Improving Detection of *Vibrio vulnificus* in *Octopus variabilis* by PCR

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ABSTRACT

PCR methods can detect foodborne pathogenic bacteria with simplicity, specificity and speed. In order to improve sensitivity and speed of PCR methods for detection of *Vibrio vulnificus* in small octopus homogenate, several media and culture conditions were compared. Modified brain heart infusion media containing 2% NaCl and adjusted to pH 8.0 and 30°C was most effective for enrichment of the bacteria. Procedures affecting the efficiency of template DNA extraction and target DNA amplification were also optimized. By these combined efforts, a PCR procedure capable of detecting *V. vulnificus* as low as 10 cells/mL within 10h was developed.

Key Words: *Vibrio vulnificus*, PCR, small octopus, rapid enrichment, DNA extraction

INTRODUCTION

THE PATHOGENIC marine bacterium *Vibrio vulnificus* occurs in raw seafoods including oysters, and fish from coastal waters (Depaola et al., 1994; Oliver et al., 1983; O'Neill et al., 1992; Tamplin et al., 1982). *V. vulnificus* has been identified as the causative agent of foodborne diseases such as gastroenteritis and life-threatening septicemia in immuno-compromised individuals. Mortality from septicemia is quite high ($>$ 50 %), and death may occur within 1 to 2 days after the first sign of illness (Klontz et al., 1988; Tacket et al., 1984).

It has been well established that the inability to recover *V. vulnificus* by standard culture techniques is not totally due to cell death, but due to change of the cells into the viable but nonculturable (VBNC) state. *V. vulnificus* in the VBNC state may be induced by lowering incubation temperature or by other natural environmental changes (Biosca et al., 1996; Oliver et al., 1991; Oliver et al., 1995). These VBNC cells of *V. vulnificus* have been reported to retain the ability to repair and to resuscitate upon incubation in favorable conditions (Nilsson et al., 1991; Oliver et al., 1995; Oliver and Bockian, 1995). Thus, the potential public health hazard presented by such nonculturable cells of *V. vulnificus* may be considerable. Consequently, a method that does not involve conventional laboratory culturing would be advantageous for detecting such nonculturable cells.

Several detection and enumeration methods based on nucleic acid hybridization have been reported for monitoring specific microorganisms in environmental samples (Bsat et al., 1994; Dodd at al., 1990; Sayler and Layton, 1990). Among them, the polymerase chain reaction (PCR), which can amplify unique sequences of DNA, provides very accurate, explicit, and rapid detection of microbial species in food or dairy products (Bsat et al., 1994; Saiki et al., 1988). PCR uses two oligonucleotide primers designed to bind specifically to the region in DNA segments of interest. However, besides designing specific primers, the development of an effective method for nucleic acid extraction and purification from food samples is necessary for successful application of PCR as a diagnostic method. For example, the sensitivity of PCR detection would be increased by developing an efficient DNA extraction method which should eliminate substances inhibitory to PCR amplification (Jacobsen and Rasmussen, 1992; Tsai and Olson, 1992; Picard et al., 1992). Furthermore, enrichment to a certain level of the foodborne pathogenic bacteria in food samples is still required for detection of the bacteria by PCR (Hill et al., 1991; Koch et al., 1993; Lee and Choi, 1995) and the time for enrichment appears to be one of the most time consuming factors. Therefore, optimizing growth media and enrichment conditions would reduce the time for identifying such microorganisms in foods.

The cited methods or procedures were specific for bacterial strains as well as for particular foods. Compared with the substantial number of reports on identification of *Vibrios* from contaminated oysters using PCR (Brauns et al., 1991; Hill et al., 1991; Koch et al., 1993; Lee et al., 1995a), only a few studies on procedures for their detection from other seafoods such as small octopus (*Octopus variabilis*) (Roper et al., 1984) has been reported (Lee and Choi, 1995). Small octopus is a popular seafood in Asia, and is often consumed in the uncooked form. Increasing numbers of outbreaks of fatal septicemia due to consumption of raw small octopus have been reported in Korea (Rhee, 1995). PCR DNA amplification coupled with enrichment in an alkaline peptone water (APW) and a guanidine isothiocyanate (GITC) method for direct extraction of DNA was an effective procedure for direct detection of *V. vulnificus* from small octopus (Lee and Choi, 1995). The primers designed for use in the PCR were very specific for all *V. vulnificus* tested (Lee and Choi, 1995). However, the sensitivity and speed of the procedure needed improvement. An initial level of *V. vulnificus* $> 10^2$ colony forming units (CFU)/mL of small octopus homogenate and incubation for $>$12h were required. The procedure required about 20h for completion.

Our objective was to improve the PCR methods for detection of *V. vulnificus* in small octopus by developing a more effective method for DNA extraction and by optimizing growth media and enrichment conditions.

MATERIALS & METHODS

Bacterial strain and culture conditions

A standard type culture of *V. vulnificus* CDC C7184 was obtained from Center for Disease Control (Atlanta, GA), and was used throughout the study. Cells of *V. vulnificus* CDC C7184 were maintained at 4°C on a modified Luria-Bertani (LB) (Sambrook et al., 1989) plate by adding salt at 2%.

Determining the most effective conditions for growth of *V. vulnificus* was initiated by growing a set of cultures with various media that had been used for growth of *V. vulnificus* (Hill et al., 1991; Massad and Oliver, 1987; Oliver et al., 1992; Sloan et al., 1992; Sun and Oliver, 1995), and by measuring specific growth rates and cell dry weights of the bacteria. The media used were ; brain heart infusion (BHI) broth (Difco), LB broth, APW and colistin-polymyxin B-cellobiose (CPC) broth. All media tested were adjusted to 2% NaCl as described (Chung et al., 1986). Cultures were inoculated to an initial cell density (OD_{600}) of $\approx$ 0.005 and inocula were from late logarithmic phase cultures which had been grown with shaking at 30°C.

Maximum specific growth rate (μ_{max}) and cell mass

Cell growth in a medium was monitored by measuring optical density of each culture at 600 nm. Based on values of cell densities, maximum specific growth rate, μ_{max}, defined as a specific growth rate at an expo-

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Table 1—Comparison of various media for their effectiveness in supporting the growth of *V. vulnificus*

Medium[a]	μ_{max} (h^{-1})[b]	Cell dry weight (mg/mL)[c]
BHI	2.488	4.7
LB	2.234	3.1
CPC	1.765	1.8
APW	2.115	1.7

[a] Details are in Materials & Methods.
[b] Specific growth rate at an exponential stage.
[c] Determined from samples harvested 10h after inoculation.

Table 2—Inhibition of growth of *V. vulnificus* by addition of exogenous glucose into medium (BHI broth)

Glucose (%)	μ_{max} (h^{-1})[b]	Cell dry weight (mg/mL)[c]	pH[d]
0.2[a]	2.414	4.3	6.50
0.5	2.398	3.7	5.16
1	2.398	3.6	5.15
2	2.250	3.4	5.12
4	2.156	3.1	5.14

[a] Brain heart infusion medium contained internal glucose at 0.2%.
b,c Same as Table 1.
[d] Measured from samples harvested 10h after inoculation.

nential phase, was calculated as described (Stanier et al., 1994). For determination of cell mass expressed as mg cell dry weight/mL culture fluid, 5 mL samples removed from cultures at indicated times were washed twice with a 0.85% NaCl solution and dried overnight at 95°C.

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 the media were purchased from Difco (Detroit, MI) and chemicals from Sigma (St. Louis, MO), at the highest purity available.

Oligonucleotide primers

The cytotoxin-hemolysin gene (Yamamoto et al., 1990), *vvhA*, was used as a target sequence to specifically detect *V. vulnificus*. The two 20-base primers, Choi-1 (5'-GACTATCGCATCAACAACCG-3', sense primer) and Choi-2 (5'-AGGTAGCGAGTATTACTGCC-3', antisense primer) (Lee and Choi, 1995) were used and were expected to generate a 704-bp-long 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, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1.0 μM of each primer, and 2 units of *Taq* polymerase per 100 μL. Forty-five cycles of amplification of the target sequence in the template DNA were conducted with initial denaturation at 94°C for 3 min, and post-amplification extension at 72°C for 10 min. Each cycle consisted of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C. A 20 μL aliquot of each PCR product was loaded and specific bands in the gel were visualized with a UV transilluminator within 1h.

Seeding and test sample preparation

Fresh small octopus were purchased from a local seafood market and washed twice with sterile saline. The homogenate of the small octopus was prepared by blending 10 g of small octopus meat with 90 mL of culture medium which had been optimized for growth of *V. vulnificus*.

An exponentially growing *V. vulnificus* culture was serially diluted in sterile saline and each dilution was added into a small octopus homogenate to a density of 10 cells/mL. The concentrations of *V. vulnificus* cells were determined by counting CFU on LB plates containing 2% salt. The seeded homogenates were incubated at 30°C for 0, 2, 4, 8h and then DNAs were extracted from 1 mL of each homogenate. A 2 μL portion of total DNA dissolved in 50 μL distilled water was used as a template for PCR amplification.

Table 3—Effects of pH of medium (BHI) on growth of *V. vulnificus*

Initial pH[a]	μ_{max} (h^{-1})[b]	Cell dry weight (mg/mL)[c]	Final pH[d]
6.0	1.792	3.2	5.01
6.5	3.299	3.5	5.60
7.0	3.016	4.7	6.94
7.5	3.102	4.8	7.20
8.0	3.342	4.7	7.31
8.5	3.000	4.8	7.41
9.0	2.448	4.6	7.51

[a] Adjusted before inoculation.
b,c Same as Table 1.
[d] Same as Table 2.

Table 4—Comparison of DNA isolated by two different extraction methods from small octopus homogenate contaminated with *V. vulnificus*

Method	Incubation (h)[a]	PCR amplification[b]
SC adsorption −elution	0	—
	1	—
	2	—
	4	+
GITC	0	—
	1	—
	2	—
	4	—

[a] Details in Results & Discussion.
[b] Determined from amplification of characteristic 704-bp DNA fragment using the recovered DNA as a template for PCR.

Total DNA extraction and purification

The GITC method, described previously elsewhere (Lee and Choi, 1995), and the silica coarse (SC) adsorption-elution method modified for this study, involved centrifugation of 1 mL of homogenate and washing of the pellet twice with 0.85% NaCl. For the SC adsorption-elution method, the washed pellet was suspended in 0.05 mL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0) and the suspension was added into a reaction vessel containing 900 μL of lysis buffer with 40 μL of silica coarse suspension. Lysis buffer and silica coarse suspension were prepared by procedures of Boom et al. (1990). The mixture was incubated at room temperature for 10 min and centrifuged to precipitate silica-nucleic acid pellets. The pellets were washed twice with the appropriate buffer (Boom et al., 1990) and then once each with 70 % ethanol and acetone respectively. From the pellets, the total DNA was purified by elution using TE buffer. Eluted DNAs were precipitated with ethanol and pellets were resuspended in 50 μL of sterile distilled water.

RESULTS & DISCUSSION

Optimum conditions for growth of *V. vulnificus*

The μ_{max} and cell dry weight of each culture were used as indexes for the effectiveness of the respective growth media and culture conditions for enrichment of *V. vulnificus* CDC C7184. *V. vulnificus* incubated in BHI showed higher μ_{max} and cell dry weight than in LB, CPC or APW (Table 1). The CPC medium has been reported as highly selective for this pathogen, and it may be effectively employed in monitoring studies to determine levels of this bacterium in seafood (Massad and Oliver, 1987; Sloan et al., 1992; Sun and Oliver, 1995). However, from our data, use of BHI medium rather than CPC medium was more advantageous for rapid enrichment. This slow growth of the bacteria with CPC medium confirmed results of Oliver et al. (1992). In our previous experiments (Lee and Choi, 1995), *V. vulnificus* seeded into octopus homogenate at an initial level of 10^2 CFU/mL, and then incubated for 12h in APW, was consistently detected using PCR. However, it appeared that BHI was more effective for the growth of *V. vulnificus* than APW. Thus, it was proposed that using BHI instead of APW for incubation could reduce the time required for completion of the PCR procedure. *V. vulnificus* also showed a growth behavior typical to other enteric group bacteria, with maximum growth rates at 30°C to 37°C (Lee et al., 1995b). Consequently, all additional studies were performed using the BHI modified by adding 2% salt with incubation at 30°C.

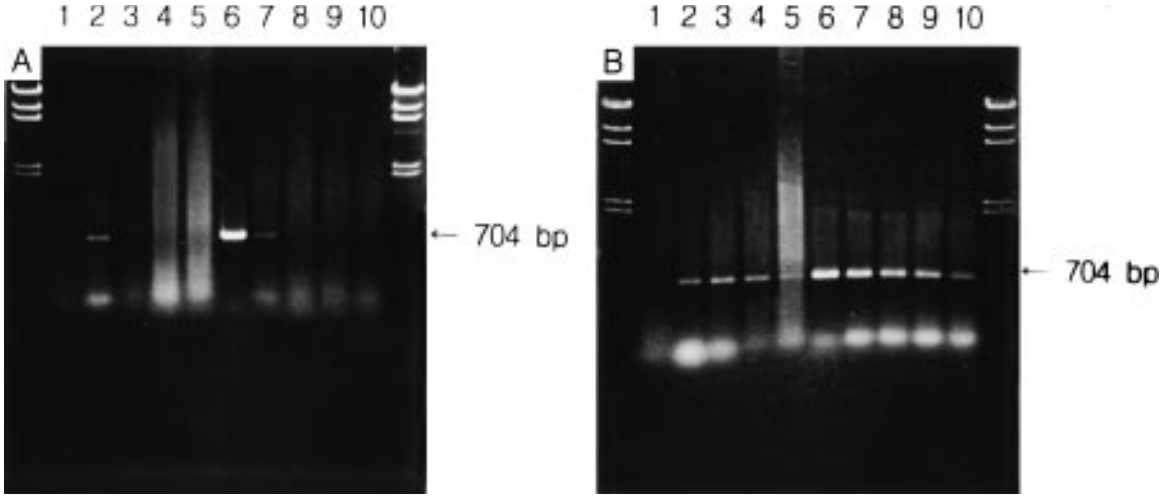

Fig. 1—Electrophoretic analysis of PCR products showing relationships between presence of inhibitors to PCR in extracts and methods used for their preparation. For lanes of both panels, 0 μL (control), 1 μL, 2 μL, 4 μL, and 8 μL of extract (lanes 1 to 5, respectively) prepared from small octopus seeded with *V. vulnificus* were used as a source of template DNA for PCR. Different amounts of octopus extract, 0 μL (control), 1 μL, 2 μL, 4 μL and 8 μL (lanes 6 to 10, respectively), prepared from unseeded small octopus, were added into genomic DNA isolated from *V. vulnificus* pure culture, and resulting mixtures were used as a source of template DNA for PCR. All extracts were prepared by GITC method (panel A), and SC adsorption-elution method (panel B). The migrations of λDNA digested with HindIII are indicated as molecular size standards.

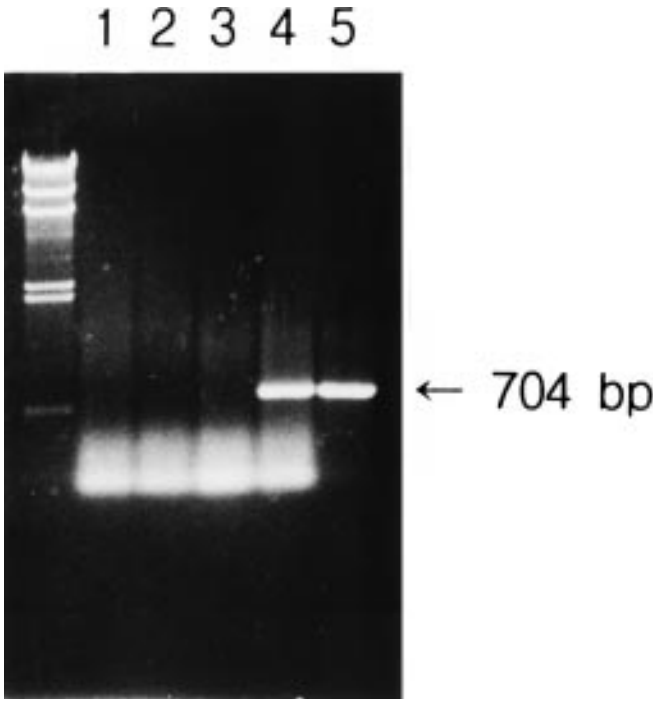

Fig. 2—Detection of *V. vulnificus* in small octopus. Extracts prepared by SC adsorption-elution from small octopus seeded with *V. vulnificus* at 10 CFU/mL initially and then incubated for different times were used as a source of template DNA for PCR. Lane 1, 0h; Lane 2, 1h; Lane 3, 2h; Lane 4, 4h; Lane 5, 8h. Molecular size markers same as Fig. 1.

For the effect of glucose on growth, *V. vulnificus* showed a growth behavior not typical of other members of enteric bacteria (Table 2). The growth rate of *V. vulnificus* at higher glucose appeared to be lower than at lower glucose concentrations. At glucose >0.5%, the pH of the culture broth decreased sharply and the cell yield was lower. However, it was not obvious whether inhibition of the μ_{max} and cell yield of *V. vulnificus* cells with increasing glucose was due to the decline of pH. Possible toxic effects of other metabolic end products such as ethanol or 2,3-butanediol (Gottschalk, 1985) should be investigated in more detail to explain the inhibitory effects of glucose.

Growth of *V. vulnificus* was highly pH dependent (Table 3) with the highest specific growth rate at pH 8.0. At acidic pH, the specific growth rate decreased sharply; in a pH 6.0 medium, cultures also showed less cell yield. This clear decline in growth rate and cell yield in acidic medium indicated that the *V. vulnificus* was acid sensitive and suggested that acid treatment could be a possible way to eliminate this pathogenic bacteria from raw seafoods. We finally used BHI medium modified by adding 2% NaCl and adjusted initially to pH 8.0 for faster enrichment of *V. vulnificus* in small octopus.

Efficiency in extracting DNA from small octopus homogenate seeded with *V. vulnificus*

We have shown that the GITC method was more effective for direct extraction of *V. vulnificus* DNA from small octopus than the phenol-chloroform method, or the freeze-thawing method used widely for isolation of bacterial DNA from natural samples (Lee and Choi, 1995). We examined here whether another applicable direct extraction method, the SC adsorption-elution method was more effective than the GITC method for recovery of *V. vulnificus* DNA from small octopus.

The efficiency of each DNA extraction method applied to 1 mL of small octopus homogenate seeded with 10 cells of *V. vulnificus* and incubated for different times was estimated by comparing qualities of extracted DNA as a PCR template (Table 4). Total DNAs extracted from homogenate with different times of incubation by each method were subjected to PCR amplification to look for existence of *V. vulnificus* DNA. Results showed that the extract prepared by the SC adsorption-elution method from homogenate incubated for as little as 4h contained *V. vulnificus* DNA, and the recovered DNA was pure and adequate for use as a template to generate the 704-bp segment (Table 4).

However, the target sequence was not amplified from the DNA extracted by the GITC method. This probability did not result from a lack of *V. vulnificus* DNA to serve as a PCR template. Possibly DNA was still sufficient in amount, but the extracts prepared by the GITC method contained compounds inhibitory to the PCR. A major obstacle in using PCR on natural samples may be the presence of components that inhibit the polymerase activities or binding of primers (Tsai and Olson, 1992). Therefore, a

good separation procedure to remove such substances from contaminated DNA is necessary to increase sensitivity in PCR.

Results indicated the presence of inhibitory substances (Fig. 1) in the extracts prepared by the GITC method. Using increasing amounts of DNA extracts, prepared from small octopus seeded with *V. vulnificus* at the level of 10 CFU/mL as a template, could inhibit the PCR (Fig. 1A). When 2 μL of extracts, prepared from unseeded small octopus homogenate were added into the PCR mixture containing 100 ng of template DNA isolated from *V. vulnificus* pure culture, the PCR was suppressed. In contrast to the extract prepared by the GITC method, that prepared by the SC adsorption-elution method showed less inhibitory effects on PCR (Fig. 1B). From these results, we could conclude that the extracts prepared by SC adsorption-elution contained a very low level of inhibitors and adequate level of template DNA.

The SC adsorption-elution method seemed to be practical for isolation of DNA from *V. vulnificus* in small octopus. Consequently, the SC adsorption-elution 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.

Sensitivity for detection of *V. vulnificus* in small octopus

The indigenous level of viable *V. vulnificus* cells was determined by using TCBS (thiosulfate-citrate-bile salts-sucrose and CPC selective media, and appeared to be <1 CFU/g of octopus prepared by the described method. The homogenate samples were seeded with the bacteria to about 10 CFU/mL to determine the detection sensitivity of PCR. From the samples incubated 4h, the targeted sequence was successfully amplified by PCR and the 704-bp DNA fragment was observed by gel electrophoresis (Fig. 2). However, samples incubated <4h did not show the characteristic 704-bp band. Although the exact concentration of *V. vulnificus* after incubation was not known, based on a detection limit of 10 CFU/mL at an initial level, the sensitivity seemed quite high. Methods based on PCR have been reported to represent sensitivies of 10^2 CFU of *V. vulnificus*/g of seeded oyster and then incubated 12–24h (Hill et al., 1991; Lee and Choi, 1995).

CONCLUSIONS

PCR CAN DETECT microbial species by amplification of unique gene sequences, and does not involve the conventional laboratory culturing step, it seems well suited for detection of such an organism that frequently remains nonculturable in natural samples. Furthermore, PCR is advantageous because of its simplicity and speed for detection of *V. vulnificus* that can produce rapidly fatal infections. The use of PCR DNA amplification coupled with enrichment in a BHI broth optimized for growth of *V. vulnificus* and the SC adsorption-elution method for direct extraction of DNA has enabled development of an improved method for specific detection of *V. vulnificus* from small octopus. The detection sensitivity was $\approx$10 CFU/mL of small octopus homogenate at an initial level and the total procedure required $<$10h.

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