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Detection of *Vibrio vulnificus* Biotypes 1 and 2 in Eels and Oysters by PCR Amplification

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DNA extraction procedures and PCR conditions to detect *Vibrio vulnificus* cells naturally occurring in oysters were developed. In addition, PCR amplification of *V. vulnificus* from oysters seeded with biotype 1 cells was demonstrated. By the methods described, *V. vulnificus* cells on a medium (colistin-polymyxin B-cellobiose agar) selective for this pathogen were detectable in oysters harvested in January and March, containing no culturable cells (<67 CFU/g), as well as in oysters harvested in May and June, containing culturable cells. It was possible to complete DNA extraction, PCR, and gel electrophoresis within 10 h by using the protocol described for oysters. *V. vulnificus* biotype 2 cells were also detected in eel tissues that had been infected with this strain and subsequently preserved in formalin. The protocol used for detection of *V. vulnificus* cells in eels required less than 5 h to complete. Optimum $MgCl_2$ concentrations for the PCR of *V. vulnificus* from oysters and eels were different, although the same primer pair was used for both. This is the first report on the detection of cells of *V. vulnificus* naturally present in shellfish and represents a potentially powerful method for monitoring this important human and eel pathogen.

Vibrio vulnificus comprises two biotypes that are distinguishable by phenotypic traits (4, 5, 14, 16, 49) and genotypic variation as revealed by ribotyping and randomly amplified polymorphic DNA analysis (9, 51). Biotype 1 is responsible for 95% of shellfish-related deaths in the United States (41). Individuals with underlying diseases, such as liver cirrhosis, that produce elevated serum iron levels are especially at risk for *V. vulnificus* infection (17, 23, 28). *V. vulnificus* may be acquired by ingestion of raw or undercooked shellfish, especially oysters, or by contact of existing skin wounds with cells naturally present in seawater (17, 23, 28). Although biotype 1 has been detected in tank water and in the gills of healthy cultured eels (13, 14, 16), it is not pathogenic for eels (3, 16).

Biotype 2 is highly virulent for juvenile European eels and produces fatal hemorrhagic septicemia following intraperitoneal injection (5, 13, 15, 16). In Europe, Japan, and Taiwan, where eels are a food crop, *V. vulnificus* biotype 2 was isolated following outbreaks in eel farms that resulted in high mortality rates (13, 16, 34, 46). A recent study indicated that encapsulated cells of biotype 2 could survive in water for up to 14 days and are thus possibly transmissible by water (2). *V. vulnificus* biotype 2 was believed to be avirulent for humans and had never been isolated from water samples or marine animals other than eels (4, 13, 34). Subsequently, studies with mice in which biotype 2 cells were injected intraperitoneally demonstrated that its virulence for mice was equal to that of biotype 1 (4, 16). In 1992, a case of biotype 2 septicemia was reported for an individual who was cleaning eels and had existing lacerations on the hands (50). The results of the studies cited above indicate that the presence of biotype 2, particularly in the eel-farm environment, may be a health hazard to humans as well as to eels.

We have been interested in developing rapid, efficient methods for detecting *V. vulnificus* cells in oyster and eel tissues, using PCR amplification of the extracted target DNA. In our laboratory, we routinely amplify DNA directly from whole bacterial cells without prior extraction (19, 20). However, when the cells are present in tissues of animals, such as oysters and eels, extraction of the target DNA is required before PCR amplification can be made possible.

In the present study, we report the adaptation of DNA extraction methods appropriate to oyster and eel tissue and PCR conditions for amplification of a portion of the hemolysin gene unique to *V. vulnificus* DNA (33, 53). To our knowledge, this is the first report in the literature of the detection of *V. vulnificus* in tissues which were not artificially seeded with cells.

MATERIALS AND METHODS

Extraction of DNA from oysters. Live oysters (*Crassostrea virginica*) harvested from the Gulf Coast of Louisiana were used in our studies. Ten oysters in good condition with shells tightly closed were selected for each trial. The oysters were shucked and homogenized in a blender by aseptic techniques and with sterile reagents and an equal amount of artificial seawater (ASW) (52). To determine the numbers of *V. vulnificus* cells naturally present in the oysters, dilutions of the oyster-ASW homogenate were made and plated onto colistin-polymyxin B-cellobiose (CPC) agar, which is specific for this species (31, 39, 43, 47).

For seeded oysters, cells of *V. vulnificus* biotype 1 (strain C7184; both opaque and translucent morphotypes [45]) were grown overnight at room temperature with shaking in heart infusion broth (Difco Laboratories, Detroit, Mich.). The cells were harvested and resuspended in ASW and added to an equal volume of an oyster homogenate. Plate counts were performed at this point to determine the number of cells which were added to the oysters. This procedure routinely resulted in a *V. vulnificus* concentration of ca. 3.0×10^7 cells per g of oyster tissue, as indicated by counts on CPC agar.

Oyster-ASW homogenates with or without added cells were used for DNA extraction by a protocol based on that originally described by Jones et al. (25). The extraction procedure we developed is described here. Following an initial centrifugation of $16,000 \times g$ for 5 min of 1 ml of oyster-ASW homogenate, the supernatant was removed and the pellet was washed twice with 1 ml of sterile, distilled, and deionized water. The resulting pellet was resuspended in 200 μl of distilled, deionized H_2O to which Chelex 100 (Bio-Rad, Hercules, Calif.) was added to a final concentration of 1%. The samples were heated in a 65°C water bath for 10 min and then immediately heated in a 100°C water bath for 20 min. Solid ammonium acetate (ca. 0.04 g) was then added to a final concentration of 1.25 M. The samples were vortexed and centrifuged at $16,000 \times g$ for 5 min, and the DNA in the supernatant was extracted with an equal volume of chloroform-isoamyl alcohol (24:1). The DNA extract was transferred to a Centricon-100 unit (Amicon, Detroit, Mich.), brought to a total volume of 2 ml with distilled,

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deionized H_2O, and centrifuged at 4°C at 1,650 × *g* for 2 h or until the retentate was approximately 200 μl. For DNA extracted from oysters to which *V. vulnificus* cells had been added, no further processing was necessary in order to produce visible electrophoretic bands following PCR. For oysters to which no cells had been added, 100 μl of the DNA retentate was processed following the Insta-Mini-Prep kit (5 Prime-3 Prime, Inc., Boulder, Colo.) protocol for plasmid DNA extraction, with the exception that no buffer was added. Extracted DNA was then subjected to PCR amplification.

Extraction of DNA from eels. European eels (*Anguilla anguilla*) with a body weight of approximately 10 g each were artificially infected with *V. vulnificus* biotype 2 (strains ATCC 33149 and E86) by intraperitoneal injection and immersion as previously described (2, 15). Infected and healthy eel tissue was preserved in 4% formalin in phosphate-buffered saline (PBS) and used in these experiments. The protocol developed for DNA extraction from these tissues is a modification of that originally described by Ali and Jameel (1). Cross-sectional slices (ca. 1.5 to 2.2 mg) of tissue from whole eels, livers, and kidneys were crushed or chopped and weighed in tared 1.5-ml microcentrifuge tubes. PBS (1 ml) at pH 7 was then added to the tissue. Tubes were vortexed and centrifuged at 16,000 × *g* for 2 min, and the PBS was decanted. The tissue specimens were soaked in 100 μl of methanol at room temperature for 10 min. The methanol was removed, and the specimens were dried with the tubes open in a 65°C oven for 20 min. To each tube was then added 120 μl of proteinase K (500 μg/ml; Sigma Chemical Company, St. Louis, Mo.). The tubes were placed in a 55°C water bath for 45 min and then into a boiling water bath for 10 min. The supernatant was used as a source of template DNA for PCR amplification.

PCR conditions. Primers 1 and 2, whose respective sequences are 5′ C GCC GCT CAC TGG GGC AGT GGC TG 3′ and 5′ GCG GGT GGT TCG GTT AAC GGC TGG 3′, were obtained from Bio-Synthesis, Inc. (Lewisville, Tex.), and used for PCR amplification. These primers are homologous to regions flanking a portion of the 1,416-bp cytolysin-hemolysin gene that is unique to *V. vulnificus* but common to both biotypes 1 and 2 (16, 27, 33).

A 5-μl sample of the extracted DNA (from oyster or eel samples) was used in the PCR. To the DNA was added 10× Buffer II (Perkin-Elmer Cetus, Norwalk, Conn.)–250 mM each deoxynucleoside triphosphate (Perkin-Elmer Cetus)–0.45 mM each primer–1.25 U Amplitaq (Perkin-Elmer Cetus). In addition, 6.4 and 2.4 μl of 25 mM $MgCl_2$ were added for final concentrations of 4.0 mM for biotype 1 and 1.5 mM for biotype 2 DNA amplifications, respectively. Diethyl pyrocarbonate-treated distilled, deionized H_2O was added to provide a final reaction volume of 40 μl. The thermocycler (Techne, Inc., Princeton, N.J.) was programmed for a denaturation and annealing cycle of 94°C for 30 s and 64.5°C for 30 s. This cycle was typically repeated 34 times for DNA from eel tissue and 50 times for DNA from oysters, with a final extension cycle in both cases of 94°C for 30 s and 64.5°C for 10 min. PCR products were visualized by electrophoresis on a 1% agarose gel and staining with ethidium bromide.

RESULTS AND DISCUSSION

Oysters. PCR is a commonly used technique for detecting microorganisms that have been seeded into environmental and food samples. PCR followed by use of a gene probe has been employed previously to detect *Escherichia coli* and *Shigella* spp. in environmental water samples to which viable cells had been added (12). Nucleic acids from enteric viruses seeded into oysters have also been detected by PCR (7, 8). A comparative investigation of DNA extraction techniques previously demonstrated the feasibility of using PCR to detect a portion of the hemolysin gene in oyster homogenates seeded with *V. vulnificus* cells and subsequently incubated in broth up to 24 h (22). We also prepared oyster homogenates to which either opaque (encapsulated) or translucent (nonencapsulated) cells of *V. vulnificus* were added. DNA extraction performed on these homogenates without cultivation produced PCR amplification (Fig. 1, lane 2). PCR detection was possible for as few as 41 cells per g of oyster homogenate without the use of gene probe hybridization (data not shown).

Studies using PCR and gene probes to detect *Salmonella* spp., commonly present in shellfish, have been conducted with both seeded and unseeded oysters (10, 11, 26). PCR-positive results were obtained by Jones et al. (26) from 26% of the unseeded oysters containing *Salmonella* spp. In our study, oysters harvested in May and June and homogenized as described above produced colonies on CPC agar. The extracted DNA produced a 344-bp product following PCR amplification (Fig. 1, lane 3). These bands represented detection of as few as 1,800 cells per g of oyster homogenate on the basis of viable counts

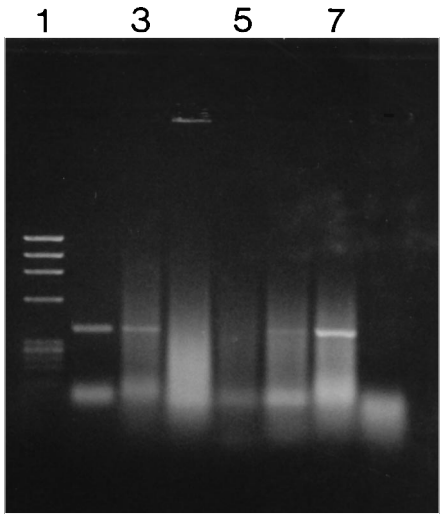

FIG. 1. Ethidium bromide-stained agarose gel of PCR-amplified DNA from *V. vulnificus* in oyster tissue. Lane 1, ϕX174 DNA digested with *Hae*III as a size standard; lane 2, oyster homogenate seeded with *V. vulnificus*; lane 3, oyster homogenate from CPC-positive, unseeded oysters harvested in June (ca. 1,800 CFU/g of oyster); lane 4, oyster homogenate from CPC-positive, unseeded oysters harvested in June (ca. 600 CFU/g of oyster); lane 5, oyster homogenate from CPC-negative, unseeded oysters (DNA prior to Insta-Mini-Prep protocol); lane 6, oyster homogenate from CPC-negative, unseeded oysters subjected to the Insta-Mini-Prep protocol and 34 amplification cycles; lane 7, homogenate as in lane 6, except subjected to 50 amplification cycles; lane 8, negative control.

on CPC agar. This is an improvement over the use of direct DNA probes of oyster homogenates that consistently could not detect fewer than 10^4 CFU/g of oyster meat (33). The minimum detection limit for amplification was not determined in these studies, but on the basis of CPC plate counts, no electrophoretic bands were produced for 600 CFU/g of oyster homogenate (Fig. 1, lane 4).

When oysters harvested in January and March were plated onto CPC agar, no colonies developed (<67 CFU/g of oyster). Therefore, it was not possible to ascertain the number of *V. vulnificus* cells providing target DNA. Attempted amplification of DNA extracts from winter studies produced no visible electrophoretic bands from unseeded oysters (Fig. 1, lane 5). However, further processing of the DNA extracted from unseeded oysters (prior to PCR) by the Insta-Mini-Prep protocol described above produced faintly visible bands (Fig. 1, lane 6). The visibility of these bands was improved by increasing the number of amplification cycles from 34 to 50 (Fig. 1, lane 7).

Results for unseeded oysters, regardless of harvest date, were sometimes inconsistent, as different batches of oysters from the same shipment and subjected to the same protocol did not always result in visible PCR amplification. We expected the quality of the PCR amplification products of oysters harvested in May and June, which through use of CPC agar could be shown to harbor cells of *V. vulnificus*, to improve noticeably over those obtained from oysters taken during the winter, for which no culturable cells could be detected. Surprisingly, the electrophoretic bands obtained from summer oysters were not as strong. An increase to 50 cycles produced bands only slightly better than bands produced by CPC culture-negative DNA subjected to 34 cycles (Fig. 1, lanes 3 and 6).

We are uncertain why the expected improvement in detection did not occur; however, the inconsistency observed may be related to oyster-to-oyster variations in *V. vulnificus* content. Tamplin (48) has examined the levels of *V. vulnificus* naturally occurring in oysters and found that great variation exists. Lack

of improved detection may also be due to factors that make it difficult to detect bacteria occurring naturally in environmental samples, such as entry of cells into the viable but nonculturable (VBNC) state, low concentrations of cells, and the presence of substances that inhibit DNA extraction and PCR amplification.

At 5°C, *V. vulnificus* enters into a VBNC state in which no colonies form when the bacterium is plated onto standard culture media (30, 37, 40, 52). This probably accounts for the absence of colonies on CPC agar with winter oyster homogenates that were PCR positive. *V. vulnificus* cells in the VBNC state are metabolically active (30, 52) and potentially capable of causing infection (35–38, 40, 41). PCR amplification is able to detect the target DNA from VBNC cells (18, 20); however, approximately 400 times more DNA is required to produce a visible electrophoretic band (18). Decreased sensitivity of the PCR in detecting VBNC *V. vulnificus* DNA may be related to novel DNA-binding proteins produced during the nonculturable state (32).

In addition to being nonculturable at low water temperatures, *V. vulnificus* is present in low numbers in the environment (42). Filters to concentrate microorganisms from environmental samples have been used previously when microorganisms were present in water (11). However, when bacteria are present in a eukaryotic host, filtration is not feasible and other means must be employed to increase cell numbers for analysis. A period of enrichment during which the number of cells increases exponentially can increase the amount of target DNA (10, 22, 29). However, enrichment may require up to 24 h to produce a sufficient amount of target for consistently PCR-positive results (22), thus adding a day to the time required for detection. Furthermore, this is an option only for harvested oysters with culturable *V. vulnificus* cells and is therefore not useful for detecting cells from winter oysters, in which only VBNC cells may be present. We utilized 10 oysters for each experiment in an effort to include sufficient numbers of *V. vulnificus*-containing oysters. This could have led to the dilution of the pathogen if only a few of the 10 oysters contained *V. vulnificus* cells.

Studies comparing the efficacy of PCR detection of *Vibrio cholerae* seeded to homogenates of a variety of foods demonstrated that oyster tissue or fluids in some way inhibit PCR amplification (29). Similar interference has been noted when different amounts of oyster homogenate extracts were added to a fixed amount of *V. vulnificus* DNA (22). Type II oyster glycogen in concentrations of >3.13% (wt/vol) decreased or completely inhibited reverse transcription-PCR amplification of enteric virus RNA in oysters (8). In our studies, DNA was extracted from homogenates containing oyster tissue and ASW at ratios of 1:1 and 1:10 (wt/vol). The 1:1 ratio produced better results, qualitatively speaking, for DNA extracted from seeded oysters (data not shown), and this was the concentration employed for the unseeded oysters. Dilution of extracted DNA to 10% (vol/vol) in order to reduce the concentration of inhibitors in the unseeded samples apparently also reduced the number of copies of DNA below the detectable number as there was no visible PCR amplification for these samples.

Eels. In a recent study, samples of isolated eel DNA inoculated with *V. vulnificus* DNA, biotypes 1 and 2, and subjected to nested-PCR amplification resulted in detection of DNA sequences within 23S rRNA genes of *V. vulnificus* (6). In our study, primers specific for the hemolysin gene of *V. vulnificus* were employed. PCR consistently resulted in amplification of target DNA isolated from biotype 2 cells in all three types of artificially infected tissue (whole eel, liver, and kidney), whereas uninfected tissues were PCR negative (Fig. 2).

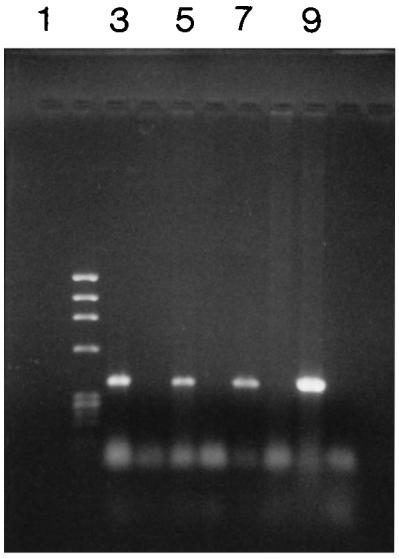

FIG. 2. Ethidium bromide-stained agarose gel of PCR-amplified DNA from *V. vulnificus* in eel tissue. All positive bands represent biotype 2 infection of eels by intraperitoneal injection or immersion, except as noted. Lane 1, blank; lane 2, ϕX174 DNA digested with *Hae*III as a size standard; lane 3, body tissue from infected eel; lane 4, body tissue from uninfected eel; lane 5, kidney tissue, infected; lane 6, kidney tissue, uninfected; lane 7, liver tissue, infected; lane 8, liver tissue, uninfected; lane 9, *V. vulnificus* biotype 2 grown in heart infusion broth overnight at room temperature; lane 10, negative control (heart infusion broth substituted for template DNA in PCR mixture).

All eel specimens examined were preserved in 4% formalin. Although formalin has been found in some cases to cause the DNA of the tissue being preserved to leach out into the surrounding solution (24), we did not find the formalin to affect the extraction or amplification of the *V. vulnificus* DNA. We did not attempt to subject the formalin solution to PCR to detect the presence of *V. vulnificus* DNA, since amounts of DNA sufficient to produce visible bands on an electrophoretic gel were present in all cases in the tissue itself.

Methods that use a buffer solution composed of nonionic detergents and proteinase K to extract human protein C DNA from mouse tail digests have been described previously (21). In our studies, a solution of proteinase K (500 μg/ml) alone was sufficient to release the DNA from the eel tissue. Whereas glass fiber filters have been employed prior to PCR amplification of hepatitis B virus DNA from human liver tissue (1), we did not find such filters to be of value in removing the tissue from the extract. Further, the presence of eel tissue in the microcentrifuge tube with the extracted DNA, and even in the PCR mixture itself, did not appear to inhibit amplification and thus made the use of the filters unnecessary in these experiments.

It has been proposed that the magnesium ion (Mg^{2+}) concentration required for successful PCR is dependent on the primers employed (44). We found whole cells of *V. vulnificus* biotype 2 grown in heart infusion broth and subjected to PCR without prior DNA extraction to result in electrophoretic bands of comparable intensities at $MgCl_2$ concentrations of both 1.5 and 2.0 mM (data not shown). However, biotype 2 DNA extracted from eel tissue produced an electrophoretic band noticeably brighter at 1.5 mM than at 2.0 mM. Thus, 1.5 mM $MgCl_2$ was subsequently employed in our studies of this biotype. Unlike that of biotype 2, we have found DNA amplified from whole cells of biotype 1 to produce an electrophoretic band only with 2.0 mM $MgCl_2$. In contrast, biotype 1

DNA extracted from oysters produced a brighter band with $MgCl_2$ at 4.0 mM than at 2.0 mM. These differences in Mg^{2+} concentration likely reflect the presence of different amounts and types of the nucleic acids, proteins, and glycogen present in the oyster extracts employed for amplification of the *V. vulnificus* hemolysin DNA.

This study demonstrates for the first time that it is possible to detect *V. vulnificus* naturally present in oysters and in artificially infected eels by unique extraction methods to provide DNA which is used as the target for PCR amplification. For both oysters and eels, the extraction and PCR procedures described in this work allow analysis in one working day and can even be used to detect *V. vulnificus* in the VBNC state in oysters.

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