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Contributed Paper

Application of SYBR Green Real-Time PCR for Detection of Toxigenic *Vibrio cholerae* O1 in the Aquatic Environment

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ABSTRACT

Vibrio cholerae O1 is the causative agent of cholera disease and its natural habitat is the aquatic environment. *V. cholerae* can become a "viable but non-culturable" (VBNC) organism resulting in unsuccessful isolation from aquatic environments; therefore, sensitive, rapid and accurate detection of VBNC taken from an aquatic environment is needed. The aim of this study was to develop SYBR green real-time PCR compared to the conventional PCR and culture methods for detection of *V. cholerae* O1 in water samples. The SYBR green real-time PCR assays were developed using specific primers targeting genes for the outer membrane protein (*omp*W), cholera toxin A (*ctx*A), *rfb*O1 (serogroup O1) and *rfb*O139 (serogroup O139). The respective sensitivity of uniplex (*omp*W, *rfb*O139) and duplex (*ctx*A and *rfb*O1) SYBR green real-time PCR was 10^2 CFU/ml (3 CFU/PCR reaction) and 10^3 CFU/ml (25 CFU/PCR reaction). *V. cholerae* O1 was detected in 31.8% (27/85) of samples and all were *ctx*A positive by SYBR green real-time PCR and conventional PCR, vs. 3.5% (3/85 samples) by culture method. Our results indicate that both PCR-based assays have similar efficiency for detecting *omp*W, *ctx*A, *rfb*O1 and *rfb*O139 genes and could be applied for rapid detection of *V. cholerae* O1 in environmental water samples.

Keywords: SYBR green real-time PCR, *Vibrio cholerae* O1, aquatic environment

1. INTRODUCTION

Vibrio cholerae O1 is the causative agent of severe dehydrating diarrhea, cholera disease which is an important enteric disease in less developed and developing countries including Thailand [1]. The main transmission of *V. cholerae* is consumption of contaminated food and drinking water because aquatic environments are an important reservoir for this bacteria [2]. The outbreak of this disease is a serious public health problem, so its rapid detection in environmental samples is needed.

Currently, the culture technique is the standard method for phenotypic identification; however, it requires a long incubation, is laborious [3] and cannot detect the coccoid form or a "viable but non-culturable" (VBNC) state in stress conditions (i.e., low temperature and nutrient-poor environments), resulting in unsuccessful isolation from aquatic environments [4, 5].

Molecular methods such as conventional PCR have become the alternative methods for bacterial detection from environmental samples [6]. These methods, however, require product characterization by gel electrophoresis and low through-put. To overcome the weaknesses of conventional PCR, the real-time PCRs or quantitative PCRs (qPCR) have been developed for detection of several microorganisms in clinical and environmental samples [7]. The number of microorganisms can be determined using qPCR so that technique has value for epidemiological studies [7, 8].

In real-time PCR based assays, non-specific fluorescent dyes such as SYBR green real-time PCR [9], and sequence-specific DNA probes like TaqMan real-time PCR [10] have been widely used. The SYBR green real-time PCR uses a SYBR Green fluorescent dye that binds with the double stranded DNA, and the products are distinguished by analysis of melting temperature (T_m) curve analysis. By comparison, TaqMan real-time PCR uses an internal probe labeled with a fluorescent reporter dye and a quencher dye. During PCR amplification, TaqMan probe is cleaved by DNA polymerase, resulting in separation of the reporter and quencher dye, which manifests as increased fluorescence [11]. Although TaqMan is more specific than SYBR green real-time PCR, this limitation can be resolved by optimizing conditions [9]. Previous studies reported that the non-sequence-specific dyes assay were more cost effective than the sequence-specific DNA probes assays [12], therefore SYBR green real-time PCRs have been developed [13].

The uniplex SYBR green real-time PCR assay was used to detect the *V. cholerae* in oysters, aquatic sediments, and seawater from Mobile Bay [14] and was also used in environmental water samples [15]. The multiplex SYBR green real-time PCRs were used to detect several bacteria and virulence associated genes in food and waterborne pathogens [16] such as *E. coli* O157:H7 [12] and *V. cholerae* [17].

For detection of *V. cholerae,* the SYBR green real-time PCRs using many target genes such as *dna*J have been used for detection of this microorganism in both clinical and environmental samples [17]; however, some of these target genes were not specific [18-20] and did not apply to natural water samples spiked with *V. cholerae* [7].

In the present study, we developed SYBR green real-time PCR assays capable of providing rapid, sensitive, specific and quantitative detection of *V. cholerae* O1 and cholera toxin in aquatic environments. We then compared the protocol to conventional PCR and culture methods. The uniplex SYBR green real-time PCR assays were developed using primer pairs by specifically encoding the outer membrane protein of highly conserved species specific (*omp*W) and serogroup O139 (*rfb*O139). By contrast, the duplex SYBR green real-time PCR was developed using primer pairs of cholera toxin (*ctx*A) and the serogroup O1 (*rfb*O1). We also determined the concentration of *V. cholerae* O1 in various types of water in order to predict the risk of transmission of toxigenic *V. cholerae*.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

The bacterial strains used in this study were obtained from various sources, including: the American type culture collection (ATCC), the Department of Medical Sciences Thailand Culture Collection (DMSC), and clinical and environmental sources stored at Srinagarind Hospital and the Department of Microbiology Laboratory, Faculty of Medicine, Khon Kaen University, Thailand (Table 1). All strains from bacterial stock culture stored at -70°C were cultured in blood agar (Oxiod, England). After incubation at 37°C for 18-24 h, a single colony of each bacterial strain was grown in Brain heart infusion broth (BBL, USA) and incubated at 37°C for 18-24 hr. One ml of each bacterial suspension was used for DNA extraction by boiling method.

Table 1. Bacterial species specificity of SYBR green real-time PCR of *Vibrio cholerae*.

Bacterial strains	Number of isolate	Presence (+) or absence (-) of PCR product			
		*omp*W	*ctx*A	*rfb*O1	*rfb*O139
Vibrio cholerae O1(clinical isolate)	6	+	+	+	-
Vibrio cholerae nonO1 (3 clinical isolate and 2 environmental isolates)	5	+	-	-	-
Vibrio cholerae O139 (environmental strain)	1	+	+	-	+
Vibrio mimicus(ATCC 33653)	1	-	-	-	-
Vibrio parahaemolyticus(ATCC 17802)	1	-	-	-	-
Vibrio vulnificus(ATCC 27562)	1	-	-	-	-
Vibrio fluvialis (DMST 19347)	1	-	-	-	-
Vibrio alginolyticus(DMST 14800)	1	-	-	-	-
Escherichia coli(ATCC 25922)	1	-	-	-	-
Salmonella spp.(clinical isolate)	1	-	-	-	-
Shigella dysenteriae(DMST 15111)	1	-	-	-	-
Klebsiella spp.(clinical isolate)	1	-	-	-	-
Shigella sonnei(ATCC 11060)	1	-	-	-	-
Enterobacter spp.(clinical isolate)	1	-	-	-	-
Aeromonas hydrophila(clinical isolate)	1	-	-	-	-
Pseudomonas aeruginosa(clinical isolate)	1	-	-	-	-
Campylobacter jejuni(clinical isolate)	1	-	-	-	-
Staphylococcus epidermidis(clinical isolate)	1	-	-	-	-
Staphylococcus pyogenes(clinical isolate)	1	-	-	-	-
Listeria monocytogenes(clinical isolate)	1	-	-	-	-
Bacillus spp.(clinical isolate)	1	-	-	-	-
Enterococcus spp.(clinical isolate)	1	-	-	-	-

2.2 PCR Primer Specificity

The four target genes and the oligonucleotide primers used in SYBR green real-time PCR for detection of *V. cholerae* O1/ O139, cholera toxin and serogroups are listed in Table 2. The primer pair *rfb*O1 newly designed by using Premier Primer 3.0 (Premier Biosoft International, Palo Alto, CA) was tested for primer specificity using BLAST software from the NCBI nucleotide public database (http:/ blast.ncbi.nlm.nih.gov/Blast.cgi). The primer pairs *ctx*A and *omp*W were previously verified by Senachai *et al.* [21], and *rfb*O139 by Alam *et al.* [22]. Specificity of the primers was also evaluated by PCR assay using 100 ng of DNA template of the strains listed in Table 1.

Table 2. Oligonucleotide primers used in this study.

Target genes	Primer sequence (5'->3')		Product size(bp)	T_m of product	Ref.
*omp*W	Forward	GTACTTGCAGCCCTAAGCTC	307	79.4±0.4C°	[21]
	Reverse	GGACCATAAAGGTAGGTGGC			
*ctx*A	Forward	TGGTCTTATGCCAAGAGGACA	517	76.6±0.3C°	[21]
	Reverse	ATCTTGGAGCATTCCCACAAC			
*rfb*O1	Forward	TAATTCATCTTGGCGTGGAG	171	79.4±0.4C°	In this study
	Reverse	CCCCGAAAACCTAATGTGAG			
*rfb*O139	Forward	AGCCTCTTTATTACGGGTGG	449	79.4±0.2C°	[22]
	Reverse	GTCAAACCCGATCGTAAAGG			

2.3 DNA Preparation

The sensitivity of SYBR green real-time PCR for detection of *V. cholerae* and cholera toxin genes was performed using the boiling method and a genomic DNA purification kit (Puregene DNA Purification System, Gentra Systems, USA), according to the manufacturer's instructions [21]. In brief, a mid-log phase of *V. cholerae* O1 and O139 strains underwent ten-fold serial dilution using sterile distilled water to obtain 10^6- 10^0 CFU/ml. One ml of each dilution was boiled for 10 min followed by cooling on ice for 5 min. Five microliters of the prepared aqueous boiled samples (100 - 150 ng) were used as the template DNA.

2.4 Conventional PCR Assay

The amplification reaction of the target genes for *V. cholerae* O1 and O139 was conducted in a 30-µl of PCR mixture containing 5 µl of DNA template, 0.3 mM of each dNTP (Amresco, Ohio, USA), 1X PCR buffer, 3.0 mM $MgCl_2$, 2.0 units of *Taq* polymerase (RBC bioscience, Taipei, Taiwan), 0.15 µM of primer *ctx*A, and 0.35 µM in each primers of *omp*W, *rfb*O1 and *rfb*O139. Amplification was conducted with a thermal cycler (Veriti, ABI system) programmed as follows: 94°C for 10 min; 35 cycles of 94°C for 1 min; 59°C for 1 min; 72°C for 2 min; and 72°C for 10 min. Amplified products were subjected to 2% agarose gel electrophoresis and visualized under UV after ethidium bromide staining.

2.5 SYBR Green Real-Time PCR Assay

The uniplex SYBR green real-time PCR assay was conducted in a 20-µl reaction volume containing 10 µl of 2X Maxima™ SYBR Green/ROX qPCR Master Mix

(Thermo scientific, USA), 0.2 µM of *omp*W and *rfb*O139 primers and 7 µl of DNA template (100 ng of genomic DNA). The duplex SYBR green real-time PCR was performed in a 20-µl reaction volume containing 12.5 µl of 2X Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo scientific, USA), 0.2 µM of *rfb*O1 and 0.8 µM *ctx*A primers, and 7 µl of DNA template (100 ng of genomic DNA). The nuclease-free water was used for no template control (NTC). Real-time PCR amplification was conducted using an ABI 7500 thermocycler (Applied Biosystems). Cycle threshold (C_t) values were determined employing a thermocycling program: 2 minutes at 50°C; 10 minutes at 95°C; 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds and 72°C for 32 seconds. The limit of detection was determined using the cycle number threshold (C_t). A C_t of > 33 was considered negative for the uniplex (*omp*W) whereas the C_t of > 32 was considered negative for the uniplex (*rfb*O139) gene and the duplex (*ctx*A, *rfb*O1) gene. For melting curve analysis, the mean melting temperature (T_m) of *omp*W, *rfb*O1 and *ctx*A PCR product showing the peak at 79.4 ± 0.4°C, 76.6 ± 0.3°C and 79.0 ± 0.2°C, respectively (Figure 1).

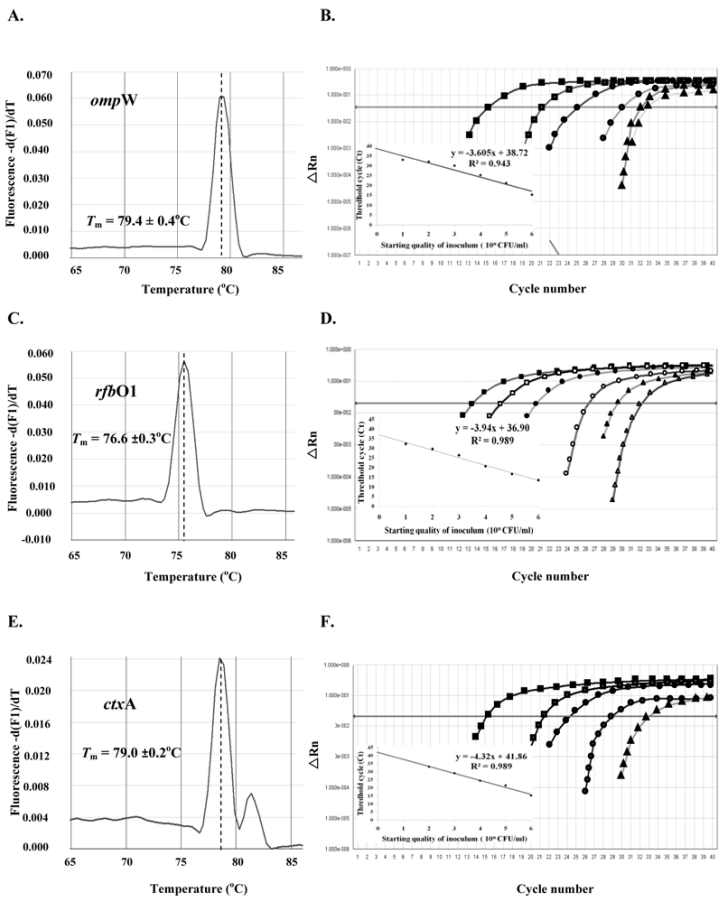

Figure 1. Melting curve analysis of uniplex SYBR green real-time PCR (A, C, E) and fluorescent amplication curves showing sensitivity of uniplex SYBR green real-time PCR for *omp*W, *rfb*O1 and *ctx*A. Symbols represent serial dilution of *V. cholerae* strains: -■-10^6 -□-10^5; -●-10^4; -○-10^3; -▲-10^2; -△-10^1CFU/ml.

2.6 Detection of *V. cholerae* in Environmental Water Samples

Eighty-five environmental water samples were collected from the municipality of Khon Kaen, Northeastern Thailand, from 3 ponds, 7 canals and 2 wastewater reservoirs, and aseptically transported to the laboratory [23]. In brief, each 400 ml of water samples was filtered through Whatman No.1 filters and the filtrate was again passed through a 0.2-μm pore size (Millipore). The membrane was placed in 15 ml of alkaline peptone water (APW) (Oxoid, England) at pH 8.4 and incubated at 37°C for 6 h. The samples resolved in APW were used for the culture (5 μl) and 1.5 ml each was boiled for 10 min followed by cooling on ice for 5 min and was used as the template DNA.

2.7 Culture Method and Serogroup

A 5-μl aliquot of each sample in APW were streaked onto thiosulfate citrate bile-salts sucrose (TCBS) agar and incubated at 37°C for 18 h. The yellow colonies that were oxidase-positive were further characterized by biochemical tests [24]. Colonies presumptively identified as *V. cholerae* were serogrouped by the slide agglutination test using polyvalent O1/O139 specific antisera (Oxoid, USA).

3. RESULTS AND DISCUSSION

3.1 Specificity of PCR Primers

The specificity of all primers was determined using the NCBI database and was confirmed by using the DNA templates from the 31 bacterial species (Table 2). The results demonstrated that only the Genomic DNA from six strains of *V. cholerae* O1 was positive for all three products while that from the other bacteria gave negative results.

In this study, we used the *omp*W gene as a marker for detection of *V. cholerae* even though other researchers used the hemolysin gene (*hly*A) [18, 19]. Our rationale was that some *V. cholerae* El Tor strains do not produce hemolysin [20], resulting in failure detecting those pathogens.

For duplex real-time PCR, the combination of two target genes including *ctx*A and *rfb*O1 can be used for differentiation of toxigenic *V. cholerae* O1 from non-toxigenic *V. cholerae* non O1 because *ctx*A gene are mainly responsible for watery diarrhea of toxigenic *V. cholerae* [25]. These results indicate that real-time PCR can be applied for detection of toxigenic *V. cholerae* O1 in water samples. We also used *rfb*O139 gene for distinguishing *V. cholerae* O139 from other serogroups of *V. cholerae*.

3.2 Sensitivity of Conventional PCR and SYBR Green Real-Time PCR

The sensitivity of the uniplex (*omp*W or *rfb*O139) and duplex (*ctx*A and *rfb*O1) conventional PCR, and the uniplex and duplex SYBR green real-time PCR in pure cultures were equal with 10^2 CFU/ml (3 CFU/PCR reaction) and 10^3 CFU/ml (25 CFU/PCR reaction), respectively, by boiling method. The results indicated that our SYBR green real-time PCR had as high a sensitivity as the conventional PCR, and nearly equal sensitivity as some previous studies using boiled DNA template [7, 18].

When the DNAs were extracted using a genomic DNA purification kit, the sensitivity of the uniplex (*omp*W or *rfb*O139 gene) and duplex (*ctx*A and *rfb*O1 gene) conventional PCR were 10^2 CFU/ml (3 CFU/PCR reaction) and 10^3 CFU/ml (25 CFU/PCR reaction), respectively. By comparison, the sensitivity of the uniplex and duplex SYBR green real-time PCRs was 10^1 CFU/ml (1 CFU/PCR reaction) (Figures 1 and 2). Although the sensitivity of real-time PCR of

DNA extracted using a commercial kit was higher than DNA using boiled DNA, the DNA extracted from the commercial kit took longer time and had more expensive than the boiling method. We, therefore, suggest the boiling method is a simple, rapid and cost-effective method for the preparation of DNA from water samples for the detection of *V. cholerae* in the environment. When comparing the sensitivity with previous reports using DNA template from a commercial kit, our SYBR green real-time PCR is in accord. For example, Blackstone *et al*. reported that the sensitivity of the uniplex real-time PCR was less than 10 CFU per reaction using pure culture [14]. By comparison, Lyon reported that the sensitivity of the uniplex TaqMan real-time PCR assay for detection of *V. cholerae* O1 and O139 pure culture were 7.3 and 8.2 CFU/ml, respectively [11]. Finally, Huang *et.al* reported that the sensitivity of tetraplex assay was 2 CFU/PCR reaction [19].

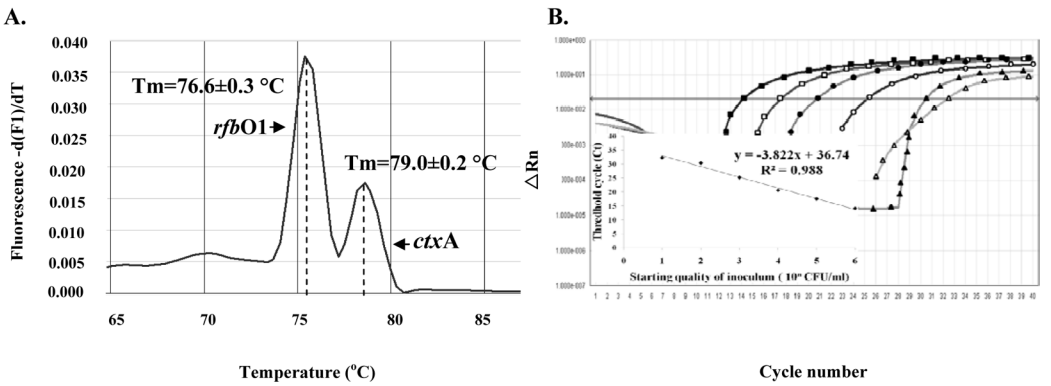

Figure 2. Melting curve analysis of the duplex SYBR green real-time PCR showing PCR product for *rfb*O1 and *ctx*A (A) and Amplification plot show sensitivity of duplex real-time SYBR green PCR of *V. cholerae* strains ranging from 10^6-10^0 CFU/ml were used as template (B). Symbols indicate ten-fold serial dilution of *V. cholerae* strains: -■-10^6; -□-, 10^5; -●-,10^4;-○-, 10^3;-▲-, 10^2;-△-, 10^1 CFU/ml.

3.3 Detection of *V. cholerae* O1/O139 in Water Samples

V. cholerae O1 was detected by both conventional PCR and SYBR green real-time PCR assay in 31.8% (27/85) of cases whereas *V. cholerae* O139 was not. Among 27 *V. cholerae* O1 positive samples, all were *ctx*A positive (100%) (Table 3). Three samples (3.5%) detected by culture method were positive for *V. cholerae* O1. The higher sensitivity of PCR over the culture method allows detection of bacteria in a viable but non-culturable (VBNC) state [25-27]. The conventional and real-time PCR assays are both more sensitive and rapid than the culture method; therefore, they can serve as alternative methods for detecting environmental *V. cholerae*. A previous study showed that, in 2007, *V. cholerae* was detected in the water samples in Northeast Thailand using duplex conventional PCR with targeting *ctx*A and *tcp*A genes detecting of the toxigenic *V. cholerae* in 50% of water samples [23]. In the present study, the toxigenic *V. cholerae* was detected in 31.8% of water samples, which is less than previously report [23]. The difference may be due to (a) the different period of time,

(b) different kinds of water samples and (c) different genes detected. Our results confirm, however, that the aquatic environment can be a source of transmission of cholera.

Previous studies showed that toxigenic *V. cholerae* was present in the aquatic environments of many Asian and African countries, where cholera is endemic [28]. For example, toxigenic *V. cholerae* was isolated from freshwater lakes and ponds in eastern Calcutta [29], while the toxigenic *V. cholerae* O1, serotype Inaba, was isolated from patients and stored household water in Africa [30]. Lipp *et al.* reported that 62.5% of seawater and plankton samples in the coast of Peru contained *V. cholerae* O1 [25].

Table 3. Detection of *V. cholerae* in water samples by SYBR green real-time PCR.

Bacterial strains	SYBR green real-time PCR				Total (%)
	Uniplex		Duplex		
	*omp*W	*rfb*O139	*ctx*A	*rfb*O1	
Toxigenic *V. cholerae* O1	+	-	+	+	27/85 (31.8)
Non-toxigenic *V. cholerae* non-O1	+	-	-	-	31/85 (36.5)
Total					58/85 (68.2)

3.4 Quantitative Detection of *V. cholerae* in Various Water Samples by Real-Time PCR

The concentration of contaminated *V. cholerae* O1 in each water sample after enrichment in APW for 6 hours is presented in Table 4. *V. cholerae* O1 was detected at 10^4 CFU/ml in both the wastewater and the water from canals as compared to 10^5 CFU/ml in pond water. Since, the concentration of *V. cholerae* O1 was determined after enrichment for 6 hours, therefore, the initial number of this bacteria should be approximately 1 CFU/ml. To the best of our knowledge, this is the first report on detection of the concentration of *V. cholerae* O1 in water samples.

Table 4. Quantitative detection of *V. cholerae* in various types of water sample by real-time PCR.

Type of water samples	No. of *V. cholerae* O1 positive samples (%)	Quantitative contaminated *V. cholerae* O1 (CFU/ml)*
Wastewaters	13/27 (48.1)	10^4
Canals	11/27 (40.8)	10^4
Ponds	3/27 (11.1)	10^5

* Average number of cells after 6 h enrichment in alkaline peptone water

4. CONCLUSION

Owing to the limitations of the culture method, we developed conventional and SYBR green real-time PCR assays for the detection of *V. cholerae* in water samples. The primer pairs used (a) for the detection of *V. cholerae* species in water samples was the *omp*W gene and (b) for distinguishing toxigenic strains of *V. cholerae* O1/O139 from non-toxigenic strains were the *ctx*A, *rfb*O1 and *rfb*O139 genes.

The uniplex and duplex real-time PCR assay achieved 100% specificity. The respective sensitivity of uniplex SYBR green real-time PCR vs. duplex SYBR green real-time PCR (using DNA extracted by the boiling method) was 10^2 CFU/ml (3 CFU/PCR reaction) vs. 10^3 CFU/ml (25 CFU/PCR reaction).

In summary, both PCR-based assays have the same efficiency for detecting *V. cholerae* O1 in aquatic environments. SYBR green real-time PCR assay was, however, more rapid and could be applied for quantication of *V. cholerae* in environmental samples. Real-time PCR can, therefore, be used as an alternative tool for direct detection of *V. cholerae* in water samples and for rapid monitoring of transmission of toxigenic *V. cholerae*, thereby enabling appropriate prevention of cholera outbreaks.

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