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Polymerase Chain Reaction Identification of *Vibrio vulnificus* in Artificially Contaminated Oysters†

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DNAs extracted from *Vibrio vulnificus* seeded into oyster homogenates were evaluated as templates for the polymerase chain reaction. Several extraction procedures were examined, and it was determined that DNA recovered from cells lysed by guanidine isothiocyanate, extracted with chloroform, and precipitated with ethanol was most suitable for use as a polymerase chain reaction template. The region targeted was a 519-bp portion of the cytotoxin-hemolysin gene of *V. vulnificus*. This region was amplified only when DNA from this species was present in the homogenate. *V. vulnificus* seeded into oyster homogenates at an initial level of 10^2 CFU/g of oyster meat was consistently observed after 24 h of incubation in alkaline peptone water.

Appropriate responses of public health agencies to outbreaks of food-borne disease are often hampered by the length of time required to identify and characterize etiologic agents in contaminated foods. If suspect foods are not obtained and processed quickly, bacterial and viral contaminants may be inactivated and no longer recoverable by standard microbiological methods.

Although DNA hybridization techniques are sensitive for identifying food-borne bacterial pathogens (5, 8), the assays are hampered by the length of time needed for growth and selective enrichment before molecular methods can be applied. The polymerase chain reaction (PCR), which uses a thermostable DNA polymerase and automated thermocycling, is an efficient and rapid procedure for amplifying specific segments of DNA (19, 20). It has already been used to detect pathogenic microorganisms, especially enterotoxigenic *Escherichia coli*, in clinical specimens (6, 17, 18) and in the environment (1). The PCR was recently used to identify *Shigella flexneri* seeded into lettuce (13). However, there are obstacles to the widespread application of both DNA hybridization and PCR techniques. Difficulties encountered in preparing food, clinical, or environmental specimens for analysis require the development of new sample-handling techniques. Rapid and efficient methods for purifying DNA for use in the PCR will allow a broader application of the PCR in the identification of food-borne bacterial pathogens. The techniques needed to prepare a complex food matrix, such as oysters, may yield insights into new methods for the handling of samples for nucleic acid analysis.

Vibrio vulnificus is a halophilic bacterium often recovered from seafood harvested from U.S. coastal waters (10, 11). Shellfish, particularly raw oysters contaminated with this bacterium, have been implicated as a vehicle for gastroenteritis (9) and life-threatening septicemia in susceptible individuals (12). The first signs of illness often occur less than 24 h after ingestion (12), and death may follow within 1 to 2 days. Rapid identification of *V. vulnificus* in seafoods, therefore, is essential to reduce the potentially hazardous effects of widespread consumption of contaminated oysters.

V. vulnificus elaborates a cytotoxin-hemolysin (7), and DNA hybridization has shown that the gene for this protein is unique to this species (16, 23). The structural gene for the cytotoxin-hemolysin has been sequenced (24), and oligonucleotide primers have been synthesized for use in the PCR.

This report investigates the use of a chaotropic salt and compares it with other methods for the preparation of *V. vulnificus* DNA from oysters, a challenging food matrix. The DNA obtained is suitable for amplification by the PCR.

MATERIALS AND METHODS

Strains. The strains used in this study and their sources are listed in Table 1.

Enzymes. Restriction enzymes were obtained from New England BioLabs, Beverly, Mass., and *Taq* polymerase was obtained from Perkin-Elmer/Cetus, Norwalk, Conn., and New England BioLabs.

Seeding and test sample preparation. Fresh retail shellstock oysters were opened aseptically, and 10 g of oyster meat was blended with 90 ml of alkaline peptone water. Indigenous microfloras were enumerated by plating dilutions of oyster homogenate (unseeded) on plate count agar (Difco, Detroit, Mich.) and Trypticase soy agar (BBL, Cockeysville, Md.) containing an additional 1.5% NaCl and then incubating the plates at 35°C for 24 h; 1 ml of unseeded homogenate was incubated to serve as a negative control.

V. vulnificus was grown overnight in alkaline peptone water, and dilutions ranging from 10^2 to 10^7 CFU/g of oyster meat was added to an oyster homogenate. Aliquots (5-ml) from the homogenate and from each dilution were incubated at 33 to 35°C for 0, 4, or 24 h and then stored at 4°C until analyzed.

DNA purification. *V. vulnificus* DNAs to be used as PCR templates were extracted from oyster homogenates by three methods. For each method, 1 ml of oyster homogenate was first pelleted and then washed twice with physiological saline. One of the protocols described below was followed, and the DNA was precipitated with ethanol, resuspended in 25 μl of sterile deionized water, and stored at −20°C.

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† This paper is dedicated to the memory of the late Ralston B. (Pete) Read, former Director, Division of Microbiology, Food and Drug Administration, Washington, D.C.

TABLE 1. Bacterial strains used to test the sensitivity and specificity of PCR in identifying *V. vulnificus*

Species	Strain	Source
Vibrio vulnificus	A1402	CDC,[a] via J. Oliver, University of North Carolina, Charlotte
	C7183	CDC, via J. Oliver
	LA M624	Clinical isolate; R. Murray, California State Dept. of Health Services, Berkeley
	85E1171	Oyster; R. Murray
	27-1C	Oyster; Alabama
V. alginolyticus	39 Q3	A. DePaola, Fishery Research Branch, FDA,[b] Dauphin Island, Ala.
V. cholerae	O1 V69	E. Elliot, FDA, Washington, D.C.
	ATCC 14033	
	C8701	Water; California
V. fluvialis	CDC 1959-83	CDC, via A. DePaola
V. furnisii	CDC 1958-83	CDC, via A. DePaola
V. hollisae	89A4206	Clinical isolate; J. Janda, California State Dept. of Health Services, Berkeley
	89A1960	Clinical isolate; J. Janda
V. mimicus	ATCC 33053	
V. parahaemolyticus	832850 K+	Clam; F. Stanley, FDA, Bothell, Wash.
	NY 477	Food, New York; R. Twedt, FDA, Washington, D.C.
	832998	Shrimp, Ecuador; J. Jagow, FDA, Bothell, Wash.
Aeromonas hydrophila	CA 11	Oyster; D. Hanes, FDA, Washington, D.C.
	MMJJ5	Lettuce; D. Hanes
	VL9515	Clinical isolate; D. Hanes
Yersinia enterocolitica	417-10-1C	Oyster; S. Weagant, FDA, Bothell, Wash.
	8081	D. Portnoy, Rockefeller University, New York, N.Y.
Pseudomonas aeruginosa	ATCC 27853	
Proteus vulgaris	ATCC 13315	
Staphylococcus epidermidis	ATCC 12228	
Escherichia coli	ATCC 25922	
Salmonella typhimurium	ATCC 14028	

[a] CDC, Centers for Disease Control.
[b] FDA, Food and Drug Administration.

(i) Phenol-chloroform method. Pellets were resuspended in 600 μl of Tris-buffered saline (pH 7.4); cells were lysed with 2% sodium dodecyl sulfate–0.4μg of proteinase K (Bethesda Research Laboratories [BRL], Gaithersburg, Md.) per ml (3). The lysate was extracted once with 500 μl of phenol (Sigma, St. Louis, Mo.) and saturated with 1.0 M Tris, pH 8.0; the aqueous layer was extracted again with an equal volume of chloroform.

(ii) GITC method. Cells were disrupted by adding 25 μl of 5.9 M guanidine isothiocyanate (GITC; BRL) (4, 14), and the suspension was incubated for up to 90 min at 60°C. The suspension was diluted to 0.3 M GITC with sterile deionized water, and sodium acetate was added to a concentration of 0.3 M.

(iii) GITC-chloroform method. GITC was used to lyse the cells; the lysate was diluted as described above and extracted twice with chloroform.

Ethanol precipitation. To determine the effect of GITC on DNA recovery, purified bacteriophage lambda DNA (BRL) was precipitated with 95% ethanol at −20°C with several GITC and sodium acetate concentrations. Recoveries were estimated by image analysis of photographs of ethidium bromide-stained 0.7% agarose gels run for 1.5 h at 150 V.

PCR. Primers were based on the nucleotide sequence of a 1.8-kb *Hin*cII region containing the cytotoxin-hemolysin gene (24). The two 19-base primers, VVp1 and VVp2, delineate a 519-bp region within the open reading frame (Fig. 1) and were synthesized with an automated DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.) by the phosphoramidite method. VVp1 (5′-CCGGCGGTACAGGT TGGCGC-3′, sense primer) starts at base 69, and VVp2 (5′-CGCCACCCACTTTCGGGCC-3′, antisense primer) starts at base 569 of the *Hin*cII region. Both primers have a calculated dissociation temperature of 66°C (22).

A GeneAmp kit (Perkin-Elmer/Cetus Corp.) was used to carry out the PCR. The manufacturer's recommendations were followed with the following modifications. Each 100-μl reaction mixture included 100 pmol of each primer and approximately 2 ng of template DNA. To minimize the synthesis of regions catalyzed by low-stringency annealing of primers, the deoxyribonucleotide triphosphates were added to the reaction mixtures after the tubes were heated to more than 65°C and before a 10-min incubation at 94°C before cycle 1. Each of the 30 PCR cycles consisted of 1.75 min at 94°C, 2 min at 67 to 69°C, and 2 min at 72°C. DNA concentrations were estimated with the DNA Dipstick (Invitrogen, San Diego, Calif.).

Electrophoresis. A 20-μl portion of the PCR product was analyzed by electrophoresis at 150 V for 1 h in a 1% agarose

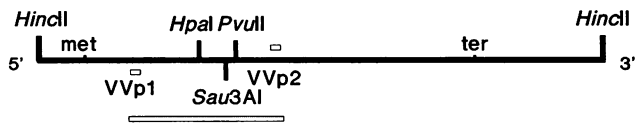

FIG. 1. Map of PCR-amplified region of the *V. vulnificus* cytotoxin-hemolysin gene. A 1.8-kb region bounded by *Hin*cII restriction enzyme sites contains the structural gene for the cytotoxin-hemolysin. VVp1 and VVp2 (□) show the location of the PCR primers that amplify a 519-bp segment (▩). The enzymes *Hpa*I, *Pvu*II, and *Sau*3AI cleave this region near the middle. The initiation and termination codons for the open reading frame (met and ter, respectively) are shown.

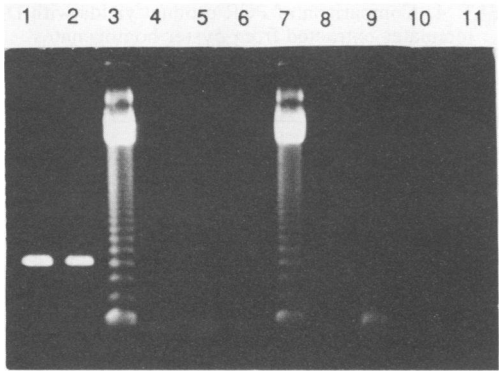

FIG. 2. Agarose gel electrophoresis of PCR products from different template DNAs. The sources of the DNA in the lanes are as follows: lane 1, *V. vulnificus* LA M624; lane 2, *V. vulnificus* 27-1C; lane 4, *Vibrio cholerae* O1 V69; lane 5, *V. cholerae* ATCC 14033 O1 Inaba; lane 6; *Vibrio parahaemolyticus* 832850 Kanagawa+; lane 8, *Proteus vulgaris* ATCC 13315; lane 9, *Staphylococcus epidermidis* ATCC 12228; lane 10, *E. coli* ATCC 25922; lane 11, *Salmonella typhimurium* ATCC 14028. Lanes 3 and 7 are 123-bp molecular weight standard ladders.

gel in Tris-borate-EDTA buffer (15) containing 0.2 μg of ethidium bromide per ml. A 123-bp ladder (BRL) was used as a molecular weight standard.

Sequencing. PCR products were sequenced by the dideoxy method (21) with a Sequenase 2.0 kit (U.S. Biochemicals, Cleveland, Ohio). An 18-base sequencing primer located 28 bases 3′ to the sense PCR primer was used. The primer was synthesized as described above and purified by using Bio-Spin 6 columns (Bio-Rad, Richmond, Calif.) with Tris-EDTA buffer according to the manufacturer's instructions. PCR products were purified with Centricon-100 microconcentrators (Amicon, Danvers, Mass.); the manufacturer's recommendations were followed.

RESULTS

PCR specificity. The PCR-generated molecule was the predicted size (Fig. 2), and restriction endonuclease digestion with *Hpa*I, *Pvu*II, and *Sau*3A yielded fragments of predicted sizes (data not shown), supporting the contention that the amplified material is a portion of the cytotoxin-hemolysin gene. Partial sequencing of the PCR product (50 bases in the middle of the 519-bp PCR product) confirmed its identity to the published sequence of the cytotoxin-hemolysin gene (24).

The specificity of the primers was tested by performing the PCR on DNA isolated from the strains listed in Table 1. Only reaction mixtures containing template DNA extracted from *V. vulnificus* strains generated the characteristic 519-bp band (Fig. 2). None of the other reactions generated visible bands.

DNA precipitation. The relative recovery efficiencies of DNA precipitated by ethanol under various conditions are listed in Table 2. The presence of 0.3 M GITC does not significantly affect the recovery of DNA precipitated by ethanol in 0.3 M sodium acetate (84 versus 82%). When DNA from seeded oyster extracts was precipitated in the presence of 5.9 M GITC, a gelatinous mass was observed. This substance was very difficult to resuspend, and DNA in this material could not be amplified. In all subsequent extractions, extracts were diluted to 0.3 M GITC before they were precipitated with ethanol.

TABLE 2. Relative recovery of ethanol-precipitated DNA under various conditions

Molarity		Recovery (%)[a]
Sodium acetate	GITC	
0	0	<5
0.3	0	82
0	0.3	70
0	5.9	68
0.3	0.3	84
0.3	5.9	73

[a] An equivalent amount of ethanol-precipitated phage lambda DNA was used as the 100% recovery standard.

DNA extraction from homogenates. Several column-based systems are commercially available for rapidly purifying bacterial DNA, but they may not be suitable for use with oyster homogenates. For example, the Extractor (Molecular Biosystems, San Diego, Calif.) became clogged by the considerable amount of particulate matter in the homogenates (data not shown).

To determine whether oyster homogenates inhibit the PCR, we added different amounts of extract to a constant amount of *V. vulnificus* DNA, and we used gel electrophoresis to estimate the amount of product generated (Fig. 3). Unseeded oyster homogenates extracted by the GITC method had the highest level of inhibitors. No PCR-amplified product was observed when 2 or more μl of oyster extract was added to the reaction mixture. The extracts prepared by the phenol-chloroform method were inhibitory only when 20 μl was added. The yield of *V. vulnificus* DNA after amplification was affected least by extracts prepared by the GITC-chloroform method, because PCR product was observed when 20 μl of oyster extract was added to the reaction mixture. The product yields are presented in Table 3.

To estimate the effect of inhibition by oyster extracts on the sensitivity of PCR detection of *V. vulnificus* DNA, we compared the three extraction methods. Various amounts of *V. vulnificus* 85E1171 DNA were added to extracted oyster homogenates. For oyster homogenates extracted by the GITC method, amplified DNA was observed only when 3×10^7 target copies were added (Fig. 4 and Table 4). The yields of amplified DNA were about twofold higher than those of the other methods used when DNA was prepared by using

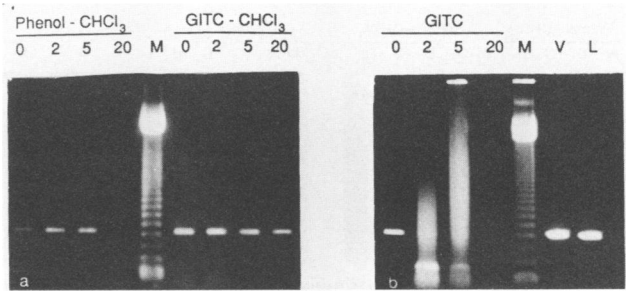

FIG. 3. Agarose gel electrophoresis of PCR products from *V. vulnificus* 85E1171 template DNA, showing inhibitory effect of oyster extracts on product yield. The method used for extraction of oyster homogenates is shown above the lanes. Lanes 0, 2, 5, and 20 indicate the number of microliters of oyster extract added to each reaction mixture. Lanes M, 123-bp ladder molecular weight standard; lanes V and L, purified *V. vulnificus* DNA and purified bacteriophage lambda DNA, respectively, used as controls.

TABLE 3. Effect of oyster homogenate on PCR product yield

Extraction method	Oyster extract (μl)	Estimated no. of final copies	Estimated fold increase[a]
Phenol-chloroform	0	2.6×10^{11}	8.8×10^{5}
	2	4.4×10^{11}	1.5×10^{6}
	5	4.4×10^{11}	1.5×10^{6}
	20	$<1.0 \times 10^{11}$[b]	$<5.0 \times 10^{5}$
GITC-chloroform	0	8.8×10^{11}	2.9×10^{6}
	2	8.8×10^{11}	2.9×10^{6}
	5	6.6×10^{11}	2.2×10^{6}
	20	5.3×10^{11}	1.8×10^{6}
GITC	0	8.8×10^{11}	2.9×10^{6}
	2	$<1.0 \times 10^{11}$	$<2.9 \times 10^{6}$
	5	$<1.0 \times 10^{11}$	$<2.9 \times 10^{6}$
	20	$<1.0 \times 10^{11}$	$<2.9 \times 10^{6}$

[a] The initial number of target DNA copies was 3.0×10^{5}.
[b] Below level of detection by gels (10 ng per band, which is equivalent to about 2×10^{10} copies of a 519-bp fragment).

TABLE 4. Comparison of PCR product yields with DNA templates extracted from oyster homogenates by different procedures

Extraction method	Estimated no. of copies[a]		Estimated fold increase
	Target	Final	
Phenol-chloroform	3.0×10^{7}	4.4×10^{12}	1.5×10^{5}
	3.0×10^{5}	6.2×10^{11}	2.0×10^{6}
	3.0×10^{3}	1.8×10^{10}	6.0×10^{6}
GITC-chloroform	3.0×10^{7}	4.4×10^{12}	1.5×10^{5}
	3.0×10^{5}	1.8×10^{12}	6.0×10^{6}
	3.0×10^{3}	4.4×10^{10}	1.5×10^{7}
GITC	3.0×10^{7}	1.3×10^{12}	4.0×10^{4}
	3.0×10^{5}	$<8.8 \times 10^{10}$	$<2.9 \times 10^{7}$
	3.0×10^{3}	$<8.8 \times 10^{10}$	$<2.9 \times 10^{9}$

[a] Estimated minimum number of copies visible on gels is 2×10^{10}.

GITC for cell lysis followed by chloroform extraction. Template DNA from subsequent seeding experiments was extracted exclusively by this method.

Sensitivity. To determine the sensitivity of the PCR for detecting *V. vulnificus*, homogenates were seeded with 10^2 to 10^7 CFU of *V. vulnificus* per g of oyster meat. After incubation for up to 24 h in alkaline peptone water at 35°C, DNA was extracted from 1 ml of each homogenate by the GITC-chloroform method, amplified by PCR, and analyzed by gel electrophoresis. Although incubation for 0 or 4 h gave variable results, the 519-bp band was always observed after seeded homogenates were incubated for 24 h, extracted, and then analyzed (data not shown).

DISCUSSION

Specificity. Gene probes targeted to the cytotoxin-hemolysin gene of *V. vulnificus* have been used for species identification (23). We constructed a pair of PCR primers to amplify a 519-bp region within an open reading frame of a *Hin*cII fragment encoding the cytotoxin-hemolysin gene. The PCR product had the predicted molecular weight (Fig. 2) and the expected restriction endonuclease digestion pattern (data not shown). Partial sequencing of the PCR product agreed with previously published data (24). The PCR primers generated the 519-bp DNA molecule only when *V. vulnificus* DNA was present (partial results shown in Fig. 2).

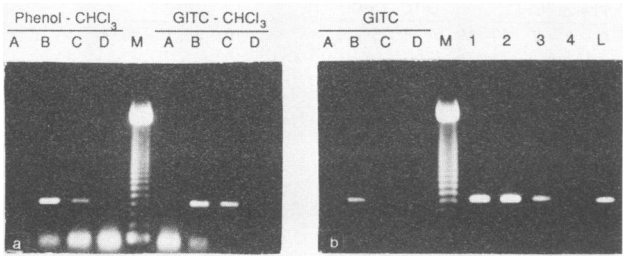

FIG. 4. Agarose gel electrophoresis of PCR products, showing the effect of template level and the inhibitory effect of oyster extracts prepared by three methods on product yield. Oyster extract (2 μl) prepared by the method indicated was added to each reaction mixture. Lanes A, B, C, and D are from reactions initially containing 0, 3×10^{7}, 3×10^{5}, and 3×10^{3} copies of template DNA, respectively; lanes M and L are as described for Fig. 3; control lanes 1, 2, 3, and 4 are from reactions initially containing 6×10^{7}, 3×10^{7}, 3×10^{5}, and 3×10^{3} copies of template, respectively.

DNA extraction. Although the nature of DNA hybridization is reasonably well understood and the principles governing the PCR have been established, widespread application of this new technology awaits the development of rapid and reliable means for preparation of test samples. The identification of invasive *S. flexneri* in artificially contaminated lettuce by the PCR was relatively straightforward (13); a plasmid extraction technique (2) was used to isolate and purify template DNA for amplification by the PCR. However, this method may not be practical for isolating chromosomal DNA from large numbers of very viscous food samples. Commercially available columns for rapidly isolating DNA are ineffective because they are easily clogged by oyster homogenates; therefore, we sought to develop an alternative protocol.

A method based on the lysis of bacteria using GITC followed by chloroform extraction took less than 3 h and yielded DNA that was suitable for amplification by the PCR. The DNA prepared by this method yielded about twice as much PCR product as did DNA extracted with phenol-chloroform. For foods seeded with 3×10^{5} copies of template, a 6.7×10^{7}-fold amplification would be needed to visualize the product on a gel. This represents a 68% amplification efficiency for each of 30 cycles.

Sensitivity. For these experiments, it was not practical to process more than 1 ml of oyster homogenate for each analysis, and only 2 of 25 μl (about 10%) of the final extract was used in the PCR to minimize inhibition of the reaction by oyster homogenates. Inconsistent PCR results were obtained with growth periods of less than 24 h with fewer than 10^2 CFU/g of oyster seeded into homogenates; however, initial levels as low as 10^2 CFU/g of oyster were observed consistently after overnight incubation. Morris and coworkers (16) reported a sensitivity of 10^4 CFU of *V. vulnificus* per g of oyster when they used a ^{32}P-labeled, cloned cytotoxin-hemolysin gene probe. Their method required overnight incubation as well as time for autoradiography.

Summary. PCR primers directed to the *V. vulnificus* cytotoxin-hemolysin gene can be used to identify this species. After 24 h of incubation in alkaline peptone water, positive PCR results can be obtained with DNA extracted from oyster homogenates seeded with 10^2 CFU of *V. vulnificus* per g of oyster.

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