



ORIGINAL ARTICLE

Multiplex Real-time Polymerase Chain Reaction Assays for Simultaneous Detection of *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*

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Received: March 6, 2013
Revised: March 25, 2013
Accepted: April 5, 2013

KEYWORDS:

TaqMan probe multiplex
real-time PCR,
Vibrio cholerae,
Vibrio parahaemolyticus,
Vibrio vulnificus

Abstract

Objectives: A multiplex real-time polymerase chain reaction (RT-PCR) method was developed for the identification of three *Vibrio* species: *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*.

Methods: Specific primers and probes targeting the *hlyA*, *tlh*, and *vwA* genes were selected and used for multiplex real-time PCR to confirm the identification of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, respectively. This method was applied to screen *Vibrio* species from environmental samples and combining it with a culture-based method, its effectiveness was evaluated in comparison with culture-based methods alone.

Results: Specific PCR fragments were obtained from isolates belonging to the target species, indicating a high specificity of this multiplex real-time PCR. No cross-reactivity with the assay was observed between the tested bacteria. The sensitivity of the multiplex real-time PCR was found to have a lower limit of 10^4 colony-forming units/reaction for all three *Vibrio* species. The combination strategy raised the isolation ratio of all three *Vibrio* species 1.26- to 2.75-fold.

Conclusion: This assay provides a rapid, sensitive, and specific technique to detect these three *Vibrio* species in the environment.

1. Introduction

Vibrio is a genus of Gram-negative bacteria that possess a curved rod shape and naturally inhabits aquatic environments worldwide [1–5]. Within the genus *Vibrio*, several species are known to be important human pathogens [6–8]. Among these, *Vibrio cholerae*, *Vibrio vulnificus*, and *Vibrio parahaemolyticus* are the

major pathogenic *Vibrio* species. *V. cholerae* and *V. parahaemolyticus*, contracted through consumption of contaminated seafood and seawater, can cause gastroenteritis whereas *V. vulnificus* can cause septicemia by exposure of an open wound to seawater or consumption of contaminated seafood [9–11]. In Korea especially, owing to the common practice of raw seafood consumption, gastroenteritis caused by infection

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with *V. parahaemolyticus* and septicemia caused by infection with *V. vulnificus* occur frequently.

Because cases of infection by *Vibrio* spp. are commonly found in coastal areas, it is prudent to investigate the distribution of the pathogenic *Vibrio* spp. in the coastal environment of Korea. However, the isolation of bacteria using conventional microbiological method is a time-consuming and laborious process that presents potential chance for error resulting in nondetection of *Vibrio* spp. present in environmental samples.

Real-time PCR is a rapid, sensitive, and highly specific technique. In many reports, real-time PCR has been used to detect various human and animal pathogens [12–19].

In the present study, we developed a simple multiplex PCR method based on TaqMan real-time PCR to detect *V. parahaemolyticus*, *V. vulnificus*, and *V. cholerae* in a single PCR reaction. In this assay, we selected specific primers and probes targeting the hemolysin genes of three *Vibrio* spp. for their identification. The *hlyA*, *tlh*, and *vvhA* genes are species-specific markers for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, respectively [20–23].

This PCR method allowed for quick and easy isolation of three *Vibrio* species from environmental samples. Owing to its sensitivity, accuracy, and the potential increase in isolation rate in conjunction with other isolation methods, such as the use of chromogenic selective media, the method provides a rapid and effective detection tool for research and diagnostics.

2. Materials and Methods

2.1. Bacterial strains and DNA extraction

A total of 56 strains from the Korean Centers for Diseases Control and Prevention strain collection were

used in this study (Table 1). These included five reference strains and 51 clinical and environmental strains. All strains were cultured on tryptic soy agar (Becton Dickinson and Company, Sparks, MD, USA) with overnight incubation at 37 °C.

Genomic DNA extraction was performed by boiling. Briefly, one colony was suspended in 200 µL of sterile distilled water and boiled for 10 minutes to lyse the bacteria. After boiling, the suspension was centrifuged for 2 minutes at 14,000 rpm to sediment the cell debris. The supernatant was collected and used as a template for PCR.

2.2. Primer and TaqMan probe design

Suitable primers and probes for the multiplex TaqMan real-time PCR (amplifying the *hlyA*, *tlh*, and *vvhA* genes of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*, respectively) were designed using Primer Express (Applied Biosystems, Foster City CA, USA) and were synthesized by Applied Biosystems (Table 2). Each set of primers was used to amplify its respective target gene from the purified genomic DNA of three *Vibrio* species reference strains (*V. cholerae* ATCC 14033, *V. parahaemolyticus* ATCC 17802, and *V. vulnificus* ATCC 27562). All PCR products were verified by confirming their expected sizes via electrophoresis in a 3% Nusieve 3:1 agarose gel Lonza Group Ltd., Basel, Switzerland.

2.3. Multiplex TaqMan real-time PCR for detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*

The real-time PCR reactions were performed on a Roche LightCycler 480 (Roche Diagnostics Ltd., Penzberg, Germany). Typical reactions contained 10 µL of 2× probes Master (Roche Diagnostics Ltd.), 90 nM each

Table 1. Strains tested for assay specificity

Species (No. isolates)	Gene target		
	<i>hlyA</i>	<i>tlh</i>	<i>vvhA</i>
<i>Vibrio cholerae</i> (10)	+	–	–
<i>Vibrio parahaemolyticus</i> (10)	–	+	–
<i>Vibrio vulnificus</i> (10)	–	–	+
<i>Vibrio mimicus</i> (2)	–	–	–
<i>Vibrio alginolyticus</i> (1)	–	–	–
ETEC ATCC 43896	–	–	–
ETEC (5)	–	–	–
EHEC ATCC 43895	–	–	–
EAEC (3)	–	–	–
<i>Shigella</i> spp. (2)	–	–	–
<i>Shigella flexneri</i> (3)	–	–	–
<i>Shigella sonnei</i> (2)	–	–	–
<i>Salmonella</i> spp. (2)	–	–	–
<i>Salmonella enteritidis</i> (2)	–	–	–
<i>Salmonella typhimurium</i> (2)	–	–	–

EAEC = enteroaggregative *Escherichia coli*; EHEC = enterohemorrhagic *Escherichia coli*; ETEC = enterotoxigenic *Escherichia coli*.

Table 2. Target genes, primers, and probes used for multiplex real-time PCR detection of the three *Vibrio* spp

<i>Vibrio</i> spp.		Primer or probe sequence (5' to 3')	Target gene	Amplicon size (bp)
<i>V. cholerae</i>	VCF	GCGTTGGGAGTGGCGTAA	<i>hlyA</i>	57
	VCR	GGACTCGCCGCTGTAGACA		
	VCP	FAM-AGCACAGATGAATTGACCAMG-BNHQ		
<i>V. parahaemolyticus</i>	VPF	AACCGTGGCGTTCCAGAA	<i>tlh</i>	58p
	VPR	CCGTCAAACGAATCAGTGCTT		
	VPP	VIC-TGAAAGCGGATTATGC-MGB		
<i>V. vulnificus</i>	VVF	GATCGTTGTTTGACCGTAAACG	<i>vvhA</i>	79p
	VVR	TGCTAAGTTCGCACCACACTGT		
	VVP	NED-CAAAACGCTCACAGTCG-MGB		

F = forward primer; P = probe; R = reverse primer.

of the *hlyA*, *tlh*, and *vvhA* forward and reverse primers, 200 nM of the TaqMan MGB probes for *hlyA*, *tlh*, and *vvhA* (Table 2), and 1 uL of DNA template in a total volume of 20 µL. The cycling conditions were as follows: 2 minutes at 50 °C and 5 minutes at 95 °C, followed by 40 cycles, each consisting of 15 seconds at 95 °C and 1 minute at 60 °C. Data were analyzed using the LightCycler480 software (Roche Diagnostics Ltd.). PCR amplification was detected directly by monitoring the increase in fluorescence of each dye-labeled probe. Samples with cycle threshold (C_t) value above 35 were considered as negative.

2.4. Specificity and sensitivity of detection by multiplex TaqMan real-time PCR

The specificity of each set of primers and probes for its respective target gene was determined by PCR amplification of the purified genomic DNA from 33 *Vibrio* spp. strains and 23 other bacterial strains.

To identify the lower limit of detection and generate standard curves, 10-fold serial dilutions from 10^1 to 10^7 colony-forming units (CFU)/mL of each reference strain were used. Reference strains were cultured in tryptic soy broth media at 37 °C to yield 10^9 CFU/mL. Bacterial growth was monitored by observation of the absorbance at 600 nm using a spectrophotometer (GeneQuant pro; Amersham Pharmacia Biotech Inc., Cambridge, UK). Ten-fold serial dilutions of bacteria in physiological saline, from 10^1 to 10^7 CFU/mL, were used to generate templates for multiplex real-time PCR. To determine the number of cells in the samples, 100 µL of each 10-fold serial dilution was spread on a trypticase soy agar plate and incubated at 37 °C for 16 hours, and the grown bacterial colonies were counted. The standard curves were determined by plotting the C_t values against the log CFU/reaction. Amplification efficiency was calculated using the equation $E = 10^{(-1/\text{slope})} - 1$.

2.5. Application of multiplex real-time PCR as a screening method

Prior to the isolation of *Vibrio* species strains from the samples, real-time PCR was performed to detect the presence of *V. cholerae*, *V. parahaemolyticus*, and

V. vulnificus in the samples. From May 2007 to December 2007, 2729 marine environmental samples (seawater, sediments, and plankton etc.) were collected from 11 Korean coastal areas. Ten milliliters or 10 g of the samples was added to 90 mL of alkaline peptone water (1% peptone, 1% NaCl, pH 8.4) and incubated 6–8 hours at 37 °C. After enrichment of the samples, a 1 mL portion of alkaline peptone water was removed, and the total DNA extracted by the boiling method previously described. The extracted DNA was used as a template for multiplex real-time PCR, as described above.

A loopful of each enrichment culture was also streaked onto thiosulfate citrate bile salts sucrose (TCBS; Beckton Dickinson and Company) agar plates and incubated for 18–20 hours at 37 °C. After incubation, candidate colonies from the plate corresponding to the PCR positive culture were replica plated onto CHROMagar *Vibrio* (CV; CHROMagar, Paris, France) and TCBS agar plates. By comparing the growth on the TCBS and CV agar plates, a candidate species could be identified for each colony: a colony that was green on TCBS and blue on CV was thought to be *V. vulnificus*; a colony that was green on TCBS and mauve on CV was thought to be *V. parahaemolyticus*; and a colony that was yellow on TCBS and blue on CV was thought to be *V. cholerae*. The colonies were further identified biochemically using an API 20E identification kit (bioMérieux, Inc., Hazelwood, MO, USA).

3. Results

3.1. Specificity and sensitivity of detection

The primers and probes were specifically designed to identify the hemolysin genes present in *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*: the *hlyA*, *tlh*, and *vvhA* genes, respectively. PCR amplification with these primers yielded amplicons of the expected molecular weights (57 bp for *hlyA*, 58 bp for *tlh*, and 79 bp for *vvhA*; Figure 1, Table 2).

The specificity of the primers and probes against 33 *Vibrio* spp. strains and 23 other bacterial strains was

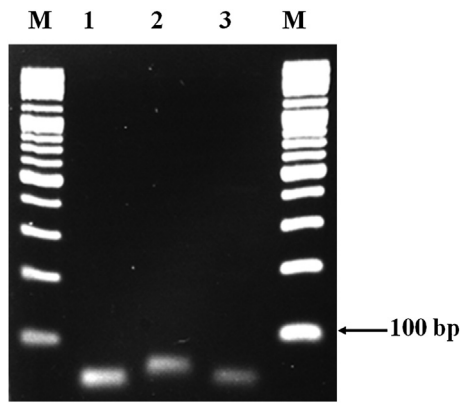


Figure 1. The molecular size of polymerase chain reaction (PCR) fragments amplified with species-specific primers. Agarose gel electrophoresis showing the results from a PCR amplification of genomic DNA from three *Vibrio* spp. Lane M: 100 bp DNA ladder; Lane 1, Lane 2, and Lane 3: amplified DNA using oligonucleotide primers specific for the *hlyA*, *vvhA*, and *tlh* genes showing specific bands of DNA of the expected sizes of 57 bp, 79 bp, and 58 bp, respectively.

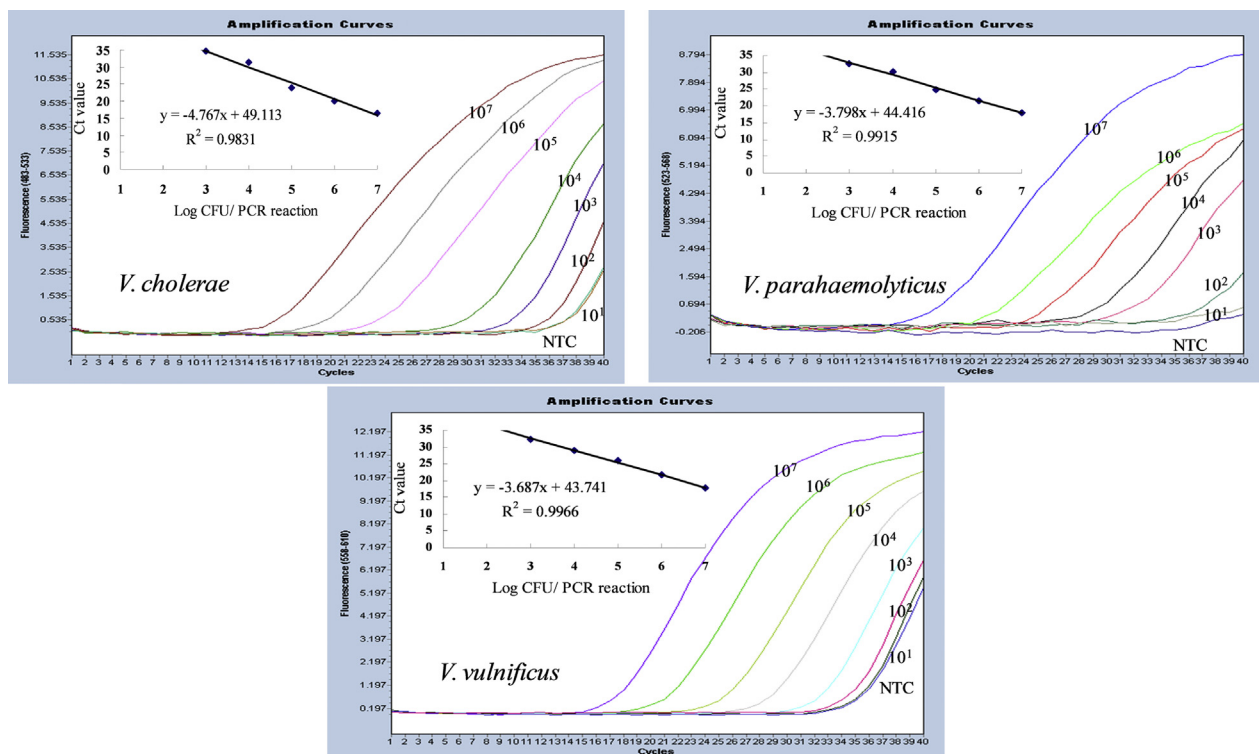
tested by multiplex real-time PCR. Specificity was confirmed by PCR amplification of the hemolysin genes of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*,

respectively, but no amplification was detected for any other *Vibrio* spp. or non-*Vibrio* spp. (Table 1).

The aim of this study was to develop and evaluate a method for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* using multiplex real-time PCR in a single sample. During development of the assay, no cross-reactivity between the tested bacteria was observed.

To determine the detection limit of the multiplex real-time PCR and to establish a standard curve for quantification, DNA extracted from 10-fold serial dilutions of three *Vibrio* spp. at final concentration of $10^1 - 10^7$ CFU/reaction was analyzed by real-time PCR. Standard curves were constructed for three *Vibrio* spp. using a single target DNA in the mixture of three primer sets and three fluorescent probes. When only one *Vibrio* species target was amplified by the multiplex real-time PCR assay, the detection limits were 10^3 CFU/reaction. The amplification efficiencies were 98% for *V. cholerae*, 97% for both *V. parahaemolyticus*, and *V. vulnificus*, resulting in highly accurate standard curves (Figure 2).

Next, simultaneous detection of three *Vibrio* species was attempted using a mixture of DNA from the same three *Vibrio* species. As a consequence, the detection limit decreased by 10-fold (10^4 CFU/reaction) for all



three *Vibrio* spp. However, the amplification efficiency was similar to the 98% achieved with a single target for all three *Vibrio* spp., indicating that this assay effectively quantified each target (Figure 3).

3.2. Application of real-time multiplex PCR as a screening method

The multiplex real-time PCR assay developed in this study was used to screen samples from a pilot surveillance study of marine *Vibrio* in Korea. Of the 2729 marine environmental samples tested by the multiplex real-time PCR assay, 2085 (64.7%) were positive for the *tlh* gene, 621 (19.3%) were positive for the *vhA* gene, and 330 (10.3%) were positive for the *hlyA* gene (Table 3).

Isolation of the three *Vibrio* spp. was attempted from samples that had tested positive in the multiplex real-time PCR assay. The most prominent *Vibrio* species, *V. parahaemolyticus*, was isolated from 1501 (37%) of the 2769 samples, whereas *V. cholerae* and *V. vulnificus* were found in 228 (5.6%) and 180 (4.4%) of the samples, respectively. *V. parahaemolyticus* was the most frequently detected of the three *Vibrio* species using both culture-based methods and multiplex real-time PCR.

The same surveillance project was performed in 2006, during the same seasonal period from May to December, prior to the development of multiplex PCR screening. In that study, *V. parahaemolyticus* was isolated from 1893 (29.3%) of the 5445 samples, followed by *V. cholerae* from 213 (3.3%), and *V. vulnificus* from 106 (1.6%; Table 3).

4. Discussion

With climate change, the threat of huge outbreaks of disease caused by *Vibrio* species such as *V. cholerae* is increasing. Consequently, the importance of surveillance of pathogenic *Vibrio* species present in the environment is also growing [24]. However, isolation of specific *Vibrio* species from the environment is not easy due to the difficulty associated with discriminating between *Vibrio* species on selective media. Additionally, in culture-based methods, colony confirmation is usually carried out using a panel of biochemical tests. In a previous study, O'Hara et al reported that the API 20E identification kit possessed an accuracy >90% for *V. parahaemolyticus* identification, but it was able to

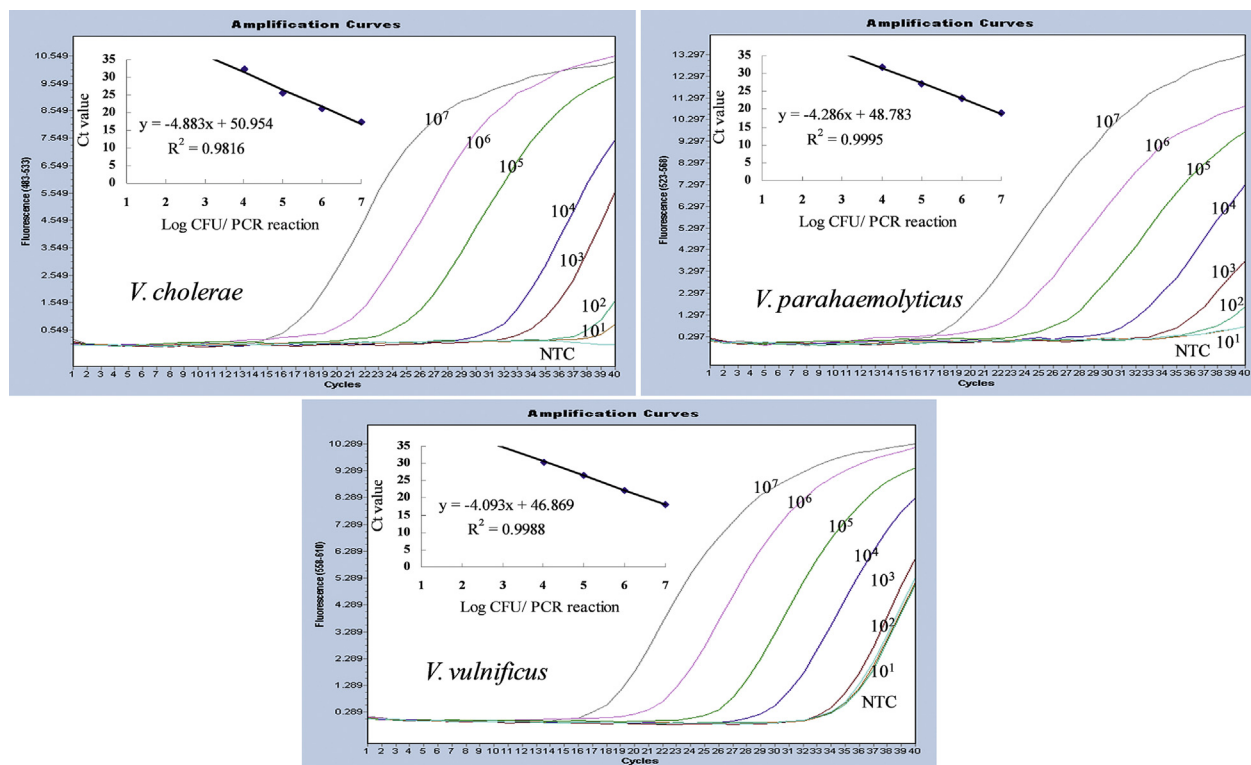


Figure 3. Multiplex real-time polymerase chain reaction (PCR) amplification plots and standard curves of three target genes for *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, respectively. Standard curves were plotted for the log cell number of bacteria versus the number of cycles required to reach C_t. Samples were derived from DNA extracted from 10-fold dilutions of cells at concentrations of 10¹ – 10⁷ colony-forming units/reaction. Although all samples were assayed in duplicate, only a single replicate is displayed for each sample for clarity. The equations of the lines obtained for *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* were $y = -4.883x + 50.954$ ($R^2 = 0.9816$), $y = -4.286x + 48.783$ ($R^2 = 0.9995$), and $y = -4.093x + 46.869$ ($R^2 = 0.9988$), respectively.

Table 3. Test results for the multiplex real-time PCR assay and isolation of *Vibrio* spp. from environmental samples

<i>Vibrio</i> spp.	No. positive (%)		
	2007		2006
	RT-PCR (<i>n</i> = 2,729)	Isolates (<i>n</i> = 2,769)	Isolates (<i>n</i> = 5,445)
<i>V. cholerae</i>	330 (10.3)	228 (5.6)	213 (3.3)
<i>V. parahaemolyticus</i>	2085 (64.7)	1501 (37)	1893 (29.3)
<i>V. vulnificus</i>	621 (19.3)	180 (4.4)	106 (1.6)

Data are presented as *n* (%).

identify only 50% and 60% of *V. cholerae* and *V. vulnificus* strains, respectively [25]. Because commercial bacterial identification systems are used for clinical isolates and a comprehensive evaluation of the ability of these systems to identify bacteria accurately from environmental samples has not been performed, commercial systems may misidentify *Vibrio* spp. as other bacterial species [26–28]. The phenotypic variability of environmental strains and the close relationship among *Vibrio* spp. account for the failure to identify *Vibrio* variants accurately. It has been reported that some *V. cholerae* strains are unable to ferment sucrose [29]. In 2004–2006, the *V. cholerae* variants isolated from four outbreaks in Taiwan were misidentified as *V. mimicus* and *V. alginolyticus* using an API 20E identification kit. However, these strains were correctly identified by several molecular techniques, including PCR [30].

For these reasons, many molecular biological tools have been developed to detect *Vibrio* species more easily, and most of these are PCR-based methods [15,20,31]. These PCR methods are usually employed to detect only one species, and, even when multiplex PCR assays are used to detect multiple species, they are performed using conventional PCR methods, which have limited sensitivity and are time consuming [32,33].

In the present study, we developed and tested a TaqMan probe-based multiplex real-time PCR assay to detect *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* in environmental samples after enrichment. Because this multiplex real-time PCR utilizes three fluorescent probes simultaneously in a single PCR reaction, the speed at which three *Vibrio* spp. can be detected is greatly increased. This assay provides results within approximately 3 hours of enrichment because there is no need for postamplification analysis, such as agarose gel electrophoresis, for confirmation of real-time detection. In addition, the threshold cycles affords a further advantage of semiquantitation.

We applied this multiplex real-time PCR method to screen marine environmental samples and performed culture-based isolation for the PCR-positive samples only. We compared the effectiveness of the combination of this multiplex real-time PCR and culture-based methods (samples collected in 2007) to culture-based methods alone (samples collected in 2006). The

isolation ratios of *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* were higher with the combination method than with the culture-based methods alone. The isolation ratio of *V. vulnificus* using combination methods was 2.75-fold higher than that of culture-based methods alone. The combination methods provided a higher isolation ratio than that of culture-based methods alone because we could neglect the samples that were PCR-negative and focus on which species to isolate from each of the PCR-positive samples.

In conclusion, we developed a multiplex real-time PCR assay for the simultaneous detection of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in a single reaction. The multiplex real-time PCR provides a rapid, sensitive, and highly specific means for the detection of three *Vibrio* spp. from environmental and clinical samples. This technique might be useful to detect the three pathogenic *Vibrio* spp. during mass outbreaks and sporadic cases of vibriosis, as well as in contaminated seafood and wastewater. It could facilitate monitoring of pathogenic *Vibrio* contamination, thereby improving hygiene.

Acknowledgments

This research was supported by a grant from the Korea Centers for Disease Control and Prevention.

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