Research Article

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Multipurpose assessment for the quantification of *Vibrio* spp. and total bacteria in fish and seawater using multiplex real-time polymerase chain reaction

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Abstract

BACKGROUND: This study describes the first multiplex real-time polymerase chain reaction assay developed, as a multipurpose assessment, for the simultaneous quantification of total bacteria and three *Vibrio* spp. (*V. parahaemolyticus*, *V. vulnificus* and *V. anguillarum*) in fish and seawater. The consumption of raw finfish as sushi or sashimi has been increasing the chance of *Vibrio* outbreaks in consumers. Freshness and quality of fishery products also depend on the total bacterial populations present.

RESULTS: The detection sensitivity of the specific targets for the multiplex assay was 1 CFU mL^{-1} in pure culture and seawater, and 10 CFU g^{-1} in fish. While total bacterial counts by the multiplex assay were similar to those obtained by cultural methods, the levels of *Vibrio* detected by the multiplex assay were generally higher than by cultural methods of the same populations. Among the natural samples without *Vibrio* spp. inoculation, eight out of 10 seawater and three out of 20 fish samples were determined to contain *Vibrio* spp.

CONCLUSION: Our data demonstrate that this multiplex assay could be useful for the rapid detection and quantification of *Vibrio* spp. and total bacteria as a multipurpose tool for surveillance of fish and water quality as well as diagnostic method.

Keywords: multiplex real-time PCR; *Vibrio* spp.; total bacteria; fish; seawater; seafood safety

INTRODUCTION

Vibrios are opportunistic human and fish pathogens present in marine and estuarine environments worldwide.[1,2] *Vibrio* infections are usually associated with the consumption of seafood or exposure of wounds to inhabited seawater.[2] The incidence of *Vibrio* infections has increased by 43% between 2006 and 2012 in the US.[3] While consumption of raw oysters has been a major cause of *Vibrio* infections in several countries, such as the US,[4,5] increasing the occasions of eating raw or undercooked finfish has also increased the possibility of *Vibrio* infection and, in some Asian countries, are considerably associated with consumption of finfish.[6] In one case, fish-balls contaminated with *Vibrio parahaemolyticus* caused a food poisoning outbreak in Thailand.[7] In Japan, *sashimi* and *sushi*, raw fish foods, were major causes of infection and occupied 26% and 23% of *V. parahaemolyticus* outbreaks in humans, respectively.[8] In the US, the sushi industry has been growing by 1.6% annually through 2008–2013 and current numbers of sushi restaurants total 4135.[9]

At least 14 species of pathogenic *Vibrio* have been reported. *Vibrio parahaemolyticus* and *V. vulnificus* are the leading cause of illness due to seafood.[2] According to the Centers for Disease Control and Prevention (CDC), 112 cases of *V. parahaemolyticus* and 25 cases of *V. vulnificus* infections occurred in 2012 and constituted 75% of all *Vibrio* spp. illnesses combined.[3] *Vibrio parahaemolyticus* may cause acute gastroenteritis and septicemia; especially in people with liver or immune deficiencies.[10] *V. parahaemolyticus* carries the thermolabile hemolysin (*tlh*) gene which has been used as a species-specific marker for *V. parahaemolyticus*.[11,12] *Vibrio vulnificus* is recognized as a highly invasive bacterium and leads to septicemia in immunocompromized individuals[13,14] as it has the highest mortality rate, greater than 50% for primary septicemia, and has led to approximately 40 deaths from seafood in the US annually.[15] Some virulence genes of *V. vulnificus*, i.e. *vvh*A, *vvp*E, and *rtx*A1, have been reported but their mechanisms of action are not fully understood.[13] *Vibrio anguillarum* causes vibriosis with lethal hemorrhagic septicemia in fish and shellfish that result in substantial economic losses in aquaculture farming worldwide[16] as it has a homologue of *V.*

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cholerae El Tor hemolysin[17] and a virulence factor of *V. cholerae* and *V. vulnificus* such as *vah*1 and *rtx*A, respectively.[18–20]

A rapid method for the detection and quantification of vibrio pathogens is crucial in aquaculture for the detection of infected fish and to monitor seafood quality in marine environments. Although *Vibrionaceae* have generally been detected using selective medium, like thiosulfate citrate bile salts sucrose agar (TCBS),[21–23] this method does not indicate the species of *Vibrio*. Conventional polymerase chain reaction (PCR) assays provide rapid, specific, and sensitive analysis of targeted vibrios, but cannot make quantitative measurements.[24–26] In comparison to conventional PCR, a current real-time PCR method is quantitative, more rapid, and about 100 times more sensitive.[27,28]

High bacterial populations in and on aquaculture products affect their quality, shelf-life,[29] and suggests the possibility of increased numbers of potential human pathogens.[30] The goal of the current study was to develop and evaluate a multiplex real-time PCR assay that would provide a rapid, sensitive, and quantitative method for the detection of *V. anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, and total bacteria in fish fillets and seawater. While there exist methods to detect *V. cholerae, V. parahaemolyticus*, and *V. vulnificus* using multiplex real-time PCR,[31,32] there are no methods to detect *V. anguillarum* and the other *Vibrio* spp. at the same time. Moreover, this study is the first attempt at multiplex real-time PCR to detect and quantify *Vibrio* spp. and total bacteria simultaneously. We describe a four-target multiplex real-time PCR assay using the Taqman system. This assay includes novel primers and probes for species-specific target genes, namely, *tlh* for *V. parahaemolyticus*, *tox*R for *V. anguillarum*, *vvh*A for *V. vulnificus,* and detection of a universal target, 16S rDNA for total bacteria.

MATERIALS AND METHODS

Bacterial cultures and DNA template preparation

For the optimization of multiplex real-time PCR and generation of standard curves, *V. anguillarum* HB155721 (Carolina Biological Supply Co., Burlington, NC, USA), *V. parahaemolyticus* O1:Kuk, and *V. vulnificus* MLT1009 (U.S. Department of Agriculture – Agricultural Research Service, Dover, DE; USDA-ARS) were used. Mixed bacteria from sea bass were used to generate a total bacterial sample, which was prepared by overnight culture at 29°C in tryptic soy broth (TSB; Carolina Biological Supply Co.) with shaking (130 rpm). A 1:50 dilution of *V. anguillarum* and a 1:100 dilution of *V. vulnificus* were maintained in TSB supplemented with additional 20 g L^{-1} NaCl (TSB+) and incubated for 4–5 h at 29°C with shaking (130 rpm) to achieve an early-exponential phase culture. A 1:100 dilution of *V. parahaemolyticus* overnight culture was incubated for 2–3 h under the same conditions as the other *Vibrio* species. A 1:100 dilution of total bacteria was cultured for 4–5 h at 29°C in TSB. *Vibrio* cultures in 8.5 g L^{-1} saline buffer were spread at ten-fold dilutions on tryptic soy agar supplemented with 20 g L^{-1} NaCl (TSA+) and incubated for 24 h at 29°C. Decimal diluted total bacterial culture was plated on TSA and cultured for 3 days at 29°C to determine the CFU mL^{-1} of each strain.

Approximately 1×10^7 CFU mL^{-1} of diluted bacterial cultures (1 mL) were centrifuged for 5 min at $10\,000 \times g$ and the supernatant was discarded. The pellets were suspended in 400 μL of TZ buffer (20 g L^{-1} Triton X-100 and 2.5 mg mL^{-1} sodium azide in 0.1 mol L^{-1} Tris-HCl, pH 8.0) and boiled for 10 min.[30,33] Cell debris were discarded by centrifugation for 5 min at $10\,000 \times g$ and supernatants containing crude DNA extract were transferred to a fresh tube. Twenty microliters of Quick-Precip Plus Solution (EdgeBio, Gaithersburg, MD, USA) and 800 µL of absolute ethanol (Fisher Scientific, Fair Lawn, NJ, USA) were added to the supernatants to precipitate genomic DNA. After centrifugation (5 min, $13\,000 \times g$), the pellets were washed with 700 mL L^{-1} ethanol and dried at room temperature. The pelleted DNA was dissolved in nuclease-free water.

Design and evaluation of primers and probes for multiplex real-time PCR

At least six ORF sequences of each species-specific target gene, *tlh*, *tox*R and *vvh*A, were obtained from GenBank and aligned using the Clustal W program. To detect total bacteria, universal primers and a probe were designed using the conserved regions of 16S rDNA following the alignment of 32 sequences from a host of bacteria based on *Bergey's Manual of Determinative Bacteriology*.[34] The 16S rDNA sequences (GenBank accession numbers in parentheses) were aligned using Clustal W for the following strains arranged by differentiation via Gram stains and cell morphology:

- Gram-positive cocci: *Staphylococcus* (GQ911564.1), *Micrococcus* (JQ726627.1), *Peptococcus* (AB644260.1), *Streptococcus* (EU156766.1), *Peptostreptococcus* (AY359243.2), *Enterococcus* (AB681177.1)
- Gram-positive bacilli: *Listeria* (JF967624.1), *Erysipelothrix* (AB685261.1), *Bacillus* (JQ905096.1), *Clostridium* (GQ911558.1), *Streptomyces* (AB184358.1), *Lactobacillus* (AB680529.1), *Eggerthella* (HQ455039.1)
- Gram-negative cocci: *Veillonella* (HM007566.1)
- Gram-negative bacilli: *Acinetobacter* (EF672504.1), *Brucella* (JN571438.1), *Bordetella* (AB682670.1), *Bacteroides* (HE608159.1), *Haemophilus* (EU909679.1), *Pasteurella* (HE800437.1), *Francisella* (JQ277265.1), *Eikenella* (JN713283.1), *Cardiobacterium* (M35014.1), *Streptobacillus* (JQ087393.1), *Vibrio* (AY292952.1), *Campylobacter* (GQ167677.1), *Aeromonas* (JQ781582.1), *Alcaligenes* (FJ151629.1), *Chromobacterium* (FJ753567.1), *Escherichia coli* (JQ904752.1), and *Salmonella* (JQ074173.1).

The Primer3 program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) was used to design oligonucleotide primers and TaqMan probes targeting highly conserved regions of each genes. The PriDimerChecking program was used to check dimerization among primers and probe and the species-specific primers and probes were validated for high specificity by Blast analysis (NCBI). The primer and probe sequences are listed in Table 1.

Optimization of multiplex real-time PCR

Primers, probe concentrations, and the real-time PCR protocol including annealing temperatures, reaction times, and cycling numbers were optimized for the multiplex detection and quantification of the 16S rDNA, *tlh*, *tox*R, and *vvh*A genes using the StepOnePlus real-time PCR platform (Applied Biosystems, Foster City, CA, USA). For comparison of PCR performance, the real-time PCR was conducted using three types of commercial real-time PCR master mixes. Those were IQ Multiplex Powermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), Express qPCR Supermix Universal (Invitrogen, Carlsbad, CA, USA), and Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan) and they were randomly represented as master mix A, B and C, respectively. The optimal reaction mixture components for a 25 µL volume were 12.5 µL of 2X master mix, 800 nmol L^{-1} each of the *tox*R forward and reverse primers and probe; 200 nmol L^{-1} each of the 16S rDNA forward and reverse primers

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Table 1. Primer and probe sequences used in this study

Name (accession #)	Start	Sequence	Modification
16S-F (JQ904752.1)[a]	226	5′-cccagatgggattagcttgt-3′	–
16S-R	331	5′-tctggaccgtgtctcagttc-3′	–
16S-P	270	5′-cgacgatccctagctggtctgaga-3′	5′ ROX to 3′ BHQ2
tlh-F (GU971655.1)	237	5′-ctactggtggagctccgttt-3′	–
tlh-R	354	5′-cgtaatgtctgcgttctcgt-3′	–
tlh-P	277	5′-accaacacgtcgccaaacgttatc-3′	5′ JOE to 3′ BHQ1
toxR-F (AJ299739.1)	162	5′-acactgcaaagcaaattgatg-3′	–
toxR-R	295	5′-tgatgggcgtattcacaact-3′	–
toxR-P	212	5′-tggctcttctattgactagccctgca-3′	5′ TAMRA to 3′ BHQ2
vvhA-F (JF718659.1)	273	5′-ccaagtttggggcctagata-3′	–
vvhA-R	365	5′-actgtgagcgttttgtctgc-3′	–
vvhA-P	315	5′-agtggcatccgatcgttgtttgac-3′	5′ FAM to 3′ BHQ1

[a] This accession number represents 32 alignment species to design 16S rDNA primers.

and probe; and 50 nmol L^{-1} each of the *tlh* and *vvh*A forward and reverse primers and probe. The remainder of the reaction volume consisted of DNA template and nuclease-free water. The optimal cycling parameters consisted of a 95°C initial denaturation hold for 2 min followed by 35 cycles of amplification, with each cycle consisting of denaturation at 95°C for 15 s and a combined annealing/extension step at 60°C for 50 s. For negative control, nuclease-free water was added to the reaction mixture as a template for every real-time PCR assay and fluorescence was read at the end of each amplification cycle.

Generation of standard curves

For the determination of detection and quantitative capabilities from fish and seawater samples, standard curves of the four target genes (*16S, tlh, tox*R, and *vvh*A) were generated during multiplex real-time PCR amplification. DNA templates of each strain were prepared from a ten-fold dilution series of three *Vibrio* spp. and total bacteria in 8.5 g L^{-1} saline buffer. The dilution series for the PCR assay were run in triplicate for three independent experiments. The standard curves were determined by plotting the Ct values against the log CFU reaction^{-1} and the slope was generated by linear regression of plotted points.

Specificity and sensitivity test

The multiplex real-time PCR assay was tested for specificity against a panel of 37 bacterial strains including 11 *Vibrio* spp., three *Bacillus* spp., two *E. coli* strains, three *Pseudomonas* spp., and 18 other strains (Table 2). These strains were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), Carolina Biological Supply Co., the USDA-ARS, and the previous study.[35] The sensitivity of the multiplex assay from artificially inoculated seawater and fish samples was tested from log 5 to log 0 CFU mL^{-1} as well as from log 3 to log 0 CFU g^{-1}, respectively.

Fish sample collection and processing

In total, 30 fish were used for this study. Ten fish fillet samples were purchased from local retail or fresh fish markets in Dover, Delaware, USA. Samples consisted of sea bass (*Centropristis striata*), cod fish (*Gadus morhua*), flounder (*Paralichthys*), and haddock (*Melanogrammus aeglefinus*). The fish fillets were cut into small pieces and 25 g were put into stomacher bags (Interscience, St. Nom, France). A low level (Log 2 CFU g^{-1}) of each *Vibrio* culture in PBS was added to fish samples and were maintained for 1 h in the refrigerator (4°C). In the stomacher bags, 75 mL of PBS was added and the fish samples were homogenized using a Bag Mixer (Interscience) for 10 min. Ten milliliters of each homogenized fish fillet were centrifuged at 150 × *g* for 5 min to remove larger pieces. Each supernatant was re-centrifuged at 5000 × *g* for 10 min and the supernatants were discarded. The pellets were resuspended in 1 mL of PBS and used for quantitative detection of *Vibrio* spp. and total bacteria by the plate count method and by multiplex real-time PCR.

In addition, 20 fish fillets were obtained from aquaculture facility at Delaware State University (DSU) and local retail sources in Dover, Delaware, USA. The fillets used in this study were: sea bass (*Centropristis striata*), cod fish (*Gadus morhua*), flounder (*Paralichthys*), haddock (*Melanogrammus aeglefinus*), hybrid striped bass (*Morone Chrysops* × *Morone saxatilis*), mummichog (*Fundulus heteroclitus*), and tilapia (*Oreochromis mossambicus*). The processing was the same as above excluding the inoculation of *Vibrio* spp.

Seawater collection and processing

A total of 15 different seawater samples were collected from February to November of 2013. Artificial seawater was made according to instructions of the manufacturer (Instant Ocean sea salt; Aquarium System Inc., Blacksburg, VA, USA) and autoclaved at 121°C for 17 min. Fourteen seawater samples were collected at different locations and on different days. Four Atlantic Ocean water samples were collected from the Indian River inlet, Delaware. Four Delaware Bay seawater samples were collected from Bowers beach, Delaware. Six aquaculture seawater samples were collected from the aquaculture facility at DSU. Within 24 h of collection, water samples were vacuum filtered through Whatman #40 filter paper (Whatman, Buckinghamshire, UK) to remove algae, debris, and grit. One artificial seawater and 4 natural seawater filtrates were artificially inoculated with low levels (Log 2 CFU mL^{-1}) of *Vibrio* cultures (*V. anguillarum*, *V. parahaemolyticus*, and *V. vulnificus* in phosphate-buffered saline; PBS) and maintained for 1 h at room temperature. Both of the five *Vibrio*-inoculated and 10 non-inoculated seawater were used for quantitative detection of *Vibrio* spp. and total bacteria by the plate count method and multiplex real-time PCR.

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Table 2. Bacterial strains used to test the specificity of multiplex real-time polymerase chain reaction (PCR)

Species	Strain	Source	Real-time PCR results (+/–)			
			16S	*tlh*	*tox*R	*vvh*A
Vibrio anguillarum	HB155721	Carolina	+	–	+	–
Vibrio anguillarum	–	USDA-ARS	+	–	+	–
Vibrio cholerae 01	–	USDA-ARS	+	–	–	–
Vibrio fischeri	–	USDA-ARS	+	–	–	–
Vibrio harveyi	–	USDA-ARS	+	–	–	–
Vibrio mimicus	ATCC 33654	ATCC	+	–	–	–
Vibrio parahaemolyticus O1:Kuk	–	USDA-ARS	+	+	–	–
Vibrio parahaemolyticus O3:K6	–	USDA-ARS	+	+	–	–
Vibrio vulnificus	15	USDA-ARS	+	–	–	+
Vibrio vulnificus	MLT 367	USDA-ARS	+	–	–	+
Vibrio vulnificus	MLT1009	USDA-ARS	+	–	–	+
Aeromonas sobria	D36	Hickey *et al.*[35]	+	–	–	–
Bacillus cereus	# 5056	USDA-ARS	+	–	–	–
Bacillus cereus	HB154870	Carolina	+	–	–	–
Bacillus subtilis	HB154921	Carolina	+	–	–	–
Clostridium butyricum	HB154965	Carolina	+	–	–	–
Enterococcus faecalis	HB155600	Carolina	+	–	–	–
Escherichia coli DH5a	ATCC67876	ATCC	+	–	–	–
Escherichia coli O157:H7	–	USDA-ARS	+	–	–	–
Lactococcus lactis	HB155610	Carolina	+	–	–	–
Photobacterium kishitanni	–	USDA-ARS	+	–	–	–
Pseudomonas aeruginosa	ATCC 10145	ATCC	+	–	–	–
Pseudomonas fluorescens	ATCC 13525	ATCC	+	–	–	–
Pseudomonas putida	155265	Carolina	+	–	–	–
Salmonella enteritidis	HB155350A	Carolina	+	–	–	–
Salmonella enteritidis	SA 36	USDA-ARS	+	–	–	–
Salmonella heidelberg	SA 42	USDA-ARS	+	–	–	–
Salmonella montevideo	SA 40	USDA-ARS	+	–	–	–
Salmonella newport	SA 41	USDA-ARS	+	–	–	–
Salmonella typhimurium	HB155351A	Carolina	+	–	–	–
Salmonella typhimurium	SA 34	USDA-ARS	+	–	–	–
Serratia marcascens	SM51	USDA-ARS	+	–	–	–
Shewanella algae	ATCC 51192	ATCC	+	–	–	–
Shewanella baltica	NCTC 10735	USDA-ARS	+	–	–	–
Shewanella baltica	D10	Hickey *et al.*[35]	+	–	–	–
Staphylococcus aureus	ATCC 12598	ATCC	+	–	–	–
Staphylococcus aureus	HB155554A	Carolina	+	–	–	–

ATCC, American Type Culture Collection; USDA-ARS, US Department of Agriculture – Agricultural Research Service.

Quantification of *Vibrio* spp. and total bacteria from the samples by culture-dependent bacterial enumeration

The processed seawater and fish samples were serially diluted ten-fold in PBS and spread on TCBS (Difco, Sparks, MD, USA) and TSA plates. The TCBS plates were incubated for 24 h at 29°C and the TSA plates were cultured for 3 days at 29°C. The colonies on the TCBS plates were enumerated to quantify *Vibrio* spp. and those on the TSA were counted to quantify total bacteria.

Quantification of *Vibrio* spp. and total bacteria using multiplex real-time PCR

The processed water samples were vacuum filtered through polyethersulfone membrane filters (0.22 μm pore size, 25 mm diameter; Whatman) and the filters were put into microcentrifuge tubes to extract bacterial DNA. Fish samples described above were centrifuged at 13 000 × *g* for 5 min and the pellets were used for template DNA preparation. Two hundred microliters of 2 × TZ buffer was added to microcentrifuge tubes containing a filter from the water samples or resuspended pellets from the fish samples. Six percent Chelex solution (Bio-Rad) was added and the microcentrifuge tubes were vortexed for 2 min. The tubes were boiled for 10 min, centrifuged at 10 000 × *g* for 5 min, and the supernatants were retained. The following precipitation processes of genomic DNA were the same as the DNA template preparation from the pure culture and the multiplex real-time PCR assay were decribed above. Quantification of *Vibrio* spp. and total bacteria were evaluated using the strandard curves as previously described.

Statistical analysis

The *T*-test was used to determine statistically significant differences between the Ct values resulted from multiplex real-time PCR

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assays for PCR optimization. The SPSS 12.0K for windows software (SPSS Inc., Chicago, IL, USA) was used to explore the statistical significance of results obtained. A confidence interval at the 95% level ($P < 0.05$) was considered in all cases.

RESULTS

Optimization of the PCR assay

Blast analyses showed that the three specific TaqMan probes and pairs of primers designed in this study were highly specific for detection and quantification of *Vibrio* spp. This study assessed three different manufacturers' PCR master mixes for optimization of multiplex real-time PCR. Among them, 16S target (ROX dye) was not consistently detected in the PCR mixture using master mix C, whereas, master mix B showed lower Ct values compared with master mix A (Table 3). Therefore, master mix B was chosen for this multiplex assay.

Primer and probe concentrations were optimized for the detection of low numbers of bacteria as well as maximization of primer and probe efficiencies. While the Ct value of the 16S rDNA gene was higher with reduced primer and probe concentrations, the Ct values of *tlh* and *vvh*A were not significantly different even with lower concentrations of primer and probe in the multiplex assay (Table 3). Therefore, the concentration of 16S primers and probe was fixed at 200 nM and primer and probe concentrations of *tlh* and *vvh*A were reduced to 50 nM (Table 3). *Tox*R was best detected with primers and probe at 800 nM (Table 3). The relative fluorescence curve of *tox*R was retarded at primer and probe concentrations up to 400 nM (Fig. 1A). As shown in Table 3, *tox*R amplification signal was influenced by the ratio of final concentration of *tlh* and *tox*R primers and probes. When the molar ratios of *tlh* and *tox*R were higher than 1:4 (*tlh*:*tox*R), *tox*R was not detected. In addition, the lower ratio of *tlh*/*tox*R (1:16) gave the lowest Ct value for *tox*R targets (Table 3).

To optimize the annealing/extension temperature, we performed temperature gradient assays from 56 to 64°C. The lowest temperature used for 16S, *tlh* and *vvh*A targets (56°C) produced the lowest Ct values (Table 4). On the other hand, *tox*R targets were lowest at 60°C in the PCR mixture using master mix B. Additionally, the amplification curves of *tox*R were not suppressed at 60 and 62°C when master mix B was used (Fig. 1B).

The real-time PCR reaction time was optimized for rapid detection. The Ct values of all four targets showed no significant differences ($P > 0.05$) with denaturation for 15–20 s and annealing/extension for 50 s to 1 min. Ct values with combined annealing/extension for 40 s were the least desirable with the highest Ct values detected (16S, 24.94 ± 0.33; *tlh*, 19.66 ± 0.37; *tox*R, not detected; *vvh*A, 24.80 ± 0.18, used master mix B). The optimal conditions for the multiplex real-time PCR in a 25 µL reaction volume requires the following: 800 nmol L^{-1} of each primer and probe for *toxR*, 200 nmol L^{-1} of each of primer and probe for the 16S rDNA, and 50 $nmol^{-1}$ of each primers and probe for *tlh* and *vvh*A forward. The optimal cycling parameters consisted of a 95°C hold for 2 min for the initial denaturation followed by 40 cycles of DNA amplification, for each cycle followed by denaturation at 95°C for 15 s and a combined annealing/extension step at 60°C for 50 s.

Standard curve

In order to verify the sensitivity of the method and the consistency of quantitative measurement, DNA was prepared from a 10-fold diluted pure cultures of *Vibrio* spp. and in total bacteria to be

Table 3. Ct values of four gene targets (*16S*, *tlh*, *toxR* and *vvhA*) obtained from different primers and probe concentrations by multiplex real-time polymerase chain reaction (PCR)

Primer and probe conc. (nmol L^{-1})				Ratio,	*16S*			*tlh*			*toxR*			*vvhA*		
16S	*tlh*	*toxR*	*vvhA*	*tlh*/*toxR*	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C
200	200	200	200	1/1	22.09 ± 0.21[a]	20.06 ± 0.08	ND[b]	17.18 ± 0.23	19.00 ± 0.06	18.45 ± 0.18	ND	ND	ND	16.99 ± 0.62	18.68 ± 0.10	19.52 ± 0.08
200	200	200	100	1/1	22.19 ± 0.48	20.13 ± 0.32	ND	17.48 ± 0.28	18.90 ± 0.24	18.60 ± 0.40	ND	ND	ND	17.87 ± 0.25	18.48 ± 0.20	19.48 ± 0.08
200	200	200	50	1/1	22.54 ± 0.50	19.63 ± 0.15	ND	17.25 ± 0.02	19.01 ± 0.01	18.73 ± 0.21	ND	ND	ND	19.62 ± 0.36	19.10 ± 0.65	19.42 ± 0.03
200	100	200	50	1/2	22.34 ± 0.13	19.83 ± 0.42	ND	17.77 ± 0.13	18.81 ± 0.24	19.17 ± 0.04	ND	ND	ND	19.67 ± 0.04	18.51 ± 0.23	19.59 ± 0.19
200	50	200	50	1/4	22.50 ± 0.05	19.53 ± 0.4	ND	21.10 ± 0.21	18.87 ± 0.11	19.40 ± 0.28	34.97 ± 0.4	16.24 ± 0.08	18.88 ± 0.38	19.24 ± 0.2	18.45 ± 0.03	19.29 ± 0.34
200	50	400	50	1/8	22.63 ± 0.09	19.66 ± 0.11	ND	20.45 ± 0.34	18.99 ± 0.18	19.46 ± 0.43	16.85 ± 0.03	16.09 ± 0.16	18.58 ± 0.09	20.19 ± 0.03	18.53 ± 0.05	19.31 ± 0.20
200	50	800	50	1/16	22.57 ± 0.35	19.59 ± 0.21	ND	20.24 ± 0.36	19.07 ± 0.10	19.47 ± 0.25	16.42 ± 0.34	15.86 ± 0.13	18.52 ± 0.28	20.20 ± 0.27	18.64 ± 0.11	19.40 ± 0.04

[a] Mean ± standard deviation.
[b] No amplicon detected.
MM, Master mix.

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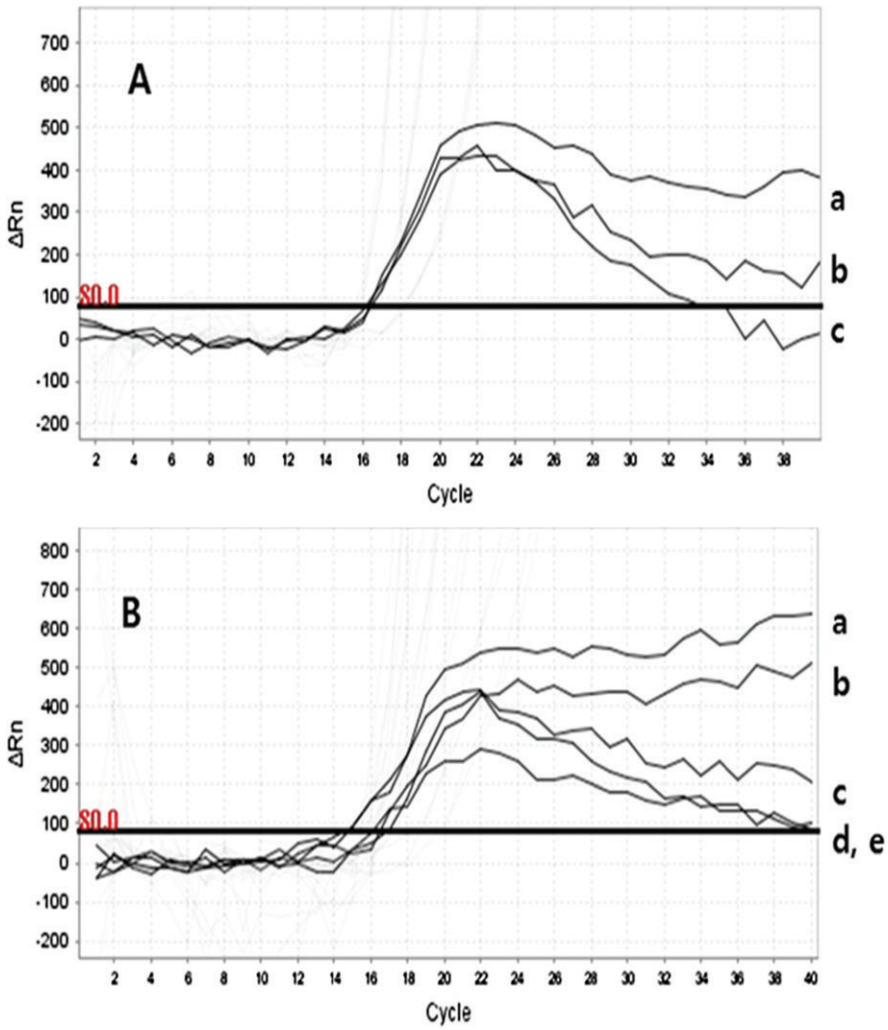

Figure 1. Multiplex real-time PCR amplification curves of a *tox*R target obtained from (A) different primers and probe concentration, (curve a) 800 nM, (curve b) 400 nM, and (curve c) 200 nM of *tox*R primers and probe; and (B) different annealing and extension temperature.; annealing and extension temperatures at (curve a) 60°C, (curve b) 62°C, (curve c) 58°C, and (curves d and e) 56 and 64°C.

analyzed using the multiplex real-time PCR. The three standard curves of specific targets obtained from each *Vibrio* strain in the range of log 0 to log 5 CFU per PCR assay and the standard curve of 16S was generated in the range of log 0.6 to log 5.6 CFU per reaction (Fig. 2). The standard curves of specific targets showed excellent correlations of linear regression with r^2 coefficients: *tlh*, *tox*R, and *vvh*A were 0.990, 0.997, and 0.993, respectively, from three independently performed experiments and the coefficient for the 16S standard curve was 0.978.

Specificity and sensitivity of PCR

The multiplex real-time PCR assay was tested for specificity against 37 bacteria (Table 2). *Vibrio anguillarum* was positive for *tox*R and 16S but negative for the other specific targets and *V. parahaemolyticus* generated Ct values for only *tlh* and 16S. Additionally, *V. vulnificus* showed positive results for *vvh*A and 16S and negative for the two other targets. No positive amplification signals were obtained with any of the other species for *tlh*, *tox*R and *vvh*A targets but all other strains were positive for a 16S target.

This multiplex assay detected 1 CFU mL^{-1} of pure cultured *V. anguillarum*, *V. parahaemolyticus* and *V. vulnificus* and 4 CFU mL^{-1} of total bacteria. Also, the detection limits of *Vibrio* spp. were 1 CFU mL^{-1} in seawater and 10 CFU g^{-1} in fish and sensitivities of total bacteria were 4 CFU mL^{-1} in seawater and 40 CFU g^{-1} in fish.

Quantification of *Vibrio* spp. and total bacteria in fish and seawater

Multiplex real-time PCR was applied to a total of 30 fish and 15 water samples, 10 *Vibrio* spp. inoculated fish and five water samples, and 10 non-inoculated natural water and 20 fish samples. The results were compared with bacterial counts by a plate count method. Overall, for seawater and fish samples, total bacterial counts were similar between TSA plates and 16S real-time PCR, whereas *Vibrio* counts on TCBS were comparatively lower than the total *Vibrio* spp. counts for the three targets as determined by multiplex real-time PCR.

Total bacteria and *Vibrio* spp. were detected in all *Vibrio* spp. inoculated samples using both plate count and multiplex real-time PCR methods. Fish I-e (6.57 ± 0.06 vs. 6.61 ± 0.07 Log CFU g^{-1}; TSA vs. 16S PCR) and seawater I-b (3.54 ± 0.16 vs. 3.69 ± 0.04 Log CFU mL^{-1}; TSA vs. 16S PCR) showed the closest similarity among total bacteria counts between the two methods (Table 5). The sum of three *Vibrio* spp. counts detected by real-time PCR were higher than the number of colonies on TCBS. However *Vibrio* counts of

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Table 4. Ct values of four gene targets (*16S*, *tlh*, *tox*R and *vvh*A) obtained from different annealing/extension temperature by multiplex real-time polymerase chain reaction (PCR)

	16S			*tlh*			*toxR*			*vvhA*		
Annealing temp (°C)	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C	MM-A	MM-B	MM-C
56	21.01 ± 0.32[a]	18.49 ± 0.35	ND[b]	19.33 ± 0.21	18.52 ± 0.52	17.99 ± 0.43	ND	16.64 ± 0.03	18.34 ± 0.18	20.31 ± 0.19	18.16 ± 0.06	18.70 ± 0.07
58	21.24 ± 0.11	19.50 ± 0.17	ND	19.82 ± 0.04	19.07 ± 0.04	17.68 ± 0.11	ND	16.96 ± 0.33	19.29 ± 0.21	20.63 ± 0.28	18.45 ± 0.08	18.59 ± 0.03
60	21.33 ± 0.39	20.51 ± 0.22	ND	20.43 ± 0.02	18.90 ± 0.24	18.32 ± 0.51	16.67 ± 0.19	15.85 ± 0.12	17.98 ± 0.01	20.82 ± 0.01	18.94 ± 0.21	18.85 ± 0.2
62	23.16 ± 0.10	22.14 ± 0.06	ND	20.66 ± 0.11	20.24 ± 0.14	19.63 ± 0.69	18.28 ± 0.32	16.75 ± 0.07	19.59 ± 0.47	21.09 ± 0.00	19.06 ± 0.04	19.34 ± 0.14
64	23.38 ± 0.03	23.09 ± 1.01	ND	20.97 ± 0.08	21.96 ± 0.25	23.46 ± 0.47	18.53 ± 0.17	17.47 ± 0.25	19.74 ± 0.06	21.07 ± 0.05	19.15 ± 0.10	22.36 ± 0.16

[a] Mean ± standard deviation.
[b] No amplicon detected.
MM, Master mix.

fish I-a were exceptionally higher on TCBS (4.33±0.09 Log CFU g^{-1}) than real-time PCR (Table 5).

In the natural samples without *Vibrio* spp. inoculation, closest data between the two methods were fish 3 (6.21 ± 0.19 vs. 6.20 ± 0.02 Log CFU g^{-1}; TSA vs. 16S PCR), fish 17 (3.61 ± 0.09 vs. 3.60 ± 0.21 Log CFU g^{-1}; TSA vs. 16S PCR), and seawater sample 3 (2.95 ± 0.07 vs. 2.92 ± 0.03 Log CFU mL^{-1}; TSA vs. 16S PCR, Table 6). While *Vibrio* spp. was detected in fish 3, 13, 15, and seawater 3 through 10 from the multiplex real-time PCR assay, *Vibrio* spp. also was detected in fish 1, 5, 8, and seawater 3, 4 and 9 by the culture-dependent method. At the species level, *V. parahaemolyticus* was detected from seawater 4 and 9, *V. anguillarum* was possessed in seawater 3, and *V. vulnificus* was quantified in fish 3, 13, 15, seawater 4–10 (Table 6).

DISCUSSION

This study presents a rapid and reliable method for the simultaneous quantification of pathogenic *Vibrio* spp. and total bacteria using a multiplex real-time PCR assay. Rapid detection and quantification of *Vibrio* spp. becomes more crucial in the US due to the increase of *Vibrio* spp. infection from seafood.[3] Also, the management of the number of total bacteria is very important to maintain the quality of seafood for both of seafood manufacturer and consumers. The enumeration of specific *Vibrio* spp. in seafood or the environment is more challengeable when they exist at low levels.

We tested three different manufacturer's real-time PCR master mix for efficient multiplex assays and one of the master mixes could not detect a 16S target. While a 16S target was detected by ROX fluorescence dye in this study, ROX was assigned to a passive reference dye as a default value in the StepOne Plus real-time PCR system. Though we manually operated the ROX channel as a reporter dye, the system could not detect the 16S target with a probe labeled ROX dye in master mix C PCR mixture, but could detect the 16S target in the PCR mixture using the other two master mixes.

Cross-talk between the fluorescence dyes having close emission wavelengths is a concern in a multiplex real-time PCR assay. In this study, the amplification signal of a *tox*R target represented by a TAMRA reporter dye was interfered by *tlh* and 16S having reporter dyes JOE and ROX, especially *tlh* (JOE) signal strongly suppressed the amplification curve of *tox*R. This could be explained by the emission wavelengths of the two fluorescence dyes being quite proximate as the wavelength of JOE is about 548 nm and that of TAMRA is 576 nm.[36] In addition, the relative fluorescence intensity of JOE was 10 times higher than that of TAMRA (data not shown), when the same concentration of primer and probe were used. In order to reduce this signal interference, the ratio of primer and probe concentrations were optimized and we demonstrated that the limiting of the concentration of *tlh* primer and probe permitted the detection of a *toxR* target. Consequently, there was no signal interference at the concentration ratio of 1:16 (*tlh*:*tox*R).

The three probe and primer sets for the detection of the three *Vibrio* spp. were designed to detect species-specifically and to avoid the amplification of other *Vibrio* spp. The target genes, *tlh* and *vvh*A, for detection of *V. parahaemolyticus* and *V. vulnificus* were clarified by their species-specificity in many previous reports.[11,12,31,37,38] The *Tox*R gene used for detection of *V. anguillarum* was verified using the universal presence in *V. anguillarum* but not detected in various other genus and species including 34 *Vibrio* spp.[39] In this study, the specific targets were successfully amplified without any false positives. Universal primer and probe

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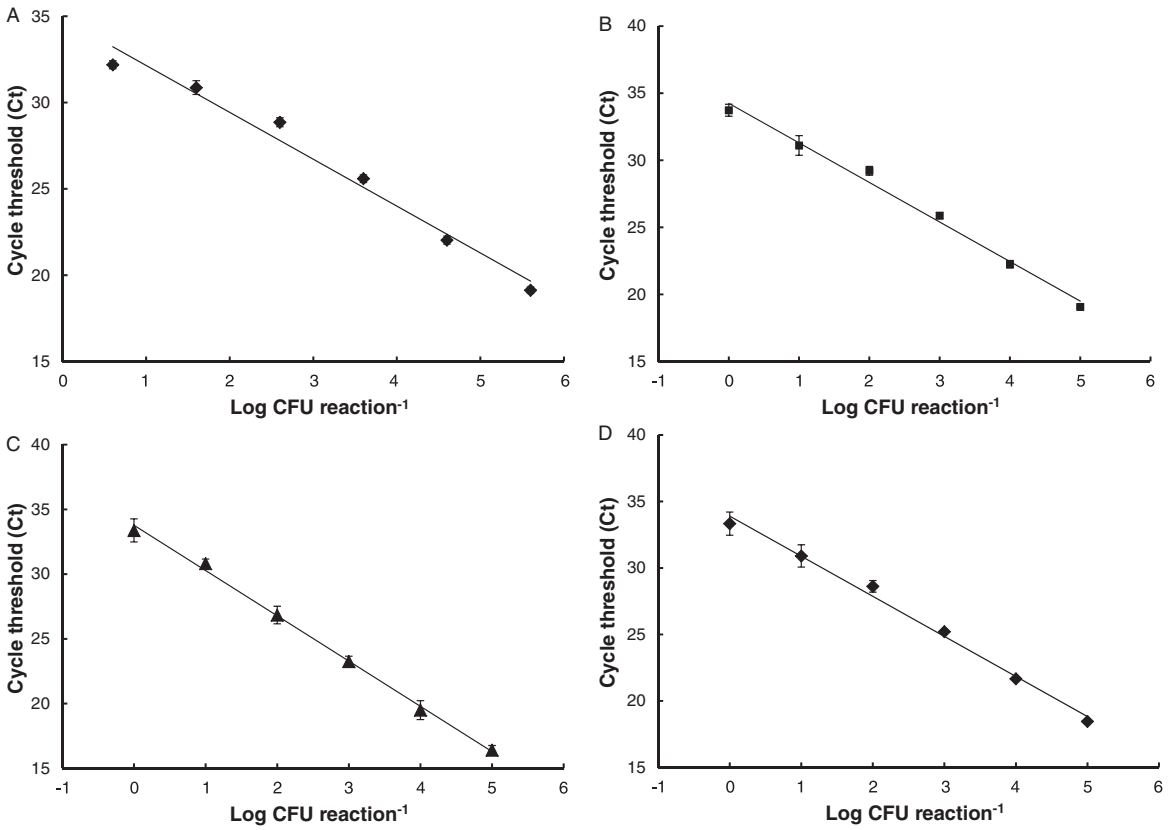

Figure 2. Standard curves of four targets: (A) 16S, (B) *tlh*, (C) *tox*R, and (D) *vvh*A for quantification, plotting Ct values and Log CFU. Pure cultured *Vibrio anguillarum*, *V. parahaemolyticus*, and *V. vulnificus* were used ranges from Log 0 CFU to Log 5 CFU and total bacteria was used ranged from Log 0.6 CFU to Log 5.6 CFU.

Table 5. Quantification of *Vibrio* spp. and total bacteria in *Vibrio* spp. inoculated seawater and fish samples by culture methods and multiplex real-time polymerase chain reaction (PCR) assay

	Total bacteria (Log CFU ml^{-1} or g^{-1})[a]		*Vibrio* spp. (Log CFU ml^{-1} or g^{-1})			
	Culture plate	Real-time PCR	Culture plate	Real-time PCR		
Sample	TSA	*16S*	TCBS	*tlh*	*tox*R	*vvh*A
Fish I-a	7.90 ± 0.05[b]	8.21 ± 0.18	4.33 ± 0.09	2.98 ± 0.07	2.90 ± 0.08	2.75 ± 0.07
Fish I-b	5.85 ± 0.21	5.93 ± 0.03	1.98 ± 0.03	2.21 ± 0.05	2.13 ± 0.11	1.95 ± 0.08
Fish I-c	6.06 ± 0.15	6.13 ± 0.09	1.59 ± 0.16	2.13 ± 0.30	1.85 ± 0.01	2.86 ± 0.04
Fish I-d	8.55 ± 0.09	8.29 ± 0.05	2.61 ± 0.32	2.88 ± 0.06	2.64 ± 0.10	2.78 ± 0.08
Fish I-e	6.57 ± 0.06	6.61 ± 0.07	1.39 ± 0.12	2.00 ± 0.03	1.84 ± 0.44	2.71 ± 0.03
Fish I-f	6.61 ± 0.09	6.44 ± 0.18	2.70 ± 0.00	2.22 ± 0.02	2.56 ± 0.37	2.05 ± 0.19
Fish I-g	6.76 ± 0.14	6.92 ± 0.01	2.00 ± 0.00	2.99 ± 0.01	2.99 ± 0.03	2.42 ± 0.01
Fish I-h	6.93 ± 0.05	7.76 ± 0.02	2.14 ± 0.34	2.45 ± 0.01	2.53 ± 0.09	2.61 ± 0.08
Fish I-i	5.50 ± 0.14	6.17 ± 0.09	1.93 ± 0.04	2.02 ± 0.26	2.65 ± 0.23	1.93 ± 0.08
Fish I-j	5.65 ± 0.07	5.89 ± 0.08	1.89 ± 0.16	2.55 ± 0.03	1.82 ± 0.04	2.15 ± 0.09
Artificial seawater	2.47 ± 0.20	2.33 ± 0.04	2.02 ± 0.03	1.96 ± 0.20	1.49 ± 0.04	2.14 ± 0.01
Seawater I-a	4.20 ± 0.10	4.56 ± 0.03	1.83 ± 0.16	2.66 ± 0.24	1.07 ± 0.34	2.96 ± 0.04
Seawater I-b	3.54 ± 0.16	3.69 ± 0.04	2.52 ± 0.06	2.59 ± 0.13	2.98 ± 0.09	2.78 ± 0.03
Seawater I-c	3.43 ± 0.24	3.65 ± 0.13	2.07 ± 0.16	2.59 ± 0.13	2.56 ± 0.20	2.23 ± 0.00
Seawater I-d	4.65 ± 0.07	4.48 ± 0.09	2.15 ± 0.32	2.91 ± 0.02	1.97 ± 0.19	2.08 ± 0.06

[a] Fish, Log CFU g^{-1}; seawater, Log CFU ml^{-1}.
[b] Mean ± standard deviation.
TCBS, thiosulfate citrate bile salts sucrose; TSA, tryptic soy agar.

Table 6. Quantification of *Vibrio* spp. and total bacteria in natural seawater and fish samples by culture methods and multiplex real-time polymerase chain reaction (PCR) assay

	Total bacteria (Log CFU ml^{-1} or g^{-1})[a]		*Vibrio* spp. (Log CFU ml^{-1} or g^{-1})			
	Culture plate	Real-time PCR	Culture plate	Real-time PCR		
Sample	TSA	*16S*	TCBS	*tlh*	*tox*R	*vvh*A
Fish 1	7.81 ± 0.05[b]	8.30 ± 0.01	3.64 ± 0.08	ND	ND	ND
Fish 2	5.77 ± 0.40	5.99 ± 0.04	ND	ND	ND	ND
Fish 3	6.21 ± 0.19	6.20 ± 0.02	ND	ND	ND	1.70 ± 0.13
Fish 4	8.55 ± 0.10	8.32 ± 0.08	ND	ND	ND	ND
Fish 5	6.64 ± 0.04	6.66 ± 0.06	1.00 ± 0.00	ND	ND	ND
Fish 6	6.31 ± 0.19	6.65 ± 0.07	ND	ND	ND	ND
Fish 7	6.84 ± 0.08	7.13 ± 0.05	ND	ND	ND	ND
Fish 8	6.93 ± 0.04	7.61 ± 0.03	1.45 ± 0.21	ND	ND	ND
Fish 9	5.45 ± 0.21	5.84 ± 0.10	ND	ND	ND	ND
Fish 10	5.59 ± 0.16	5.89 ± 0.02	ND	ND	ND	ND
Fish 11	4.49 ± 0.04	4.83 ± 0.28	ND	ND	ND	ND
Fish 12	4.01 ± 0.05	4.06 ± 0.03	ND	ND	ND	ND
Fish 13	4.19 ± 0.03	4.21 ± 0.08	ND	ND	ND	1.99 ± 0.14
Fish 14	4.17 ± 0.01	4.39 ± 0.03	ND	ND	ND	ND
Fish 15	4.52 ± 0.03	4.70 ± 0.10	ND	ND	ND	1.81 ± 0.07
Fish 16	4.35 ± 0.05	4.72 ± 0.13	ND	ND	ND	ND
Fish 17	3.61 ± 0.09	3.60 ± 0.21	ND	ND	ND	ND
Fish 18	3.56 ± 0.03	3.31 ± 0.21	ND	ND	ND	ND
Fish 19	3.60 ± 0.03	3.51 ± 0.23	ND	ND	ND	ND
Fish 20	3.32 ± 0.00	3.20 ± 0.02	ND	ND	ND	ND
Seawater 1	4.67 ± 0.01	4.50 ± 0.01	ND	ND	ND	ND
Seawater 2	2.70 ± 0.00	3.00 ± 0.15	ND	ND	ND	ND
Seawater 3	2.95 ± 0.07	2.92 ± 0.03	1.24 ± 0.34	ND	1.99 ± 0.32	ND
Seawater 4	3.85 ± 0.21	4.17 ± 0.16	1.10 ± 0.17	1.17 ± 0.27	ND	0.3 6 ± 0.16
Seawater 5	4.10 ± 0.03[c]	4.14 ± 0.15	ND	ND	ND	2.22 ± 0.09
Seawater 6	4.07 ± 0.01	4.13 ± 0.13	ND	ND	ND	2.37 ± 0.12
Seawater 7	3.87 ± 0.01	3.21 ± 0.03	ND	ND	ND	1.88 ± 0.02
Seawater 8	3.92 ± 0.09	3.57 ± 0.18	ND	ND	ND	2.04 ± 0.12
Seawater 9	4.19 ± 0.07	3.59 ± 0.10	2.69 ± 0.21	1.49 ± 0.12	ND	3.02 ± 0.00
Seawater 10	3.08 ± 0.09	2.79 ± 0.13	ND	ND	ND	1.66 ± 0.05

[a] Fish, Log CFU g^{-1}; seawater, Log CFU ml^{-1}.
[b] Mean ± standard deviation.
ND, not detected; TCBS, thiosulfate citrate bile salts sucrose; TSA, tryptic soy agar.

were also designed for the quantification of total bacteria. All experimental species showed positive results, the range of Ct values was variable between 19 and 27.

For the quantification of the bacterial levels in food or environmental samples by PCR assay, proper DNA sample preparation is crucial. To eliminate PCR inhibitors and prepare effective genomic DNA extractions with simple and fast methods, we evaluated several DNA extraction methods: (1) boiling with a TZ buffer, (2) boiling with chelex-100 solution, (3) boiling with a TZ buffer and chelex-100 solution, and (4) commercial DNA extraction kits for food or seawater (data not shown). In the previous studies, TZ buffer enhanced the yield of genomic DNA and extracted DNA from low levels of bacterial numbers.[33] Chelex-100 is a specialized resin that chelates metal ions and other contaminants as well as it is often used for DNA extraction in preparation for PCR.[40] Chelex-100 has been applied to prepare bacterial DNA from fishes as well as seawater due to its excellent chelating effects, especially in seawater.[41–44] Also, additional DNA clean up processes were tried using a DNA cleaning column or precipitation solution. Through assessment of those approaches in this study, a combination method using TZ buffer, chelex-100 solution, and further precipitation was the most effective DNA preparation for the multiplex real-time PCR assay. When using this DNA preparation method, the specific targets were detected up to 1 CFU mL^{-1} in seawater and 10 CFU g^{-1} in fish.

The total bacterial populations in fish and seawater quantified by a culture method and multiplex real time PCR assay was similar and indicates that both methods have a close correlation. Nutritionally rich bacterial media, TSA, was used for total bacterial cultivation from fish and seawater samples. The number of colony on TSA may generally represent actual bacterial counts. Therefore the multiplex real-time PCR assay would be very practical to enumerate total bacteria in fish and seawater. The total numbers of the three *Vibrio* spp. by multiplex real-time PCR assay was consistently higher than those of TCBS plates. TCBS agar is highly selective for the isolation of *V. cholerae* and *V. parahaemolyticus*, and other *Vibrio* spp.,[45] but has some limitations. Viable but injured bacteria in environments will not grow on TCBS agar because of

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selective agents;[46,47] however, those bacteria could be recovered on non-selective rich media. In addition, some *Vibrio* spp. such as *V. hollisae* and *V. metschnikovii* may not grow on TCBS agar.[48] In our preliminary test, *V. parahaemolyticus* and *V. vulnificus* strains used in this study were grown on TCBS agar except for *V. anguillarum* strain; results indicated that *V. anguillarum* inoculated in fish and seawater could not be detected on TCBS agar. Also, when *Vibrio* spp. in artificial seawater, which was sterile before *Vibrio* spp. inoculation, were spread on TSA^+ as well as TCBS plates, the colony counts on TSA^+ were higher than on TCBS agar and were approximate to the number of *Vibrio* spp. quantified by this PCR assay. Especially, when the low levels, Log 1 CFU g^{-1}, of *Vibrio* spp. inoculated in fish samples, TCBS agar did not detect the vibrios. *Vibrio* spp. counts on TCBS agar must be under-estimated in contrast to the actual numbers of *Vibrio* spp. From the results obtained in this study, the quantification of *Vibrio* spp. in fish and seawater by this multiplex real-time PCR assay might be more accurate than the culture methods.

Temperature is considered the critical factor concerning *Vibrio* spp. abundance and distribution in seawater.[49,50] Our study also showed the level of *Vibrio* spp. had a high correlation with water temperature; *Vibrio* spp. were not detected in seawater samples 1 or 2, which were collected in early spring, but were present in other seawater samples, which were collected from early summer.

In a few fish samples, some colonies were counted on TCBS, but the specific *Vibrio* spp. were not detected by the multiplex real-time PCR assay (e.g. fish 1). TCBS agar may support growth of some other genus and there was a report that only 11.9–47.9% of strains on TCBS agar were identified as *Vibrio* spp. in aquatic samples.[51] Therefore, the strains on TCBS are considered to be other species or genus with exception of the three tested *Vibrio* spp.; which supports the application of this PCR assay for high detection specificity. Meanwhile, *V. vulnificus* was detected in a fish or seawater sample by the PCR assay but was not detected by the agar media. This could be caused by the detection limit of the PCR assay as it was more sensitive than the culture method or the *V. vulnificus* might be non-culturable on agar media.

CONCLUSION

To the best of our knowledge, our study describes the first multiplex real-time PCR assay for the simultaneous detection of these three species. Furthermore, this assay quantified total bacteria in the seafood and seawater samples at the same time. To prevent pathogenic *Vibrio* infection to humans and fish, their presence in seafoods and seawater should be accurately monitored and total bacteria counts could be used as an indicator for measuring the quality of fishery products.[52] This multiplex real-time PCR assay will facilitate the rapid surveillance of fishes and seawater for *Vibrio* spp. and total bacteria as well as could be applicable, as diagnostic method, to outbreak situations.

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