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Application of the *rpoS* Gene for Species-Specific Detection of *Vibrio vulnificus* by Real-Time PCR

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***Vibrio vulnificus* is a causative agent of serious diseases in humans, resulting from the contact of wound with seawater or consumption of raw seafood. Several studies aimed at detecting *V. vulnificus* have targeted *vvh* as a representative virulence toxin gene belonging to the bacterium. In this study, we targeted the *rpoS* gene, a general stress regulator, to detect *V. vulnificus*. PCR specificity was identified by amplification of 8 *V. vulnificus* templates and by the loss of a PCR product with 36 non-*V. vulnificus* strains. The PCR assay had the 273-bp fragment and the sensitivity of 10 pg DNA from *V. vulnificus*. SYBR Green I-based real-time PCR assay targeting the *rpoS* gene showed a melting temperature of approximately 84°C for the *V. vulnificus* strains. The minimum level of detection by real-time PCR was 2 pg of purified genomic DNA, or 10^3 *V. vulnificus* cells from pure cultured broth and 10^3 cells in 1 g of oyster tissue homogenates. These data indicate that real-time PCR is a sensitive, species-specific, and rapid method for detecting this bacterium, using the *rpoS* gene in pure cultures and in infected oyster tissues.**

Keywords: *Vibrio vulnificus*, *rpoS* gene, real-time PCR, oyster

Vibrio vulnificus is an estuarine halophilic bacterium that produces fatal septicemia, gastroenteritis, and wound infections in humans. This bacterium is known to cause death in susceptible individuals after wound exposure to seawater or when ingested through the consumption of raw or uncooked contaminated seafood. Contaminated seawater and shellfish are especially prone to causing serious infections that can lead to life-threatening septicemia [19]. Therefore, the importance of a rapid and accurate method to detect *V. vulnificus* from oysters and oyster-harvesting environments cannot be overemphasized.

Classical methods of microbial pathogen detection by biochemical and microbiological tests, such as the most-probable-number technique, the use of selective agar media, or DNA-DNA colony hybridization targeting the specific gene, are time and labor intensive and involve analyzing a large number of samples [15, 31]. For this reason, modern detection methods that are more efficient and faster have been developed. The conventional PCR method for detection of microbial pathogens in food, clinical, and environmental samples has been shown to be highly specific and relatively less time-consuming than classical methods. Furthermore, multiplex PCR assays using several target genes have also been successfully applied to the detection of multiple organisms, or to the differentiation of related strains. These PCR approaches, however, require products to be analyzed by agarose gel electrophoresis, which again is time-consuming and laborious.

Real-time PCR offers a quicker and more sensitive method for detecting a diverse range of pathogens. Real-time PCR amplification is suitable for quantifying bacteria, and SYBR Green I real-time PCR provides a precise and sensitive method [6, 33]. The products of real-time PCR need no other method of detection since the nonspecific double-stranded DNA fluorescent binding dye allows monitoring of the amplified product at each PCR cycle, and the products are then distinguished by analysis of their melting temperatures. Real-time quantitative PCR is therefore being extensively used for pathogen detection and quantification in foods [26]. This method has been reported for the detection of various microbial pathogens such as *Escherichia coli* O157:H7 [12], *Legionella* [32], *Salmonella* [13], *Listeria* [13], *Campylobacter* [21], *V. cholerae* [22], *V. parahaemolyticus* [4], and *V. vulnificus* [29].

The purpose of this study was to develop a rapid and accurate technique to quantify *V. vulnificus*. The gene we

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targeted for the detection of *V. vulnificus* was the *rpoS* gene, a housekeeping gene and general stress regulator commonly found in proteobacteria. This gene also regulates the expression of various virulence genes [7, 9, 17] and is associated with host tissue colonization [25, 27]. Based on the sequences of various regions on the *rpoS* genes for several *Vibrio* spp., specific primers for the detection of *V. vulnificus* were designed and used in real-time quantitative PCR with SYBR Green I dye.

Table 1. Strains used in this study.

Species	Source or reference
V. alginolyticus	KCTC 2472
V. anguillarum (O1 type)	[10]
V. campbellii	KCCM 40864
V. carchariae	KCCM 40865
V. cholerae	ATCC 14547
V. cincinnatiensis	KCTC 2733
V. damselae	E
V. diazotrophicus	KCCM 41666
V. fisheri	KCCM 41685
V. fluvialis	KCTC 2473
V. furnissii	KCTC 2731
V. harveyi	KCCM 40866
V. hollisae	KCCM 41680
V. logei	KCTC 12281
V. mediterranei	KCCM 40867
V. metschnikovii	KCTC 2736
V. mimicus	KCTC 2737
V. natriegens	KCCM 40868
V. navarrensis	KCCM 41682
V. nereis	KCTC 2722
V. ordalii	KCCM 41669
V. orientalis	KCTC 2725
V. parahaemolyticus	KCCM 11965
V. proteolyticus	KCTC 2730
V. salmonicida	KCCM 41663
V. tubiashii	KCTC 2728
V. vulnificus	KCTC 2959 (corresponded to ATCC 27562)
V. vulnificus	KCTC 2962
V. vulnificus	KCTC 2981
V. vulnificus	KCTC 2982
V. vulnificus	KCTC 2983
V. vulnificus	KCTC 2985
V. vulnificus	KCTC 2986
V. vulnificus	KCTC 2987
Aeromonas hydrophila	KCTC 2358
Escherichia coli BL21(DE3)	L
E. coli XLI-blue	L
Enterobacter cloacae	E
Edwardsiella tarda	E
Klebsiella oxytoca	E
K. pneumoniae	E
Salmonella typhi	E
Shigella flexneri	E
S. sonnei	E

L, laboratory collection; E, environmental source.

Materials and Methods

Bacterial Strains and Growth Medium

All *Vibrio* strains including *V. vulnificus* and non-*Vibrio* strains used in this study are listed in Table 1. Bacteria listed in Table 1 were grown at their optimum temperatures on brain heart infusion (BHI, Difco), heart infusion (HI; Difco), and Luria-Bertani (LB; Difco) agars, respectively.

Vibrio vulnificus-Specific Primers

A segment of the *rpoS* gene sequence was used as the PCR target for specific detection of *V. vulnificus*. Potential primer sequences were analyzed for specificity by comparison with known *rpoS* gene sequences of *Vibrio* spp. (*vulnificus* ATCC 29307, *cholerae* N16961, *parahaemolyticus* BB22, *anguillarum* ATCC 19264, and *harveyi* ATCC 14126) retrieved from the Entrez database (Accession No.: AY187681, AE004139, AF144608, AY695434, and AF321124, respectively) using the National Center for Biotechnology Information (NCBI) GenBank database BLAST search program. An oligonucleotide primer set, vulrpoS-up and vulrpoS-rp (Table 2), was used in each PCR to test the specificity of detection for all bacterial strains used in this study. Two other primers, 16S1 and 16S2 corresponding to 16S rRNA, were selected for PCR amplification as a positive control (Table 2).

DNA Extractions and PCR Analysis

Total genomic DNA from all bacterial strains listed in Table 1 was purified as described by Ausubel *et al.* [3]. The PCR reactions with purified DNA were performed in a 50-μl mixture that contained 250 μM of each dNTP, 10 pmol of each primer, *Taq* buffer with $MgCl_2$ (Takara Bio, Japan), EX *Taq* polymerase (Takara Bio, Japan), and sterile distilled water up to 50 μl. The PCR thermal cycling with isolated DNA followed with 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, and final elongation at 72°C for 7 min. The PCR products were confirmed by 1.5% agarose gel electrophoresis.

Specificity and Sensitivity of Detection

The specificities of the primers for detecting *V. vulnificus* were tested by PCR amplification of the purified genomic DNA. Purified genomic DNA from *V. vulnificus* was 10-fold serially diluted in sterile distilled water, and the PCR amplification was performed under the determined optimal conditions.

Real-Time PCR and Cycling Parameters

Quantitative PCR was performed with the FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) in a LightCycler real-time PCR system (Roche). Fluorescence emitted by the SYBR Green I dye bound to dsDNA was measured at the end of each PCR cycle. The cycle threshold (C*t*), defined as the cycle number at which the reaction begins the exponential phase (calculated by the Roche LightCycler software version 3), was used to create the standard curve (C*t* values against the logarithm of serial 10-fold diluted standard concentration gives a linear relationship) and to

Table 2. Description of the PCR primer sequences, location lengths, and amplicon sizes used in this study

Target	Primer	Sequence	Positions within ORF (bp)	Length (bp)	Amplicon size (bp)
RpoS	vulrpoS-up	CATGCGTGTTTCCTTGATTC	753–772	20	273 bp
	vulrpoS-rp	TCCATAGCCTTTTTTCTATTGG	1,004–1,025	22	1,466 bp
16S rDNA	16S1	AGAGTTTGATCMTGGCTCAG	27–46	20	
	16S2	TACGGYTACCTTGTTACGACTT	1,471–1,492	22	

evaluate the *V. vulnificus* DNA concentration within the samples. Each sample for real-time PCR contained 20 µl of the template. For the negative control, the template was replaced with PCR-grade water.

Preparation of Artificially Contaminated Oysters

Fresh retail oysters were washed with sterilized water and prepared following standard methods [1]. Briefly, oyster meat was homogenized, and the homogenates were exposed to UV light for 1 h and then subjected to three cycles of freezing at -80°C followed by thawing at room temperature to reduce the indigenous target microbial population. *V. vulnificus* was grown in BHI medium, and then serially diluted, with the oyster homogenate as the diluent, to final concentrations ranging from 0 to 10^9 CFU/g. These artificially contaminated oyster homogenate microcosms (10 ml each) were incubated at 30°C with shaking for 5 h to enrich the *V. vulnificus* cell population. After enrichment, the inoculated samples were stored at -20°C until they were analyzed. Aliquots (1 ml) of seeded homogenates were centrifuged, and DNA was extracted for real-time PCR quantification.

RESULTS

Sequence Analysis of the *rpoS* Gene and Primer Design

To design a primer pair, we aligned the known *rpoS* gene sequences from 5 strains of *Vibrio* spp. using ClustalW. Alignment of the nucleotide sequence with the *rpoS* genes from *V. cholerae*, *V. harveyi*, *V. parahaemolyticus*, and *V. anguillarum* showed 86%, 82%, 81%, and 79% identities, respectively. Few differences were observed between the central regions of the *rpoS* genes from various *Vibrio* spp., but the homologies at the amino-terminal and carboxy-terminal ends were reduced. We found variable regions that were appropriate for designing primers for specific detection. The vulrpoS-up and vulrpoS-rp primer pair, located between bp 753 and 1,026 on the *rpoS* gene of *V. vulnificus*, exhibited the highest specificity among the primer set in the PCR reaction (Fig. 1).

Specificities of PCR Detection for *V. vulnificus*

We expected the *rpoS*-specific primer set designed for this study to produce a single PCR product of 273 bp for *V. vulnificus*. To evaluate the specificity of the PCR assay, PCR amplifications using *rpoS* and 16S specific primers were performed with 36 other bacterial strains by purified chromosomal DNA as a template (Fig. 2A) and 7 strains of *V. vulnificus* (Fig. 3). Amplification of genomic DNA isolated from all *V. vulnificus* with primers of vulrpoS-up and vulrpoS-rp resulted in a product with the predicted length of 273 bp, whereas no products were obtained from those of non-*V. vulnificus* bacterial strains. In the case of PCR

```
GATGGAGAGAAGGCGTTATTAGATATTATTCCGGATGCAAACAACTCTGACCCAGAGGTTTCAACGCAAGATGAAGACATGCGTGT  761  V. vulnificus
..T..A..T..G..AC.GC.G.....T.G..A..CT.TC.C..TG.C..T..T..GT.T..A..T.....T..T..C..TCGTGA  773  V. cholerae
..C..C..G..A..GT.AC.G.....A.T..T..TG.AA.C..CT.G..T..T..AG.T..C..T.....C..T..C..CAAATC  752  V. harveyi
..C..T..A..A..GC.TC.G.....A.T..T..CG.AA.T..CT.T..T..A..AG.G..G..T.....T..T..C..CAAATC  758  V. parahaemolyticus
..T..T..C..A..AC.AC.C.....A.C..A..TA.GA.C..CT.T..T..C..AA.T..T..G.....T..T..T..CCGTGA  770  V. anguillarum

vulrpoS-up
TTCCTTGATTCATTGGTTGGAAGAGCTCAATCCGAAACAAAAAGAAGTGTTGGCACGTCGCTTTGGTTTGTTGGGTTATGAGCCTT  847  V. vulnificus
A..GC.GC.CA.C...T.G..T..AC.T..T..A..G..A.....A..GC.T..G..T..C.....GC.TC.T..C.....A..A.  859  V. cholerae
T..AT.GA.CC.T...C.A..A..GC.G..T..A..A..G.....A..GC.G..A..T..T.....TC.GC.T..T.....G..A.  838  V. harveyi
G..TT.GA.CC.T...T.G..A..GT.G..T..A..A..A.....A..GC.T..A..C..T.....TT.GC.T..T.....G..G.  844  V. parahaemolyticus
A..AC.AC.GA.T...T.A..T..AC.G..C..A..A..A.....G..AC.T..A..C..T.....AC.AT.A..T.....A..G.  856  V. anguillarum

                                                                                vulrpoS-rp
GAAGTTCTGATCAAACAAGGTCTGAATATGGAAAATTTGTTTGATATCGTATG-GATTGAATTTGCATTCTCCAATAGAAAAAAGG  1018  V. vulnificus
..GA.TT.GG.G........TT.G..T......GCGC....TA.CG.C.A.TAC..CAACTAA----------------------  1008  V. cholerae
..GA.CT.GA.T........TC.G..C......AACT....CA.CG.A.A.GAC..CTAA-------------------------   984  V. harveyi
..AA.CC.AA.T........CC.A..T......AACT....TA.CG.T.A.GAC..TTAA-------------------------   966  V. parahaemolyticus
..GA.CT.GG.T........CT.G..C......GCGC....TA.TG.T.A.TAC..CTAA-------------------------  1002  V. anguillarum

CTATGGAATCATAG 1032   V. vulnificus
-------------- -      V. cholerae
-------------- -      V. harveyi
-------------- -      V. parahaemolyticus
-------------- -      V. anguillarum
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Fig. 1. Nucleotide sequence alignment of the *rpoS* of *V. vulnificus* (Accession No. AY187681), *V. cholerae* (Accession No. AE004139), *V. harveyi* (Accession No. AF321124), *V. parahaemolyticus* (Accession No. AF144608), and *V. anguillarum* (Accession No. AY695434).

Nucleotides identical to those of *V. vulnificus* are indicated with dots.

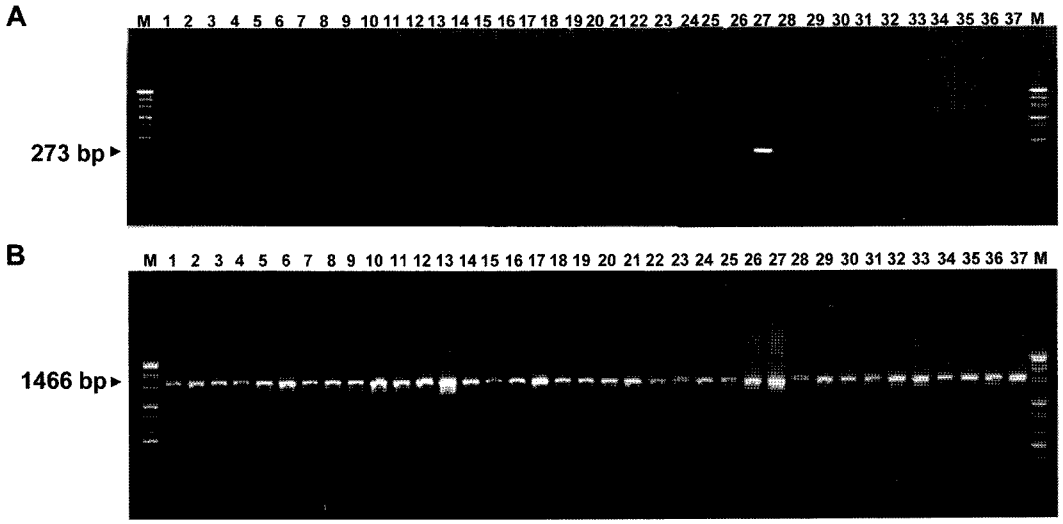

Fig. 2. A. Agarose gel electrophoresis of *V. vulnificus*-specific DNA products amplified in PCR using primers vulrpoS-up and vulrpoS-rp. M, 100-bp DNA ladder. **B.** Agarose gel electrophoresis of 16S rDNA products amplified by PCR using primers 16S1 and 16S2 as a positive control.
M, 100-bp DNA ladder; lane 1, *V. alginolyticus* ATCC 17749; lane 2, *V. anguillarum* O1 type; lane 3, *V. campbellii* ATCC 25920; lane 4, *V. carchariae* ATCC 35084; lane 5, *V. cholerae* ATCC 14547; lane 6, *V. cincinnatiensis* ATCC 35912; lane 7, *V. damselae*; lane 8, *V. diazotrophicus* ATCC 33466; lane 9, *V. fisheri* ATCC 7744; lane 10, *V. fluvialis* ATCC 33809; lane 11, *V. furnissii* ATCC 35016; lane 12, *V. harveyi* ATCC 14126; lane 13, *V. hollisae* ATCC 33564; lane 14, *V. logei* ATCC 29985; lane 15, *V. mediterranei* ATCC 43341; lane 16, *V. metschnikovii* ATCC 700040; lane 17, *V. mimicus* ATCC 33653; lane 18, *V. natriegens* ATCC 14048; lane 19, *V. navarrensis* ATCC 51183; lane 20, *V. nereis* ATCC 25917; lane 21, *V. ordalii* ATCC 33509; lane 22, *V. orientalis* ATCC 33934; lane 23, *V. parahaemolyticus* ATCC 17802; lane 24, *V. proteolyticus* ATCC 15338; lane 25, *V. salmonisida* ATCC 43839; lane 26, *V. tubiashii* ATCC 19109; lane 27, *V. vulnificus* ATCC 27562; lane 28, *Aeromonas hydrophila* ATCC 7966; lane 29, *Escherichia coli* BL21(DE3); lane 30, *E. coli* XL1-blue; lane 31, *Enterobacter cloacae*; lane 32, *Edwardsiella tarda*; lane 33, *Klebsiella oxytoca*; lane 34, *K. pneumoniae*; lane 35, *Salmonella typhi*; lane 36, *Shigella flexneri*; lane 37, *S. sonnei*.

with 16S1 and 16S2 primers, located between bp 27 and 1,492 of the 16S rDNA segment, as a positive control, the amplicon could be seen in every strain used in this study (Fig. 2B).

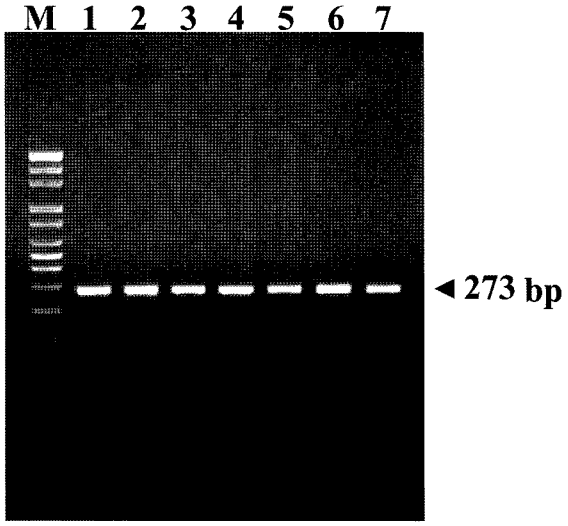

Fig. 3. Agarose gel electrophoresis of *V. vulnificus*-specific DNA products amplified in PCR using primers vulrpoS-up and vulrpoS-rp.
M, 100-bp DNA ladder; lane 1, KCTC 2962; lane 2, KCTC 2981; lane 3, KCTC 2982; lane 4, KCTC 2983; lane 5, KCTC 2985; lane 6, KCTC 2986; lane 7, KCTC 2987.

Sensitivity of PCR Detection for *V. vulnificus*

A dilution series of genomic DNA of *V. vulnificus* was prepared as described in the Materials and Methods. Aliquots of each 10-fold serial dilution were used as templates for PCR amplification with 25 cycles. A minimum of 10 pg of purified genomic DNA generated a detectable level of an amplified *rpoS* DNA band with the expected length of 273 bp in an agarose gel (Fig. 4). This detection level of 10 pg of genomic DNA, assuming 100% extraction efficiency, is equivalent to approximately 10^3 *V. vulnificus* cells. These results were found to be consistent within all three replicates for each of the extraction methods.

Real-Time PCR Assay

Species specificity was confirmed by positive signal amplification of 7 *V. vulnificus*, and the lack of a product from all other non-*V. vulnificus*, bacterial strains. The limits of sensitivity for *V. vulnificus* detection by real-time PCR were determined from endpoint titration of DNA extracted from pure culture. Linear values for PCR amplification were achieved for dilutions of purified DNA concentrations ranging from 200 ng/μl to 2 pg/μl. The minimum level of detection of the *rpoS* gene target from purified genomic DNA was 2 pg with a C*t* value of 32.81±0.04 and an expected melting temperature of approximately 84°C (Table 3). Real-time PCR amplification of DNA obtained from serial dilutions of pure culture in

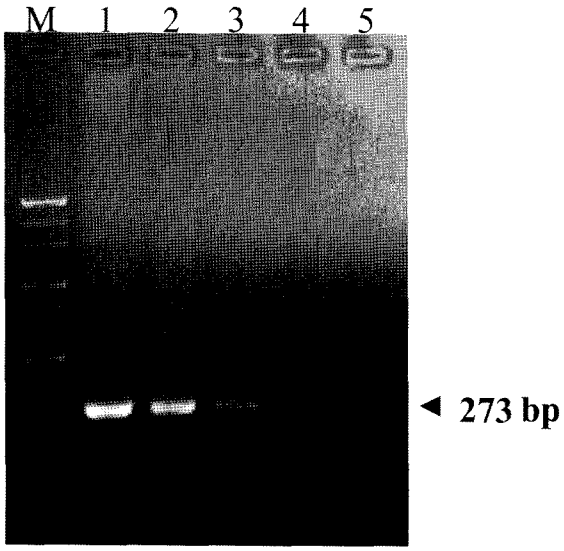

Fig. 4. Sensitivity of vulrpoS-up and vulrpoS-rp PCR primers for the amplication of the *V. vulnificus*-specific amplicon at various DNA concentrations.
DNA was extracted from overnight cultures in BHI medium by phenol-chloroform extraction and ethanol preparation. DNA concentrations were measured with a spectrophotometer, and DNA was serially diluted in TE buffer to obtain appropriate concentrations. Lanes contained *V. vulnificus* DNA: M, 100-bp DNA ladder; lane 1, 500 pg of DNA; lane2, 100 pg of DNA; lane 3, 10 pg of DNA; lane 4, 1 pg of DNA; lane 5, 0.1 pg of DNA.

BHI medium was detected at $<10^3$ CFU/ml (Table 4). The comparison of bacterial concentrations, as determined by plate counts on BHI agar and real-time PCR results, showed a good linear correlation between C*t* values and concentrations of purified DNA or *V. vulnificus* cells in pure cultures (r^2=0.99).

Enumeration of *V. vulnificus* in Artificially Inoculated Oyster Homogenates

Oyster homogenates were seeded with pure cultures of *V. vulnificus* ranging from 0 to 10^9 CFU/ml. The results of real-time PCR amplification showed that 10^3 copies of *V. vulnificus* were detected in 1 g of seeded oyster tissue homogenate following 5 h of enrichment (Table 5). An increase in the C*t* values from 20.58±0.06 to 32.48±0.08 with an increment of three cycles was observed for samples that were seeded with 10^7 to 10 *V. vulnificus* CFU/ml. All positive samples had a melting temperature of approximately 84°C. A good linear correlation was observed between C*t* values and concentration of *V. vulnificus* cells seeded in 1 g of oyster tissue homogenate. The C*t* values for the seeded oyster tissue homogenate were one to two cycles higher than the values for the cells of pure culture, indicating that the oyster tissue matrix had an inhibitory effect on the real-time PCR.

Table 3. Sensitivity of detection of purified DNA from a pure culture of *V. vulnificus* by using real-time PCR and SYBR Green I fluorescent dye.[a]

Sample	Amt of DNA	C*t* value	Melting temp (°C)
2 (positive control)		15.52±0.03	84.82±0.06
3	200 ng	16.74±0.06	84.82±0.08
4	20 ng	20.19±0.03	84.65±0.09
5	2 ng	23.85±0.04	84.91±0.19
6	0.2 ng	27.34±0.08	84.91±0.23
7	0.02 ng	30.53±0.06	84.52±0.16
8	2 pg	32.81±0.04	84.48±0.21
9	0.2 pg	33.21±0.03	
10	0.02 pg	32.80±0.04	

[a]The data are means±standard deviations for 3 independent experiments.

Table 4. Sensitivity of detection of a stationary grown pure culture of *V. vulnificus* with SYBR Green I dye.[a]

Sample	Amt of bacteria (CFU/ml)	C*t* value	Melting temp (°C)
2	10^8	14.58±0.06	84.48±0.06
3	10^7	18.46±0.07	84.47±0.03
4	10^6	21.67±0.08	84.44±0.04
5	10^5	25.22±0.15	84.47±0.01
6	10^4	28.62±0.06	84.47±0.03
7	10^3	31.45±0.07	84.56±0.07
8	10^2	32.01±0.17	
9	10^1	32.67±0.24	
10 (negative control)		37.52±0.42	76.32±0.64
11 (positive control)		11.65±0.09	84.56±0.08

[a]The values are means±standard deviations for 3 independent experiments.

DISCUSSION

V. vulnificus is a leading cause of serious human wound infections and septicemia, with seawater and raw oysters being the major source for human *V. vulnificus* infections.

Table 5. Sensitivity of detection of *V. vulnificus* from seeded oyster tissue homogenate following 5 h.[a]

Sample	Conc. (CFU/g)	C*t* value	Melting temp (°C)
1	10^7	20.58±0.06	84.60±0.02
2	10^6	23.68±0.07	84.60±0.02
3	10^5	27.42±0.12	84.60±0.03
4	10^4	30.50±0.09	84.36±0.15
5	10^3	32.08±0.05	84.36±0.17
6	10^2	32.64±0.12	
7	10^1	32.48±0.08	
8 (positive control)		18.97±0.11	84.56±0.06
9 (negative control)[b]		32.48±0.07	76.32±0.08

[a]The values are means±standard deviations for 3 independent experiments.
[b]Unseeded oyster tissue homogenate.

Therefore, sensitive, specific, and rapid methods for detection of *V. vulnificus* are one of the most important for the leisure seawater activities and seafood industries.

Various PCR methods have been developed for detecting pathogenic bacteria, and several genes are used for identification of *Vibrio* spp. These PCR methods using various gene sequences have been used to detect *V. vulnificus* from seawater and seafood. Arias *et al.* [2] develop a nested PCR method utilizing universal external primers targeting the 23S rRNA and *V. vulnificus*-specific internal primers. Multiplex PCR for identification of the foodborns pathogen *V. vulnificus* has also been carried out with the *vvhA* gene [35]. The *toxR* gene, a transmembrane DNA-binding regulatory gene, is known as a species-unique gene in various *Vibrio* spp. [20, 28], and the sequences of different regions of *toxR* from *Vibrio* spp. have been used for the detection of *V. vulnificus* [34]. Parvathi *et al.* [30] and Kumar *et al.* [18] reported that the application of a PCR method targeted the *gyrB* gene for the detection of *V. vulnificus* from oyster. Various regions of 16S rRNA of *V. vulnificus* were used for designing of *V. vulnificus* detection primers [16, 24].

However, 16S rRNA was not suitable for discrimination of the strain because of the slow rate of 16S rRNA evolution [18]. Moreover, some strains of *V. vulnificus* were not detected by 23S rRNA-specific primers [30]. The detection method that targeted the *gyrB* gene successfully identified *V. vulnificus*, but was tested in a very limited number of species and strains. Until now, the most commonly used method for detecting *V. vulnificus* has been to target the *vvhA* gene. With this method, however, because of mutation or rearrangements within the cytolysin-haemolysin gene and lack of this nonessential virulence gene, the sensitivity or detection of *V. vulnificus* cells is reduced or failed [5, 24]. Therefore, *vvhA* is not absolutely suitable for detection of *V. vulnificus*. However, housekeeping genes like *toxR* and *gyrB* appear to be useful for detection of *V. vulnificus*.

In this study, we examined a method for detecting *V. vulnificus* in pure culture and oyster tissue homogenates using real-time PCR with specific primers targeting the *rpoS* gene. The *rpoS* gene encodes a sigma factor of RNA polymerase and is itself induced in the stationary phase as a stationary regulator involved in adaptation to various environmental stresses, including high or low temperature, osmotic shock, nutrient starvation, and oxidative damage [8]. The *rpoS* gene of *V. vulnificus* plays a role in the low-temperature stress [11]. This low-temperature adaptation is important for survival since the oyster and bacterium are faced with seawater under winter conditions. The abundance of *V. vulnificus* has also been related with water temperature [24]. In Korea, oysters have been harvested during winter months. We therefore used the *rpoS* gene to detect *V. vulnificus* since it acts as a housekeeping gene essential for the bacterium. Through comparative analysis of the known *rpoS* gene sequences of *Vibrio* spp., we identified two variable regions that could be useful as target sequences to design specific primers for amplification of the *rpoS* gene in *V. vulnificus*. Several studies have shown that real-time PCR with SYBR Green I dye can be a rapid, reliable, and cost-effective method for the detection of microorganisms in various environmental samples [6, 12, 32, 33]. The use of SYBR Green I dye and primers targeting the *rpoS* gene in real-time PCR resulted in a predicted 273-bp amplicon with a consistent melting temperature of approximately 84°C, supporting the reliability of this study.

We detected *V. vulnificus* successfully by the *rpoS* gene from pure cultured broth and artificially contaminated oyster samples. We consider that the DNA from dead cells may be detected by the sensitive PCR system. However, according to the results of real-time PCR using *toxR* and *vvhA* genes for the probes, DNA from dead cells of *V. vulnificus* was in the degree of the nondetectable [14, 23]. Because the same method was used for our experiment, we assumed that the result may show the reliable level.

This technique should prove to be useful for the detection and quantification of *V. vulnificus* in marine organisms inhabiting a cold environment, and can be done within 2–3 h rather than the days required for conventional methods. Furthermore, we will test more environmental samples in order to confirm this method.

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