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Rapid Identification of *Vibrio vulnificus* in Seawater by Real-Time Quantitative TaqMan PCR

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In order to identify *Vibrio vulnificus* in the Yellow Sea near Gunsan, Korea during the early and late summers, the efficiency of the real-time quantitative TaqMan PCR was compared to the efficiency of the conventional PCR and Biolog identification system™. Primers and a probe were designed from the hemolysin/cytolysin gene sequence of *V. vulnificus* strains. The number of positive detections by real-time quantitative TaqMan PCR, conventional PCR, and the Biolog identification system from seawater were 53 (36.8%), 36 (25%), and 10 strains (6.9%), respectively, among 144 samples collected from Yellow Sea near Gunsan, Korea. Thus, the detection method of the real-time quantitative TaqMan PCR assay was more effective in terms of accuracy than that of the conventional PCR and Biolog system. Therefore, our results showed that the real-time TaqMan probe and the primer set developed in this study can be applied successfully as a rapid screening tool for the detection of *V. vulnificus*.

Key words: *Vibrio vulnificus*, conventional PCR, real-time quantitative TaqMan PCR, Biolog™ system

Vibrio vulnificus is widely distributed in the marine environment, where some strains come into contact with humans and cause infections. This species has been associated primarily with two disease syndromes: primary septicemia and wound infection (Blake *et al.*, 1979). Primary septicemia is a very serious infection with a fatality rate of about 50%. Most patients with primary septicemia caused by *V. vulnificus* have preexisting liver disease (Blake *et al.*, 1979), but there have been cases of infection in healthy individuals (Tison and Kelly, 1984). In most cases, the disease onsets several days after the patient has consumed raw oysters. *V. vulnificus* also causes severe wound infections, usually after trauma and exposure to marine animals or the marine environment (Blake *et al.*, 1979). Its morality rate is not nearly as high as in the case of primary septicemia (about 7% compared to about 50%). Other infections from which *V. vulnificus* has been isolated include pneumonia in a drowning victim and endometritis which developed in a woman after exposure to seawater (Tison and Kelly, 1984).

The genus *Vibrio* includes more than 30 species, 12 of these are human pathogens or have been isolated from human clinical specimens. Eight of the 12 human-associated *Vibrio* species have been isolated from extraintestinal clinical specimens (Park *et al.*, 1991; McLaughlin, 1995; Paik *et al.*, 1995). For a definitive diagnosis, *V. vulnificus* should be differentiated from at least seven other extraintestinal *Vibrio* species. Even with the most sophisticated and high-tech equipment or rapid presumptive detection methods, more than 2 days is needed for the definitive identification of *V. vulnificus*. Therefore, the importance of developing rapid diagnostic measures that can identify *Vibrio* species within hours cannot be overemphasized.

In this study, the efficiency of the real-time quantitative TaqMan PCR for the identification of *V. vulnificus* was compared with that of the conventional PCR and BioLog identification system™ .

Materials and Methods

Bacterial species and culture

The strains examined were members of the *Vibrio* species and enteric bacteria. Table 1 shows the lists of bacterial strains used as a positive control in this study and their sources. Seawater samples collected from 6 sites in the Yellow Sea near Gunsan, Korea were examined to compare the identification efficiency.

Isolated strains, which were cultured in Bacto-TCBS agar plates at 37°C for 2 days, were identified down to the species level by the BioLog identification system™ (MicroLog™ system, Release 4.0., USA). The cultures were maintained in LB (Luria-Bertani) broth (Difco,

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Table 1. Bacterial strains used to test the sensitivity and specificity of PCR for identifying *V. vulnificus*

Strains	Source
Vibrio vulnificus	ATCC 27562
Vibrio parahaemolyticus	ATCC 17802
Vibrio cholerae	ATCC 14547
Vibrio fluvialis	ATCC 33809
Vibrio furnissii	ATCC 35016
Vibrio mimicus	ATCC 33653
Vibrio hollisae	ATCC 33654
Vibrio alginolyticus	ATCC 17749
Vibrio harveyi	ATCC 14126
Shigella flexneri	ATCC 12022
Salmonella enteritidis	KCCM 12021

ATCC: American Type Culture Collection, KCCM: Korea Culture Center of Microorganisms.

USA) and TCBS (Thiosulfate-Citrate-Bile salts Sucrose, Difco, USA) on a shaker at 200×g at 37°C.

Preparation of DNA

Chromosomal DNA was extracted using the Qiagen DNeasy 96 Blood Kit (Qiagen Corp., USA). Briefly, 5 ml of bacterial culture which was selected from TCBS media was centrifuged. Twenty µl of proteinase K and 200 µl of kit buffer AL were added and vortexed. The tubes were incubated at 70°C for 30 min, and 200 µl of ethanol was added. After centrifugation for 10 sec at 13,000×g, 450 µl portions of the lysates were transferred to a DNeasy 96 plate and QIAamp mini column, and then centrifuged again at 13,000×g for 10 min. The column was removed, and 600 µl of kit buffers AW1, AW2 were added separately to each well. Solutions were centrifuged at 13,000×g for 10 min. The DNA was eluted by adding 50 µl of kit buffer AE (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0), and centrifuged at 13,000×g for 3 min.

Design of primers and probes

The hemolysin/cytolysin gene has been sequenced from *V. vulnificus* strains. All of the available partial and full-length hemolysin/cytolysin gene sequences from GenBank accession No. M34670 were aligned. The approximate location of the specific probe was first determined. This was done by searching the GenBank databases for uniqueness of the probe to the *V. vulnificus* hemolysin/ cytolysin gene, using the Basic Local Alignment Search Tool (BLAST) database search (http://www.ncbi.nlm.nih.gov/BLAST/) program from the National Center for Biotechnology Information (NCBI). The final selection of the primers and the exact length of the probe were determined using the ABI Primer Express program (PE Applied Biosystems version 1.5). The program selected probe and primer sets with optimized melting temperatures, secondary structure, base composition, and amplicon lengths were designed. The forward primer, vvh-F, and the reverse primer, vvh-R, were 5'-CGCTGTTTAACGGCCAGCTA-3' and 5'-GGTTGTCATTCTCGTCGGTG-3', respectively. Using the PCR, a fragment of 276 bp spanning nucleotides 382 to 657 of the hemolysin/cytolysin gene was amplified. The TaqMan fluorescent probe, vvh-P, was 5'-6FAM-ACAGCAACCGAGACGAAATCACTCAAG-TAMRA-3', which was derived from 435 to 462 nucleotides (reverse complement) of the hemolysin/cytolysin gene of *V. vulnificus*. FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethylrhodamine) served as the reporter dye and the quencher dye, respectively. The 3' end was phosphorylated to prevent extension by Taq polymerase.

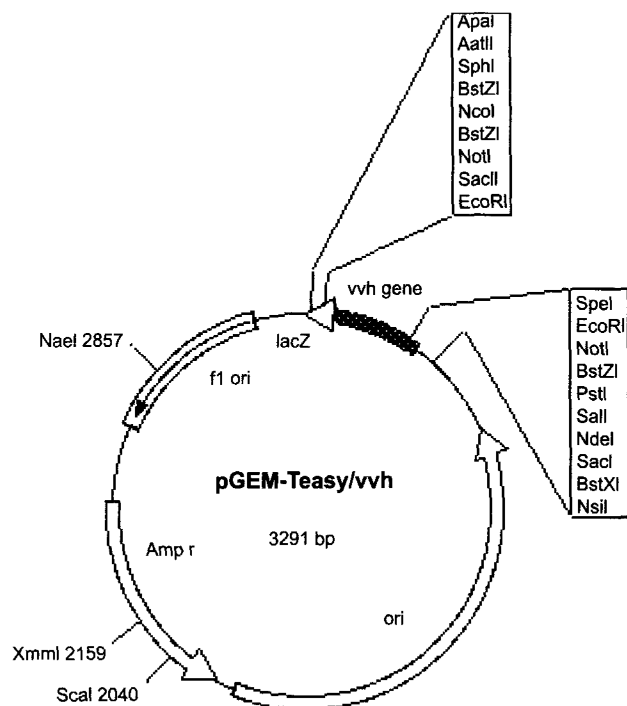

Fig. 1. pGEM-T Easy/vvh vector used in this study.

Real-time quantitative TaqMan PCR

Reactions were performed in 25 µl volumes using 0.5 ml optical-grade PCR tubes (PE Applied Biosystems, USA). The real-time PCR reaction mix consisted of 12.5 µl of TaqMan universal PCR Master Mix (PE Applied Biosystems, USA) containing dUTPs, $MgCl_2$, reaction buffer, AmpliTaq Gold, 100 nM of each primer, and 100 nM of fluorescence-labeled TaqMan probe. DNA and water were added to reach a final volume of 25 µl. Cycling conditions consisted of an initial single cycle at 95°C for 10 min to activate AmpliTaq Gold, followed by 40 to 50 cycles of two-temperature cycling consisting of 15 s at 95°C and 1 min at 55°C. PCR was performed with the ABI 7700 sequence detector according to the instrument's manual. Data were analyzed by the Sequence Detector software

(PE Biosystems, USA).

Conventional PCR

Template DNA was added to the final volume of 20 μl, which consisted of SuperTaq 10× reaction buffer I, SuperTaq 10× reaction buffer II, 2.5 mM dNTP, 10 pmol of each vvh primer, and 250 U of Taq polymerase. Amplification was performed in a PTC-200 thermocycler (MJ Research, USA). The cycle program was set for 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR product was detected by first adding 10 μl of sample, which was mixed with 1 μl of loading buffer and dye, and running the mixture in a 2% agarose gel at 140 volts.

Cloning

Cloning of the vvh gene was performed to obtain the standard positive control template DNA and to compare the sensitivity and specificity between real-time quantitative TaqMan PCR and conventional PCR. Amplified DNA was purified with the Qiagen purification kit (Qiagen Corp., USA) according to the manufacturer's instruction and was inserted into *Eco*RI sites of pGEM-T Easy Vector. The positive clones were grown in LB containing ampicillin (50 μg/ml), and the plasmids were purified with the Wizard mini-prep kit. The size of the insert was confirmed by 1% agarose gel electrophoresis after the *Eco*RI digestion (KOSCO, Korea) at 37°C for 2 h.

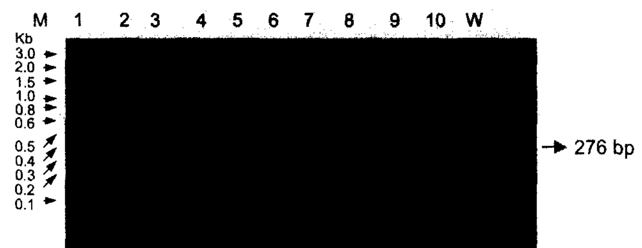

Fig. 2. The specificity of the primers in the PCR with the DNAs purified from various strains of bacteria. M, 100 bp DNA ladder; Lane 1, *V. vulnificus*, Lane 2, *V. parahaemolyticus*; Lane 3, *V. cholerae*; Lane 4, *V. fluvialis*; Lane 5, *V. furnissii*; Lane 6, *V. mimicus*; Lane 7, *V. hollisae*; Lane 8, *S. flexneri*; Lane 9, *S. enteritidis*; Lane 10, *S. choleraesuis*; Lane 11, negative control (water).

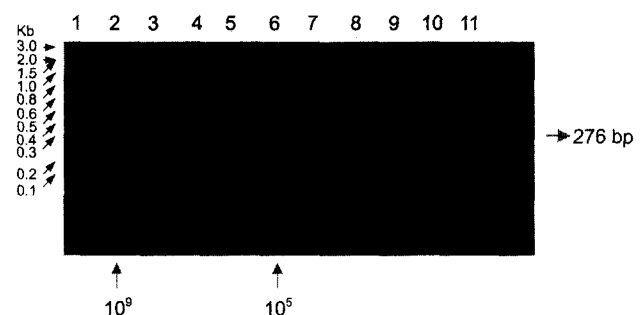

Fig. 3. Sensitivity of the PCR with the vvhF-vvhR primer set for the detection of purified chromosomal DNA from *V. vulnificus*. M, 100 bp DNA ladder; Lanes 1-10, purified chromosomal DNA serially diluted 10-fold from 10^9 to 10^0 copy; Lane 11, negative control (water).

Results

Comparison of conventional PCR, real-time quantitative TaqMan PCR, and biolog identification system™

Efficiencies of *V. vulnificus* detection from seawater by the conventional PCR, real-time quantitative TaqMan PCR with TaqMan primers and a probe, and Biolog Identification System™ were compared. Detection by each method was 36 (25%), 53 (36.8%), and 10 strains (6.9%), respectively, among the 144 samples collected from 6 sites in the Yellow Sea near Gunsan, Korea. As shown in Table 2, the real-time quantitative TaqMan PCR assay showed better identification accuracy than that of conventional PCR and Biolog Identification System™.

Specificity of primers and probe

The results of specificity of the primers were tested by performing the PCR with bacterial DNAs from *V. vulnificus, V. parahaemolyticus, V. cholerae, V. fluvialis, V. furnissii, V. mimicus, V. hollisae, S. flexneri, S. enteritidis, S. choleraesuis*, and *V. vulnificus* (Fig. 2). The amplified product was only detected in *V. vulnificus*. The probe region was chosen for optimized specificity and amplification efficiency. The primer DNA sequences were subjected to the BLAST database searches in order to avoid any sequence similarities. The specificity of selected primers and the probe was evaluated. Seventy-eight *V. vulnificus* strains were tested, and DNA from each strain was amplified with the TaqMan primers and probe. Ct values, ranging from 15.25 to 27.05, were obtained with 54 samples of the *V. vulnificus* (Table 3). When 100 ng samples of chromosomal DNA from the related species, such as *V. cholerae, V. parahaemolyticus, V. mimicus*, and *V. hollisae*, were used, the Ct values were 40 (Table 3). The result indicated that TaqMan Probe vvh-P was extremely specific to *V. vulnificus* and showed 100% accuracy in recognizing *V. vulnificus*.

Table 2. Identification of *V. vulnificus* by real-time quantitative TaqMan PCR, by conventional PCR, and by BioLog identification system in seawater (P<0.04)

Source	TaqMan PCR	PCR	BioLog
Seawater	53/144(36.8%)	36/144(25%)	10/144(6.9%)

To determine the sensitivity of the assay, conventional PCR was performed by using 10^9 to 10^0 copies/ml of plasmid DNA as a template with the primers and probes described above. The limitation of detection by conventional PCR was shown to be 10^5 copies of *V. vulnificus* DNA (Fig. 3). In TaqMan PCR, a DNA concentration could be detected for 10^8 to 10^1 copies (Fig. 4). In the 10^8

Table 3. The specificity of a probe in bacterial strains evaluated with the real-time quantitative TaqMan PCR for the detection of *V. vulnificus* DNA, Ct values generated during PCR amplification

Bacteria species	No of isolates	Source of isolation	Ct value	Interpretation
Vibrio vulnificus	54	ATCC 27562 (1), Seawater (53)	19.25-27.66	Positive
Vibrio Parahaemolyticus	2	ATCC 17802 (1), Seawater (1)	40.00	Negative
Vibrio cholerae	1	ATCC 14547(1)	40.00	Negative
Vibrio fluvialis	2	ATCC 33809 (1), Seawater (1)	40.00	Negative
Vibrio furnissii	3	ATCC 35016 (1), Seawater (2)	40.00	Negative
Vibrio mimicus	1	ATCC 33653 (1)	40.00	Negative
Vibrio hollisae	1	ATCC 33654 (1)	40.00	Negative
Vibrio alginolyticus	3	ATCC 17749 (1), Seawater (1)	40.00	Negative
Vibrio harveyi	2	ATCC 14126 (1), Seawater (1)	40.00	Negative
Vibrio mediteranei	2	Seawater (2)	40.00	Negative
Vibrio proteloyticus	1	Seawater (1)	40.00	Negative
Vibrio metschnikovii	1	Seawater (1)	40.00	Negative
Vibrio carchariae	2	Seawater (1)	40.00	Negative
Vibrio campbelli	1	Seawater (1)	40.00	Negative
Vibrio spendidus	1	Seawater (1)	40.00	Negative
Vibrio aestuarianus	1	Seawater (1)	40.00	Negative
Listonella anguillarum	2	Seawater (2)	40.00	Negative
Vibrio natriegens	1	Seawater (1)	40.00	Negative
Salmonella enteritidis	1	KCCM 12021 (1)	40.00	Negative
Aeromonas hydrophila	1	Seawater (1)	40.00	Negative
Aeromonas veronii	1	Seawater (1)	40.00	Negative
Aeromonas trota	1	Seawater (1)	40.00	Negative
Enterobacter gergoviae	1	Seawater (1)	40.00	Negative
Serratia Marcescens	1	Seawater (1)	40.00	Negative
Shigella flexneri	1	ATCC 12022 (1)	40.00	Negative

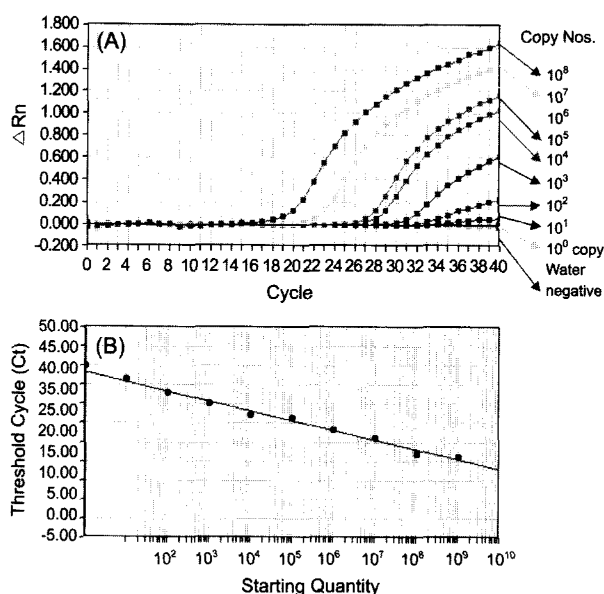

Fig. 4. Sensitivity test for the amplification of *Vibrio vulnificus* positive DNA by TaqMan real-time quantitative PCR using vvh primer and probe. The curves represent fluorescence changes over cycles. (A) A minimum of 10^1 copy of standard plasmid DNA from *V. vulnificus* could be detected after 40 cycles. △Rn (fluorescence units) was plotted against each cycle. (B) A standard curve with a linear range (correlation coefficient=0.988); Ct was plotted against the input of *V. vulnificus* DNA quantity (repeated 40 times). The intensity of fluorescence was given on the y axis (△ Rn=reporter signal[FAM]/passive reference signal[TAMRA])(R=0.988, P<0.038).

copy, the measurements change in fluorescence intensity in 16 amplification cycles. In the 10^1 copy, measurements change in fluorescence intensity in 35 amplification cycles (Fig. 4).

Detection limit by conventional PCR and real-time quantitative TaqMan PCR

The real-time quantitative TaqMan PCR assay is potentially much more sensitive than the conventional PCR that uses an agarose gel detection method because the real-time quantitative TaqMan PCR assay employs 40 amplification cycles, measures change in fluorescence intensity, and can detect template concentrations in the 10^1 copy range (standard curve with a linear range; correlation coefficient=0.988). The detection of real-time quantitative TaqMan PCR was 1,000 times better than that of conventional PCR (R=0.988, P<0.038) (Fig. 4).

If there was 1 copy of the hemolysin/cytolysin gene per cell, this would translate to 1 template, or 10 templates if there was 10 copies of the gene per cell, and so on. The 276-bp PCR fragment was cloned into the *E. coli* strain TOP10F' using the pGEM-T Easy vector that had a length of 3.01 kb (Promega, USA). The plasmid pvvh (276 bp), having a length of 3291 kb (3.01+0.276 kb) and containing the cloned insert, was used as a template. Thus, the primers and the probe combination as well as the length of the amplicon were optimal, since the greatest TaqMan PCR sensitivity could be achieved.

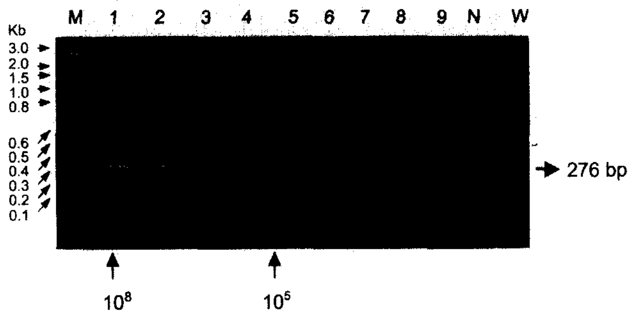

Fig. 5. Sensitivity of the PCR with the vvhF-vvhR primer set for the detection of *Vibrio vulnificus* positive DNA collected from infected human DNA (50 ng). M, 100 bp DNA ladder; Lanes 1-9, purified chromosomal DNA serially diluted 10-fold from 10^8 to 10^0 copy; N, negative control (human DNA); W, negative control (water).

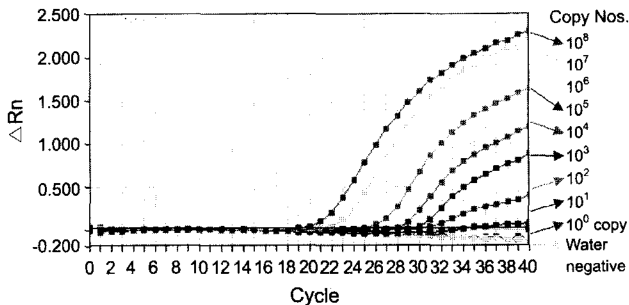

Fig. 6. Sensitivity test for the amplification of *Vibrio vulnificus* positive DNA collected from infected human DNA (50 ng) by TaqMan real-time quantitative PCR. A minimum of 10^1 copy of standard plasmid DNA from *V. vulnificus* could be detected after 40 cycles. The curves represent fluorescence changes over the cycles (R=0.985, P<0.25).

Sensitivity of the TaqMan PCR for the isolation of infected V. vulnificus DNA from human

The sensitivity study was performed with an infected *V. vulnificus* DNA from a human to test the lower detection limit of the TaqMan PCR. To determine the sensitivity of the assay, pure chromosomal DNA from a human DNA was extracted by the DNeay blood kit (Qiagen Corp., USA). Conventional PCR and TaqMan PCR were performed by using the 10^8 to 10^1 copies of chromosomal DNA as a template and probes described above. Conventional PCR could detect 10^4 copy (Fig. 5). In the TaqMan PCR, a DNA concentration of 10^8 to 10^1 copies could be detected. The detection of real-time quantitative TaqMan PCR was 1,000 times better than that of conventional PCR (R=0.985, P<0.25) (Fig. 6). In the 10^8 copy, measurements change in fluorescence intensity in 16 amplification cycles. In the 10^1 copy, measurements change in fluorescence intensity in 31 amplification cycles (Fig. 6).

To determine the sensitivity of the assay, the result of the TaqMan PCR from two human chromosomal DNA samples were extracted by the DNeasy blood kit (Qiagen Corp., USA), a DNA concentration of 10^{-4} ng/μl could be detected (P<0.02) (Fig. 7a, Fig. 7b).

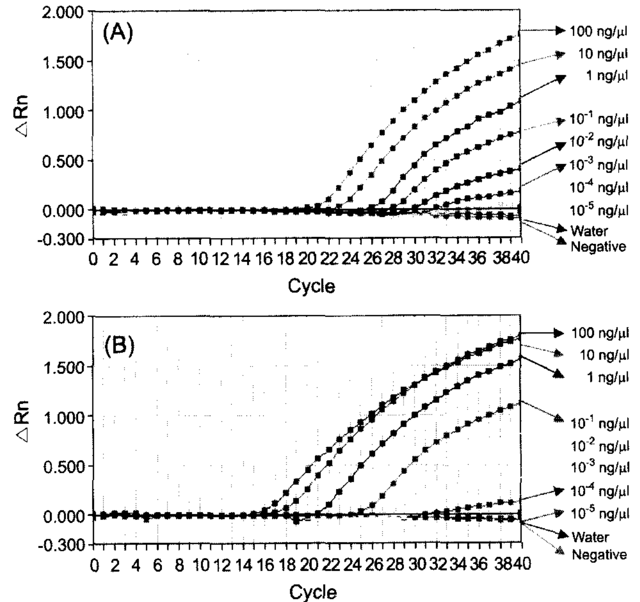

Fig. 7. Sensitivity test for the amplification of *Vibrio vulnificus* positive DNA collected from infected human DNA (50 ng) by TaqMan real-time quantitative PCR. A minimum of 10^{-4} ng/ml (arrow) of DNA from infected human could be detected after 40 cycles. The curves represent fluorescence changes over the cycles (P<0.02).

Discussion

Traditional identification methods that are currently being used are time-consuming, laborious, and require prolonged incubation time. Selective enrichment can reduce the growth of background flora. *Vibrio* cells may also enter a viable but non-culturable state, caused by nutrient starvation and physical stress. Several investigators have developed PCR and DNA probe techniques for the detection of pathogenic *Vibrio* species (Covadonga *et al.*, 1995; Shirley *et al.*, 1996; Chuang *et al.*, 1997; Athena *et al.*, 2000; Lee *et al.*, 2000; Kim *et al.*, 2001; Lyon, 2001). DNA-based methods, such as the conventional PCR, have been increasingly used as rapid and sensitive analysis, but these methods are not quantitative in their detections of *V. vulnificus*. The presence of PCR products must also be certified by subsequent procedures such as gel electrophoresis and Southern hybridization. All of these additional steps are time-consuming and laborious, and they add to the overall cost of experimentation (Lyon, 2001).

The 5'-nuclease fluorogenic TaqMan assay has been recently described (Livak *et al.*, 1995; Bassler *et al.*, 2000). The real-time quantitative TaqMan assay utilizes the 5'-nuclease activity of *Thermus aquaticus* DNA polymerase (Heid *et al.*, 1996; Kalinina *et al.*, 1997) to hydrolyze an internal TaqMan probe. This probe is labeled with a fluorescent reporter dye (FAM-6-carbooxyfluorescenin), which is attached to its 5' end, and also a quencher dye (TAMRA-6-carboxy-N,N,N',N'-tetramethylrhodamine),

which is attached to its 3' end. The probe is designed to hybridize to the DNA sequence between the PCR primers. During PCR amplification, cleavage of the TaqMan probe separates the reporter dye and quencher dye, which results in increased fluorescence. In contrast, when the probe is intact, the proximity of the reporter dye to the quencher dye results in blockage of the reporter fluorescence (Lyon, 2001). With increasing cycles of amplification, more fluorescent signal is generated by the binding of the probe to the more available targets, which can be detected in real time by the ABI 7700 sequence detector (PE Applied Biosystems, USA). The sequence detector contains a thermocycler, a laser detection system, and an analysis software system. The analysis of the signal takes only about a minute after the PCR is completed. Because the generation of the fluorescent signal depends on the hybridization of the probe to a specific template, which is amplified, there is less scope for false signals from the nonspecific amplification. TaqMan PCR eliminates the need for subsequent PCR product verification that is required by conventional PCR amplifications.

In this study, we performed the real-time quantitative TaqMan PCR that gives highly sensitive and specific results within several hours. To the best of our knowledge, the real-time quantitative TaqMan PCR has not been previously used for the detection of *V. vulnificus*. The advantages of the real-time quantitative TaqMan PCR, in comparison to conventional PCR, are shorter working time (only 3 h; 40 min for serum separation and DNA extraction from pellets centrifuged from the serum, 2 h for PCR amplification and detection checking, and gel electrophoresis is not required), determination of larger sample numbers (up to 96 samples per round), and lower risk of contamination.

The detection limits of the PCR assay were estimated to be approximately 10^{-8} CFU for milliliter in the *V. vulnificus* strain. These reported limits of detection are similar to the results in other reports obtained with a TaqMan PCR assay for endpoint detection (Bassler *et al.*, 1995; Chen *et al.*, 1997; Kalinina *et al.*, 1997; Norton *et al.*, 1999; Keya, 2000; Nogva *et al.*, 2000; Vishnubhatla *et al.*, 2000). Also, the detection limits of the TaqMan PCR assay were similar to the detection limits of the Nested PCR (Lee *et al.*, 1998). The TaqMan procedure appears to be much more sensitive than the conventional PCR method for target detection.

The use of the TaqMan PCR is a sensitive and quantitative method that is useful for estimating the number of cells of a specific pathogen in food products (Bassler *et al.*, 1995; Chen *et al.*, 1997; Norton *et al.*, 1999; Nogva *et al.*, 2000; Vishnubhatla *et al.*, 2000). Sensitivity studies were performed with infected *V. vulnificus* DNA from humans to test the lower detection limit of the TaqMan PCR. Conventional PCR could detect 10^5 copy. In TaqMan PCR, a DNA concentration of 10^8 to 10^1 copy could be detected. The efficiency of detection by the real-time quantitative TaqMan PCR was 1,000 times better than that of the conventional PCR ($R=0.985$, $P<0.25$, Fig. 6).

The real value of the TaqMan PCR lies in the potential for rapid analysis of numerous pathogen-free samples, thereby allowing laboratories the ability to screen products quickly before being released for human consumption. The detection system uses a 96-well fluorescence plate reader with optical tubes and caps, so the 96 samples can be analyzed at the same time. The plate readings do not require tubes to be opened after amplification, so the potential for carryover is reduced. Therefore, this real-time quantitative TaqMan PCR assay is more applicable than other systems for the detection of pathogenic *V. vulnificus* not only in seawater but also in food products like seafood.

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