Two-stage Nested PCR Effectiveness for Direct Detection of *Vibrio vulnificus* in Natural Samples

J. Y. Lee, Y. B. Bang, J. H. Rhee, and S. H. Choi

ABSTRCT

The nested primers designed to amplify a 222-base pair portion of the hemolysin gene, *vvhA*, were specific for all *V. vulnificus* strains tested. The nested PCR amplification, coupled with direct extraction of template DNA, revealed improved sensitivity sufficient for detection of 1 to 10 CFU *V. vulnificus* in 1 mL of seafood homogenates, and eliminated the need for enrichment culturing. Thereby, the nested PCR method achieved a broader applicability, making it effective for extensive use in identification of the pathogen in natural samples such as raw seafoods, seawater and sediments.

Key Words: *Vibrio vulnificus*, nested PCR, hemolysin gene

INTRODUCTION

VIBRIO VULNIFICUS HAS BEEN ISOLATED from various raw seafoods, and is ubiquitous in the estuarine environment (Tamplin et al., 1982; Oliver et al., 1983; O'Neill et al., 1992; DePaola et al., 1994; Wright et al., 1996). The pathogen has been identified as the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia in immuno-compromised individuals (Blake et al., 1980; Klontz et. al., 1988). Mortality from septicemia is quite high (> 50%), and death may occur as soon as 1 to 2 days after first signs of illness (Tacket et al., 1984; Oliver and Kaper, 1997). Therefore, rapid identification of *V. vulnificus* in natural samples is essential to reduce the potentially fatal effects.

The viable but nonculturable (VBNC) state of *V. vulnificus* is often induced by lowering incubation temperature or by natural environmental parameters (Oliver et al., 1995; Oliver et al., 1991). Under favorable conditions, such VBNC cells of *V. vulnificus* have been shown to retain their ability to repair and to resuscitate (Nilsson et al., 1991; Oliver and Bockian, 1995; Oliver et al., 1995; Whitesides and Oliver, 1997). Thus, there is a serious potential public health hazard presented by such nonculturable cells of *V. vulnificus*. Since such cells are not detectable by laboratory culture techniques, a method other than conventional laboratory culturing would be advantageous for detecting them.

The polymerase chain reaction (PCR), which has the ability to amplify unique sequences of DNA (Saiki et al., 1988), can be an effective means for very specific, sensitive, and rapid detection of *V. vulnificus* in seafood or other natural sources (Brauns et al., 1991; Coleman et al., 1996). However, conventional PCR procedures are usually based on one-stage DNA amplification, and their low sensitivity requires enrichment of *V. vulnificus*, which is frequently present at low numbers in seafoods, to a certain level (Hill et al., 1991; Lee and Choi, 1995; Lee et al., 1997). The length of time for the enrichment could hinder the in-time prevention of widespread consumption of seafoods contaminated with the pathogen. Application of novel procedures for effective recovery of template DNA from lower numbers of the pathogen and multi-stage amplifications of target DNA with nested pairs of primers have been attempted to enhance sensitivity (Arias et al., 1995; Coleman et al., 1996; Lee et al., 1997). In nested PCR, products of primary PCR amplifications carried out with an external set of primers are designed to serve as template DNA for the next-stage PCR, in which nested primers are used to hybridize with sequences inside the product.

Broader application of PCR procedures for identification of *V. vulnificus* in diverse seafoods also has been limited by the lack of general suitability. Most PCR procedures have been developed and optimized for identification of the pathogen in oysters (*Crassostrea virginica*), and the same efficiency may not apply in other seafood samples. Besides oysters, however, small octopus (*Octopus variabilis*), arkshell (*Anadara broughtonii*), rockfish (*Sebastes inermis*), conger eel (*Anago anago*), thin-shelled surf clam (*Ruditapes philippinarum*), blue crab (*Portunus trituberculatus*), and shrimp (*Penaeus japonicus*) which are popular in Korea, Japan and elsewhere (Ichthyological Society of Japan, 1981; Je, 1989) are often consumed raw or undercooked. Increasing numbers of outbreaks of fatal septicemia due to consumption of such seafoods have been reported in Korea (Rhee, 1995). However, only a few studies on PCR procedures for detection of *V. vulnificus* in seafoods other than oysters have been reported (Coleman et al., 1996; Lee and Choi, 1995; Lee et al., 1997). Therefore, development of a PCR procedure with improved sensitivity and suitability is needed for extensive use as a diagnostic method to identify *V. vulnificus* in different types of samples.

We demonstrated previously that a PCR method for detection of *V. vulnificus* from small octopus could be more effective by employing a method for direct extraction of DNA (Lee et al., 1997). However, the sensitivity was limited and an enrichment step was required to detect the pathogen at levels of 10^2 colony forming units (CFU)/mL of seeded small octopus homogenate. Our current objectives were to improve the general suitability as well as the sensitivity of the PCR method for detection of *V. vulnificus*, through the development and optimization of a two-stage nested PCR.

MATERIALS & METHODS

Bacterial strains and culture conditions

A total of 21 strains of *Vibrio* spp. and 13 non-*Vibrio* bacterial strains used to assess specificity of the primers, and their origins, are listed in Table 1. Among the *Vibrio* strains, a *V. vulnificus* CDC C7184 was used to seed seafoods. Halophilic *Vibrio*s were grown on Luria-Bertani (LB) plates or in LB broth containing 2% salt at 30°C, and other strains were grown on LB plates or in LB broth (0.5% salt) at 37°C.

Preparation of seafoods for sensitivity determination

Fresh small octopus, oyster, ark shell,

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Table 1—Specificity of nested PCR developed in this study for identifying *V. vulnificus*

Species	Strain	Reference or Source	PCR amplification[a]
Vibrio vulnificus	ATCC[b]29307		+
	ATCC27562		+
Vibrio vulnificus	CDC[c] C7184		+
Vibrio vulnificus	CDC H3308		+
Vibrio vulnificus	CS9133	Clinical isolate from Chonnam National University Hospital	+
Vibrio vulnificus	CNUH3	"	+
Vibrio vulnificus	*CNUH94-3*	"	+
Vibrio vulnificus	*CNUH94-8*	"	+
Vibrio vulnificus	*CNUH95-1*	"	+
Vibrio vulnificus	WK2	Clinical isolate from Wonkwang University Hospital	+
Vibrio vulnificus	WK3	"	+
Vibrio vulnificus	WK6	"	+
Vibrio vulnificus	WK15	"	+
Vibrio vulnificus	WK18	"	+
Vibrio vulnificus	V-15	Seawater isolate	+
Vibrio vulnificus	V-16	Seawater isolate	+
Vibrio alginolyticus	ATCC17749		–
Vibrio cholerae Inaba	ATCC9459		–
Vibrio cholerae	ATCC14033		–
Vibrio furnissii	ATCC35016		–
Vibrio parahaemolyticus	ATCC27519		–
Citrobacter freundii	ATCC6705		–
Enterobacter cloacae	ATCC13047		–
Escherichia coli	ATCC25922		–
Escherichia coli O157:H7	ATCC35150		–
Klebsiella pneumoniae	ATCC13883		–
Micrococcus luteus	ATCC9341		–
Proteus spp	MB838 NCMB[d]		–
Pseudomonas aeruginosa	ATCC27853		–
Salmonella enteritidis	ATCC13076		–
Salmonella typhimurium	ATCC19430		–
Shigella dysenteriae	ATCC9361		–
Staphylococcus aureus	ATCC6538		–
Staphylococcus epidemidis	ATTC12228		–

[a]Determined from observation of the characteristic 222-bp band by gel electrophoresis analysis of the PCR product.
[b]American Type Culture Collections.
[c]Center for Disease Control.
[d]National Collection of Marine Bactera.

black rockfish, and conger eel were purchased during the winter of 1995 from a local seafood market (Kwang-ju, Korea). The seafoods were washed twice with sterile saline, and kept frozen until used. Homogenates of the seafoods were prepared by blending 1g of each sample with 9 mL of modified brain heart infusion broth (Lee et al., 1997). Each homogenate was seeded with a dilution of *V. vulnificus* CDC C7184 to 1-10 CFU/mL at the initial level. The concentrations of *V. vulnificus* cells were confirmed by counting CFU appearing on LB plates containing 2 % salt. Seeded homogenates were incubated at 30°C for 0, 1, 2, 4, and 8 h and then used for determination of sensitivity of the PCR method developed.

Collection and preparation of natural samples

Natural seafoods including arkshells, thin-shelled surf clams, blue crabs, shrimps, and small octopi as well as sediment and seawater samples were obtained in the summer of 1996 from the southwestern coast of Korea. Individual samples in plastic bottles were placed in ice, and were transported to the laboratory within 2h of collection.

On arrival, 1g of each seafood sample was added to 9 mL of sterilized artificial seawater (ASW) (Nealson, 1978) at 5°C, and homogenized. Separate homogenates for intestine and muscle parts of the seafood were prepared when possible. The microbial cells from the seawater were collected by filtering 10 mL of seawater through a 25 mm dia, 0.2-μm pore-size nitrocellulose membrane filter (Millipore Corp., Bedford, MA). The cells remaining on the surface of the filter were resuspended in 200 μL of sterile distilled water. One gram of sediment was used to separate microbial cells by suspending in 9 mL of ASW and mixed vigorously using a Vortex. After waiting 3 min for particles to precipitate, 200 μL of the supernatant suspected to contain microbial cells was transferred to a sterile tube. Each of the unseeded homogenates of the natural seafoods, the filter concentrate of seawater and the supernatant of sediment suspension was examined for the presence of *V. vulnificus* by the developed PCR.

DNA extractions for PCR analysis

Total DNAs from 1 mL of pure culture of the strains listed in Table 1 and from each seeded or unseeded seafood homogenate were directly extracted by the silica coarse adsorption-elution method as described previously (Boom et al., 1990; Lee et al., 1997). A 2 μL portion of the DNA dissolved in 20 μL distilled water was used as a template DNA for PCR.

Cells collected from the seawater and sediment samples and suspended in 200 μL volume were subjected to 5 cycles of freeze-thaw lysis, frozen in a −80°C deep freezer and then thawed by transferring into tap water (Lee and Ruby, 1995). Cells were mixed vigorously using a Vortex after every cycle of freeze-thawing to release nucleic acids from the cells. A 20 μL aliquot of individual cell lysates was then directly used to provide template DNA for PCR.

Oligonucleotide primers and PCR amplification

Two sets of *V. vulnificus*-specific oligonucleotide primers were synthesized by the Bioneer Co., Seoul, Korea. The external primers, JY-1 (5'-GACTATCGCATCAACAACCG-3', sense primer) and JY-2 (5'-AGGTAGCGAGTATTACTGCC-3', antisense primer)(Lee and Choi, 1995), and inner primers, JY-3 (5'-GCTATTTCACCGCCGCTCAC-3', sense primer) and JY-4 (5'-CCGCAGAGCCGTAAACCGAA-3', antisense primer) were located within the open reading frame of *vvhA* (Fig. 1).

Primary PCR amplifications were carried out in a DNA thermal cycler (Ericomp, SingleBlock System, Bio-Pacific Corp., U.S.A.) using JY-1 and JY-2 primers and *Taq* DNA polymerase (Bioneer, Seoul, Korea), and were expected to generate a 704-bp-long DNA fragment. PCR reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.0 μM of each primer, and 2 units of Taq polymerase. The resulting mixtures, adjusted to 100 μL by adding template DNA, were preheated for 5 min at 94°C, and then 30 cycles of amplifications of target sequences were conducted. Each cycle consisted of a denaturing step (1 min, 94°C), an annealing step (1 min, 60°C), and an extension step (2 min, 72°C).

For the second round PCR, a 2 μL portion of primary PCR products, which had been purified through ethanol precipitation and resuspended in 20 μL sterile distilled water, was added to each reaction mixture as template DNA. Reaction conditions were identical to those used for the first PCR, except that 62°C was the annealing temperature and JY-3 and JY-4 were used as nested primers. Generation of a DNA fragment with 222-bp was expected by second stage PCR. To detect the presence of specific fragments, 20 μL of each PCR product was separated by agarose gel electrophoresis.

RESULTS & DISCUSSION

Specificity of the nested PCR amplification

The specificity of the nested PCR proce-

dure using JY-1, JY-2, JY-3 and JY-4 primers was assessed with DNAs recovered from pure cultures of the strains listed in Table 1. Previous works revealed that the *vvhA* gene was unique to this organism by DNA hybridization (Wright et al., 1985). However, there are regions where the nucleotide sequences of the *vvhA* gene and the *hly* gene encoding hemolysin of *V. cholerae* showed significant homologies (Yamamoto et al., 1990). Therefore, the 704-bp and 222-bp fragments within the region of *vvhA* where the nucleotide sequences mismatch with the *hly* gene in *V. cholerae* were targeted for amplification, and two sets of primers for respective amplification were designed. The PCR amplification generated the characteristic 222-bp bands for all *V. vulnificus* tested. However, when the PCR procedure was applied to other strains, including members of the genera *Vibrio*, amplifications of the characteristic band were not apparent (Table 1). These results indicated that the two sets of nested primers were designed to bind specifically to *vvhA* DNA of *V. vulnificus* but not to other DNA, and that this nested PCR procedure was specific and could identify all *V. vulnificus* including natural and clinical isolates.

V. vulnificus is frequently isolated together with other *Vibrio* species such as *V. parahaemolyticus, V. cholerae, V. furnissii*, and *V. alginolyticus* (Choi, 1997; Oliver et al., 1983). Although current laboratory techniques using selective media have proven effective in identifying *V. vulnificus* (Oliver et al., 1992; Miceli et al., 1993), these techniques still cannot provide optimal differentiation of the pathogen from other halophilic *Vibrio*s (Choi, 1997). From this result, it was apparent that the two-stage nested PCR procedure provided a very specific means to differentiate all *V. vulnificus* from other *Vibrio*s in the natural samples where the pathogen exists together with a large background of other procaryotic and eucaryotic cells. Such specificity would be especially beneficial and became a key criterion for the detection method to be developed.

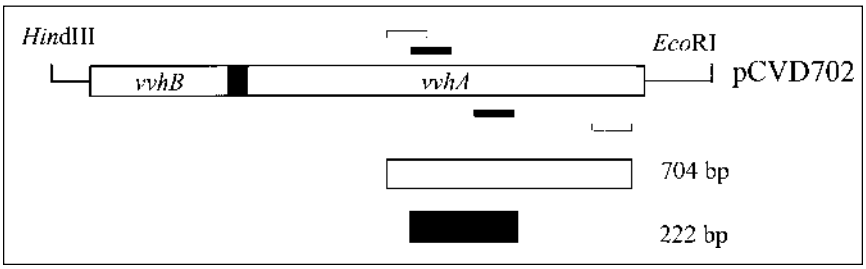

Fig. 1—Schematic representations of the *V. vulnificus* cytotoxin-hemolysin gene (*vvhA*) cloned on pCVD702 (Yamamoto et al., 1990), the locations of primers used, and the proposed products of each PCR. Hybridizing locations of oligonucleotide primers and the DNA fragments amplified by each PCR are depicted by open bars for primary PCR and by closed bars for nested PCR. The sizes of the PCR products follow the representation of each DNA segment.

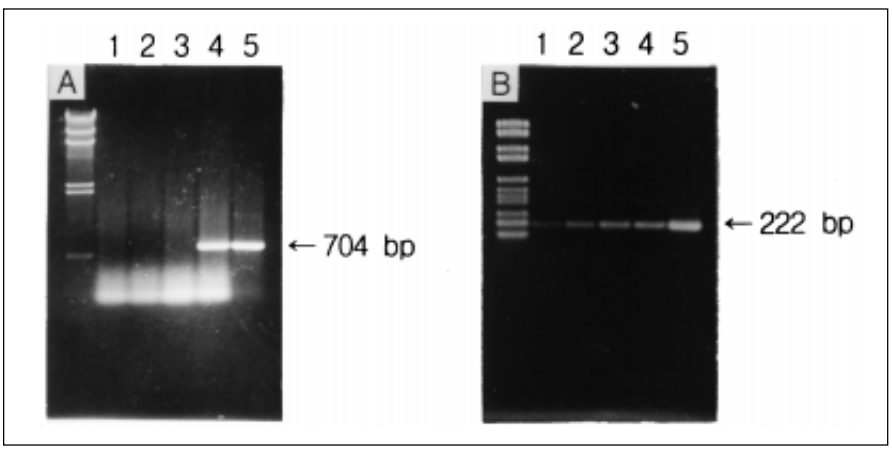

Fig. 2—Sensitivity of primary PCR and two-stage nested PCR for detection of *V. vulnificus* in small octopus. For panel A, small octopus homogenates seeded with *V. vulnificus* at the initial level of 10 CFU/mL were incubated for different periods, 0h, 1h, 2h, 4h and 8h (lanes 1 to 5 respectively), and then were used for extraction of template DNA for primary PCR. Lanes of panel B are the same as in panel A, but the recovered DNA from primary PCR products analyzed on each lane of panel A were used as template DNA for second stage nested PCR analyzed on corresponding lanes of panel B. Characteristic 704-bp bands and 222-bp bands are indicated. The migrations of lambda DNA digested with *Hin*dIII are indicated to the left as molecular size marker for panel A, and the migrations of pBR328 DNA digested with *Bgl*I and *Hin*fI are indicated to the left for panel B.

Sensitivity for detection of *V. vulnificus* in various seafoods by nested PCR

The sensitivity of the PCR detection system applied to real food samples was evaluated by determining the detection limit after seeding the small octopus homogenate with a known concentration of the pathogen. DNA extract was prepared from each homogenate, which was seeded with *V. vulnificus* at the level of 1 to 10 CFU/mL and then incubated for different lengths of time. Using the DNA as a template, the targeted sequence was successfully amplified by first round PCR and the 704-bp DNA fragment was generated from homogenates incubated for as long as 4h or more for enrichment (Fig. 2A). However, the characteristic 704-bp band did not appear from homogenates incubated for less than 4h. In contrast with these results, a 222-bp DNA fragment, amplified by the second round PCR with nested primers was consistently observed and any incubation of seeded homogenate samples for enrichment was not necessary (Fig. 2B). From these results, it was obvious that significant enhancement in sensitivity was achieved by employing two-stage nested PCR. In both PCR, however, no positive signals were obtained from control homogenates which had been unseeded with *V. vulnificus* but incubated for 4h (data not shown).

For use as a successful diagnostic method, the same sensitivity must be obtained consistently with the nested PCR procedure when applied to various seafoods other than small octopus. Therefore, detection of the pathogen in diverse seafoods including arkshells, blue crabs, and conger eels was attempted using the developed nested-PCR procedure. The target sequence from the pathogen seeded at the level of 1 to 10 CFU/mL into these seafood homogenates was successfully amplified by the PCR (Fig. 3). The results indicated that the sensitivity was not significantly affected by the types of seafood where the pathogen existed and that the PCR procedure could therefore detect *V. vulnificus* in different seafoods with a similar level of sensitivity.

It was not possible to access total indigenous cells of *V. vulnificus* including VBNC

state in the seafood homogenates used for sensitivity determination, since quantitation of VBNC cells of *V. vulnificus* differentially from other bacterial cells is still one of our greatest limitations. However, the indigenous level of viable *V. vulnificus* cells was determined by using TCBS (thiosulfate-citrate-bile salts-sucrose) and CPC (cellobiose-polymyxin B-colistin) selective media, and appeared to be less than 1 CFU/mL of the seafood homogenates prepared by our method. Although the exact concentration of *V. vulnificus* after inoculation was not known, based on a detection limit of fewer than 10 CFU/mL at the initial level, the sensitivity of the nested PCR detection system seemed quite high. This enhanced sensitivity of the nested PCR would render the identification of the pathogen, frequently present at low levels in natural samples, more rapid and more feasible.

Detection of *V. vulnificus* in natural samples by two-stage nested PCR

To demonstrate the general suitability of the nested PCR optimized under experimental conditions by using the seeded homogenates of seafoods, field trials for direct detection of *V. vulnificus* in naturally contaminated samples were carried out. Total DNAs were extracted directly from natural seafood samples of small octopus, oyster, arkshell, rock fish, and conger eel without any step for enrichment of the pathogen and were used as a PCR template. In 20% of the natural seafood samples, a 222-bp DNA fragment specific for *V. vulnificus* was amplified by nested PCR and observed by gel electrophoresis, indicating the presence of *V. vulnificus* in those samples (Fig. 4). The PCR analysis for the intestine part and flesh part of arkshell revealed the characteristic bands in both, indicating that the pathogens were in both parts of the arkshell. These results suggested that there were no specific associations between the bacteria and particular organs of marine animals. Positive PCR amplifications were also found in 2 out of 10 seawater samples and in 2 out of 6 sediment samples, indicating that *V. vulnificus* was present in those samples (Fig. 4). These results confirmed previous reports that *V. vulnificus* is ubiquitous and commonly found in temperate coastal environments (Tamplin et al., 1982; Oliver et al., 1983; O'Neill et al., 1992; Wright et al., 1996).

It has been suggested that the major obstacle to widespread application of a PCR procedure on food samples is the presence of components that may inhibit the polymerase activities or binding of primers (Rossen et al., 1992). Many components are unique to particular foods and extracted together with the DNA used as PCR templates. A good separation procedure to remove these undesirable contaminants from various natural samples is necessary to increase general

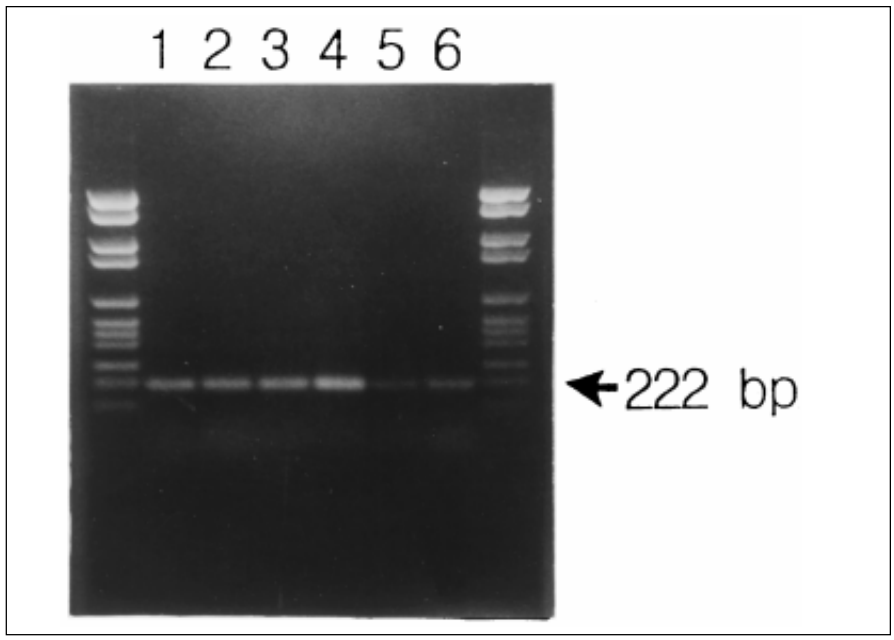

Fig. 3—General suitability of the two-stage nested PCR for detection of *V. vulnificus* in a variety of seafoods. Each seafood was seeded with *V. vulnificus* at the level of 1 to 10 CFU/mL initially and the resulting mixtures were used without any enrichment step for extraction of template DNA. Lane 1, small octopus; Lane 2, oyster; Lane 3, arkshell; Lane 4, rockfish; Lane 5, conger; Lane 6, 100 ng of genomic DNA from *V. vulnificus* pure culture. The migrations of pBR328 DNA digested with *Bgl*I and *Hinf*I are indicated to the left and right as molecular size markers.

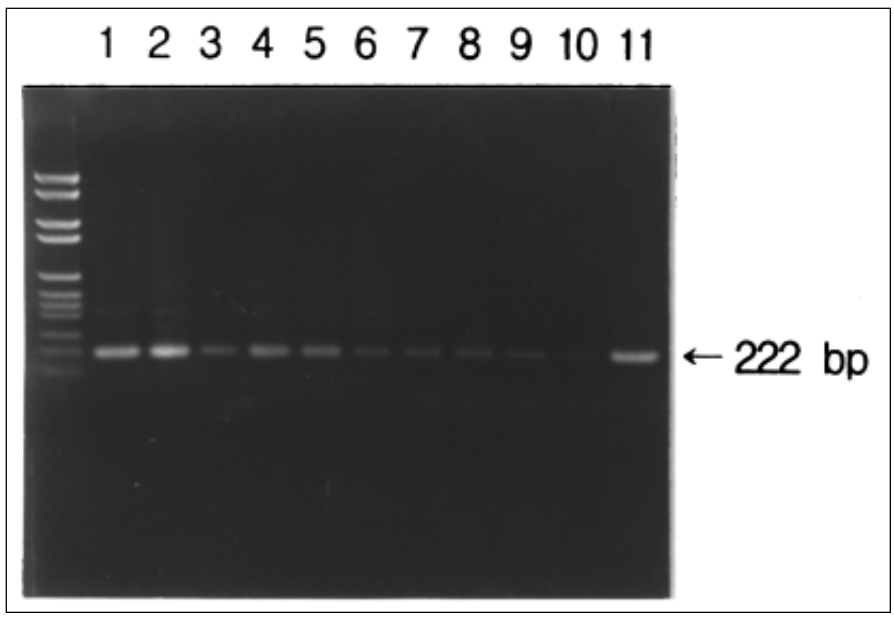

Fig. 4—Electrophoretic analysis of PCR products indicating the presence of *V. vulnificus* in various natural samples and showing wide application of the two-stage nested PCR we developed. Samples were used without any enrichment for extraction of template DNA. Lane 1, arkshell (muscle); lane 2, arkshell (intestinal organ); lane 3, thin-shelled surf clam; lane 4, blue crab; lane 5, shrimp; lane 6, small octopus; lane 7, seawater 1; lane 8, seawater 2; lane 9, sediment 1; lane 10, sediment 2; lane 11, 100 ng of genomic DNA from *V. vulnificus* pure culture. The migrations of pBR328 DNA digested with *Bgl*I and *Hinf*I are indicated to the left as molecular size markers.

applicability of a PCR procedure. However, it has been noted that an effective procedure developed for recovery of DNA from various inhibitory substances may be food specific. Only a few studies concerning extraction and purification of *Vibrio*s DNA from contaminated seafoods other than oysters have been reported (Lee and Choi, 1995; Coleman et al., 1996; Lee et al., 1997). Results presented in Fig. 4 indicated that the template DNAs extracted by the silica coarse adsorption-elution method from various seafood samples contained no notable materials that inhibit the PCR. The DNAs recovered from seawater and sediment samples by freeze-thaw methods, which involve the initial separation of bacterial cells from the natural samples, also seemed to be sufficiently pure for use as templates for the PCR.

The results showed that the nested PCR detection system was sensitive enough to directly detect *V. vulnificus* not only in seafoods but also in natural environments such as seawater and sediments, where the presence of *V. vulnificus* also may represent a serious health risk. The nested PCR procedure, by eliminating the need for enrichment culturing, was rapid enough to complete the total procedure within 10h. This speed is especially beneficial for detection of pathogens such as *V. vulnificus* which can cause rapidly fatal infections. Furthermore, the nested PCR procedure appeared to be suitable for application in identifying the pathogen in various natural samples while maintaining the highest sensitivity. However, direct detection of the pathogen in natural samples by conventional one-step PCR, using JY-1 and JY-2 primers and DNA extract prepared in the same way, was not possible (data not shown). From this observation, it is suggested that the enhanced sensitivity obtained through two-stage amplification can play a key role for the nested PCR to achieve broader applicability.

CONCLUSION

A TWO-STAGE NESTED PCR WAS DEVELoped and optimized to improve the general suitability as well as the sensitivity of the method for detection of *V. vulnificus*. This PCR was rapid and sensitive enough to complete detection of *V. vulnificus* as low as 1 to 10 CFU/mL of seafood homogenate within 10h, without any enrichment. The PCR method was also successfully applied for detection of the pathogen present in various samples, including seafoods, seawater, and sediments.

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