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Rapid and Direct Detection of *Vibrio vulnificus* in Small Octopus (*Octopus variabilis*) Using Polymerase Chain Reaction

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The cells of *Vibrio vulnificus* can be induced to the viable but nonculturable (VBNC) state by natural environmental parameters. The *V. vulnificus* cells in the VBNC state can not be recovered by ordinary laboratory techniques. This nonculturability could often hamper development of effective processing strategies to minimize the number of *V. vulnificus* in seafoods. Even with *V. vulnificus* cells in a culturable state, the length of time required to identify the bacteria in contaminated food by phenotyphic characterization may prevent appropriate in-time responses by public health agencies to infections of the bacteria. In the present study, we used polymerase chain reaction (PCR) to develop a rapid and direct detection method for *V. vulnificus* in small octopus (*Octopus variabilis*) which is consumed as a raw food in Korea. The region targeted was a 704-base pair (bp) portion of the hemolysin gene, *vvhA*, of *V. vulnificus*. The primers designed for PCR amplification were specific for all *V. vulnificus* sp. tested. Several methods were examined to extract total DNA directly from *V. vulnificus* seeded into the octopus homogenate and the guanidine isothiocyanate (GITC) method appeared to be most effective. From the octopus homogenate seeded by *V. vulnificus* at an initial level of 10^2 CFU/ml of the homogenate and then incubated for 12 h, the targeted sequence was successfully amplified by PCR and the 704-bp DNA fragment was observed by gel electrophoresis. The total completion of this assay requires less than one day.

The pathogenic marine bacterium *Vibrio vulnificus* can be isolated from seawater and the estuarine environment (8, 17, 18, 25, 26). It also occurs in raw seafoods including oysters, arkshells, small octopi, and fish harvested from coastal waters (3, 12, 18). *V. vulnificus* has been identified as the causative agent of food-born diseases such as gastroenteritis and life-threatening septicemia in immuno-compromised individuals. It also has been reported that wound infections have resulted from exposure to seawater or from the handling of shellfish contaminated with the organism. Mortality from septicemia is quite high (exceeding 50%), and death may occur as fast as within 1 to 2 days after ingestion (9, 24, 26). Therefore, rapid identification of *V. vulnificus* in seafoods is essential to reduce the potentially fatal effects of widespread consumption of seafoods contaminated with the organism.

It has been shown by several researchers that direct counts of total bacteria in the natural environments such as seafoods are typically more than one order of magnitude higher than plate counts (20). Furthermore, it has been well established that the inability to recover *V. vulnificus* by using standard culture techniques is not totally due to cell death, but due to entry of the cells into the viable but nonculturable (VBNC) state. *V. vulnificus* in the VBNC state can be induced by lowering incubation temperature or by other natural environmental parameters (15). These viable but non-culturable cells of *V. vulnificus* have been reported to retain their ability to repair themselves and to resuscitate upon incubation in favorable conditions (14). Thus, the potential public health hazard presented by such non-culturable cells of *V. vulnificus* may be significant. Consequently, developing a method that does not involve conventional laboratory culturing of microorganisms is advantageous for detecting such nonculturable cells of *V. vulnificus*. The detection method that is developed must be sensitive and specific enough to identify low numbers of *V. vulnificus* against a large background of other procaryotic and eucaryotic cells in the natural samples. In addition to sensitivity and specificity, the method must be rapid for appropriate in-time responses of public health agencies to in-

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fections of the bacteria.

Several detection and enumeration methods based on nucleic acid hybridization have been developed for monitoring specific microorganisms in environmental samples (4, 21, 30). Among them, the polymerase chain reaction (PCR), which has the ability to amplify unique sequences of DNA, has been successfully developed as a specific and sensitive diagnostic method for the direct detection of microbial species in food or dairy products, and aquatic environments (4, 10, 21, 23).

However, purification of nucleic acids from microorganisms in foods appears not to be as easy as it might be from organisms of pure cultures or from other environments. Two approaches, direct extraction and cell extraction, for isolation of DNA from bacteria in natural samples such as foods, have been applied (6, 19). Cell extraction methods involve the initial separation of bacterial cells from natural samples; then the cells are lysed with lysozyme and SDS, and the DNA is purified. A limitation of the cell extraction method is that the initial separation of bacterial cells from foods is not efficient. Recently, it has also been reported that food poisoning bacteria such as *Pseudomonas fragi, E. coli* and *Salmonella* spp. deposit, attach, and form a microbial biofilm on the food surface. This microbial attachment and biofilm formation may also be seriously detrimental to the recovering of bacteria from foods (32). Compared with cell extraction methods, direct extraction methods that involve treatment of a sample with chemicals such as SDS or mechanical forces for direct lysis of cells are less time-consuming and result in a higher yield. A major drawback of this direct extraction is the possibility of contamination with food-borne eucaryotic or extracellular DNA, as well as organic interfering substances which may be present in the sample. Appropriate elimination of these undesirable contaminants is probably required for efficient amplication of the bacterial target DNA by PCR.

In the present study, a direct extraction method has been applied for the rapid isolation of *V. vulnificus* DNA from small octopus (*Octopus variabilis*), which is one of the most popular uncooked seafoods consumed in Korea, and we have coupled the rapid direct DNA extraction method with the PCR to detect a low cell density of *V. vulnificus* in small octopus.

MATERIALS AND METHODS

Bacterial Strains

A total of 17 strains of *Vibrio* spp. and 9 non-*Vibrio* bacterial strains were used as test organisms and are listed in Table 1. Among the *Vibrio* strains, a *V. vulnificus* CDC C7184 was used to seed small octopus for this study. Other *V. vulnificus* strains, clinical isolates, were obtained from patients of Chonnam National University Hospital (Kwangju, Korea) and Wonkwang University Hospital (Iri, Korea).

Enzyme and Chemicals

The *Taq* polymerase and deoxynucleotide triphosphates (dNTPs) were purchased from Korea Biotechnology Co., Seoul, Korea, and were used as suggested by the supplier. Reagents for media were purchased from Difco, Detroit, Michigan, USA, and chemicals from Sigma, St. Louis, MO., USA, at the highest purity available.

Culture Conditions

Cultures of all bacteria were grown in LB (Luria-Bertani) broth or on LB agar containing the appropriate concentration of salt. For halophilic *Vibrios* including *V. vulnificus, V. parahemolyticus* and *V. alginolyticus*, LB with 2% salt (LBS) was used. Unless otherwise indicated, all broth cultures and plates were incubated at 37°C.

Oligonucleotide Primers

The cytotoxin-hemolysin gene (31), *vvhA*, was used as a target sequence to specifically detect *V. vulnificus* in contaminated small octopus. Two *V. vulnificus*-specific primers were synthesized by the Korea Biotechnology Co., Seoul, Korea. Primers were based on the nucleotide sequence of the *vvhA* region encoding the cytotoxin-hemolysin. The two 20-base primers, Choi-1 (5'-GACTATCGCATCAACAACCG-3', sense primer) and Choi-2 (5'-AGGTAGCGAGTATTACTGCC-3', antisense primer) were located within the open reading frame and were expected to generate a 704-bp DNA fragment by PCR.

PCR Amplification

PCR amplifications were carried out in a DNA thermal cycler (ERICOMP, SingleBlock System, Bio-pacific corp., U.S.A.) using Choi-1 and Choi-2 oligonucleotide primers and *Taq* DNA polymerase. PCR reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM of each dNTP, 1.0 μM of each primer, and 2 units of *Taq* polymerase per 100 μl. The appropriate amount of template DNA extracted from bacterial cultures or from contaminated small octopus was added. Thirty cycles of amplification of the target sequence in template DNA were conducted with initial denaturation at 94°C for 2 min, and post-amplification extention at 72°C for 10 min. Each cycle consisted of 2 min at 94°C, 1 min at 60°C and 3 min at 72°C.

PCR amplified DNAs were detected by using gel electrophoresis (22). A 20 l aliquot of each PCR product

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

Species and Strains		Reference or Source	PCR amplification[a]
Vibrio vulnificus	ATCC[b]29307		+[e]
〃	C7184	CDC[c]	+
〃	H3308	〃	+
〃	CS9133	Clinical isolate from Chonnam National University Hospital	+
〃	CNUH1	〃	+
〃	CNUH2	〃	+
〃	CNUH3	〃	+
〃	V-16	Seawater isolate	+
〃	V-19	〃	+
〃	V-23	〃	+
〃	WK3	Clinical isolate from Wonkwang University Hospital	+
〃	WK6	〃	+
V. alginolyticus	ATCC17749		-
V. cholerae	ATCC14033		-
V. cholerae	ATCC14035		-
V. furnisii	ATCC35016		-
V. parahemolyticus	ATCC27519		-
Acinetobacter calcoaceticus	ADP1	Wild type, soil isolate (7)	-
Enterobacter cloacae	ATCC13047		-
Esherichia coli	ATCC25922		-
Klebsiella pneumoniae	ATCC13883		-
Micrococcus leteus	ATCC9341		-
Proteus spp.	MB838	NCMB[d]	-
Pseudomonas aerogenosa	ATCC27853		-
Salmolella typhimerium	ATCC19430		-
Shigella dysenteriae	ATCC9316		-

[a]Determined from observation of characteristic 704-bp band by gel electrophoresis analysis of PCR product. [b]American Type Culture Collections. [c]Center for Disease Control. [d]National Collection of Marine Bacteria. +: amplified, -: not amplified.

was loaded and the separated bands in the gel were visualized with a UV transilluminator within an hour.

Seeding and Test Sample Preparation

Fresh small octopus were purchased from a local seafood market in Kwangju, Korea. The fresh small octopus were washed twice with sterile saline and then kept frozen in a sterile plastic bottle until used. The homogenate of the small octopus was prepared by blending 10 g of small octopus meat with 90 ml of alkaline peptone water (APW, 5, 10).

An exponentially growing *V. vulnificus* culture was serially diluted in sterile saline and each dilution ranging from 10^2 to 10^9 CFU/ml was added to a small octopus homogenate. The concentrations of *V. vulnificus* cells in each dilution were determined by counting colony forming units; aliquots (100 μl) of the serially diluted *V. vulnificus* cultures were plated on LBS plates and incubated at 37°C for 12 h. The seeded homogenates were incubated at 37°C for 0, 6, 12, 18 h and then kept frozen at -60°C until analyzed.

Total DNA Extraction and Purification

To provide PCR templates, genomic DNA samples from pure cultures of *V. vulnificus* were purified according to the procedure described by Ausubel *et al* (1). *V. vulnificus* DNAs from seeded homogenates of small octopus were directly extracted by four methods. Three methods, the phenol-chloroform method, the guanidine isothiocyanate (GITC) method, and the GITC-chloroform method, described previously elsewhere (5), and one freeze-thawing method developed for this study, were tested. All four methods involve centrifugation of 1 ml of the homogenate and washing of the pellet twice with saline solution followed by direct lysis of whole cells by treatment with appropriate chemical reagents such as SDS or guanidine isothiocyanate. For the freeze-thawing method, the washed pellet was suspended in 0.5 ml of saline-EDTA (0.15 M NaCl and 0.1 M EDTA, pH 8.0) and then frozen. Before thawing, 0.5 ml of Tris-SDS (0.1 M Tris and 1% SDS) solution was added. The mixture was then incubated at 50°C until it thawed and the solution had cleared. From the solution, total DNA was purified by extraction using phenol saturated with 0.1 M Tris (pH 9.0).

The extracted DNAs were precipitated with ethanol and the pellets were resuspended in 100 μl of sterile distilled water. Purity of the DNAs extracted was calculated by measurement of A_{260}/A_{280} ratios,

and DNA concentrations were obtained from the A_{260} values.

RESULTS AND DISCUSSION

Specificity of the PCR Amplification

V. vulnificus produces a cytotoxin-hemolysin. Although the protein does not appear to be a major determinant for pathogenicity, it has been implicated as a virulence factor for this organism (28, 29). A 3.4 kilobase (kb) fragment of *V. vulnificus* DNA that encodes cytotoxin-hemolysin has been cloned (Fig. 1) and the structural gene for the protein, *vvhA*, has been sequenced (27, 31). Although the *vvhA* gene has been shown to be unique to this organism by DNA hybridization, there are regions where the nucleotide sequences of the *vvhA* gene and the *hly* gene encoding hemolysin of *V. cholerae* show significant homologies (31). Therefore, a 704-base pair (bp) fragment within the region of *vvhA* where the nucleotide sequences mismatch with the *hly* gene in *V. cholerae* was targeted for PCR amplification in this study.

V. vulnificus is frequently isolated together with other *Vibrio* species such as *V. parahemolyticus, V. cholerae, V. fluvialis,* and *V. alginolyticus.* The specificity of the Choi-1 and Choi-2 primers was tested by perfoming PCR amplification of the DNA isolated from the strains listed in Table 1. The PCR amplification generated the characteristic 704-bp bands for all *V. vulnificus* tested. For the PCR amplification applied to other strains, the characteristic 704-bp band was not observed by gel electrophoresis (Data not shown). The results summarized in Table 1 indicate that the PCR amplification using oligonucleotide primers, Choi-1 and Choi-2, provide a very specific means of identifying all *V. vulnificus* including clinical isolates. Since current laboratory techniques using selective media fail to provide optimal differentiation of *V. vulnificus* from other halophilic *Vibrios,* such specificity is especially beneficial for the detection method to be developed. As a consequence of this result, it is suggested that this PCR technique using Choi-1 and Choi-2 primers could be a potentially useful tool for rapid and direct identification of *V. vulnificus* naturally present in environmental samples.

Fig. 1. Organization of *V. vulnificus* cytotoxin-hemolysin gene, *vvh* cloned on pCVD702 (26).
Location of oligonucleotide primers (closed bars near *vvhA*) and the 704-bp DNA segment (open bar) amplified by PCR are indicated within structural gene of *vvhA*.

Efficiency in Extracting DNA from Small Octopus Homogenate Seeded with *V. vulnificus*

As mentioned in the introduction, we employed direct extraction methods rather than cell extraction methods. The efficiency of each DNA extraction method applied to 1 ml of small octopus homogenate seeded with 10^8 cells of *V. vulnificus* was estimated by comparing the quantities and qualities of extracted DNA (Table 2). The GITC method was found to be more efficient than the other three methods. In terms of the amounts of DNA extracted, the yield of the GITC method was not significantly higher than those of the other methods used. However, this DNA can originate either from *V. vulnificus* seeded to the homogenate, or from eucaryotic cells of small octopus. It can also originate from bacteria which naturally exist in small octopus but are not released at the washing stage. Therefore, the total DNAs extracted by each method were subjected to PCR amplification to examine the existence of *V. vulnificus* DNA. As shown in Table 2, our results indicate that the extracts prepared by the GITC method contain *V. vulnificus* DNA and the recovered DNA is sufficiently pure for use as a template to generate the 704-bp segment.

Presumably, the failure to amplify a target sequence from the DNA extracted by the other three methods resulted from a lack of *V. vulnificus* DNA to serve as a PCR template. However, it is also possible that the DNA is still sufficient in amount, but that the extracts prepared by the other three methods may contain compounds inhibitory to the PCR. It has been suggested that the major obstacle in using PCR on food samples is the presence of components that can inhibit the polymerase activities or binding of primers (4, 10). A good separation procedure to remove the harmful substances from contaminated DNA is necessary to increase sensitivity in PCR. Al-

Table 2. Quantitative and qualitative comparison of DNA isolated by different extraction methods from small octopus contaminated with *V. vulnificus,* and used as a template for PCR.

Method	Amount of DNA[a](μg)	PCR amplification[b]
GITC	57.6	+
GITC-chloroform	56.5	-
Phenol-chloroform	51.9	-
freeze-thawing	32.0	-

[a]Extracted from 1 ml of seeded homogenate, and calculated from measument of A_{260} values. [b]Determined from observation of characteristic 704-bp band by gel electrophoresis analysis of PCR product.

though the nature of the impurities is not yet known, it is obvious that the extract prepared by the GITC method and used as a template contains no significant materials that inhibit the PCR.

Since isolating a template DNA from samples is the most time consuming factor in PCR, developing an efficient DNA extraction method will reduce the time required for completion of the total procedure of identifying microorganisms in foods. The GITC method based on the lysis of cells using GITC and followed by ethanol precipitation was less time-consuming (taking less than 3 h). Furthermore, since the GITC method was easier than the others and yielded DNA that was suitable for amplification by PCR, this method seems to be practical for isolation of DNA from *V. vulnificus* in small octopus. Consequently, the GITC method was used for all DNA extraction in subsequent studies. However, this method may be food specific and the same efficiency may not be obtained when applied to other food samples. Further studies are required for efficient isolation of DNA from *V. vulnificus* in a large number of different food samples.

Sensitivity for Detection of *V. vulnificus* Present in Small Octopus

From the experiments described above, it is apparent that PCR amplification using primers Choi-1 and Choi-2 is specific and the GITC method is effective for direct extraction of DNA from *V. vulnificus* in small octopus. In order to determine detection sensitivity of the PCR, each DNA isolated from pure cultures of *V. vulnificus* and then diluted serially was added as a template. As shown in Fig. 2, we were able to amplify and detect as few as 7 pg of DNA, corresponding to about 10^3 cells. The sensitivity of PCR for detecting *V. vulnificus* present in small octopus was tested by seeding homogenized small octopus with 10 to 10^8 CFU of *V. vulnificus* per ml of homogenate. As noted in Materials and Methods, after incubation for up to 18 h, DNA was extracted from 1 ml of each homogenate and a 2 μl portion of the total DNA dissolved in 20 μl distilled water was used as a template for PCR amplification. In homogenates seeded with 10^3 or more *V. vulnificus* CFU/ml positive signals were obtained after incubation of 18 h or less. However, it has been reported that *Vibros* exists at concentrations ranging from 10^2 to 10^3 CFU/ml in natural habitats such as seawater and coastal sediments in the South Western area of Korea (18). Therefore, it seemed impractical to optimize a PCR method to detect *V. vulnificus* from samples seeded at the level of more than 10^2 CFU/ml. From the samples inoculated with approximately 10^2 CFU/ml of the homogenate and then incubated as long as 18 h, the targeted sequence was successfully amplified by PCR and the 704-bp DNA fragment was observed by gel electrophoresis (Fig. 2). However, samples seeded by *V. vulnificus* at an initial level of 10^1 CFU/ml of the homogenate and analyzed by the procedure described above, did not show the characteristic 704-bp band regardless of an incubation period of up to 18 h (data not shown).

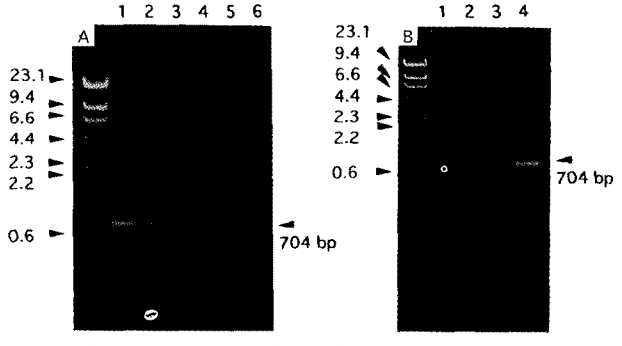

Fig 2. Electrophoretic analysis of PCR products.
A. PCR used the genomic DNA isolated from *V. vulnificus* pure culture and diluted as a template. The amounts of each template DNA are 70 ng (lane 1), 7 ng (lane 2), 700 pg (lane 3), 70 pg (lane 4), 7 pg (lane 5), and 0.7 pg (lane 6). B. Small octopus homogenate seeded with *V.vulnificus* at an initial level of 10^2 CFU/ml incubated for 0 h (lane 1), 6 h (lane 2), 12 h (lane 3), 18 h (lane 4) were used for extraction of DNA, and then each reversed DNA was used as a template for PCR. The migrations of DNA digested with *HindIII* are indicated to the left in kb as molecular size standards.

Although the exact concentration of *V. vulnificus* after incubation was not known, based on a detection limit of 10^2 CFU/ml at an initial level, the sensitivity of the procedure seems quite high. Recently, a sensitivity of 10^4 CFU of *V. vulnificus* per gram of oyster at an initial level has been reported when a ^{32}p-labeled *vvhA* gene sequence is used as a probe (13). It is also proposed that the higher sensitivity of detection for *V. vulnificus* could be obtained by optimizing the PCR method.

The current techniques for identifying microorganisms from environmental samples are enrichment with, and/or plating to, specific selective media. Enrichment involves competition with possibly more vigorously growing organisms in a mixed population, and may result in overgrowth of unwanted bacteria and thereby change in the population structure of the microbial community. Another limitation of such techniques is that the isolation of the bacteria is not easy and a large portion of the population may be lost or may die during the separation of the bacteria from the environmental samples. Therefore, it renders the detection of sparsely distributed microorganisms difficult (20, 23).

In isolating and identifying *V. vulnificus* in foods using ordinary culturing techniques, the factors mentioned above should be considered. Enrichment of *V. vulnificus* in natural samples in selective media such as colistin-polymixinB-cellobiose (CPC) agar involves the use of colistin and polymixin to suppress growth of background bacteria. However, these compounds seem also to be detrimental to the growth of *V. vulnificus*. The plating efficiency of *V. vulnificus* on CPC agar appeared to be about 10% of the plating efficiency on brain heart infusion (BHI) agar, one of the most enriched media for the organism (16). Furthermore, even the CPC agar that has been reported to be most optimal as a selective medium can not differentiate *V. vulnificus* from other halophilic *Vibrio* sp. such as *V. parahaemolyticus* (11). Therefore, for final identification of *V. vulnificus*, usually biochemical and/or seriological confirmation is required. However, biochemical tests require time-consuming subcultures of a large number of individual isolates for statistical reliability of the results (12, 16). Immunological methods using monoclonal antibodies have proven highly specific; however, subcultures of individual colonies from selective media to provide ample signal for detection is still required (2, 25). The cost and labor involved in this type of assessment can also prove prohibitive for practical use.

Recent outbreaks of food-born diseases such as gastroenteritis and life-threatening septicemia due to consumption of raw seafoods contaminated with *V. vulnificus* can cause a loss of public health confidence and may negatively affect the seafood (or fish farming) industry. Quality assurance of *V. vulnificus*-free seafoods based on credible analytic methods is demanded by consumers, and requested by the industry itself. The most important consideration in the development of the methods for detection of *V. vulnificus* in seafoods is that a large portion of *V. vulnificus* populations in natural samples frequently remain nonculturable. Since those viable but nonculturable cells of *V. vulnificus* are still potentially hazardous to public health, analysis for the presence of the organism through the use of techniques that depend entirely on colony-forming ability is not appropriate. Obviously, since PCR can detect microbial species by amplification of gene sequences unique to the organism, and does not involve the conventional laboratory culturing step, it seems well suited for detection of such an organism. Furthermore, PCR is advantageous because of its simplicity and speed for detection of *V. vulnificus* that can produce rapidly fatal infections. In the present study, we were able to apply PCR techniques to amplify specific target sequences in *vvhA* of *V. vulnificus* in small octopus. The approach of using PCR DNA amplification coupled with enrichment in an alkaline peptone water and with the GITC method for direct extraction of DNA have allowed us to develop a rapid and direct method for detection of *V. vulnificus* from small octopus. The total procedure of this assay required less than one day.

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