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Nested PCR Method for Rapid and Sensitive Detection of *Vibrio vulnificus* in Fish, Sediments, and Water

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A nested PCR for the detection of *Vibrio vulnificus* in fish farms was developed as an alternative to cultural methods by using universal primers flanking the *V. vulnificus*-specific sequences directed against 23S rRNA genes. This specific assay detected 10 fg of DNA or 12 to 120 cells in artificially inoculated samples without enrichment and within 24 h.

Molecular techniques, particularly specific oligonucleotide probes, constitute a very sensitive and specific tool for detecting very low numbers of bacteria, or even viable but nonculturable forms, that has been used satisfactorily for a variety of pathogens (9, 12–14, 16), including *Vibrio vulnificus* (1, 3, 4, 18). This marine species includes human and fish pathogens in two biotypes, is not predominant in the aquatic environment, and enters a nonculturable state (15, 17). It has been the subject of increasing interest as a consequence of its implication in food-borne diseases after consumption of different seafoods (6–8). Using amplification primers derived from the cytolysin gene sequence, Brauns et al. (3) were able to detect viable but nonculturable *V. vulnificus* in seawater by PCR. In order to circumvent the possible loss or rearrangement of nonessential genes such as the hemolysin gene, we have developed a specific and sensitive method for detection and identification of *V. vulnificus* in eel farms, combining the use of universal and specific oligonucleotides directed against 23S rRNA genes and PCR in a two-step amplification (nested PCR). The strains of *V. vulnificus* biotypes 1 and 2 used are listed in Table 1. They include reference strains of *V. vulnificus* and other species and genera, as well as wild *V. vulnificus* isolates obtained from an eel farm during different epizootic outbreaks of vibriosis in Spain (2). *Vibrio* strains were grown in brain heart infusion (Difco) supplemented with 0.5% (wt/vol) NaCl and 1.5% agar (for solid medium). Clinical strains were incubated at 37°C, and environmental isolates were incubated at 25°C for 24 h.

Specific primers complementary to *V. vulnificus*-specific sequences in three variable regions in the 23S rRNA genes previously described (1), corresponding to helices 9, 18, and 45, were designed and are referred to below as Dvu9, Dvu18, and Dvu45. These sequences were optimized to be used as primers for specific amplification and arranged as three sets of sense-antisense primers (Dvu9V-Dvu18R, Dvu18V-Dvu45R, and Dvu9V-Dvu45R). PCR sense primers Dvu9V (3′-GACC GAATACGGTCACC-5′) and Dvu18V (3′-GGCAAGCAGA TTGTGTAC-5′) were complementary to positions 129 to 147 and 286 to 303, respectively, and PCR antisense primers Dvu45R (3′-AAGATACTTGTAACCCATC-5′) and Dvu18R (3′-GCCAGACCTTTCAGGCT-5′) were complementary to positions 1169 to 1190 and 301 to 317, respectively. Sequence positions are given according to the *Escherichia coli* numbering system (5). Primers were tested for PCR amplification at different annealing temperatures (48 to 54°C), with DNA from selected strains of *V. vulnificus* and other *Vibrio* species included in Table 1. The amplification was conducted in a total reaction volume of 50 μl containing 5 μM universal or specific primer, 0.03 U of *Taq* polymerase (Promega, Serva, Heidelberg, Germany), 5 μl of *Taq* polymerase buffer (Promega, Serva), 5 μl of 25 mM $MgCl_2$ (Promega, Serva), and 0.2 mM each deoxynucleoside triphosphate. Twenty nanograms of purified DNA, unless otherwise stated, was supplied as the template for amplification in a 50-μl reaction mixture. DNAs were extracted and purified as previously described (1). The reaction mixtures were overlaid with 30 μl of mineral oil (Sigma, St. Louis, Mo.) and subjected to amplification at 94°C for 1 min followed by 35 cycles (OMNIGENE; Hybaid, Ebersberg, Germany) at 94°C for 20 s, the selected annealing temperature for 30 s, and 72°C for 45 s with an increment time of 15 s in each repeat cycle. The amplification products were electrophoresed on 1% (wt/vol) agarose gels (Pharmacia, Freiburg, Germany) with TAE (0.04 M Tris-acetate, 0.001 M EDTA) electrophoresis buffer, stained with ethidium bromide, and photographed under UV (254 nm) transillumination to visualize the corresponding amplification fragments. *Hin*dIII DNA fragments (Gibco, BRL, Eggestein, Germany) and a 123-bp ladder (Gibco, BRL) were included as molecular weight markers.

The primer combinations Dvu9V-Dvu18R and Dvu18V-Dvu45R gave specific amplification products of 181 and 897 bp, respectively, at a 52°C annealing temperature (Fig. 1a and c, lanes B to D), but, in addition, multiple amplification products were observed with both *V. vulnificus* strains and with other *Vibrio* species (lanes E to G). They appeared even when annealing temperatures were raised to 54°C (data not shown). The primer set Dvu9V-Dvu45R produced a unique and specific amplification product of 978 bp at 52°C, found only with the three *V. vulnificus* strains (Fig. 1b, lanes B to D); no amplification products were observed with the other *Vibrio* species (lanes E to G). The specificity of this primer combination was assessed by testing DNA from a total of 36 strains (Table 1), including 15 *V. vulnificus* strains, 15 *Vibrio* spp., and six strains belonging to related genera, for PCR amplification. All 15 *V. vulnificus* strains showed a single 978-bp amplification product; no amplification products were found with the rest of the bacteria tested (data not shown). On the basis of these results, the nested PCR was set up with the primer combination Dvu9V-Dvu45R as the inner set. As the outer primer set we selected two universal primers complementary to highly conserved regions of eubacterial 23S rRNA genes flanking the selected specific primers. They were PCR sense primer 118V

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TABLE 1. Strains used in this study

Organism	Source and strain[a]	Origin
Aeromonas macleodi	NCIMB 1963[T]	
Alteromonas haloplanktis	NCIMB 14393[T]	
Vibrio alginolyticus	NCIMB 1903[T]	
Listonella (*Vibrio*) *anguillarum*	NCIMB 6[T]	
Vibrio carchariae	NCIMB 12705[T]	
Vibrio cholerae O1	CECT 514[T]	
Vibrio cincinnatiensis	NCTC 12012[T]	
Vibrio diazotrophicus	NCIMB 2169[T]	
Vibrio furnissii	ATCC 35016[T]	
Vibrio fluvialis	NCTC 11327[T]	
Vibrio harveyi	NCIMB 1280[T]	
Vibrio mimicus	NCTC 11435[T]	
Vibrio orientalis	NCIMB 2195[T]	
Vibrio ordalii	ATCC 33509[T]	
Vibrio parahaemolyticus	CECT 511[T]	
Vibrio pelagius	ATCC 25916[T]	
Vibrio tubiashii	NCIMB 1340[T]	
Vibrio vulnificus biotype 1	ATCC 27562[T]	Human wound infection
	C7184[b]	Human blood
	L-180[b]	Septicemia case
	VvL1[b]	Fatal wound infection
	374[b]	Septicemia case
	UMH1[b]	Fatal wound infection
	TW1[c]	Tank water
	E114[c] and E335[c]	European eels
Vibrio vulnificus biotype 2	ATCC 33149	Japanese eels
	NCIMB 2138	Japanese eels
	NCIMB 2137	Japanese eels
	NCIMB 2136	Japanese eels
	E39[c] and E22[c]	Internal organs of European eels
Photobacterium angustum	NCIMB 1895[T]	
Photobacterium (*Vibrio*) *damsela*	CECT 626[T]	
Photobacterium logei	NCIMB 2252[T]	
Photobacterium phosphoreum	NCIMB 1282[T]	

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CECT, Colección Española de Cultivos Tipo, Valencia, España; NCIMB, National Collection of Industrial and Marine Bacteria, Torry Research Station, Aberdeen, Scotland, United Kingdom; NCTC, National Collection of Type Cultures, London, United Kingdom; T, type strain.

[b] Clinical isolate kindly supplied by J. D. Oliver (University of North Carolina, Charlotte).

[c] Environmental isolate kindly supplied by E. G. Biosca and M. Ortigosa (University of Valencia, Burjasot, Valencia, Spain).

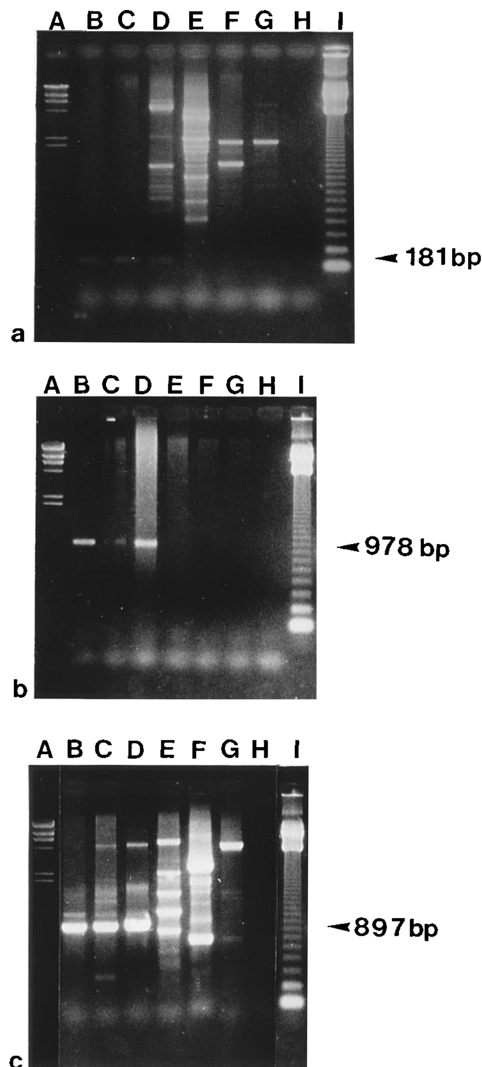

FIG. 1. PCR amplification products obtained with primers specific for *V. vulnificus* sequences within the 23S rRNA genes: Dvu9V and Dvu18R (a), Dvu9V and Dvu45R (b), and Dvu18V and Dvu45R (c). Lanes A and I, *Hin*dIII DNA fragments (sizes in kilobases) and 123-bp molecular size standards, respectively; lanes B through D, *V. vulnificus* biotype 1 and 2 strains (ATCC 27562[T], C7184, and E39, respectively); lane E, *Vibrio harveyi*; lane F, *Vibrio fluvialis*; lane G, *Vibrio parahaemolyticus*; lane H, no template (negative control).

(3′-CCGAATGGGGAAACCCA-5′, positions 112 to 130) and PCR antisense primer 1037R (3′-CGACAAGGAATTTCGC TAC-5′, positions 1930 to 1948), kindly provided by W. Ludwig (Technical University of Munich, Munich, Germany). The amplification with universal primers was conducted at an annealing temperature of 52°C and rendered a 1,828-bp fragment. A 1-μl aliquot of the first amplification mixture was subsequently used as the template for the second reaction, which was conducted with the specific primers for *V. vulnificus* at the same annealing temperature.

In order to test the sensitivity of this procedure, we used purified DNA, spectrophotometrically quantified (Genequant spectrophotometer; Pharmacia), extracted from (i) *V. vulnificus* cells, (ii) liver and glass eel homogenates free of *V. vulnificus*, and (iii) liver and glass eel homogenates free of *V. vulnificus* and artificially seeded with 10^9 to 10^2 *V. vulnificus* CFU per g. DNA from tissue samples was extracted by basically the procedure described by O'Brien et al. (16). DNA of *V. vulnificus* was mixed with DNA extracted from tissue samples in ratios of 1:10 and 1:100, and 10-fold dilutions ranging from 1 ng to 1 fg were prepared for amplification. When the specific primers were used, the amplification products (978 bp) were obtained with samples containing from 1 ng to 100 pg of *V. vulnificus* DNA either alone or mixed with fish DNA (data not shown). With nested PCR, the expected universal 23S rRNA gene amplification products (1,828 bp) were obtained with 1 ng to 100 fg of *V. vulnificus* DNA template (Fig. 2, lanes B to F). These amplification products from the gel shown in Fig. 2 (lanes B to H) were subjected to a further round of PCR with the *V. vulnificus*-specific primer combination. Figure 2 (lanes L to R) shows the results of this nested PCR. In this case, the *V. vulnificus*-eel DNA mixtures corresponding to *V. vulnificus* DNA from 1 ng to 10 fg resulted in the successful amplification of the 978-bp DNA fragment (Fig. 2, lanes L to Q). Thus, the nested-PCR strategy achieved an approximately 10,000-fold increase in sensitivity, with a detection limit of 10 fg of *V. vulnificus* DNA, corresponding to one *V. vulnificus* cell. This

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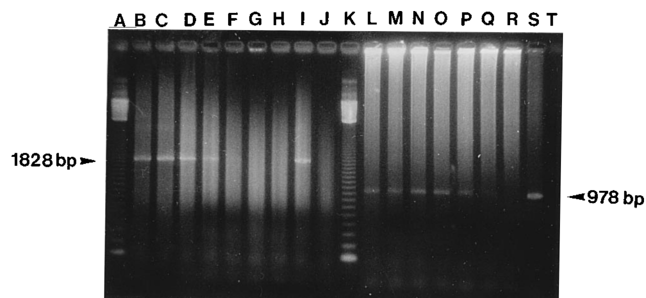

FIG. 2. Sensitivity of nested-PCR assay. The limit of detection of *V. vulnificus* with universal and specific primers in a two-step amplification of purified DNA from *V. vulnificus* mixed 1:10 with DNA extracted from glass eels is shown. Lanes A and K contain 123-bp molecular size standards. Lanes B through J show amplification with universal primers 118V and 1037R. Lanes B to H, DNA dilutions containing from 1 ng to 1 fg of DNA from *V. vulnificus*; lane I, 20 ng of *V. vulnificus* DNA (positive control); lane J, no template (negative control). Lanes L to T show amplification with specific primers Dvu9V and Dvu45R. Lanes L to R, 1 μl of the amplification products corresponding to lanes B to H; lane S, 20 ng of *V. vulnificus* DNA (positive control); lane T, no template (negative control).

detection limit was not affected by the presence of other eubacterial or eucaryotic DNA, as revealed by the results of amplification from mixed DNA. To date, the limits for detection of *V. vulnificus* by amplification of the cytotoxin-hemolysin gene are 10^2 CFU/g of oyster after overnight incubation (11) and 1 or 50 cells, depending on the set of primers, with whole-cell lysates of a pure culture (4). The last method does not require DNA extraction but does require a culture step, which increases the detection time. With the nested PCR, we were able to detect between 12 and 120 *V. vulnificus* cells in the artificially seeded samples without enrichment.

The nested-PCR procedure for the detection of *V. vulnificus* was used with different samples from an eel farm, including eel samples, tank water, and sediments. PCR inhibition was detected with samples of sediments, a finding that has already been described (16), but we could overcome it in most cases by column chromatography with commercial Sephadex S-200 HR columns (MicroSpin; Pharmacia, Biotech). In this case, the first reaction of the nested PCR constitutes a positive control for sample amplification and may indicate the presence of bacteria in internal organs of healthy animals, where they should not be found.

PCR detection procedures for other fish pathogens are based on the amplification of specific DNA fragments isolated from genomic DNA libraries for *Aeromonas salmonicida* (12) and *Renibacterium salmoninarum* (14) or genes coding for the virulence surface array protein in *A. salmonicida* (10). In contrast to the previously described methods, the nested PCR described here allows the direct and specific detection of *V. vulnificus* within 24 h without prior enrichment and has the advantage of including a simultaneous positive control for the reaction in every sample. It constitutes a powerful tool for the rapid and unequivocal diagnosis of *V. vulnificus* infection in fish farms, where the presence of either of the two biotypes represents a serious health risk both for humans and eels, apart from having economic consequences due to eel mortalities and antibiotic treatments. With a similar PCR approach, O'Brien et al. (16) have been able to correlate the presence of *A. salmonicida* in a fish farm with subsequent clinical disease. Further investigation of specific sequences for other pathogenic *Vibrio* species will allow their simultaneous detection by combining nested PCR with other PCR-based strategies (i.e., multiplex PCR).

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