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Application of duplex-PCR in rapid and reliable detection of toxigenic *Vibrio cholerae* in water samples in Thailand

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Toxigenic *Vibrio cholerae*, the cause of cholera, is a native flora of the aquatic environment which is transmitted through drinking water and still remains the leading cause of morbidity and mortality in many developing countries including Thailand. The culture method (CM), which is routinely used for assessing water quality, has not proven as efficient as molecular methods because the notorious pathogen survives in water mostly in a non-culturable state. We employed duplex-polymerase chain reaction (duplex-PCR) for detection of *tcpA* and *ctxA* genes in toxigenic *V. cholerae*, and compared PCR detection with CM in various waters of Khon Kaen Municipality, Thailand. We also evaluated the effect of different pre-PCR conditions on the results of *ctxA* and *tcpA* detection including: 1) water filtered and enriched in alkaline peptone water (APW) for 3 h before PCR, 2) water filtered without enrichment before PCR, and 3) use of only enrichment in APW for 6 h before PCR. Of the 96 water samples (taken from waste-water, potable and waste-water from patients' houses, and from rivers) tested, 48 (50%) were positive for *ctxA* and *tcpA* by duplex-PCR, whereas only 29 (30%) were positive for *V. cholerae* by CM. Of the 29 *V. cholerae* isolated by CM, 2 (7%) were toxigenic *V. cholerae* belonging to serovar O1, while the rests were non-O1/ non-O139. Results revealed, therefore, that *ctxA* and *tcpA*-targeted duplex PCR is more sensitive than CM for detection of toxigenic *V. cholerae* from water samples because CM detected much less toxigenic *V. cholerae* than the non-toxigenic *V. cholerae*. Template DNA as low as 100 fg or 23 cells of *V. cholerae* in the water sample was detected in duplex PCR. Pre-PCR filtration followed by enrichment for 3 h significantly increase in the efficiency of duplex-PCR detection of toxigenic *V. cholerae*.

Key Words—*ctxA*; duplex PCR; *tcpA*; *Vibrio cholerae*; water samples

Introduction

The waterborne pathogen *Vibrio cholerae* is the

causative agent of epidemics of cholera in many developing countries including Thailand (Dalsgaard et al., 1999; Shears, 2001). The two serogroups associated with cholera are *V. cholerae* O1 and O139 (Faruque et al., 2003; Huq et al., 1995). Although routine isolation from water is not achieved by the conventional culture method, *V. cholerae* is present in natural aquatic environments (Colwell and Huq, 1994) and the role of water in the transmission of *V. cholerae* is well established (Colwell, 1993, 2000; Islam et al., 1993a, 1994a). The toxigenic *V. cholerae* serovars O1 and

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O139 survive in water by entering into a resting state known as the viable but non-culturable (VBNC) state (Colwell, 1993; , 2000; Islam et al., 1993a). Cells in the VBNC state retain viability and infective potential (Colwell, 1993) in the environment over a year and also maintain the associated pathogenic genes along with the integrity of chromosomes (Chaiyanan et al., 2001).

Rapid and accurate identification of *V. cholerae* in the potable water and aquatic environment is important for disease management and public health protection. The conventional culture method, which is routinely used for the isolation of *V. cholerae*, is time-consuming and may not be suitable for cells that are VBNC (Huq et al., 1995; Islam et al., 1993b, 1994b; Kapley and Purohit, 2001). Recent molecular techniques such as polymerase chain reaction (PCR) offer both a rapid and reliable method for detecting toxigenic *V. cholerae* occurring in the aquatic environment (Bhanumathi et al., 2003; Chakraborty et al., 1999; Kapley and Purohit, 2001). The *ctxAB* and *tcpA* are the genes known to play key roles in the virulence of *V. cholerae* especially of serogroups O1 and O139 (Faruque et al., 1997; Herrington et al., 1988; Kovach et al., 1994). The *ctxA* gene, which encodes the subunit A of cholera toxin (CT), plays a critical role in the CT action. On the other hand, *tcpA* encodes the toxin coregulated pilus (TCP) which plays a very important role in the colonization of the pathogen in the host intestinal mucosa (Shears, 2001; Singh et al., 2001). It is presumed that CT and TCP that are exclusively associated with the toxigenic *V. cholerae* strains are acquired by the bacterium from aquatic environment (Colwell, 1993; Shears, 1994, 2001). Thus, the aquatic environment may play an important role in the ecology, transmission, and epidemiology of cholera bacteria (Colombo et al., 1997; Radu et al., 2002).

In recent years, PCR amplification of virulence marker genes such as *ctxA* and *tcpA* has become very useful for rapid and reliable detection of toxigenic *V. cholerae* in both clinical and environmental samples. However, the efficiency of PCR detection depends on several factors with the selection of an appropriate DNA extraction method and the pre-PCR procedures as very crucial factors. In the present study, we report the specificity of PCR detection using *ctxA* and *tcpA*-targeted primers. The sensitivity of detection using duplex PCR was compared with the culture method using samples from potable water, surface waters from aquatic environments, and municipal waste of Khon

Kaen, Thailand. We also report that the pre-PCR filtration and enrichment in APW for only 3 h enhances the efficiency of the detection method.

Materials and Methods

Collection of water samples. Water samples were collected over 3 seasons between 2003 and 2004 from the municipality of Khon Kaen, Thailand. Fifty-four water samples were collected from 9 aquatic environments, including 1 well, 4 canals, and 4 wastewater sites. In addition, 42 water samples were collected from patients' houses during cholera outbreaks, including 15 drinking water, 16 washing water, and 11 wastewater. Samples were collected aseptically in sterile glass bottles and transported at ambient temperature from the site of collection to the laboratory for analysis.

Processing of water samples. Four hundred fifty milliliters of each water samples was added to an enrichment medium (50 ml) containing ten times the concentration of alkaline peptone water ($10\times$ APW; Difco) at pH 8.4. The sand and other solids were allowed to settle for 15 min, and then the water samples in APW were subjected for the pre-PCR processing by 1) filtering a 400 ml aliquot of the water in APW (from sedimentation flask) through a $0.45\mu\text{m}$ pore size membrane (Millipore Corp., Bedford, Mass., USA). The membrane filter was then placed in 15 ml of APW, stirred well to dislodge any bacteria trapped on it and $5\mu\text{l}$ of the APW was streaked on a plate for culture and another 1.5 ml used for preparing template DNA for PCR; 2) further enriching the same water sample aliquots in APW with filtration was by incubating them at 37°C for 3 h, and 6 h before samples culturing and PCR; and 3) further incubating the water in APW without filtration that remained in the sedimentation flask at 37°C for 6 h before culturing and PCR.

Culture. For culture methods, $5\mu\text{l}$ of each of the water samples in APW as stated above were streaked onto thiosulfate-citrate-bile-salt-sucrose (TCBS) agar and taurocholate-tellurite-gelatin agar (TTGA) and incubated at 37°C for 18 to 24 h. Suspected *Vibrio* colonies were confirmed by using biochemical tests (Koneman, 1997).

Serotyping. *V. cholerae* colonies were further determined for serogroup by slide agglutination with polyvalent *V. cholerae* O1 antiserum. All isolates that agglutinated with the polyvalent *V. cholerae* O1 antiserum were then serotyped with specific monovalent

Table 1. Primers used for the amplification of *ctxA* and *tcpA* genes in toxigenic *V. cholerae*.

Primers	Primer sequence (5'–3')	Amplicon size (bp)	Nucleotide position
<i>ctxA</i>	CTCAGACGGGATTTGTTAGGCACG	302	309–332
	TCTATCTCTGTAGCCCCTATT		610–590
<i>tcpA</i>	GAAGAAGTTTGTAAGAAGAACAC	472	83–107
	GAAAGCACCTTCTTTCACGTTG		554–533

antisera against Inaba and Ogawa serotypes.

Template DNA preparation for PCR. DNA was extracted by following the manufacturer's protocol using a genomic DNA purification kit (Puregene DNA purification system, Gentra System, USA). Water samples (1.5 ml) in APW (directly or pre-incubated at 37°C for 3 h or 6 h, as stated above) were centrifuged at 13,000×*g* for 15 min and the pellet was washed in PBS, re-suspended in 300 µl of cell lysis solution and 1.5 µl of proteinase-K solution (20 mg/ml) and incubated at 55°C for 3 h. RNase A solution (1.5 µl, 4 mg/ml) was added to the cell lysate and incubated at 37°C for 60 min. Then, protein precipitation solution (300 µl) was added and centrifuged at 13,000×*g* for 3 min. The supernatant was collected and 400 µl of 100% isopropanol was added and centrifuged at 13,000×*g* for 5 min. The supernatant was carefully discarded. 300 µl of 70% ethanol was added to the pellet and centrifuged. The ethanol was poured off and left to dry for 2 h. 50 µl DNA hydration solution was added and incubated for 1 h at 65°C. DNA was stored at –20°C until used.

PCR assays. Specific primers designed for PCR analysis of *ctxA* and *tcpA* gene are shown in Table 1. PCR reactions were performed according to Kapley and Purohit (2001) and Shirai et al. (1991) with modification. The reaction was conducted in 50 µl of a reaction mixture containing 100–200 ng of target DNA, 200 µM of each deoxynucleoside triphosphate (dNTP) (Gibco BRL), 1.5 mM MgCl₂, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 200 nM of each primer and 2.5 units of *Taq* polymerase (Gibco BRL). Amplification was conducted using a thermal cycler (Perkin-Elmer, Gene Amp, PCR 2400) programmed with 1 cycle at 95°C for 2 min; 5 cycles of 95°C for 30 s, 54°C for 30 s, 72°C for 15 s; 10 cycles of 95°C for 30 s, 57°C for 1 min, 72°C for 15 s; and 15 cycles of 94°C for 10 s, 60°C for 1 min, 72°C for 15 s. The amplified product was subjected to 2% agarose gel electrophoresis, and visualized under UV (Imagemaster VDS, Pharmacia Biotech, USA) after

ethidium bromide staining.

DNA probe hybridization. The presence of virulence *ctxA* and *tcpA* genes was confirmed using a specific primer oligonucleotide probe (Table 1). The probes and hybridization were performed according to the manufacturer's instructions using the DIG high prime DNA labeling and detection starter kit I (Roche Diagnostic, Ltd., German).

Results

Determination of sensitivity and specificity of detection of *V. cholerae* O1 by duplex-PCR

The sensitivity of detection of *V. cholerae* O1 by duplex-PCR was investigated using a serial dilution of *V. cholerae* O1 template DNA. The result showed that as low as 100 fg of template DNA of *V. cholerae* O1 was enough for the amplification of *ctxA* and *tcpA* primers in duplex-PCR (Fig. 1). Based on the size of the complete genome of *V. cholerae*, this 100 fg of template DNA was calculated to be the amount of template DNA corresponding approximately to 23 bacterial cells.

The sensitivity of PCR detection with *V. cholerae* O1 cells as template for direct duplex-PCR was also tested. The cells as source of DNA templates were prepared from 10-fold serial dilutions of the culture suspensions. The presence of about 63 CFU of *V. cholerae* was required to be detected, compared to 100 fg DNA that was calculated to be present in only about 23 cells. The estimates on the number of *V. cholerae* cells required to be detected appeared to be smaller if extracted DNA was used as template compared to the use of whole cells without DNA extraction. This may be due to the loss of DNA during extraction steps from whole cells.

For both detection protocols, triplicate experiments were conducted using three strains of *V. cholerae*.

To determine the specificity of the primer extension for the detection of *V. cholerae* O1, ca. 100 ng DNA

Fig. 1. Sensitivity of the duplex-PCR in detection of *ctxA* and *tcpA* genes.

The amplified products obtained in PCR using different amount of DNA template were subjected to agarose gel electrophoresis (2%) and visualized under UV after ethidium bromide staining. Lanes 1–10, use of *V. cholerae* O1 DNA template from 100 ng to 100 fg, respectively; lane 11, negative control. Lane M is the 100 bp standard DNA ladder.

from each of *V. cholerae* O1, *V. cholerae* non-O1, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Escherichia coli* was used as a template for the amplification of *ctxA* and *tcpA* primers by duplex-PCR. Only the PCR using the template DNA from *V. cholerae* O1 amplified a 302 bp *ctxA* and a 472 bp *tcpA* fragments (Fig. 2). Furthermore, no amplicon was produced in PCR using template DNA from other pathogenic *Vibrio* spp. including *V. parahaemolyticus*, *V. alginolyticus*, *V. vulnificus*, *V. minicus*, and *V. fluvialis* (data not shown), confirming the specificity of primers for detecting *V. cholerae* O1.

Effect of pre-PCR conditions on efficiency of detection

The amplification of *ctxA* and *tcpA* genes in water samples by duplex-PCR using three pre-PCR conditions was evaluated. The results showed that the water samples filtered through a 0.45- μ m filter paper and enriched in APW at least for 3 h before PCR increased the efficiency of detection in the toxigenic *V. cholerae* from water samples. Of the total 59 water samples, 22 (37.3%) were positive by filtered and enriched in APW before PCR, 13 (22%) by filtered without enrichment before PCR, and 18 (30.5%) by only enrichment in APW before PCR (Table 2). The result indicates that prior filtration concentrated the cells pres-

Fig. 2. Specificity of the *ctxA* and *tcpA* genes-targeted primers for toxigenic *V. cholerae* detection.

DNA templates from different bacterial species were used in duplex-PCR. Lane M, 100 bp DNA ladder. Lanes 1–6, PCR profile using DNA template from *V. cholerae* O1, *V. cholerae* non-O1/non-O139, *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli*. Lane 7 has no template on. M is the 100 bp DNA ladder.

Table 2. Duplex-PCR detection of *ctxA* and *tcpA* under different pre-PCR processing conditions.

Method (n=59) ^a	No. of samples positive (%)
Filtrates enriched in APW before PCR	22 (37)
Filtrates without enrichment before PCR	13 (22)
Water enriched in APW before PCR	18 (30)

^a A total 59 water samples were tested.

ent in water samples and the enrichment in APW only for 3 h was enough to allow the multiplication of viable but non-culture (VBNC), thereby improving the frequency of detection of *V. cholerae* by duplex-PCR. Although the detection of *V. cholerae* was greatly enhanced after a 6 h enrichment culture, *ctxA*-positive genes were already detected after 2 h (Theron et al., 2000); therefore, the 3 h enrichment was used in the duplex-PCR detection.

Culture method versus PCR detection

Of the total 96 water samples tested by culture methods, 29 (30%) were positive for *V. cholerae*, 2 (2%) of which were positive for *V. cholerae* O1, and the rests were non-O1/ non-O139 (Table 3). Serotyping results of the *V. cholerae* O1 isolates revealed that

Table 3. Isolation and detection of toxigenic *V. cholerae* by enrichment and selective culture, and duplex-PCR methods from different water samples.

Sampling source	No. of samples	Number (%) sample positive for toxigenic <i>V. cholerae</i>			
		Culture method <i>Vibrio cholerae</i> serogroup			D-PCR
		O1	O139	Non-O1/non O139	
During cholera season:					
Drinking water	15	0 (0)	0	0 (0)	7 (47)
Washing water	16	0 (0)	0	1 (6)	9 (56)
Waste water	11	1 ^a (9)	0	4 (37)	3 (27)
During non-cholera season:					
Well water	6	0 (0)	0	1 (17)	3 (50)
River water	24	0 (0)	0	10 (42)	13 (54)
Waste water	24	1 ^a (4)	0	13 (54)	13 (54)
Total	96	2 (2)	0	29 (30)	48 (50)

^a Inaba serotype.

both belonged to serotype Inaba and were isolated from waste-waters.

In contrast, 48 (50%) of the 96 water samples that were used for preparing DNA templates for the duplex-PCR amplified the primers for *ctxA* and *tcpA* (Table 2), indicating the presence of more toxigenic *V. cholerae* in water samples. These results illustrate the greater sensitivity of duplex-PCR over the culture methods in detecting the toxigenic *V. cholerae* in water samples. The various water samples that were positive for the *tcpA* and *ctxA* genes are shown in Fig. 3.

DNA templates prepared from non-O1/ non-O139 *V. cholerae* were used as templates for *ctxA* and *tcpA* targeted duplex-PCR; as expected, no amplicons were observed in PCR using templates from the non-O1 nor non-O139 *V. cholerae* isolated (data not shown), confirming that indeed the isolates were non-toxigenic *V. cholerae*.

Specificity of duplex-PCR, specific DNA probe was hybridized with the *ctxA* and *tcpA* that were amplified against the template DNA prepared from water samples. All the samples tested positive by DNA-probe hybridization.

The amplified genes in duplex PCR were hybridized with labelled *ctxA* and *tcpA* probes, confirming that the amplicons were *ctxA* and *tcpA* gene fragments.

Fig. 3. Detection of *ctxA* and *tcpA* gene in water samples from different sources.

Lanes 1 and 8, positive and negative control, respectively. Lanes 2, 3, and 7, no *V. cholerae* detected using DNA templates from drinking water, washing water, or well water, respectively. Lanes 4, 5, and 6, *V. cholerae* detected using DNA templates from well water, waste water, and washing water, respectively.

Discussion

To evaluate the public health threat posed by *V. cholerae* occurring in water, a rapid and accurate method for detection of toxigenic *V. cholerae* is essential. Although the culture method (CM) has been widely

accepted as the gold standard, toxigenic *V. cholerae* O1 is isolated only infrequently from surface water by CM. As the toxigenic *V. cholerae* survives mostly in the viable but non-culturable (VBNC) state in the aquatic environment, only fluorescent monoclonal antibody combined with molecular genetic based methods can demonstrate the presence of toxigenic *V. cholerae* in the aquatic environment. Although the isolation of toxigenic *V. cholerae* is very rarely, if ever, achieved by employing CM, the isolation and identification of *V. cholerae* by CM is expensive, time-consuming, labor-intensive, and unable to precisely distinguish between toxigenic *V. cholerae* and non-toxigenic *V. cholerae*. To overcome all these problems, molecular genetic techniques have been developed (Kapley and Purohit, 2001; Ramamurthy et al., 1993; Theron et al., 2000). Thus, direct detection of *ctxA* and *tcpA* genes encoding cholera toxin and toxin-coregulated pilus (TCP) from samples by PCR provides a rapid and specific method for the detection of toxigenic *V. cholerae*.

Amplification of the *ctxA* and *ctxB* gene encoding cholera toxin subunits A and B, respectively, by polymerase chain reaction techniques has been shown to be successful in distinguishing the toxigenic from the non-toxigenic *V. cholerae* strains in pure cultures (Fields et al., 1992). We employed the duplex-PCR for detecting *ctxA* and *tcpA* genes of toxigenic *V. cholerae* from environmental samples collected in the municipality of Khon Kaen, Thailand and compared the result with that of CM. We have also demonstrated herein that the pre-PCR filtration and enrichment of samples for only 3 h significantly improved the efficiency of duplex-PCR in detecting *ctxA* and *tcpA* genes present in water samples.

PCR assays reported by many investigators have claimed high sensitivity and specificity of PCR detection of toxigenic *V. cholerae* (Albert et al., 1997; Lipp et al., 2003; Lyon, 2001; Rivera et al., 2003). However, such detections of targeted genes in a DNA template extracted from different samples such as stools, sea water or environmental samples do not essentially reflect the minimum number of cells required for the efficient detection (Albert et al., 1997; Lipp et al., 2003; Lyon, 2001; Rivera et al., 2003). These numbers range from as little as 3 CFU using *ctxA* as the target (50), to 100–1,000 CFU, as reported by Fields et al. (1992). With reference to multiplex PCR assays detecting *V. cholerae*, Kapley and Purohit reported the sensitivity levels of 100 CFU for their duplex PCR (Kapley and

Purohit, 2001) while Hoshino et al. reported the sensitivity levels of 65 and 200 CFU for *V. cholerae* O1 and O139 serotypes, respectively, for their triplex assay (Hoshino et al., 1998). The tetraplex assay described by Gubala and Proll detected all four gene targets at approximately 1,000 CFU (Gubala and Proll, 2006). Rivera et al. found that the concentration of DNA extracted from water samples varied from 20 ng to 480 µg/µl and approximately 10 ng of each sample was successfully amplified. The sensitivity was 100 CFU in 250 ml of water (Rivera et al., 2003). The present study reports even higher sensitivity of duplex-PCR for the detection of toxigenic *V. cholerae* using template DNA that was estimated to be 100 fg of DNA, equivalent to 23 *V. cholerae* cells against 63 CFU required for the CM. The sensitivity of PCR assays depends on many factors such as primers used, DNA extraction methodology, and the number of targeted genes to be detected.

The specificity of duplex-PCR to detect only the toxigenic *V. cholerae* in this study was determined by using of template DNA from various bacteria including other Vibrionaceae, *V. cholerae* non-O1, non O-139, and other Enterobacteriaceae. No amplicon was produced in *ctxA* and *tcpA* PCR using those templates. In addition, to confirm the specificity of *ctxA* and *tcpA* primers, the PCR products amplified from water samples were also hybridized with labelling *ctxA* and *tcpA* probes. All the PCR products show positive hybridization, indicating the specificity of primer extension for detecting the toxigenic *V. cholerae*.

When we compared the duplex-PCR with CM, *V. cholerae* O1 (toxigenic *V. cholerae*) was found in only 2% of water samples by CM, whereas when duplex-PCR was used for the same set of samples, the *ctxA* and *tcpA* genes were detected in as high as 50% of the water samples. These results are consistent with previous reports on the higher sensitivity of PCR compared to CM (Hasan et al., 1994; Hoge et al., 1990; Norazah et al., 2001; Shirai et al., 1991). Norazah et al. (2001) reported that only one of the 8 samples (12.5%) positive by PCR was positive by CM. The reason may be that the PCR can detect both culturable cells and VBNC cells (Chakraborty et al., 1999; Colwell, 2000; Lipp et al., 2003) and the VBNC state may lead to significant underestimation of the total number of *V. cholerae* population that are physically present in the environment (Colwell, 1993, 2000). The PCR method, however, may detect dead cells in addition to

viable cells (Theron et al., 2000). To minimize this situation, duplex-PCR combined with enrichment in APW was used. The enrichment procedure will not only increase the numbers of *V. cholerae* in water samples but also dilute any inhibitors for duplex-PCR that may be present. The enrichment procedure also dilutes the impact of dead cells. The assessment of water using duplex-PCR may also be affected by the types of water being tested because of the inhibitory substances present in water. However, Rivera et al. and Theron et al. reported that the difference in PCR results using samples from the different types of water was not significant (Rivera et al., 2003; Theron et al., 2000).

When the pre-PCR conditions were evaluated for determining the efficiency of PCR detection, the highest efficiency was observed with the water filtered and enriched in APW for 3 h. We presume that the filtration of water samples increases the concentration of bacterial cells that are diluted in water samples, and the enrichment in APW supports the bacterial growth. The pre-PCR condition that showed the lowest efficiency was the water filtered without enrichment before PCR. This result indicated that the extremely low number of toxigenic *V. cholerae* cells that prevail in the aquatic environment are not enough to detect by duplex-PCR. On the other hand, the pre-PCR condition that gave moderately efficient results was that water samples were enriched in APW without filtration. This result supports our supposition that filtration increases the concentration of *V. cholerae* cells. Besides, the advantages of enrichment of water in APW are not only its support of the growth of the culturable bacterial population, but also the injured or VBNC *V. cholerae* cells (Farmer and Hickman-Brenner, 1992). This study is the first to show that the pre-enrichment of water samples in APW (after filtration) for only 3 h gave a detection level that was comparable with that of the 6 h enrichment before the duplex-PCR (data not shown). This reduction of enrichment time will certainly make the PCR detection faster. Finally, the duplex-PCR described in the present study employing membrane filtration and pre-PCR enrichment water samples in APW provides a very rapid and specific means of detection of toxigenic *V. cholerae* from water samples.

We apparently found lower numbers of toxigenic *V. cholerae* compared to the non-toxigenic ones by CM as previously reported (Alam et al., 2006). This may be due to the better adaptability of the non-toxigenic *V.*

cholerae over the toxigenic *V. cholerae* in water samples. In addition, both examples of toxigenic *V. cholerae* O1 isolated in the present study were from wastewaters, perhaps because wastewater comprises higher levels of nutrients and fecal contamination than do river and potable water. Our results appear in agreement with the previous reports, which showed that the frequency of isolation of toxigenic *V. cholerae* depends mostly upon the types of water samples being tested (Norazah et al., 2001; Theron et al., 2000).

Although non-O1/ non-O139 *V. cholerae* strains are believed to be non-toxigenic, they are known to have some virulence factors like that of *V. cholerae* O1 *El Tor* hemolysin (Ichinose et al., 1987; Rivas et al., 1996; Singh et al., 2001) and a heat stable enterotoxin (NAG-ST) (Guglielmetti et al., 1994; Pal et al., 1992; Saha et al., 1996) including other toxins that have not been determined in this study. Some previous reports showed that the clinical profile of patients infected with *V. cholerae* non-O1/ non-O139 was indistinguishable from that of patients with cholera (Mukhopadhyay et al., 1995). Thus, the large numbers of CT-negative non-O1/non-O139 *V. cholerae* that we have isolated in the present study by CM may presumably have the ability to cause gastroenteritis mimicking cholera (Saha et al., 1996; Sharma et al., 1998). Consequently, the presence of both toxigenic *V. cholerae* O1 and non-toxigenic non O1/non-O139 strains that were isolated and detected from the aquatic environments of Khon Kaen, Thailand, in the present study may have potential to affect the public health.

Our results indicated that the surface water of the aquatic environment as well as the potable water of the municipality of Khon Kaen, Thailand, is a potential source of toxigenic *V. cholerae*. The toxigenic *V. cholerae* including the non-toxigenic ones are autochthonously present in the aquatic environments of many Asian and African countries, where cholera is endemic (Alam et al., 2006; Colwell and Huq, 1994). No study on the distribution of toxigenic *V. cholerae* by duplex-PCR has so far been done in water samples in Thailand. This study is the first to show the direct detection of toxigenic *V. cholerae* from the water samples in Thailand. Duplex-PCR and the pre-PCR conditions that we presented here may be of great use for the rapid and reliable detection of toxigenic *V. cholerae* and related pathogens in potable and natural waters to assess the potential health risk. Therefore, the duplex-

PCR we reported here can be adopted in cholera surveillance programs for efficient monitoring of toxigenic *V. cholerae* and related pathogens in potable and surface water. Therefore, the public health departments of Thailand and those countries where sanitation is poor and clean drinking water is not available to local populations should adopt the duplex-PCR presented here to ensure the quality water for people.

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