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Sequence of a Cloned pR72H Fragment and Its Use for Detection of *Vibrio parahaemolyticus* in Shellfish with the PCR

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The nucleotide sequence of pR72H cloned from *Vibrio parahaemolyticus* 93 was determined. We examined all *V. parahaemolyticus* gene sequences published in the GenBank-EMBL databases for homology and found that no other DNA sequence of *V. parahaemolyticus* was highly homologous to the sequence reported in this study. A pair of primers, VP33-VP32, derived from a pR72H fragment were selected to detect *V. parahaemolyticus*. The sensitivity of PCR detection for a pure culture of *V. parahaemolyticus* was 10 cells from crude bacterial lysates. Furthermore, a detection level of 2.6 fg, equivalent to 1 cell, was obtained by using purified chromosomal DNA as the template. The expected PCR products were obtained from all *V. parahaemolyticus* strains tested ($n = 124$), while no PCR amplicons were found in other vibrios or related genera ($n = 50$). High levels (10^6 to 10^{10} CFU/ml) of *Escherichia coli* cells did not affect the PCR assay sensitivity. The presence of 10^8 *V. parahaemolyticus* cells or 10^9 *E. coli* cells in the PCR mixtures completely inhibited the PCR. When oyster samples were inoculated with *V. parahaemolyticus* 93 and cultured in tryptic soy broth containing 3% NaCl for 3 h at 35°C, an initial sample inoculum level of 9.3 CFU/g was detected in a PCR assay with crude bacterial lysates. The PCR assay with enrichment culturing in salt polymyxin broth was compared with the conventional method for naturally contaminated shellfish and fish samples. We conclude that this PCR assay with enrichment culturing is a good alternative method for the detection of *V. parahaemolyticus*.

Vibrio parahaemolyticus is an enteric pathogen, and shellfish contaminated with this pathogen have been a source of disease outbreaks in Taiwan, Japan, and other coastal regions (5, 22, 25). A lethal toxin (20, 41) and a vascular permeability factor (19), as well as thermostable direct hemolysin (44) and other related hemolysins (18, 33, 35), have been identified in *V. parahaemolyticus*. The incidence of *V. parahaemolyticus* in the environment and shellfish varies greatly, depending on the season, location, sample type, level of fecal pollution, and analytical method (3, 8, 9, 48). An accurate and rapid method for identifying food products contaminated with *V. parahaemolyticus* would aid in the prevention of such outbreaks. Recently, a sensitive and rapid technique, PCR, has been used to identify the presence of *tdh* (thermostable direct hemolysin) and/or *trh* (thermostable direct hemolysin-related hemolysin) genes in *V. parahaemolyticus* (29, 30, 43). We have reported the development of a PCR technique which can specifically detect hemolysin genes (*tdh*) in strains of hemolytic *V. parahaemolyticus* associated with human gastroenteritis and have applied it successfully to stool specimens from outbreak patients (29, 30). The oligonucleotide primers used in the previously reported PCR procedure were derived from the nucleotide sequence of the *tdh* gene of *V. parahaemolyticus* (29, 30).

However, few environmental or seafood isolates of *V. parahaemolyticus* produce thermostable direct hemolysin as most cases of gastroenteritis associated with clinical isolates do (9, 33). Dai et al. (7) demonstrated that an environmental nonhemolytic strain of *V. parahaemolyticus*, CCRC12958, also exhibited lethality for mice in iron-limited cultures, which is similar to the responses of clinical strains. They concluded that hemolysin or thermostable direct hemolysin may not be the sole major lethal factor for mice and that another toxic factor or factors lethal for mice may be present in CCRC12958. Yoh et al. (52) also demonstrated that Kanagawa phenomenon-negative environmental strains of *V. parahaemolyticus* can produce thermostable direct hemolysin-related hemolysin which is physiochemically, biologically, and immunologically indistinguishable from the toxin from clinical isolates. That Kanagawa-negative *V. parahaemolyticus* is associated with wound infection and gastroenteritis has been well documented (21, 23, 25, 38). These reports have suggested that a certain percentage of environmental strains of *V. parahaemolyticus* are responsible for pathogenesis.

We are interested in developing a PCR protocol specific to *V. parahaemolyticus* from food or environmental isolates, which could be useful for epidemiological surveys, determination of distribution and source, identification, and routine monitoring of this bacterium in contaminated shellfish and other seafood. In a previous study, we cloned a 0.76-kb *Hin*dIII DNA fragment of the chromosomal DNA of *V. parahaemolyticus* 93 into pUC119. This cloned DNA fragment, designated pR72H, can be used as a species-specific DNA probe for *V. parahaemolyticus*. Although the function of this DNA fragment is still unknown, the pR72H DNA fragment is conservative in all strains of *V. parahaemolyticus* tested (28). In the present study, we determined the sequence of pR72H and developed a successful approach for detection of *V. parahaemolyticus* in shellfish by using enrichment culturing with a PCR assay.

MATERIALS AND METHODS

Bacterial strains and culture media. The *V. parahaemolyticus* isolates, other vibrios, and strains from other genera used in this study to evaluate specificity and sensitivity and their sources are listed in Table 1. Tryptic soy agar (TSA)

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TABLE 1. Bacterial strains used in this study

Species	No. of strains	Source(s)[a]
Vibrio parahaemolyticus	4	ATCC
	16	CCRC
	3	M. Nishibuchi
	36	Clinical isolates from NIPM, NLFD, PTPI, and TSGH
	65	Food isolates from NIPM, NLFD, PTPI, and TSGH
Other vibrios		
Vibrio alginolyticus	8	ATCC, K. Aoki
Vibrio campbellii	1	ATCC
Vibrio cholerae	17	PTPI
Vibrio cholerae non-O1	1	H. C. Wong
Vibrio costicola	1	ATCC
Vibrio damsela	2	
Vibrio diazotrophicus	1	ATCC
Vibrio hollisae	1	NIPM
Vibrio logei	1	ATCC
Vibrio mimicus	1	
Vibrio natriegens	1	ATCC
Vibrio pelagius	1	ATCC
Vibrio percolans	1	IFO
Vibrio vulnificus	1	ATCC
Other genera		
Aeromonas hydrophila	2	CCRC
Escherichia coli	2	IAM, CCRC
Klebsiella pneumoniae	1	ATCC
Micrococcus luteus	1	ATCC
Proteus mirabilis	1	CCRC
Pseudomonas aeruginosa	2	
Salmonella typhi	1	ATCC
Salmonella typhimurium	1	
Staphylococcus aureus	1	ATCC

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CCRC, Culture Collection and Research Center, Hsinchu, Taiwan; NIPM, National Institute of Preventive Medicine, Taipei, Taiwan; NLFD, National Laboratories of Foods and Drugs, Taipei, Taiwan; PTPI, National Ping-Tung College of Agriculture, Ping-Tung, Taiwan; TSGH, Tri-Service General Hospital, Taipei, Taiwan; IFO, Institute for Fermentation, Osaka, Japan; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan.

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   1  AGCTTAGCTA GTCATTTCAG TCTACATGCA TTCTTCTCTA CTATTTTGCT
  51  AATATCGATA CGGGACTCGC TTTCCCCAAT TTCCAATCGT CTTGAGTAAT
                                                VP33 -->
 101  ATGAGTTTGT CGATTTGATT CAATCTATAA GATTAAGCAT GCGAATTCGA
 151  TAGGGTGTTA ACCACCTTTG TCTGTTTTTT TCGAGAGTGAG CCTAGTTTC
 202  AATCCTTTAA TAATTATTTT TTAAGCCGTT CTAAGGTGCT TAGAACTAAT
      <-- VP35
 251  CAAATACCGC TCTGTATTTA TGTTTAAAAG GTGTGTAGGG CCCTTCAGGG
 301  TGAGATATGT CTTTTGAAAA ATGACTGCCG TGTACAAAAA AAGCCCAACA
 351  TAAGTTGGGC TTTCGAAAGA CAAATAAGCG GAAGATATTT AAAGGATACG
 401  CTTTAAAACA CCGTCAGCTT TTAAGCGTGT GATTTTTCTC ACGAGTTTTT
 451  GCACAGCAAG CGGGTAATCT TCGACTTTTT CCAACTGTTT GTAAGTGCAG
 501  ATTAGCTGCG TATGTTCAAG GATTCGGGCT ACTTCATCTT CAAACTTATG
                        <-- VP32
 551  AGTAAGGTTT GCGTAGTCAT CTTGAATCAA CAAACCATTT TCAAGTCGAG
 601  AGACCAAGCA CGCGGATTTA AGTTGTTACC CGTAAGCAGC ATGTATCGCT
 651  TATCAACCCA GATACCCTTT AGGTGGAAGC TGTTGTTTTC ATGCTTCCAT
 701  AAGTGCACAG A
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FIG. 1. Nucleotide sequence of pR72H. Underlining indicates the relative positions of oligonucleotides VP33, VP35, and VP32, and dashed arrows show the 5′-to-3′ orientations.

(Difco) or tryptic soy broth (TSB) (Difco) supplemented with 2.5% NaCl was used for growth of all of the *Vibrio* species. Other bacterial cultures were grown on TSA or TSB medium.

DNA sequence determination. Plasmid pR72 is a pUC119 derivative containing a 0.76-kb *Hin*dIII insert of *V. parahaemolyticus* 93, and the insert DNA fragment is designated pR72H (28). Large-scale plasmid preparation and purification were performed by a modified alkaline lysis procedure and polyethylene glycol precipitation protocol (39). The complete nucleotide sequence of both strands of the insert was determined by a modified dideoxy sequencing method (31, 40) with the Sequenase system (United States Biochemical Corp., Cleveland, Ohio). The nucleotide sequence data were analyzed for homology to the published sequences in EMBL or GenBank databases by using the GENALIGN multiple sequence alignment program from IntelliGenetics (IntelliGenetics Corp., Palo Alto, Calif.), which is based on the homology search method of Wilbur and Lipman (51).

Synthetic oligonucleotide primers. Three oligonucleotide primers (22- or 24-mer) were designed and synthesized (Bio-Synthesis, Lewisville, Tex.) according to the determined DNA sequence of pR72H, the *Hin*dIII insert of *V. parahaemolyticus* used in this study. The sequences were 5′-TGCGAATTCGATAGG GTGTTAACC-3′ (designated VP33) and 5′-CGAATCCTTGAACATACGCA GC-3′ (designated VP32) as primers and 5′-GGATTGAAACTAGGCTCACTC TCG-3′ (designated VP35) as an internal probe. The T_m values of the primers and probe were 71.3, 69.3, and 68.4°C, respectively. The homologous sites for hybridization to the pR72H are shown in Fig. 1.

PCR amplification. The PCR solution contained 1× PCR amplification buffer (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphates, 1 μM (each) primers, 1.25 U of *Taq* DNA polymerase (Promega), 5 μl (40 ng) of template DNA or lysed bacterial broth, and double-distilled water treated with 0.1% diethylpyrocarbonate to make a final volume of 50 μl. The mixtures were overlaid with 50 μl of mineral oil (Sigma) and subjected to 35 PCR cycles in a programmable temperature cycler (ASTEC PC-700, Tokyo, Japan). The parameters for the amplification cycles were denaturation for 1 min at 94°C, annealing of primers for 1 min at 60°C, and primer extension for 1 min at 72°C. After the last cycle, the PCR mixtures were incubated for 10 min at 72°C.

Detection of PCR products. PCR-amplified DNAs were detected by agarose gel electrophoresis in 2% agarose gels (Clontec). Ten microliters each of the amplification mixtures and molecular weight markers (pBR322 digest of *Hin*fI DNA) was subjected to electrophoresis and ethidium bromide staining. The specific amplified DNA fragments were visualized by UV illumination.

Southern and dot blot hybridization. For confirmation of the amplified DNA by Southern blot hybridization, 10 μl of PCR products was subjected to 2% agarose gel electrophoresis and then transferred to a Zeta-probe membrane (Bio-Rad) by alkaline Southern blotting (36). An internal oligonucleotide probe (VP35) was labeled with [γ-^{32}P]ATP by 5′ end labeling with T4 polynucleotide kinase (Bethesda Research Laboratories) as a probe (10^6 cpm/ml). The membranes were air dried and hybridized at 40°C overnight in 1.5× SSPE (1× SSPE is 0.18 M NaCl, 0.01 M sodium phosphate, 0.01 M disodium EDTA), 1% sodium dodecyl sulfate (SDS), 0.5% BLOTTO (nonfat dry milk; Carnation), and 0.5 mg of denatured herring sperm DNA per ml (Boehringer Mannheim). After hybridization, the membranes were rinsed with 2× SSPE and 2× SSPE–0.1% SDS, followed by 0.5× SSPE–0.1% SDS and 0.1× SSPE–0.1% SDS, at 25°C for 15 min. The last wash was done in 0.1× SSPE–1% SDS at 40°C for 30 min, followed by rinsing with 0.1× SSPE. After the hybridization, the colony blot or dot blot membranes were autoradiographed with X-Omat film (Eastman Kodak) with intensifying screens and exposure at −80°C for an appropriate length of time.

For confirmation of the PCR products by dot blot hybridization, PCR products were heated at 100°C for 10 min and then loaded on a Zeta-probe membrane with a Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was air dried and stored in a plastic bag until used. Dot blot hybridization followed the same procedure as Southern blot hybridization.

Preparation of artificially contaminated oysters. Fresh retail oysters were washed with sterilized water, and 5 g of oyster meat was blended with 45 ml of TSB containing an additional 2.5% NaCl. The oyster sample was heated at 60°C for 10 min and was then filtered with cheesecloth. Samples of 10 ml of homogenate were inoculated with *V. parahaemolyticus* at 9,300, 930, 93, 9.3, and 0 CFU/g. One milliliter of each homogenate was then mixed with 9 ml of TSB containing an additional 2.5% NaCl in a 50-ml flask and incubated at 35°C for 0, 3, 6, or 9 h in a shaker (YIHDER LM-570R, Taipei, Taiwan) at 50 rpm. After enrichment, the inoculated samples were stored at −20°C until they were analyzed.

DNA extractions for PCR analysis. All pure culture DNAs were prepared with the mini-preparation of chromosomal DNA method described by Ausubel et al. (2). Bacterial chromosomal DNA was extracted by SDS-proteinase K lysis, followed by hexadecyltrimethyl ammonium bromide (CTAB) treatment, two phenol-chloroform-isoamyl alcohol extractions, and absolute ethanol precipitation.

The purified DNA was then suspended in small volumes of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA).

V. parahaemolyticus DNAs to be used as PCR templates were extracted from oyster homogenates by three methods. For the first method, 0.5 ml of oyster homogenate was heated at 100°C for 10 min (protocol I). The second method simply involved the addition of 50 μl of 10% Triton X-100 (Riedel-deHaën) to 0.5 ml of oyster homogenate and then heating of the sample at 100°C for 10 min (47) (protocol II). The third method involved the lysing of 0.5-ml samples of the bacterial suspension with 1 mg (final concentration) of lysozyme per ml (Sigma) at room temperature for 15 min, followed by digestion with 200 μg (final concentration) of proteinase K per ml (Boehringer Mannheim) at 60°C for 30 min, followed by boiling for 10 min (29) (protocol III). All treated oyster samples were stored at −20°C until they were analyzed. When PCR amplification was applied, the treated samples were undiluted or diluted 10-fold.

Procedure for analysis of food samples by PCR. Food samples were obtained from local retail shops and supermarkets. Twenty five grams of food samples was homogenized in a Stomacher 400 (Seward, London, England) with 225 ml of 3% NaCl for 60 s. One milliliter of homogenate was mixed with 9 ml of salt polymyxin broth (per liter containing 10.0 g of peptone, 3.0 g of yeast extract, 20.0 g of sodium chloride, and 250,000 U of polymyxin B [pH 7.4]; Nissui, Tokyo, Japan) (SPB) and tested for the presence of *V. parahaemolyticus* by the classical culture method described below. Five milliliters of homogenate was added to 45 ml of SPB and incubated for 6 h at 35°C. Afterwards, 1 ml of enrichment broth was lysed as described in protocol III and then was subjected to PCR amplification both undiluted and diluted 10- or 100-fold.

Classical culture method. Isolation and enrichment steps were performed in accordance with procedures in the *Bacteriological Analytical Manual* (46). One loopful of enrichment broth was then plated onto thiosulfate citrate bile salts agar and incubated at 35°C overnight. Green colonies thought to be *V. parahaemolyticus* were further characterized by using the minimal number of biochemical tests described in the *Bacteriological Analytical Manual* (46). Further tests, including a motility test, ornithine decarboxylase production, and lactose and mannitol fermentation, were also carried out for identification of *V. parahaemolyticus*.

Nucleotide sequence accession number. The nucleotide sequence data presented in this paper will appear in the EMBL and GenBank nucleotide sequence databases under accession number L30116.

RESULTS

Nucleotide sequence of pR72H. The DNA nucleotide sequence of pR72H isolated from *V. parahaemolyticus* 93 is shown in Fig. 1. The molecular G+C content for the 711 bp in a length of pR72H is 39%, compared with 46 to 47% for the chromosomes of *V. parahaemolyticus*. Neither potential translational start sites, possible open reading frames, nor the consensus Shine-Dalgarno sequence was found. The nucleotide sequence of pR72H was analyzed for homology to the known sequences in the EMBL and GenBank databases with the GENALIGN program from IntelliGenetics. There was no significant homology of the pR72H sequence to *V. parahaemolyticus* hemolysin genes *dvh*, *tdh*, *trh*, or *tlh*; *V. parahaemolyticus* 5S or 16S rRNA; or *V. parahaemolyticus* insertion sequence-like elements.

Development of the PCR assay. Primer pair VP33-VP32 was designed on the basis of the nucleotide sequence of pR72H determined in this study (Fig. 1). The nucleotide sequences of the primers and localizations on the pR72H sequence are shown in Fig. 1. Primer pair VP33-VP32 was used to amplify target sequences in genomic DNA from five strains of *V. parahaemolyticus* and eight strains of other vibrios and other genera. An expected product with a length of 387 bp was found from these five strains of *V. parahaemolyticus*, while nonspecific products were not found (Fig. 2A). Southern blot hybridization with an internal oligonucleotide DNA probe (VP35) showed that the 387-bp DNA fragments from *V. parahaemolyticus* could hybridize with the oligonucleotide probe that was derived from the sequence located between the two primers VP33 and VP32 (Fig. 2B).

Specificity of the PCR. To evaluate the specificity of the PCR assay, PCR amplification was performed with 124 strains of *V. parahaemolyticus* and 50 other bacterial strains with purified chromosomal DNA as a template. The PCR amplification with the VP33 and VP32 primers generated 387-bp-amplified DNA bands for all *V. parahaemolyticus* strains tested, while no amplification was apparent for non-*V. parahaemolyticus* bacterial strains. The ^{32}P-labeled internal oligonucleotide probe VP35 showed positive hybridizations with the amplified DNA from the *V. parahaemolyticus* strains, and some of the dot blot hybridization results are shown in Fig. 3. These results indicate that the oligonucleotide primers VP33 and VP32, as well as the PCR procedure, were adequate for specific detection of *V. parahaemolyticus*. These results are also in agreement with our previous DNA hybridization results (28), which indicated that the reported nucleotide sequence of the insert of *V. parahaemolyticus* 93, pR72H, is conserved in all *V. parahaemolyticus* strains.

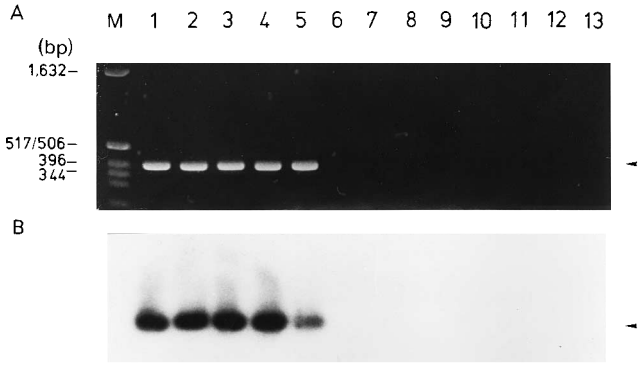

FIG. 2. Specific amplification of the 387-bp fragment (arrows) from the total DNA of *V. parahaemolyticus* and other genera. (A) Two percent agarose gel electrophoresis of PCR product. (B) Southern blot analysis of the products probed with ^{32}P-labeled VP35. Lanes: M, molecular size marker pBR322/*Hin*fI; 1 to 5, *V. parahaemolyticus* strains 93, A92, 175, 176, and 178, respectively; 6 and 7, *Vibrio alginolyticus* ATCC 17749 and 179, respectively; 8, *V. vulnificus* ATCC 33147; 9, *Vibrio campbellii* ATCC 25920; 10, *Vibrio costicola* ATCC 33508; 11, *Staphylococcus aureus* ATCC 65389; 12 and 13, *E. coli* IAM C-600 and A15, respectively.

Sensitivity of PCR. A dilution series of genomic DNA of *V. parahaemolyticus* 93 was prepared as described in Materials and Methods. Aliquots of each dilution were used as templates for PCR amplification with 25 or 35 cycles. Aliquots containing 2.6 pg of genomic DNA could be detected successfully after

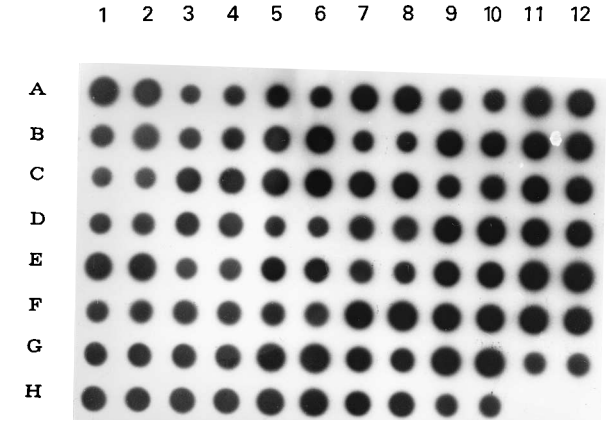

FIG. 3. Results of dot blot hybridization of *V. parahaemolyticus* with ^{32}P-labeled VP35. Dots A1 and A2, *V. parahaemolyticus* 93 as a positive control; dots H11 and H12, salmon sperm DNA as a negative control. The other 92 dots represent 46 strains of *V. parahaemolyticus* (each strain is duplicated) as indicated in Table 1.

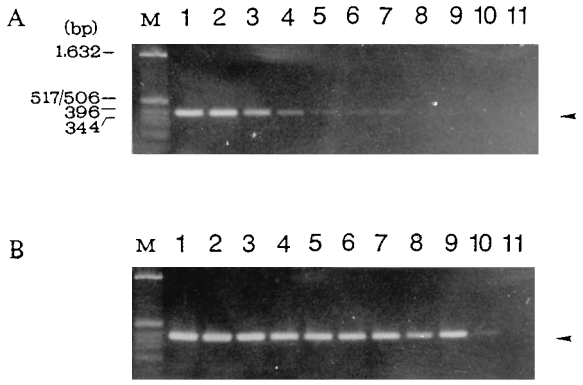

FIG. 4. Analysis of the limit of *V. parahaemolyticus* detection by PCR amplification with 25 (A) and 35 (B) cycles by agarose gel electrophoresis. Lane M, molecular size marker pBR322/*Hin*fI. The amounts of DNA template in the initial PCR mixtures were as follows (by lane): 1, 2.6 μg; 2, 260 ng; 3, 26 ng; 4, 2.6 ng; 5, 260 pg; 6, 26 pg; 7, 2.6 pg; 8, 260 fg; 9, 26 fg; 10, 2.6 fg; and 11, 0 fg.

amplification with 25 cycles, while the detection limit could be increased to 2.6 fg once amplification with 35 cycles was performed (Fig. 4). Thus, 35 cycles of PCR were performed throughout the experiments in this study.

PCR sensitivity tests for *V. parahaemolyticus* seeded to oysters. When oyster samples were cultured in TSA–3% NaCl for 0 h, the initial sample inocula of 9.3 to 9.3 × 10^3 CFU of *V. parahaemolyticus* 93 per g were always PCR negative. After 3 h of incubation, an initial inoculum of 9.3 CFU of this *V. parahaemolyticus* strain per g was detected by PCR assay with DNA extracted according to protocol III (Fig. 5). Comparison of the three protocols of DNA extraction was performed, and protocol III provided the highest level of sensitivity for *V. parahaemolyticus* detection. After the culturing, the bacterial lysates subjected to PCR amplifications needed 10-fold dilution; false-negative results were given without such dilution (Table 2).

Detection of *V. parahaemolyticus* by PCR in the presence of *Escherichia coli*. To investigate whether the presence of other bacteria with the target bacteria would inhibit the PCR, we used mixtures of *V. parahaemolyticus* and *E. coli* cultures as PCR templates to evaluate the sensitivity of this PCR protocol for detection of *V. parahaemolyticus*. Fresh, growing cultures of *V. parahaemolyticus* and *E. coli* cells were diluted separately and then mixed with each other to equal volumes. Five microliters of mixed-cell suspensions was lysed as described in protocol III and then was subjected to PCR amplification. As shown in Table 3, the sensitivity of PCR for the detection of *V. parahaemolyticus* for a pure culture of *V. parahaemolyticus* was 2 × 10^3 cells per ml (10 cells in 50 μl of PCR mixture). The PCR was inhibited when the number of *V. parahaemolyticus* cells increased to 2 × 10^{10} cells in 1 ml of mixed-cell suspension (10^8 cells in 50 μl of PCR mixture). High levels (10^6 to 10^{10} CFU/ml) of *E. coli* cells did not affect the PCR-culture assay sensitivity and specificity. However, the presence of 10^{11} *E. coli* cells per ml of mixed-cell suspension (10^9 cells in 50 μl of PCR mixture) inhibited the PCR, as found from observation of ethidium bromide-stained agarose gel electrophoresis (Table 3).

Application of PCR for detection of *V. parahaemolyticus* in natural shellfish and fish samples. The PCR assay with enrichment culturing and the conventional culture method were used to examine 18 natural shellfish and fish samples for the presence of *V. parahaemolyticus*. From each sample, a 25-g sample was subjected to enrichment culturing in SPB for 6 h and assayed by PCR. Because SPB is much more selective than

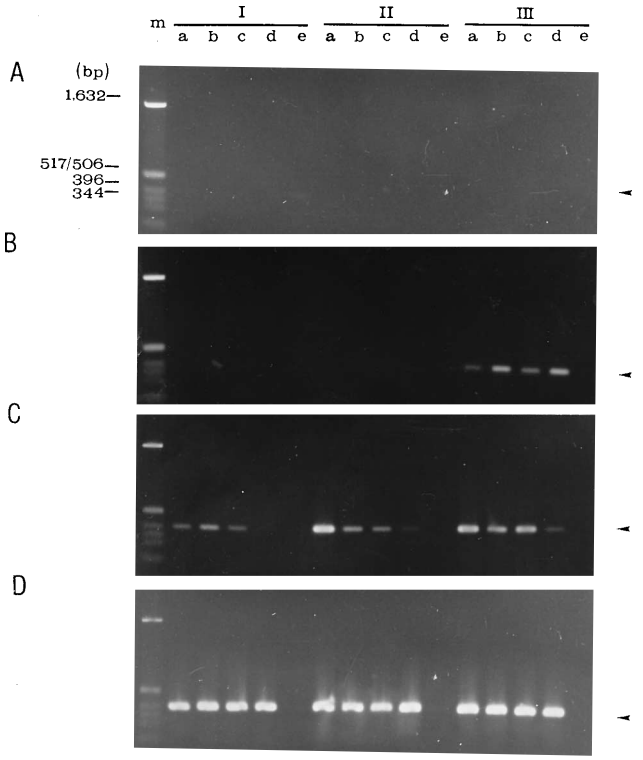

FIG. 5. Agarose gel electrophoresis of 10-fold-diluted amplification products obtained from oyster samples contaminated with serial 10-fold dilutions of *V. parahaemolyticus*. The oyster samples were enriched for 0 (A), 3 (B), 6 (C), or 9 (D) h, and then the DNAs were extracted from the enriched oyster homogenates by protocol I, II, or III. Lanes: m, molecular size marker pBR322/*Hin*fI; a to e, PCR products of oyster homogenate lysates with initial contamination levels of *V. parahaemolyticus* 93 of 9300, 930, 93, 9.3, and 0 CFU/g, respectively.

TSB–3% NaCl broth for *V. parahaemolyticus*, we chose SPB as the enrichment broth for screening of *V. parahaemolyticus* in natural shellfish samples (unpublished data). Sixteen samples were found to be positive by the method involving both the SPB enrichment culture and PCR assay. *V. parahaemolyticus* was found to be present in 12 of these samples by the culture method (Table 4). All food samples considered to be naturally contaminated by *V. parahaemolyticus* with the conventional culture method were detected with the PCR culture assay as well.

DISCUSSION

In this report, we sequenced pR72H, a cloned DNA fragment of chromosome, from *V. parahaemolyticus* 93 (Fig. 1). On the basis of this sequence, we designed and established a PCR assay with enrichment culturing to detect *V. parahaemolyticus* in food samples. This PCR assay was found to be highly specific for *V. parahaemolyticus*, with the primers VP33 and VP32 yielding an amplified 387-bp DNA fragment unique to *V. parahaemolyticus* (Fig. 2). Moreover, it was possible to identify *V. parahaemolyticus* from food samples in only 10 h with this assay, including 6 h of enrichment, 3 h of PCR, and 1 h of agarose gel electrophoresis. The PCR assay we have described could reliably detect 2.6 fg of purified *Vibrio* DNA amplified through 35 cycles (Fig. 4), which corresponds to approximately 1 cell. This level of sensitivity was higher than those reported by others: 400 fg of cellular DNA by PCR amplification (43) and 28 fg of DNA by a microtiter-based nonisotopic method

TABLE 2. Effect of enrichment length and DNA preparation protocol on PCR detection limit

Enrichment duration (h)	Fold PCR dilution	Response to initial contamination tested (CFU/g) for protocols given[a]:														
		9,300			930			93			9.3			0		
		I	II	III	I	II	III	I	II	III	I	II	III	I	II	III
0	10^0		−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10^1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	10^0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10^1	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−
6	10^0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10^1	+	+	+	+	+	+	+	+	+	−	+	+	−	−	−
9	10^0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10^1	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−

[a] Three different protocols (I, II, and III) were used to extract DNAs from oyster homogenates as described in Materials and Methods. +, specific 387-bp fragment found; −, specific 387-bp fragment not found.

for the detection of amplicons (42). By using crude cell extracts of *V. parahaemolyticus* prepared by proteinase K treatments, a 10-CFU (2×10^3 CFU/ml) level of detection of *V. parahaemolyticus* whole cells was found (Table 3).

The three methods used for template DNA preparation, protocols I, II, and III, were compared by PCR assay for the detection of artificially contaminated oysters seeded with various numbers of *V. parahaemolyticus* cells. The boiling method (protocols I and II) was simpler than the lysis method (protocol III) for the preparation of PCR templates. However, our results indicated that the lysis method was a more sensitive detection method for *V. parahaemolyticus* by PCR assay (Table 2 and Fig. 5). This result agreed with that of Makino et al. (32), who detected *Bacillus anthracis* DNA in animal samples and suggested using the lysis method to prepare samples. Therefore, the method of proteinase K treatment was used to prepare PCR templates throughout the subsequent experiments.

Wang et al. (47) recently reported that PCR assays could give false-negative results if more than 10^5 or 10^6 CFU of target cells in a 25-μl PCR mixture was used for the assay. Niederhauser et al. (34) also reported that high concentrations of bacterial DNA could lead to inhibition of enzymatic amplification. Other researchers found that the sensitivity of PCR detection for target bacteria was reduced in mixed cultures with *E. coli* (10, 16), while Giesendorf et al. (15) found that high levels (10^6 to 10^8 CFU/g) of other contaminating bacteria in chicken products did not affect the specificity of the PCR-culture assay for detection of *Campylobacter* spp. In this study, we observed that the PCR products produced with 10^7 CFU (2×10^9 CFU/ml) of *V. parahaemolyticus* were less intense than those produced with 10^6 CFU (2×10^8 CFU/ml) of *V. parahaemolyticus*, regardless of the absence or presence of different numbers of *E. coli* cells (data not shown). The PCR products were not observed in ethidium bromide-stained agarose gel electrophoresis when the number of *V. parahaemolyticus* cells in a 50-μl PCR mixture increased to 10^8 CFU (2×10^{10} CFU/ml) (Table 3). This result suggested that when the number of target cells of *V. parahaemolyticus* in the PCR mixtures was greater than 10^6 CFU, there was a negative effect on the reaction. As shown in Table 3, the presence of 10^9 *E. coli* cells in the PCR mixtures (2×10^{11} CFU/ml) completely inhibited amplification of target DNA. However, the detection limit of *V. parahaemolyticus* was not reduced when 10^4 to 10^8 *E. coli* cells were present in the PCR mixtures.

Analysis of artificially contaminated samples indicated that protocol III with only 3 h of enrichment enabled reliable detection of a small number of cells (Table 2). However, it has been reported that *V. parahaemolyticus* can be injured or inactivated by environmental challenges (4, 14, 27, 37). Niederhauser et al. (34) also proposed that, in contrast to fully viable food-borne pathogens seeded in food samples, food-borne pathogens present in natural food samples have reduced viability. Therefore, when the PCR assay was applied to the naturally contaminated food samples, we extended the enrichment length to 6 h for recovery of stressed cells. In the experiments with the application of PCR-culture assay in nat-

TABLE 3. Detection of *V. parahaemolyticus* by PCR in the presence of *E. coli*

No. of *V. parahaemolyticus* cells/ml of mixed suspension	Detection with no. of *E. coli* cells/ml of mixed suspension[a]						
	2×10^{11}	2×10^{10}	2×10^9	2×10^8	2×10^7	2×10^6	0
2×10^{10}	−	−	−	−	−	−	−
2×10^9	−	+	+	+	+	+	+
2×10^8	−	+	+	+	+	+	+
2×10^7	−	+	+	+	+	+	+
2×10^6	−	+	+	+	+	+	+
2×10^5	−	+	+	+	+	+	+
2×10^4	−	+	+	+	+	+	+
2×10^3	−	+	+	+	+	+	+
2×10^2	−	−	−	−	−	−	−
0	−	−	−	−	−	−	−

[a] +, specific 387-bp fragment found by gel electrophoresis; −, specific 387-bp fragment not found.

TABLE 4. PCR and culture methods for detection of *V. parahaemolyticus* in naturally contaminated food samples

Food sample	No. of samples positive/no. tested by:	
	PCR with SPB enrichment	Culture method
Oyster	2/3	2/3
Shrimp	2/2	2/2
Clam	4/5	4/5
Fish	3/3	0/3
Squid	5/5	4/5
Total	16/18	12/18

ural shellfish and fish samples, three fish samples and one squid sample were positive by the PCR assay for *V. parahaemolyticus*, while they were negative with the culture method (Table 4). We conclude that these food samples were contaminated with *V. parahaemolyticus* which was not detectable under the conditions tested. Brauns et al. (4) have demonstrated that PCR is potentially able to detect the presence of viable but nonculturable *Vibrio vulnificus* cells.

Direct detection of pathogens in food samples by PCR presents some technical problems, as reported by several authors (1, 10, 13, 16, 17, 24, 34, 45, 47, 49, 50). It is concluded that components of food, enrichment media, or a high concentration of DNA may lead to inhibition of the PCR. However, these difficulties could be overcome by performing nucleic acid extraction or by diluting the lysate before PCR amplification. Recently, a new detection system, the magnetic immuno-PCR assay, has been developed to detect pathogens in food. This method separates target cells from food samples by using magnetic beads coated with specific monoclonal antibodies (11, 12, 26). However, this method requires antibodies specific to various serogroups of the target pathogen (26). In this study, the proposed PCR-culture assay could increase the number of target cells and dilute out non-*Vibrio* DNA and other inhibitors of PCR which may exist in food samples. It also ensures that only *V. parahaemolyticus* DNA obtained from viable cells is detected.

Because *V. parahaemolyticus* represents a marine bacterium that is often found in marine and estuarine water samples and shellfish (3, 9, 48), surveillance of contamination in food samples, in shelf stock of retail seafood markets, and in seafood plants is important (6, 9). Although the multiplication of environmental strains of *V. parahaemolyticus* in oysters does not necessarily indicate a human health risk, the PCR-culture assay with VP33 and VP32 primers established in this study can facilitate the monitoring of bacterial contamination by detecting the 387-bp DNA fragment in seafood samples and can reduce any potential health risk. A further study to design both a colorimetric method for detection of PCR-amplified DNA sequences in solid phase and an internal standard for the PCR assay is in progress.

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