* (clinical strain), *Pseudomonas aeruginosa* (clinical strain) and *Escherichia coli* ATCC 25922 were used for testing of the triplex RT-PCR.

Collection of environmental water samples

Eighty environmental water samples were collected from 2 ponds, 7 canals and 2 wastewater reservoirs in the municipality of Khon Kaen, Northeast Thailand. Aliquot of 450 ml of environmental water sample was added with 50 ml of 10X alkaline peptone water (APW) pH 8.4 (Oxoid, Hamshire, England). Aliquot of 400 ml was filtered through Whatman No.1 filter and then through a 0.2- m pore size filter (Millipore, Billerica, MA). The filtrate was placed in 15 ml of APW and incubated at 37ºC for 6 hours. The samples then underwent the following analysis: 1) 5 l aliquot was used for isolation and detection of *V. cholerae*, and 2) 1.5 ml aliquot was used to prepare template RNA for PCR detection of *V. cholerae*. In order to determine the efficiency of the detection process, the filtration and 100 ml of unfiltered water samples in APW were analyzed by triplex RT-PCR and culture methods.

Isolation and identification of *V. cholerae*

The 5 l aliquot obtained as described above was streaked onto thiosulfate citrate bile-salts sucrose (TCBS) agar (Eiken, Tokyo, Japan) and incubated at 37ºC for

18-24 hours. Ten yellow sucrose fermenting colonies were further identified by biochemical and serological tests (Chomvarin *et al*, 2007). *V. cholerae* colonies were sero-grouped and sero-typed by the slide agglutination test with polyvalent anti-*V. cholerae* O1/O139 antiserum and monovalent antiserum against Inaba and Ogawa strains (Oxoid, Hamshire, UK), respectively.

Triplex RT-PCR assay

Primer pairs employed for amplification of *V. cholerae* (GenBank accession no. CP000626) *omp*W, as described by Senachai *et al* (2013), of *V. cholerae* (GenBank accession no. EU48778) *ctx*A, designed by Wongboot *et al* (unpublished), and *V. cholerae* (GenBank accession no. X64098) *tcp*A from this study are listed in Table 1. Specificities of the primers were checked using BLAST program and PCR assay performed using 200 ng of DNA from the test bacterial strains listed above.

Template RNA was prepared using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI), converted to cDNA using Superscript III reverse transcriptase (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The concentration of DNA template was quantified by measuring the UV-induced emission of fluorescence from intercalated ethidium bromide using a spectrophotometer at A260 nm. PCR was conducted in a 25 l reaction mixture containing 0.2 mM dNTPs (Amresco, Solon, OH), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.15 M *ctx*A primers, 0.3 M *tcp*A primers, 0.35 M *omp*W primers, 1.25 U *Taq* DNA polymerase (RBC Bioscience, Taipei, Taiwan) and 500 ng of cDNA (described above). Thermocycling (Veriti Thermal Cycler, Applied Biosystems, Foster City, CA) was performed as follows: 94°C for 10 minutes; 35 cycles of 94°C for 1 minute, 59°C for 1 minute, and 72°C for 2 minutes; with a final heating 72°C for 10 minutes. Amplicons were separated by 2% agarose gel-electrophoresis, stained with ethidium bromide and examined under a UV light. Positive control was *V. cholerae* O1 and O139 positive for *omp*W, *ctx*A, and *tcp*A, while negative control was sterile distilled water.

Triplex RT-PCR sensitivity determination

Pure cultures of *V. cholerae* O1, O139 and non-O1/non-O139 were prepared as previously described (Goel *et al*, 2007). Log phase cultures were serially diluted ten-fold in sterile saline water (0.85% NaCl) to obtain 10^7-10^0 cfu/ml. One ml aliquot of each dilution was subjected to triplex RT-PCR assay.

In order to determine the sensitivity of triplex RT-PCR assay for environmental water samples, 1 ml aliquots of the mixed culture of *V. cholerae* O1, O139 and non-O1/non-O139 containing 10^0 to 10^7 cfu were added to 450 ml of sterilized water samples and 50 ml of 10X APW (Oxoid, Hamshire, UK). The spiked water samples were filtered as described above and the membranes were transferred into 15 ml of APW (Oxoid, Hamshire, UK) and incubated at 37°C for 0, 3 and 6 hours, at which time 1 ml aliquots were subjected to triplex RT-PCR assay.

RESULTS

Specificity and sensitivity of triplex RT-PCR for *V. cholerae* detection

The designed primers amplified the expected *V. cholerae* O1/O139 *omp*W, *ctx*A and *tcp*A, and non-specific PCR bands from the other bacteria and other *Vibrio*

Table 1
Primers used for the amplification of *V. cholerae* genes.

Gene	Primer sequence (5'->3')	Amplicon size (bp)	References
*ctx*A	F - TGGTCTTATGCCAAGAGGACA R - ATCTTGGAGCATTCCCACAAC	517	Wongboot W, unpublished
*tcp*A	F- CAATACTGGGAGGTGGAGCCTA R-GCAAACTGGAGCTTATTCTGGTCG	335	This study
*omp*W	F- GTACTTGCAGCCCTAAGCTC R- GGACCATAAAGGTAGGTGGC	307	Senachai *et al*, 2013

Table 2
Detection and isolation of *V. cholerae* in 80 environmental water samples by triplex RT-PCR and culture method.

Detection of *V. cholerae*	No. of positive samples (%)	
	Culture	Triplex RT- PCR[a]
V. cholerae positive	23 (28.7)	35 (43.7)
Toxigenic *V. cholerae*	3 (3.7)	15 (18.7)
Non-toxigenic *V. cholerae*	20 (25.0)	20 (25.0)
V. cholerae negative	57 (71.3)	45 (56.3)

[a]After enrichment for 6 hours.

spp were not detected (Fig 1). The detection limit of triplex RT-PCR for pure *V. cholerae* culture was $3.2x10^5$ cfu/ml ($3.2x10^4$ cfu/PCR assay), and that for water spiked with *V. cholerae* (with filtration) after enrichment for 6 hours was $1x10^2$ in 15 ml of APW or 6.7 cfu/ml (1 cfu/PCR assay) compared to $1x10^5$ in 15 ml of APW or $6.7x10^3$ cfu/ml ($6.7x10^2$ cfu/PCR assay) with no enrichment (Fig 2).

Comparison between triplex RT-PCR and culture methods in detecting *V. cholerae* in environmental water samples

After filtration and enrichment, 80 environmental water samples were examined by triplex RT-PCR, which showed that 35 samples (44%) were positive for *omp*W only, while 15 (19%) positive for *omp*W, *ctx*A and *tcp*A, thus identified as containing toxigenic *V. cholerae* (Fig 3). Among these 15 toxigenic *V. cholerae*-positive samples only 3 (20%) were positive by the culture method and identified as *V. cholerae* O1 serotype Inaba, and positive for *omp*W, *tcp*A and *ctx*A (Table 2). Of the 80 samples, 20 (25%) were triplex RT-PCR negative for *ctx*A, *tcp*A and thus identified as containing non-toxigenic *V. cholerae* (Fig 3). All of these 20 samples were *V. cholerae* culture positive and were not agglutinated by anti-O1/O139 antisera, and so were identified as *V. cholerae* non O1/non-O139 (Table 2).

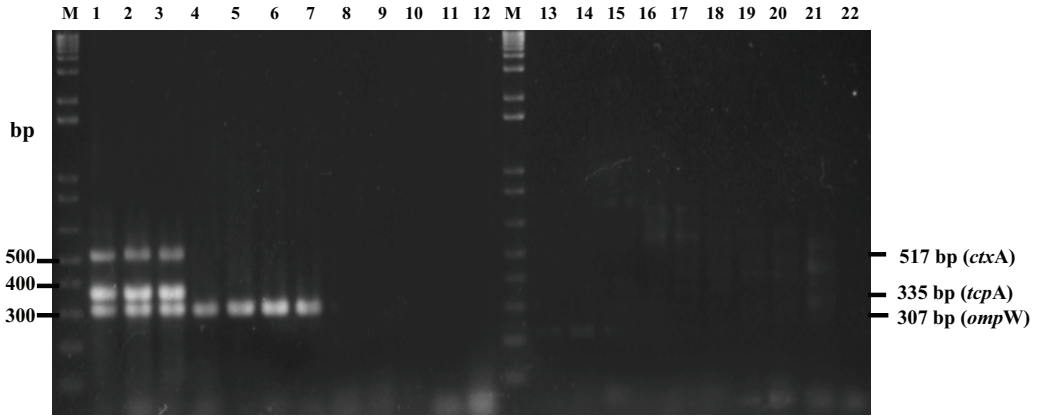

Fig 1–Specificity of the triplex RT-PCR for detection of *V. cholerae*. Primers and PCR conditions are described in Materials and Methods and Table 1. Lane M, 1 kb DNA markers; Lanes 1-2, toxigenic *V. cholerae* O1 from patients; lane 3, toxigenic *V. cholerae* O139 (MO10); lanes 4-7, non-toxigenic *V. cholerae* O5, O6, O8 and O24; lane 8-21, *V. parahaemolyticus* (ATCC 17802), *V. mimicus* (ATCC 33653), *V. vulnificus* (ATCC 27562), *V. fluvialis* (DMST 19347), *V. alginolyticus* (DMST 14800), 3 *Salmonella* spp (clinical and environmental strains), *Shigella dysenteriae* (DMST 15111), *Shigella flexneri* (DMST 4423), *Shigella boydii* (DMST 28180), *Aeromonas hydrophila* (clinical strain), *Pseudomonas aeruginosa* (clinical strain), *Escherichia coli* (ATCC 25922), respectively; lane 22, negative control.

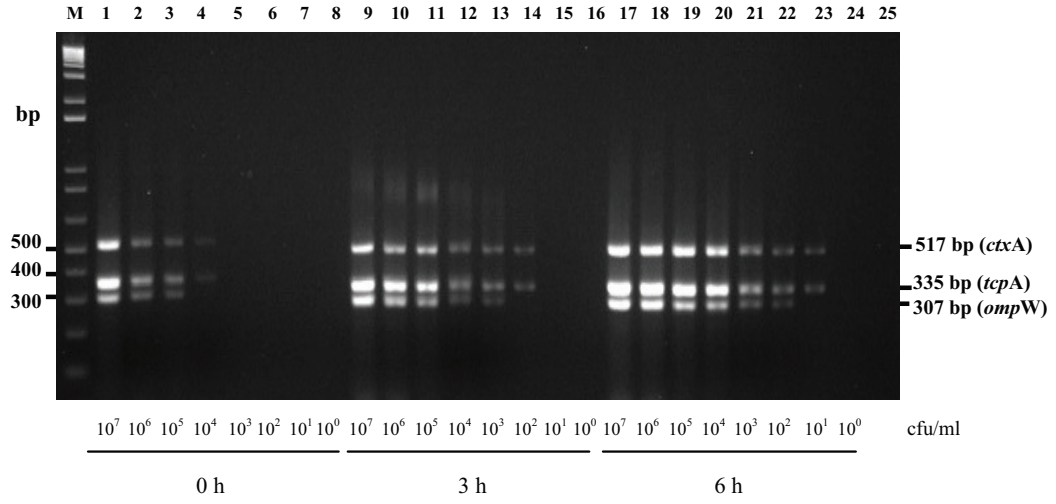

Fig 2–Sensitivity of triplex RT-PCR for detection of *V. cholerae* specific genes in spiked water samples at 10^7 - 10^0 cfu/ml after filtration and enrichment in 15 ml of APW for 0, 3 and 6 hours. Lane M, 1 kb DNA markers. Lanes 1-8, 0 hour; lane 9-16, 3 hours; lane 17-24, 6 hours; lane 25, negative control. Primers and PCR conditions are described in Materials and Methods and Table 1.

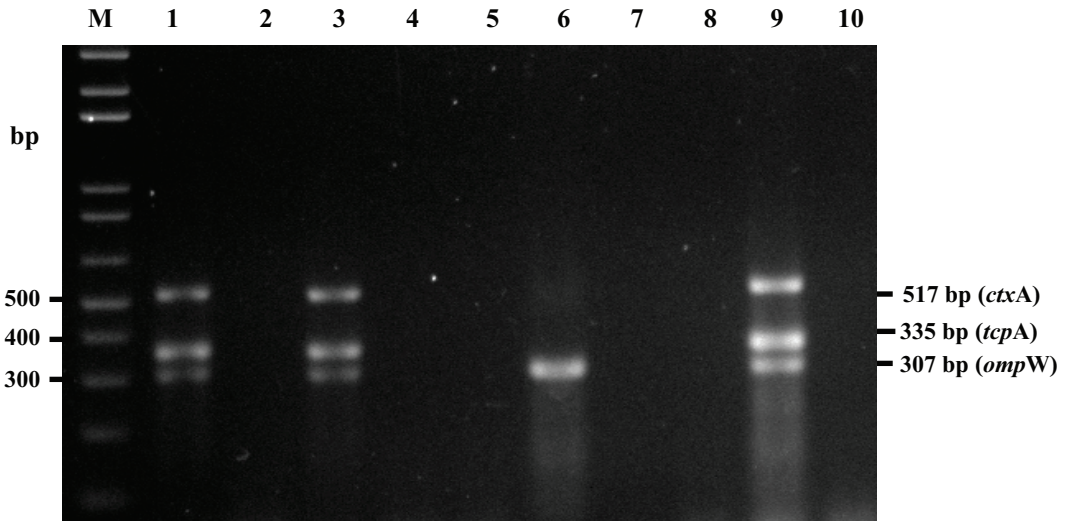

Fig 3–Triplex RT-PCR for detection of *V. cholerae* in environmental water samples. Samples were fitered and enriched in 15 ml for 6 hours. Primers and PCR conditions are described in Materials and Methods and Table 1. Lane M, 1 kb DNA marker. Lanes 1 and 3, positive sample for viable toxigenic *V. cholerae*; lanes 2, 4, 5, 7 and 8, samples negative for viable *V. cholerae*; lane 6, sample positive for viable non-toxigenic *V. cholerae*; lane 9, positive control; lane 10, negative control.

DISCUSSION

We successfully developed triplex RT-PCR for simultaneous detection in a single reaction tube of the virulence genes of viable toxigenic *V. cholerae* with the sensitivity of *V. cholerae* pure culture of $3.2x10^5$ cfu/ml ($3.2x10^4$ cfu/PCR assay). The sensitivity of uniplex RT-PCR for the detection of *ctx*A of viable *V. cholerae* has been previously reported to be 10^3 cfu/ml using pure culture (Bej *et al*, 1996). Morin *et al* (2004) reported that the sensitivity of mRT-PCR for *E. coli* O157:H7 and *Salmonella* Typhi detection was 30 cells/50 l of PCR assay. The sensitivity of RT-PCR for detection of *Vibrio cholerae*, *V. parahaemolyticus* and *Campylobacter jejuni* was 10^3 cfu/g stool specimen, but the specificity of these primers could not differentiate among closely related bacterial species, *viz*., *V. cholerae* from *V. mimicus*, *V. parahaemolyticus* from *V. alginolyticus*, and *C. jejuni* from *C. coli* (Kurakawa *et al*, 2012). In the current study, we used specific *V. cholerae* primers that can differentiate among species of *V. mimicus*, *V. parahaemolyticus* and *V. vulnificus* (Senachai *et al*, 2013).

After filtration and enrichment in APW for 6 hours, triplex RT-PCR had a high sensitivity of detecting viable *V. cholerae* (6.7 cfu/ml) from environmental water samples. Our results confirmed that 6 hours of enrichment were sufficient for improving the detection rate of *V. cholerae* in environmental water samples (Goel *et al*, 2007; Senachai *et al*, 2013).

Previous studies demonstrated that RNA is a potential viability marker of live bacterial cells (Lleo *et al*, 2000) because it is present only in viable cells and degrades quickly after cell death (Sheridan *et al*, 1998). The higher sensitivity of RT-PCR over the culture method may be because

the bacteria have entered the VBNC state (Coutard *et al*, 2005; Goel *et al*, 2005; Senachai *et al*, 2013) which is not a limitation for the RT-PCR assay, as it can detect both culturable and VBNC cells (Colwell, 2000; Coutard *et al*, 2005). Most *V. cholerae* O1/O139 carry *ctx*A and *tcp*A and can become VBNC, which is important because they can still produce toxins and, therefore, have potential for pathogenesis (Colwell *et al*, 1996; Colwell, 2000; Baffone *et al*, 2003). In this study, 3 colonies of toxigenic *V. cholerae* O1 were detected by both the culture method and RT-PCR, indicating that the viable toxigenic *V. cholerae* O1 was present in the environmental waters of Northeastern Thailand. Notably, all of the non-toxigenic *V. cholerae* were detected by both culture and RT-PCR (25%), suggesting that most non-toxigenic *V. cholerae* are viable and culturable whereas most toxigenic *V. cholerae* were VBNC organisms.

In conclusion, we developed specific and rapid triplex RT-PCR for detection of both culturable and VBNC toxigenic *V. cholerae* in environmental water samples. Since the detection of toxigenic *V. cholerae* in the environment is important for public health risk assessment, the developed method could serve as an adjunct tool for environmental surveillance of cholera outbreaks.

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