Comparison of Conventional, Nested, and Real-Time PCR Assays for Rapid and Accurate Detection of *Vibrio vulnificus*[▽]

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We conducted a prospective study to target *toxR* in the blood of patients with skin and soft tissue infections who were admitted to four tertiary hospitals to assess the clinical usefulness of real-time quantitative PCR (Q-PCR) as a diagnostic technique. We performed conventional PCR (C-PCR), nested PCR (N-PCR), and Q-PCR assays and compared the results to those obtained using the “gold standard” of microbiological culture. The lower detection limit for the Q-PCR assay was 5×10^0 copies/ μ l. By use of blood samples of patients with skin and soft tissue infections, the sensitivities of the C-PCR and N-PCR assays against the target *toxR* gene of *V. vulnificus* as diagnostic tools were determined to be 45% and 86%, respectively. The C-PCR and N-PCR assays had specificities of 100% and 73%, respectively. When we adopted a crossing-point (cp) cutoff value of <38 cp as a positive result, the Q-PCR assay had 100% sensitivity and specificity. Q-PCR to detect *V. vulnificus*-specific genes is not only the most sensitive and specific of the techniques but also the most rapid diagnostic method. Therefore, the appropriate application of the Q-PCR assay using blood is useful for the rapid diagnosis and subsequent treatment of *V. vulnificus* sepsis.

Vibrio vulnificus can cause severe and life-threatening disease in those who eat contaminated seafood or have a wound that is exposed to seawater (2, 11, 15, 24). The disease develops rapidly and mortality is high. Hence, these patients require rapid diagnosis and subsequent treatment. Microbiological culture methods for the identification of causative organisms take several days; they are time-consuming and laborious but have good specificity (13). PCR assays have proven useful for early diagnosis. Conventional PCR (C-PCR) has been used to detect specific target genes in various microorganisms (5, 6, 13). Nested PCR (N-PCR) was developed to improve sensitivity but can give erroneous positive results due to DNA contamination (1). Multiplex PCR has the advantage of detecting several target genes at the same time, but it is time-consuming and laborious like C-PCR and N-PCR (3, 14). Real-time quantitative PCR (Q-PCR) can detect *V. vulnificus*-specific genes within 2 h (4, 15); there is no agarose gel-loading step (23), and the assay is not laborious and has high sensitivity and specificity (22).

Up to now there has been little comparative evaluation of these three PCR methods, namely, C-PCR, N-PCR, and Q-PCR, for targeting *V. vulnificus*-specific genes. The *toxR* gene is known as a gene encoding a transmembrane DNA binding regulatory protein in *Vibrio* species. The partial sequences of *toxR* differ among *Vibrio* species. The difference in *toxR* se-

quences among *Vibrio* species has been used as an effective marker for the identification of *Vibrio* species (22). To assess the clinical usefulness of Q-PCR as a diagnostic technique, we conducted a prospective study targeting the *toxR* gene of *V. vulnificus* in blood samples of patients with skin and soft tissue infections who were admitted to four tertiary-care hospitals. We carried out C-PCR, N-PCR, and Q-PCR assays and compared the results to those obtained using the “gold standard” of microbiological culture.

MATERIALS AND METHODS

Bacterial strains and media. The type strains used for positive or negative controls in this study are listed in Table 1. These strains were obtained from the American Type Culture Collection (ATCC), the Korea Culture Center of Microorganisms (KCCM), and the Korean Culture Type Collection (KCTC).

The clinical *V. vulnificus* strains were obtained from blood, bulla aspiration, and other skin and soft tissue samples from patients with skin and soft tissue infections in Chosun University Hospital, Chonnam University Hospital, Chonbuk University Hospital, and Pusan University Hospital in 2006 and 2007. The clinical strains were identified with a Vitek automated system (bioMérieux, Marcy l'Etoile, France). All strains were cultured in LB (Luria-Bertani) or brain heart infusion broth (Difco) or agar (Difco) containing 2% NaCl. The cells were stored at -70°C . The negative control used for these assays was water and genomic DNA from sera of patients with diseases other than *V. vulnificus* sepsis, while the positive control was extracted genomic DNA of cultured *V. vulnificus*.

Patient selection. We enrolled adult patients (ages of ≥ 18 years) suffering from skin and soft tissue infections such as cellulitis or necrotizing fasciitis. Informed consent was obtained from all patients or their guardians. Patients were admitted in 2006 or 2007 to four tertiary hospitals. Whole-blood samples for PCR were collected for this study. Clinical strains were isolated from the blood, bulla, and skin and other soft tissues. The identification of the clinical isolates was initially performed with a Vitek II automated system. PCRs were performed at Chosun University Hospital (Gwang-ju, Republic of Korea). The

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TABLE 1. Results of C-PCR, N-PCR, and Q-PCR assays for the detection of *V. vulnificus* in 30 type strains and 41 blood samples of patients with *V. vulnificus* and other infections

Strain or patient no.	Pathogen	Result by indicated assay			
		C-PCR	N-PCR	Q-PCR	
				cp value ^a	Interpretation
Strains					
1	<i>Aeromonas hydrophila</i> subsp . <i>hydrophila</i> KCTC 2358	—	—	38.35	—
2	<i>Vibrio alginolyticus</i> KCCM 40513	—	—	35.67	—
3	<i>Vibrio cholerae</i> KCCM 41626	—	—	37.37	—
4	<i>Vibrio fluvialis</i> KCCM 40827	—	—	35.8	—
5	<i>Vibrio furnissii</i> KCCM 41679	—	—	37.2	—
6	<i>Vibrio mimicus</i> KCCM 42257	—	—	34.37	—
7	<i>Vibrio proteolyticus</i> KCCM 11992	—	—	37.52	—
8	<i>Vibrio vulnificus</i> ATCC 27562	+	+	10.2	+
9	<i>Streptococcus agalactiae</i> KCCM 40417	—	—	>35	—
10	<i>Streptococcus mitis</i> KCTC 3556	—	—	35.15	—
11	<i>Streptococcus mutans</i> KCTC 3065	—	—	>40	—
12	<i>Streptococcus pyogenes</i> KCTC 3208	—	—	>40	—
13	<i>Streptococcus salivarius</i> KCTC 3960	—	—	35.25	—
14	<i>Streptococcus sobrinus</i> KCTC 3288	—	—	>35	—
15	<i>Staphylococcus aureus</i> subsp. <i>aureus</i> (MRSA ^b) KCCM 40510	—	—	36.25	—
16	<i>Staphylococcus aureus</i> (MRSA ^b) KCTC 29213	—	—	>35	—
17	<i>Staphylococcus epidermidis</i> KCTC 1917	—	—	>35	—
18	<i>Staphylococcus saprophyticus</i> subsp. <i>saprophyticus</i> KCTC 3345	—	—	>35	—
19	<i>Salmonella pneumoniae</i> KCTC 1925	—	—	37.08	—
20	<i>Klebsiella pneumoniae</i> KCTC 2242	—	—	>40	—
21	<i>Shigella sonnei</i> KCTC 2518	—	—	33.5	—
22	<i>Pseudomonas aeruginosa</i> KCTC 27853	—	—	39.64	—
23	<i>Aeromonas caviae</i> KCTC 1653	—	—	33.78	—
24	<i>Aeromonas salmonicida</i> subsp. <i>salmonicida</i> KCTC 12266	—	—	38.27	—
25	<i>Aeromonas hydrophila</i> subsp. <i>anaerogenes</i> KCTC 12487	—	—	>40	—
26	<i>Vibrio hollisae</i> KCCM 41680	—	—	36.46	—
27	<i>Vibrio parahaemolyticus</i> KCCM 11965	—	—	36.17	—
28	<i>Streptococcus pneumoniae</i> KCTC 3932	—	—	33.81	—
29	<i>Streptococcus sanguinis</i> KCTC 3299	—	—	36.95	—
30	<i>Clostridium difficile</i> KCTC 5009	—	—	36.67	—
Patients					
1	<i>Vibrio vulnificus</i>	+	+	30.25	+
2	<i>Vibrio vulnificus</i>	—	+	36.1	+
3	<i>Vibrio vulnificus</i>	—	—	35.08	+
4	<i>Vibrio vulnificus</i>	+	+	31.7	+
5	<i>Vibrio vulnificus</i>	+	+	31.61	+
6	<i>Vibrio vulnificus</i>	—	+	34.87	+
7	<i>Vibrio vulnificus</i>	+	+	25.85	+
8	<i>Vibrio vulnificus</i>	+	+	31.85	+
9	<i>Vibrio vulnificus</i>	—	+	34.3	+
10	<i>Vibrio vulnificus</i>	—	+	36.91	+
11	<i>Vibrio vulnificus</i>	—	+	32.09	+
12	<i>Vibrio vulnificus</i>	—	—	34.96	+
13	<i>Vibrio vulnificus</i>	—	—	33.75	+
14	<i>Vibrio vulnificus</i>	+	+	29.16	+
15	<i>Vibrio vulnificus</i>	+	+	27.16	+
16	<i>Vibrio vulnificus</i>	—	+	34.14	+
17	<i>Vibrio vulnificus</i>	+	+	30.77	+
18	<i>Vibrio vulnificus</i>	—	+	33.16	+
19	<i>Vibrio vulnificus</i>	+	+	29.89	+
20	<i>Vibrio vulnificus</i>	—	+	33.68	+

Continued on following page

TABLE 1—Continued

Strain or patient no.	Pathogen	Result by indicated assay			
		C-PCR	N-PCR	Q-PCR	
				cp value ^a	Interpretation
21	<i>Vibrio vulnificus</i>	—	+	18.02	+
22	<i>Vibrio vulnificus</i>	+	+	28.92	+
23	<i>Shewanella putrefaciens</i>	—	—	>40	—
24	<i>Staphylococcus aureus</i>	—	—	>40	—
25	<i>S. pyogenes</i>	—	—	>40	—
26	<i>Cellulomonas</i> spp.	—	+	>35	—
27	<i>Streptococcus viridans</i>	—	+	>35	—
28	<i>Streptococcus equi</i> subsp. <i>equi</i>	—	+	>35	—
29	<i>Staphylococcus hominis</i>	—	+	>35	—
30	<i>Streptococcus dysgalactiae</i>	—	—	>40	—
31	<i>Aeromonas hydrophila</i>	—	—	>40	—
32	<i>Pseudomonas aeruginosa</i>	—	—	>40	—
33	<i>Peptostreptococcus prevotii</i>	—	—	>40	—
34	<i>Staphylococcus aureus</i>	—	+	>40	—
35	<i>Aeromonas hydrophila</i>	—	—	>40	—
36	<i>Aeromonas</i> spp.	—	—	>40	—
37	<i>Fusobacterium necrophorum</i>	—	—	>40	—
38	<i>S. pyogenes</i>	—	—	>40	—
39	<i>Streptococcus anginosus</i> , <i>S. epidermidis</i>	—	—	>40	—
40	<i>S. mitis</i>	—	—	>40	—
41	<i>S. dysgalactiae</i>	—	—	>40	—

^a the cp cutoff value in Q-PCR is <38 cp for blood samples and <30 cp for bacterial isolates.

^b MRSA, methicillin-resistant *S. aureus*.

laboratory personnel who carried out the PCR assays were not aware of any of the clinical information or diagnoses, and the physician who treated the patients did not know the result of the PCR. The study was approved by the Ethics in Human Research Committee of each of the four tertiary university hospitals.

toxR cloning. The *toxR* gene of *V. vulnificus* was cloned according to the method of Takahashi et al. (22). Briefly, *V. vulnificus* ATCC 27562 was cultured in tryptic soy broth (Bacto) containing 2% NaCl, and genomic DNA for PCR was extracted using a QIAamp DNA mini kit (Qiagen, Hilden, Germany).

The primers designed to target the *toxR* gene of *V. vulnificus* (GenBank accession no. AF170883) are given in Table 2. PCR was conducted in 20-μl mixtures containing 1 μl of template DNA, 0.2 μl of 2.5 U of Takara *Taq* DNA polymerase (Takara Bio, Shiga, Japan), 1 μl each of 10 μM of forward primer

and reverse primer, 2 μl of deoxynucleoside triphosphates (dNTPs), 2 μl of 10× PCR buffer, and 12.8 μl of water. PCR was performed with predenaturation at 94°C for 5 min followed by 39 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min by use of an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA). The elongation step was prolonged to 7 min in the last cycle. The PCR product was electrophoresed on a 1.2% agarose gel (SeaKem LE agarose) with ethidium bromide at 100 V (0.5× Tris-borate-EDTA buffer). The amplified DNA was eluted from the gel by use of a QIAquick gel extraction kit (Qiagen, Hilden, Germany). The target DNA was then ligated into the pGEM-T easy vector and transformed into *Escherichia coli*. After confirmation of the insertion of the correct *toxR* sequence, the positive clones were cultured in LB broth containing

TABLE 2. Oligonucleotide primers and probe used in this study

Primer or probe name (sequence)	Location in bp (length)	Amplicon size (bp)	Temp (°C)	Reference	PCR assay function(s)
ToxAll 1 (5'-GAG CAG GGG TTT GAG GTG GAT GAT-3')	1–24 (24-mer)	573	63.1	1	Cloning primer
ToxAll 2 (5'-GTT TTG GCC CCC CGT CGC GAT CAC-3')	550–573 (24-mer)		72.7	1	
Tox-130 (5'-TGTTTCGGTTGAGCG CATTAA-3')	130–149 (20-mer)	70	56.4	1	Q-PCR primer, C-PCR primer, N-PCR internal primer
Tox-200 (5'-GCTTCAGAGCTGCG TCATTC-3')	180–200 (21-mer)		56.3	1	
Tox-152 (5'-FAM-CGCTCCTGTC AGATTCAACCAACAACG-B HQ1- 3' ^a) (probe)	152–188 (27-mer)	70	63.8	1	Q-PCR probe
Tox-100 (5'-ACGGTTCCAAAACG TGGTTA-3')	100–119 (20-mer)	204	60		N-PCR external primer
Tox-303 (5'-TGTTGACGTGCCAG CATTAT-3')	284–303 (20-mer)		60		

^a FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher-1.

FIG. 1. Diagram of primer positions in the *V. vulnificus* transmembrane regulatory protein *toxR* gene (GenBank accession no. AF170883).

ampicillin (50 mg/ml), and the plasmid DNA was extracted with a Gene All quick plasmid kit (General Biosystems, Republic of Korea).

Primers and probe. The primers and probe used in this study are listed in Table 2. The primers Tox-130 and Tox-200 and the probe Tox-152 were used as described by Takahashi et al. (22). Tox-130 and Tox-200 were used in the C-PCR, second-round N-PCR, and Q-PCR assays. Primers Tox-100 and Tox-303 were designed using the Basic Local Alignment Search Tool (BLAST) database search program of the National Center for Biotechnology Information (NCBI) and the Primer 3 program (7). The Tox-100 and Tox-303 primers were used in the first-round N-PCR assay. The 5' and 3' ends of probe Tox-152 were labeled with 6-carboxyfluorescein and Black Hole Quencher-1, respectively. The positions of the primers and the probe sequence are shown in Fig. 1.

PCRs. (i) C-PCR. DNA was extracted from whole blood, clinical isolates, and type strains as described in the manual supplied with the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Primers Tox-130 and Tox-200 were used in the C-PCR assay (Table 2). Template DNA was mixed with 1 μ l each of 10 pmol/ μ l of forward primer and reverse primer, 10 μ l of 2 $\times$ Excel *Taq* premix (2 U *Taq* polymerase, 400 μ M dNTPs, 2.0 mM $MgCl_2$, and KCl and Tris-HCl at proprietary concentrations) (CoreBioSystem, Republic of Korea), and the volume was adjusted to 20 μ l with distilled water. PCR conditions were as follows: preincubation at 94°C for 5 min and three steps of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min (35 cycles). The final extension step was 7 min and was done by use of an Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA). The PCR product was electrophoresed (Embi Tec) on a 2% agarose gel (SeaKem LE agarose; Cambrex Bio Science, Rockland, ME) with ethidium bromide for 30 min at 100 V (0.5 $\times$ Tris-borate-EDTA).

(ii) N-PCR. The internal primers Tox-130 and Tox-200 are listed in Table 2. The external primer was designed by use of the Primer 3 program (Table 2). The

first PCR assay was performed with the Tox-100 and Tox-303 primers (10 pmol/ μ l), 2 $\times$ Excel *Taq* premix (2 U *Taq* polymerase, 400 μ M dNTPs, 2.0 mM $MgCl_2$, and KCl and Tris-HCl at proprietary concentrations) (CoreBioSystem, Republic of Korea), template DNA, and distilled water in a total volume of 50 μ l. The first-round PCR conditions were the same as the C-PCR assay conditions. The second-round PCR mixture was the same as used for the first-round PCR assay, except for the use of primers Tox-130 and Tox-200 (10 pmol/ μ l). The template DNA for the second PCR was the product of the first PCR (2 μ l of a 50- μ l total volume). The second amplification was performed using the following cycles: predenaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. An Applied Biosystems Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA) was used. PCR products were electrophoresed on a 2% agarose gel at 100 V.

(iii) Q-PCR. The principle of Q-PCR is the detection of the fluorescent dye emitted during the PCR assay. Plasmid DNA was quantified using a spectrophotometer (DU 530 life science UV/visible-light spectrophotometer; Beckman Coulter). Units were converted from ng/ μ l to number of copies/plasmid molecule by use of the formula in the Promega protocol.

Plasmid DNA was serially diluted 10-fold after the concentration of the standard plasmid DNA was set to 1×10^8 copies/ μ l. The Q-PCR assay was conducted in 20- μ l reaction mixtures containing 5 μ l of template DNA, 1 μ l each of 5 pmol/ μ l forward primer and reverse primer (Tox-130 and Tox-200), 1 μ l of 2 pmol/ μ l probe, 4 μ l of master mix (reaction buffer, FastStart *Taq* DNA polymerase, $MgCl_2$, and dNTP [with dUTP instead of dTTP]) (Roche Diagnostics, Indianapolis), and water. The amplification conditions consisted of preincubation at 95°C for 10 min and two steps (45 cycles) at 95°C for 10 s and 60°C for 30 s followed by cooling at 40°C for 30 s. The results were analyzed using LightCycler software 4.0 (Roche, Basel, Switzerland).

Data analysis. We computed the sensitivities and specificities and the 95% confidence intervals (95% CI) for the three kinds of PCR assay. For all the statistical analyses, *P* values of <0.05 were considered as statistically significant. The data were analyzed using SPSS 10.0 software (SPSS Inc., Chicago, IL). Mutual relations between sensitivities and specificities of the methods used were presented according to the receiver operating characteristic (ROC) curve concept, with microbiological culture used as the gold standard (10). We applied the ROC curve to analyze and compare the diagnostic accuracies of three kinds of PCR assay by use of the MEDCALC software program (MedCalc Software, Mariakerke, Belgium) (21).

RESULTS

Standard bacterial strains. (i) Detection sensitivity. To determine the detection sensitivity, we performed C-PCR, N-PCR, and Q-PCR assays on serial dilutions of plasmid DNA of from 5×10^8 to 5×10^0 copies/ μ l (Fig. 2). C-PCR (70 bp) using Tox-130 and Tox-200 primers could detect down to $5 \times$

FIG. 2. Sensitivities of C-PCR (A), first-round N-PCR with external primers Tox-100 and Tox-303 (B), and second-round N-PCR with internal primers Tox-130 and Tox-200 (C) to detect *V. vulnificus* in plasmid DNA. Lanes: 1, 100-bp ladder marker (Bioneer); 2, negative control (sterile distilled water); 3, positive control (*Vibrio vulnificus*); 4 to 12, plasmid DNA serially diluted from 5×10^8 copies/ μ l to 5×10^0 copies/ μ l.

FIG. 3. Standard curves (5×10^8 to 5×10^0 copies/ μ l) from the Q-PCR assay. Plasmid DNA was used as the template. Circled numerals: 1, negative control (sterile distilled water); 2 to 10, plasmid DNA serially diluted from 5×10^8 copies/ μ l to 5×10^0 copies/ μ l.

10^3 copies/ μ l. The first-round N-PCR also detected down to 5×10^3 copies/ μ l, while the second-round N-PCR could detect as few as 5×10^2 copies/ μ l. Figure 3 shows the detection sensitivity of the Q-PCR assay. The Q-PCR assay using Tox-130 and Tox-200 primers and the Tox-152 probe could detect as few as 5×10^0 copies/ μ l.

(ii) Detection specificity. To confirm the specificities of the primers (Tox-130 and Tox-200) and probe (Tox-152) for *toxR*, we conducted C-PCR, N-PCR, and Q-PCR assays with the type strains shown in Table 1. C-PCR and N-PCR assays of the type strains did not give rise to any bands except with *V. vulnificus* (Table 1). The Q-PCR assay also produced high cp values (> 30 cp), except for *V. vulnificus* (10.2 cp) (Table 1); the cp value is the number of cycles at the point where fluorescence rises prominently above background noise, and it is similar to the threshold cycle. Hence, if we consider a cp value of more than 30 as negative (12, 16, 18), the test has 100% specificity.

Clinical isolates. C-PCR, N-PCR, and Q-PCR assays were performed on the clinical isolates identified as *V. vulnificus* by

the Vitek II system. Twenty-nine out of the 30 clinical isolates identified as *V. vulnificus* by the Vitek II system gave positive results in the C-PCR assay. All gave positive results in the N-PCR assay (data not shown). If we adopt a negative cutoff value of 30 cp for the Q-PCR assay (12, 16, 18), 29 of the 30 clinical isolates gave positive results; the exception was one isolate (CHU-A-47 [cp value of > 36]) which was initially identified as *V. vulnificus* by the Vitek II system but was finally identified as *Pseudomonas aeruginosa* by 16S rRNA gene sequence analysis and conventional microbiological methods (data not shown). In fact, all of the 29 positive clinical isolates of *V. vulnificus* gave positive results in the C-PCR and Q-PCR assays, and the tests were 100% specific. The positive result in the N-PCR assay of the CHU-A-47 clinical isolate finally identified as *Pseudomonas aeruginosa* turned out to be a false positive.

Clinical usefulness of Q-PCR using blood as a diagnostic method for *Vibrio vulnificus*. A total of 86 patients with skin and soft tissue infections were enrolled in our study. Pathogens were isolated from sterile fluids of 41 of these patients by

FIG. 4. ROC curve analysis of the sensitivities and specificities of the methods used, with microbiological culture as the gold standard.

microbiological methods. The three kinds of PCR assays were performed using extracted DNA from 200- μ l blood samples from these 41 patients. We detected 10 of the 22 *V. vulnificus* infections by C-PCR assay and 19 by N-PCR assay (Table 1). Comparison of the results of the C-PCR assay of the blood with the results of conventional microbiological methods yielded a sensitivity of 45% (95% CI, 25 to 67%) and a specificity of 100% (95% CI, 79 to 100%) as to diagnostic accuracy. The N-PCR assay had a sensitivity of 86% (95% CI, 64 to 96%) and a specificity of 73% (95% CI, 49 to 90%). If we adopt a cutoff value of 38 cp as a negative result (9, 20), the Q-PCR assay of blood samples has a sensitivity of 100% (95% CI, 82 to 100%) and a specificity of 100% (95% CI, 79 to 100%) (data not shown). The area under the curve for the Q-PCR assay was 1 (95% CI, 1 to 1) for the highest sensitivity and specificity. This difference was statistically significant (Q-PCR versus C-PCR, $P = 0.001$; Q-PCR versus N-PCR, $P = 0.004$) (Fig. 4).

DISCUSSION

V. vulnificus sepsis is a rapidly progressing and lethal disease entity which causes mortality at a rate that is reported as $\geq 50\%$ (2, 11, 15, 24). It is very important to administer adequate antibiotics immediately after early diagnosis. It takes several days to isolate causative infectious organisms from patients' samples, such as those of blood, bulla, cerebrospinal fluid, and skin tissue. For the PCR assays, it takes at least 6 h to detect the *V. vulnificus*-specific gene, but the sensitivity of the C-PCR assay is not good. Although N-PCR requires longer than C-PCR (9 h), its sensitivity is known to be better than that of C-PCR (8). The Q-PCR assay can give results within 2 h from genomic DNA extraction to data analysis after amplification (start to finish), and it is very useful for establishing an early diagnosis, has the potential for automation for high-throughput analysis, and yields quantitative information for assessing prognosis or responses to treatment. Until now, there have been no data on the evaluation of three PCR methods (C-PCR, N-PCR, and Q-PCR) against the same target gene specific for *V. vulnificus*. We selected the *toxR* gene, which is known to be a housekeeping regulatory gene regarded as an

effective taxonomic marker for the identification of *Vibrio* species (22). For the type strains used here, the specificities of the three kinds of PCR assays for the various microorganisms were very good. No positive results were detected in the C-PCR and N-PCR assays, except from samples containing *V. vulnificus*. The Q-PCR assay also had a high cp value (>30) for various microorganisms other than *V. vulnificus*. Only *V. vulnificus* gave a low cp value (10.2 cp).

Takahashi et al. reported that Q-PCR targeted to *toxR* for the detection of *V. vulnificus* was very sensitive, detecting as few as 10 microbes per milliliter of seawater or oyster homogenate (22). In our study, the detection sensitivities of the three kinds of PCR assays differed. The lower limit of detection of the C-PCR assay was 5×10^3 copies/ μ l, and that of the N-PCR assay was 5×10^2 copies/ μ l. However, the lower limit of detection of the Q-PCR assay was five copies/ μ l, and it had the best detection sensitivity.

The cp values of cultured microorganisms are lower than those from clinical specimens such as blood, tissue fluids, biopsy samples, and so on. Negative results using cp values for cultured microorganisms generally have been determined by use of cp values of >30 or >28 , as described previously (12, 16–18). The cp's for clinical specimens from stool and biopsy samples were reported to agree, with values of >40 and >38 , respectively (19). The cp value of blood was higher than 38 (9, 20). In our study, when the cp value was more than 38, sequencing of the PCR products revealed them to be dimers, not *V. vulnificus* genes; when the cp value was less than 38, the product was *toxR*. Therefore, in the Q-PCR assay, we adopted a cp value of >38 as a negative result for blood samples. We adopted a cutoff cp value in the Q-PCR assay of >30 when we performed the Q-PCR assay with cultured bacterial isolates.

To assess the clinical usefulness of the Q-PCR assay in practice as a diagnostic technique, we blindly compared the Q-PCR assay results obtained using blood samples from the patients with skin and soft tissue infections with the other PCR assays and the results of microbiological culture. If we used cutoff values of <38 cp as positive results, the results of the Q-PCR assay using blood samples showed 100% sensitivity and specificity. The area under the curve for Q-PCR was 1 (95% CI, 1 to 1) with the highest sensitivity and specificity. This difference was statistically significant (Fig. 4). Furthermore, 5 out of the 22 patients with *V. vulnificus* infection showed positive Q-PCR assay results from blood with a negative blood culture result but isolation of *V. vulnificus* from skin and soft tissue specimens. Therefore Q-PCR was not only the most sensitive and specific technique for detecting *V. vulnificus*-specific genes but also the most rapid diagnostic method. Rapid diagnosis and adequate treatment of *V. vulnificus* sepsis might make a contribution to reducing mortality.

In conclusion, Q-PCR to detect *V. vulnificus*-specific genes is not only the most sensitive and specific technique but also the most rapid diagnostic method. Therefore, our study suggests that appropriate use of the Q-PCR assay using blood is useful for the rapid diagnosis and subsequent treatment of *V. vulnificus* sepsis.

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