Detection of Pathogenic *Vibrio* spp. in Shellfish by Using Multiplex PCR and DNA Microarrays

Gitika Panicker,[1] Douglas R. Call,[2] Melissa J. Krug,[2] and Asim K. Bej[1]*

Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama,[1] and Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington[2]

This study describes the development of a gene-specific DNA microarray coupled with multiplex PCR for the comprehensive detection of pathogenic vibrios that are natural inhabitants of warm coastal waters and shellfish. Multiplex PCR with *vvh* and *viuB* for *Vibrio vulnificus*, with *ompU*, *toxR*, *tcpI*, and *hlyA* for *V. cholerae*, and with *tlh*, *tdh*, *trh*, and open reading frame 8 for *V. parahaemolyticus* helped to ensure that total and pathogenic strains, including subtypes of the three *Vibrio* spp., could be detected and discriminated. For DNA microarrays, oligonucleotide probes for these targeted genes were deposited onto epoxysilane-derivatized, 12-well, Teflon-masked slides by using a MicroGrid II arrayer. Amplified PCR products were hybridized to arrays at 50°C and detected by using tyramide signal amplification with Alexa Fluor 546 fluorescent dye. Slides were imaged by using an arrayWoRx scanner. The detection sensitivity for pure cultures without enrichment was 10^2 to 10^3 CFU/ml, and the specificity was 100%. However, 5 h of sample enrichment followed by DNA extraction with Instagene matrix and multiplex PCR with microarray hybridization resulted in the detection of 1 CFU in 1 g of oyster tissue homogenate. Thus, enrichment of the bacterial pathogens permitted higher sensitivity in compliance with the Interstate Shellfish Sanitation Conference guideline. Application of the DNA microarray methodology to natural oysters revealed the presence of *V. vulnificus* (100%) and *V. parahaemolyticus* (83%). However, *V. cholerae* was not detected in natural oysters. An assay involving a combination of multiplex PCR and DNA microarray hybridization would help to ensure rapid and accurate detection of pathogenic vibrios in shellfish, thereby improving the microbiological safety of shellfish for consumers.

The warm coastal waters of temperate regions, including the Gulf of Mexico (gulf water), support a diverse aquatic biota and a robust seafood industry. This environment, however, also provides suitable conditions for the growth of pathogenic bacteria that pose problems for human health. Molluscan shellfish, especially oysters, concentrate microorganisms from surrounding waters during the filter-feeding process. Consequently, filter feeders are recognized as reservoirs for various microbial pathogens, including *Vibrio* spp. (36). Among the various *Vibrio* spp. that inhabit marine and estuarine environments, *Vibrio vulnificus*, *V. parahaemolyticus*, and *V. cholerae* are primarily involved in causing gastrointestinal illnesses or, in some cases, septicemia. Infections arise due to the consumption of raw, undercooked, or improperly processed shellfish or other seafood containing significant levels of bacteria.

Infections caused by *V. vulnificus* often result in fatal consequences, with a mortality rate of up to 60%, primarily in individuals who are immunocompromised or have liver disease (27, 31). Due to an increased number of disease incidents, the state of California in 2003 released emergency restrictions on the sale of all oysters harvested between April and October from the Gulf of Mexico unless they are treated with a scientifically validated process to reduce *V. vulnificus* to a nondetectable level (<30 CFU/g of oyster) (California Department of Health Services, April 2003, [http://www.dhs.ca.gov]). Besides illnesses caused by *V. vulnificus*, sporadic outbreaks of

gastrointestinal illnesses have also been caused by *V. parahaemolyticus*. The first reported outbreak in the United States occurred in 1982, affecting 10 individuals, followed by the occurrence of a relatively larger outbreak in the Pacific Northwest, affecting 209 people and including 1 death (14). Recently, a newly emerging serotype of *V. parahaemolyticus*, O3:K6, marked the largest reported food-borne outbreak in the United States, affecting 416 individuals (19, 20).

Although *V. vulnificus* and *V. parahaemolyticus* are considered the two major causative agents for shellfish-associated bacterial illnesses, incidents of cholera in the United States have also been reported in recent years (11–13, 41). Sporadic cases of *V. cholerae*-associated illnesses in the United States from the consumption of raw or undercooked shellfish suggest the reintroduction of this pathogen into the U.S. marine and estuarine environments, primarily from South America and Asia (10, 30). Pathogenic strains of *V. parahaemolyticus* and *V. cholerae* have been involved in epidemics worldwide (16, 29, 33, 39, 45) The bacterial load in shellfish, including pathogenic strains, increases in coastal waters during summer months and thereby increases opportunities for outbreaks of food-borne illnesses, which are a cause for concern for the seafood industry. Recently, the Centers for Disease Control and Prevention estimated a 126% increase in the incidence of *Vibrio*-associated infections between 1996 and 2002, despite efforts directed at seafood consumers (especially high-risk consumers) to warn them of the potential hazards of eating raw shellfish (15). Targeted efforts to reduce the rate of food-borne illnesses should therefore include steps to decrease the prevalence of pathogenic bacteria in seafood as well as the development of rapid, specific, and sensitive detection methods.

* Corresponding author. Mailing address: Department of Biology, University of Alabama at Birmingham, 1300 University Blvd., Birmingham, AL 35294-1170. Phone: (205) 934-9857. Fax: (205) 975-6097. E-mail: abej@uab.edu.

Conventional methods of pathogen detection, such as the most-probable-number technique in association with biochemical tests or colony blot hybridization with gene-specific probes, are limited by the time and labor involved in analyzing a large number of samples (25). Detection of pathogens by PCR has been shown to be highly specific and relatively less time-consuming than conventional methods. However, discrimination of potentially virulent strains by PCR demands the targeting of multiple genes. During the last decade, several reports have described PCR detection and discrimination of total and pathogenic *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* strains, including selected serotypes (4, 23, 34, 38). However, comprehensive detection of all of the aforementioned pathogenic vibrios by the targeting of at least 10 genes would be unreliable by conventional PCR. Moreover, the Taqman probe-based real-time PCR is limited by the ability to detect only 4-fluorophores in a single reaction. In most situations, postamplification detection methods for each of the pathogens must be applied to ensure that the amplicons were in fact generated from targeted gene segments during the PCR process. Comprehensive detection of the amplicons from all of the pathogenic vibrios in a single assay would significantly increase the speed of detection and thereby improve the microbiological safety of seafood.

Recently, the DNA microarray hybridization approach was shown to be effective for the detection of amplicons generated by PCR from multiple food-borne microorganisms, including pathogenic strains (17, 46). In this format, the DNA array involves the immobilization of numerous oligonucleotide DNA probes at high densities on a solid support to which fluorescence-labeled PCR-amplified DNAs are hybridized. It allows for the identification of microbial pathogens that are present in clinical as well as environmental samples, such as water, soil, or food products, and that could be hazardous to human health. The application of a DNA microarray hybridization approach for the detection of total and pathogenic *Listeria*, *Campylobacter*, *Shigella*, and *Escherichia coli* strains has been reported (8, 26, 42, 43). In these studies, various serovars, pathotypes, and virulence genes of each of these pathogens were successfully identified by multigene hybridizations on a DNA array. In separate studies, PCR-amplified DNAs in various combinations from 18 microbial pathogens, including bacteria, viruses, and eukaryotes, were detected on a single DNA array with high sensitivity and accuracy; in addition, 15 bacterial species were discriminated based on 16S ribosomal DNA polymorphisms (44, 46).

With the increased incidence of seafood-related illnesses caused by vibrios, rapid detection and discrimination of total and pathogenic strains by multiplex PCR amplification followed by analysis of all amplicons together on a single DNA array would help to ensure a steady supply of oysters that are safe to eat and thereby protect consumer health and financially benefit the seafood industry. In this study, we coupled multiplex PCR with a DNA array for the comprehensive detection of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in shellfish.

MATERIALS AND METHODS

Bacterial strains and media. The following bacterial strains were used in the present study: *V. cholerae* 154 serovar O:1 Inaba Tox (ATCC 14100), *V. vulnificus* MO6-24 (O), *V. parahaemolyticus* F113-A, and *V. parahaemolyticus* serotype O3:K6 BAC 98–03372. *V. vulnificus* was maintained on 0.5× (18.7 g/liter) marine agar (Becton Dickinson, Franklin Lakes, N.J.), *V. cholerae* was maintained on

Luria-Bertani agar (Difco), and *V. parahaemolyticus* was maintained on nutrient agar (Becton Dickinson) supplemented with 3% (wt/vol) NaCl. All cultures were grown at 37°C. The seeded oyster tissue homogenates were enriched with gulf water (salinity, 18 ppt) supplemented with 0.2% (wt/vol) Bacto Peptone (GWP-18) (Becton Dickinson). Also, modified cellobiose-polymyxin-colistin (mCPC) agar and thiosulfate-citrate-bile-sucrose (TCBS) agar were used to obtain viable plate counts for the three vibrio species (2).

Genomic DNA extraction. Genomic DNA was extracted from exponential cultures (1 ml) of the organisms by centrifugation ($9,000 \times g$) followed by resuspension of the cell pellet in 567 μl of TE buffer (10 mM Tris-Cl, 1.0 mM EDTA [pH 8]). Alkaline lysis was performed with 30 μl of 10% (wt/vol) sodium dodecyl sulfate (SDS) and 3 μl of proteinase K (20 mg/ml; Sigma, St. Louis, Mo.) by using a procedure adapted from that of Ausubel et al. (3). After 1 h of incubation, 100 μl of 5 M NaCl was added along with 80 μl of cetyltrimethyl-ammonium bromide (CTAB)-NaCl solution to complex with polysaccharides. DNA was purified from proteins and other cellular constituents with an equal volume (780 μl) of chloroform-isoamyl alcohol (24:1) and was centrifuged at $10,000 \times g$ for 5 min. The supernatant was transferred to a new tube with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). The resulting supernatant was transferred to a new tube with 300 μl of isopropanol and was centrifuged at $10,000 \times g$ for 5 min to pellet DNA. The pellet was washed once with 1 ml of cold 70% (vol/vol) ethanol before being dried under vacuum. Purified DNA was resuspended in 50 μl of TE buffer.

Oligonucleotide primers and probes. The targeted genes, oligonucleotide primer sets, and DNA probes used for the detection of each of the three pathogens are listed in Table 1. The oligonucleotide probes were designed based on the nucleotide sequences internal to the amplified segments of the respective targeted genes. All oligonucleotide probes were selected by using Primer Quest software and custom synthesized along with the primers at Integrated DNA Technology, Inc. (Coralville, Idaho). The oligonucleotides synthesized at Integrated DNA Technology were deprotected and desalted. Potential probe sequences were analyzed for specificity by comparison with known gene sequences in GenBank by using the BLAST N search program provided by the National Center for Biotechnology Information.

Multiplex PCR and nick translation. Each multiplex PCR amplification was performed with a 50-μl reaction volume consisting of 10 ng of purified genomic DNA, 200 μM deoxynucleoside triphosphates (Sigma), 3 U of AmpliTaq DNA polymerase (Promega Corporation, Madison, Wis.), and PCR buffer F [60 mM Tris-Cl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$] or buffer C [50 mM Tris-Cl (pH 8.9), 50 mM KCl, 2.5 mM $MgCl_2$]. Amplification of the targeted genes for *V. cholerae* and *V. parahaemolyticus* was carried out with 1 μM each oligonucleotide primer. However, for the detection of *V. vulnificus*, 1 μM each of oligonucleotide primers F-vvh and R-vvh and 2 μM each of oligonucleotide primers F-viuB and R-viuB were used in order to achieve optimum multiplex PCR amplification of *vvh* and *viuB*, respectively. All PCR amplifications were performed by using a GeneAmp 2400 thermocycler (Perkin-Elmer, Shelton, Conn.) with parameters specific for the gene sets to be amplified. Reaction parameters included initial denaturation at 94°C for 3 min; 30 cycles of amplification with denaturation at 94°C for 1 min and primer annealing for 1 min at 55°C for *V. parahaemolyticus*, 65°C for *V. vulnificus*, or 60°C for *V. cholerae*; primer extension at 72°C for 1 min; and final extension of the incompletely synthesized DNA at 72°C for 5 min. Amplified products (5 μl) were analyzed by agarose gel electrophoresis (2% [wt/vol] Nusieve or 1% [wt/vol] SeaKem agarose [FMC Bioproducts, Rockland, Maine]) or SDS-polyacrylamide gel electrophoresis (5% [wt/vol] polyacrylamide). Gels were stained with ethidium bromide (0.005% [wt/vol]) and visualized under a Photoprep I UV transilluminator (Fotodyne, Inc., Hartland, Wis.).

Multiplex PCR mixtures (50 μl) were subjected to nick translation by using a nick translation kit (Amersham Biosciences Corp., Piscataway, N.J.). Following the incorporation of biotinylated dCTP, the reactions were terminated with 5 μl of 0.2 M disodium EDTA, and the reaction mixtures were denatured at 95°C for 5 min. The nick translation-PCR products were dried by using a Speed Vac DNA 110 instrument (Savant, Sunnyvale, Calif.) and were stored at −80°C until used for hybridization with DNA arrays.

DNA array preparation and use. Teflon-masked, 12-well microscope slides (Erie Scientific, Porthsmouth, N.H.) were cleaned, acid washed, and derivatized with epoxysilane (3-glycidoxypropyltrimethoxysilane; Sigma-Aldrich, Milwaukee, Wis.) as described by Call et al. (9). Oligonucleotide probes were diluted in spotting buffer (0.1 M NaH_2PO_4, 0.2 M NaCl, 0.01% SDS) to a final concentration of 60 μM and transferred to a 96-well polystyrene plate (Robbins Scientific Corp., Sunnyvale, Calif.) for spotting. Twelve independent DNA arrays (1 per masked well) were spotted onto each slide by using a MicroGrid II arrayer (BioRobotics Inc., Woburn, Mass.). Each probe was spotted as four replicates on

TABLE 1. Oligonucleotide primers and probes used for multiplex PCR and DNA array hybridization

Pathogen	Target gene	Primer and probe sequences[a]	Oligonucleotide length	% GC content	T_m[b] (°C)	Amplicon size (kbp)	Reference
V. vulnificus	*vvh*	F-VVH: 5′-TTCCAACTTCAAACCGAACTATGAC-3′	25	40.0	61.3	0.205	6
		R-VVH: 5′-B-ATTCCAGTCGATGCGAATACGTTG-3′	24	45.8	62.8		
		P-VVH: 5′-GAAGCGCCCGTGTCTGAAACTGGCGTAACG-3′	30	60.0	72.3		
	viuB	F-VIUB1728: 5′-GGTTGGGCACTAAAGGCAGATATA-3′	24	45.8	62.8	0.504	34
		R-VIUB2231: 5′-B-CGGCAGTGGACTAATACGCAGC-3′	22	59.9	66.4		
		P-VIUB: 5′-GAGAAAGCGACCTCGCTGTCTGCTTTTATC-3′	30	50.0	68.7		
V. parahaemolyticus	*tlh*	F-TLH: 5′-AAAGCGGATTATGCAGAAGCACTG-3′	24	45.8	62.8	0.45	4
		R-TLH: 5′B-GCTACTTTCTAGCATTTTCTCTGC-3′	24	41.6	61.1		
		P-TLH: 5′-ACGGACGCAGGTGCGAAGAACTTCATGTTG-3′	30	53.3	70.1		
		P-TLH2: 5′-TTGCGCTCTGAGTGTGCAGCGTCTGGTGCT-3′	30	60.0	72.8		
	tdh	F-TDH: 5′-GTAAAGGTCTCTGACTTTTGGAC-3′	23	43.5	57.7	0.269	4
		R-TDH: 5′B-TGGAATAGAACCTTCATCTTCACC-3′	24	41.7	61.1		
		P-TDH: 5′-GCGGTGTCTGGCTATAAGAGCGGTCATTCT-3′	30	53.3	70.0		
	trh	F-TRH: 5′-TTGGCTTCGATATTTTCAGTATCT-3′	24	33.3	57.7	0.5	4
		R-TRH: 5′B-CATAACAAACATATGCCCATTTCCG-3′	25	40.0	61.3		
		P-TRH: 5′-AAAACTGAATCACCCAGTTAACGCAATCGTT-3′	30	36.6	63.2		
		P-TRH2: 5′-ACAAGCCTAAATCAGAACTATTCTTCTGTTAG-3′	32	34.3	63.3		
		P-TRH3: 5′-TTGACCTACCATCCATACCTTTTCCTTCTCCA-3′	32	43.7	67.2		
	ORF8	F-O3MM824: 5′-AGGACGCAGTTACGCTTGATG-3′	21	52.4	62.5	0.369	32
		R-O3MM1192: 5′-B-CTAACGCATTGTCCCTTTGTAG-3′	22	45.4	60.8		
		P-ORF8: 5′-CTCACTCCTGCTGTACTTTTAGCTCGGTTA-3′	30	46.6	67.3		
V. cholerae	*ompU*	F-OMPU: 5′-ACGCTGACGGAATCAACCAAAG-3′	22	50.0	62.6	0.869	37
		R-OMPU: 5′-B-GCGGAAGTTTGGCTTGAAGTAG-3′	22	50.0	62.6		
		P-OMPU: 5′-TCACTGACTTCACCGATATCATGTCTTACC-3′	30	43.3	66.0		
	toxR	F-TOXR: 5′-CCTTCGATCCCCTAAGCAATAC-3′	22	50.0	62.6	0.779	37
		R-TOXR: 5′-B-AGGGTTAGCAACGATGCGTAAG-3′	22	50.0	62.6		
		P-TOXR: 5′-GAATCAAGCAGTGTGCCTTCATCATCCACT-3′	30	46.6	67.3		
	tcpI	F-TCPI: 5′-TAGCCTTAGTTCTCAGCAGGCA-3′	22	50.0	62.6	0.862	37
		R-TCPI: 5′B-GGCAATAGTGTCGAGCTCGTTA-3′	22	50.0	62.6		
		P-TCPI: 5′-ATCTGATCTCACTCACAGTACCTTAATTGG-3′	30	40.0	64.6		
Classical or El Tor	*hlyA*	F-HLYA: 5′-GGCAAACAGCGAAACAAATACC-3′	22	45.4	60.8	0.738 or 0.727	37
		R-HLYA: 5′-B-CTCAGCGGGCTAATACGGTTTA-3′	22	50.0	62.6		
		P-HLYA: 5′-AGCCAAACTAGAGGCGAGAGCAAGTTATAC-3′	30	46.7	67.4		

[a] F, forward primer; R, reverse primer (biotinylated [B] at the 5′ end); P, oligonucleotide probe. The oligonucleotide probes for the respective genes were selected for this study.
[b] T_m, melting temperature.

each array. Every array included an arbitrary oligonucleotide probe (25-mer) conjugated with biotin. These biotin pseudoprobes served as positive controls for the detection chemistry and as orientation points for image processing. A schematic diagram of the positions of the probes spotted on the array is shown in Fig. 1A. After spotting was completed, slides were baked for 1 h at 130°C and stored at room temperature until used.

Biotinylated targets (biotin conjugated to primers or incorporated by nick translation; 10 µl) were diluted to a total volume of 70 µl in hybridization buffer (4× SSC [1× SSC is 15 mM NaCl plus 0.15 mM sodium citrate] [pH 7.0], 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin]), heat denatured at 98°C for 3 min, and hybridized overnight at 50°C to replicate arrays on the same slide. Slide preblocking and detection protocols were carried out as described by Call et al. (7). Briefly, after hybridization, slides were washed three times for 1 min each time with 100 mM Tris-HCl (pH 7.5)–150 mM NaCl–0.05% Tween 20 buffer with agitation. A tyramide signal amplification biotin system (Perkin-Elmer, Boston, Mass.) was used in conjunction with Alexa Fluor 546 (Molecular Probes, Eugene, Oreg.) to detect target-probe hybrids. Upon completion of detection chemistry, arrays were imaged by using an arrayWoRx scanner (Applied Precision, Issaquah, Wash.), and images were processed by using softWoRx software. The assay was repeated on replicate arrays from the same print lot.

Detection of *Vibrio* spp. in unenriched pure cultures. The *Vibrio* spp. were grown separately in 10 ml of GWP-18 (autoclaved at 121°C and 15 lb/in² for 20 min) to an optical density at 600 nm of 0.2. The cultures were 10-fold serially diluted, and viable plate counts were determined. Aliquots (1 ml) of each of the undiluted cultures were mixed together and 10-fold serially diluted to extinction in 10 ml of GWP-18. Aliquots (1 ml) were centrifuged at 12,000 × g for 10 min, and the cell pellets were treated with 200 µl of Instagene (Bio-Rad Laboratories, Hercules, Calif.) matrix (35). The samples were incubated at 56°C for 10 min, boiled for 20 min, cooled to room temperature, and centrifuged at 3,000 × g for 3 min to collect DNA. A 3-µl aliquot of each sample was used for PCR amplification.

PCR inhibition study with oysters. Live oysters were purchased from a local seafood store and processed by standard methods (1). The inhibition control experiments were performed with unseeded oyster tissue homogenates that were enriched for 5 h at 37°C in a rotary shaker incubator (Innova 4000; New Brunswick Scientific Co. Inc., Edison, N.J.). Following enrichment, 5-ml aliquots were centrifuged and treated with Instagene matrix as described above. Next, 10-fold serial dilutions of purified genomic DNA ranging from 1.0 µg to 0.1 pg of *V. cholerae* 154 serovar O:1 Inaba Tox (ATCC 14100), *V. vulnificus* MO6-24 (O), *V. parahaemolyticus* F113-A, and *V. parahaemolyticus* BAC 98–03372 were added to 3-µl aliquots of the Instagene matrix-treated unseeded oyster tissue homoge-
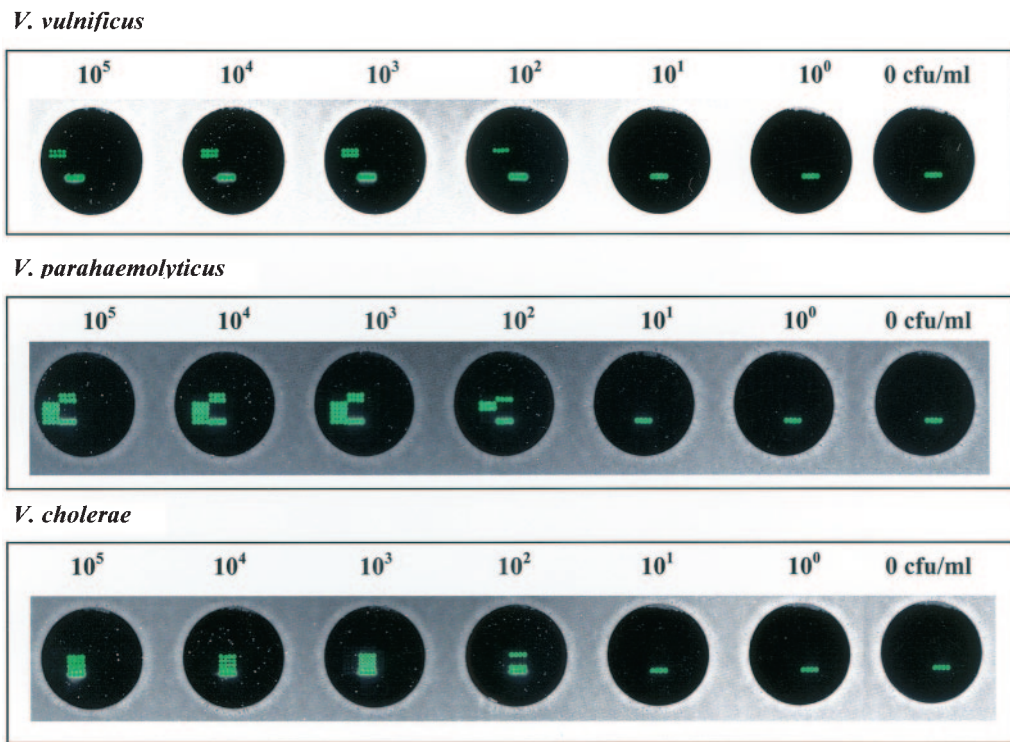
A.
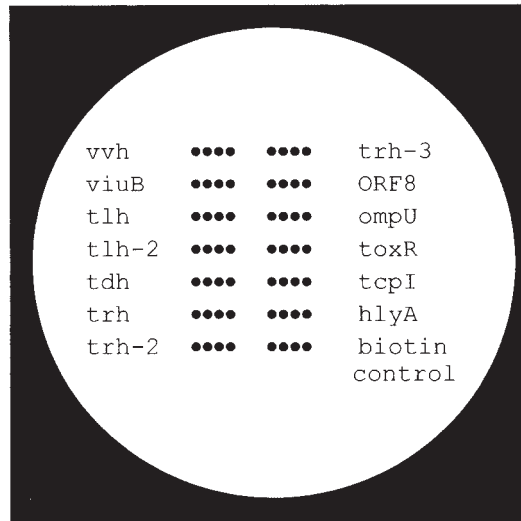
B.

FIG. 1. (A) Schematic diagram showing the positions of the immobilized probes spotted in quadruplicate within a single well of the 12-well Teflon-masked glass slides. (B) Sensitivity of detection of genomic DNAs extracted from 10-fold serially diluted pure cultures of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* prior to enrichment. The range of dilutions used for these cultures extended from 10^5 CFU/ml to extinction. The results shown here are representative of experiments carried out in duplicate.

nates and used for PCR. The cycling and reaction parameters used for PCR were as stated above. The sensitivity of detection obtained with multiplex PCR of oyster tissue homogenates was compared with the sensitivity of detection obtained with purified DNA serially diluted in MilliQ water.

Detection of seeded microbial pathogens in oysters. Three aliquots (1 g each) of market-oyster tissue homogenates were 10-fold serially diluted in GWP-18 and plated on mCPC agar for *V. vulnificus* and TCBS agar for *V. cholerae* and *V.*

parahaemolyticus to determine viable plate counts. Individual yellow cellobiose-positive colonies on mCPC agar plates for *V. vulnificus* or yellow or blue colonies on TCBS agar for *V. cholerae* or *V. parahaemolyticus*, respectively, were PCR amplified with species-specific primers and the parameters stated above to confirm the actual numbers of viable vibrio cells in the samples. Cells of each of the microbial strains were grown separately in 10 ml of GWP-18 to an optical density at 600 nm of 0.2, and viable plate counts were determined. Equal samples of each

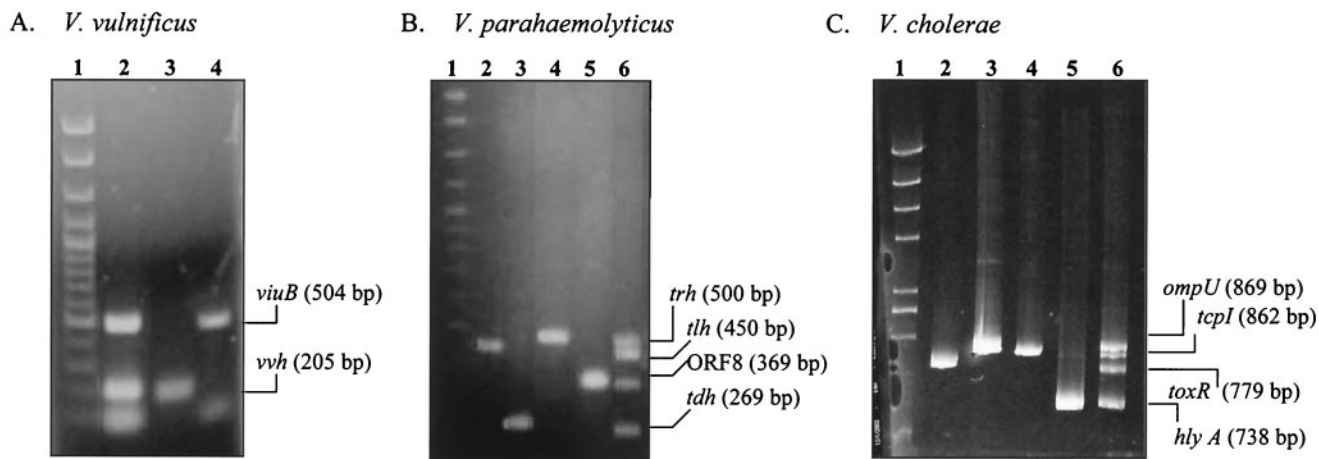## A. *V. vulnificus*   B. *V. parahaemolyticus*   C. *V. cholerae*

FIG. 2. Agarose gel electrophoresis showing the results of multiplex PCR amplification of three *Vibrio* spp. (A) *V. vulnificus*: lane 1, DNA size standard (Clone-sizer; Norgen Biotek); lane 2, multiplex PCR of both *vvh* and *viuB* targets; lane 3, 205-bp *vvh* amplicon; lane 4, 504-bp *viuB* amplicon. (B) *V. parahaemolyticus*: lane 1, DNA size standard; lane 2, 450-bp *tlh* amplicon; lane 3, 269-bp *tdh* amplicon; lane 4, 500-bp *trh* amplicon; lane 5, 369-bp ORF8 amplicon; lane 6, multiplex PCR amplification of all four targeted gene segments. (C) *V. cholerae*: lane 1, DNA size standard; lane 2, 779-bp *toxR* amplicon; lane 3, 869-bp *ompU* amplicon; lane 4, 862-bp *tcpI* amplicon; lane 5, 738-bp *hlyA* amplicon; lane 6, multiplex PCR amplification of all four targeted gene segments.

of the cultures were mixed together and 10-fold serially diluted to extinction in 50 ml of GWP-18 with 1 g of oyster tissue homogenate. The seeded oyster tissue homogenates were enriched for 5 h at 37°C in a rotary shaker incubator (Innova 4000). Following enrichment, 5-ml aliquots were centrifuged at $12,000 \times g$ for 10 min, and the supernatants were discarded. The cell pellets were treated with 200 µl of Instagene matrix as described above, and DNA was released (35). A 3-µl aliquot of each sample was used for PCR amplification.

Detection of *Vibrio* spp. in natural oysters. Oysters were collected from the Gulf of Mexico near Bayou LaBatre, Ala., from June through August 2003. The temperature was recorded, and the salinity was determined by using a salinity refractometer (Reichert Scientific Instruments, Buffalo, N.Y.) during collection. Each collection included three samples, and each of these samples consisted of 10 oysters. The oysters from each sample were chilled immediately after collection, the shell surfaces were cleaned and shucked, and the oysters were homogenized for 2 min in a Waring blender (Fisher Scientific) according to the procedure outlined by the American Public Health Association (1). One gram of oyster tissue homogenate from each sample was added to 50 ml of GWP-18 and enriched for 5 h as described above. For testing of the samples, 5-ml aliquots were centrifuged at $12,000 \times g$ for 10 min, and the cell pellets were treated with 200 µl of Instagene matrix as described above to release DNA (35). A 3-µl aliquot of each sample was used for PCR amplification. All reactions were performed in triplicate.

RESULTS

Multiplex PCR. The oligonucleotide primers selected for PCR amplification of the three *Vibrio* spp. were specific for their respective targeted gene segments, as shown in previous studies (4, 32, 34, 35, 37, 38). At an annealing temperature of 65°C and with 2.5 mM MgCl₂, PCR amplification of purified genomic DNA from *V. vulnificus* MO6-24 yielded DNA fragments of 205 and 504 bp for *vvh* (total *V. vulnificus*) and *viuB* (pathogenic strains), respectively (Fig. 2A). Using the PCR conditions described above, we successfully multiplexed four targeted genes for *V. parahaemolyticus* strains (Fig. 2B): *tlh* (450 bp) for total *V. parahaemolyticus* and *tdh* (269 bp), *trh* (500 bp), and open reading frame 8 (ORF8) (369 bp), representing pathogenic strains. Also, targeted genes for total and pathogenic *V. cholerae* strains were amplified at an optimum primer annealing temperature of 60°C and with 2 mM MgCl₂ (Fig. 2C): *hlyA* (738 bp) (toxigenic and nontoxigenic *V. cholerae* O1

and O139 and *V. cholerae* non-O1 and non-O139), *tcpI* (862 bp) (toxigenic and nontoxigenic *V. cholerae* O1 and non-O139), *toxR* (779 bp) (all *V. cholerae* serogroups), and *ompU* (869 bp) (*V. cholerae* O1 and O139 and environmental *V. cholerae* non-O1 and non-O139). These targeted genes were selected based on differences in distributions in various *V. cholerae* serogroups as described by Rivera et al. (37). Multiplex PCR targeting the simultaneous amplification of 10 targeted gene segments from the three *Vibrio* spp. in a single reaction tube produced inconsistent results (data not shown).

Specificity of oligonucleotide probes used in DNA array hybridization. PCR products were labeled with biotinylated PCR primers or by nick translation. Both individual and multiplexed targets from the three *Vibrio* spp. hybridized to their complementary probes (100% specificity) at a hybridization temperature of 50°C (Fig. 1B). For *V. parahaemolyticus*, three out of the four targets (*tlh*, *tdh*, and ORF8) could not be detected unless the products were nick translated prior to hybridization. This was true for both individual and multiplexed PCR products. Similar results were observed with the *ompU* target in *V. cholerae*. Both *vvh* and *viuB* gene segments in *V. vulnificus* hybridized to their respective probes with or without labeling by nick translation.

Sensitivity of detection in pure cultures. Unenriched cultures of *Vibrio* spp. grown in GWP-18 exhibited a detection level of 10^3 CFU/ml for all targets in a multiplex PCR followed by DNA array hybridization (Fig. 1B). However, certain targets, such as *vvh*, *tlh*, ORF8, *ompU*, and *hlyA*, were detected individually at levels as low as 10^2 CFU/ml in the multiplex PCR.

PCR inhibition control study. The results from the inhibition control experiments indicated that an oyster tissue matrix spiked with purified DNA had an inhibitory effect on the multiplex PCR, resulting in a level of detection that was 10-fold lower than that seen with purified DNA (data not shown). The level of detection for *vvh* and *viuB* was 10 pg for DNA mixed with oyster tissue, whereas it was 1 pg for purified DNA. For
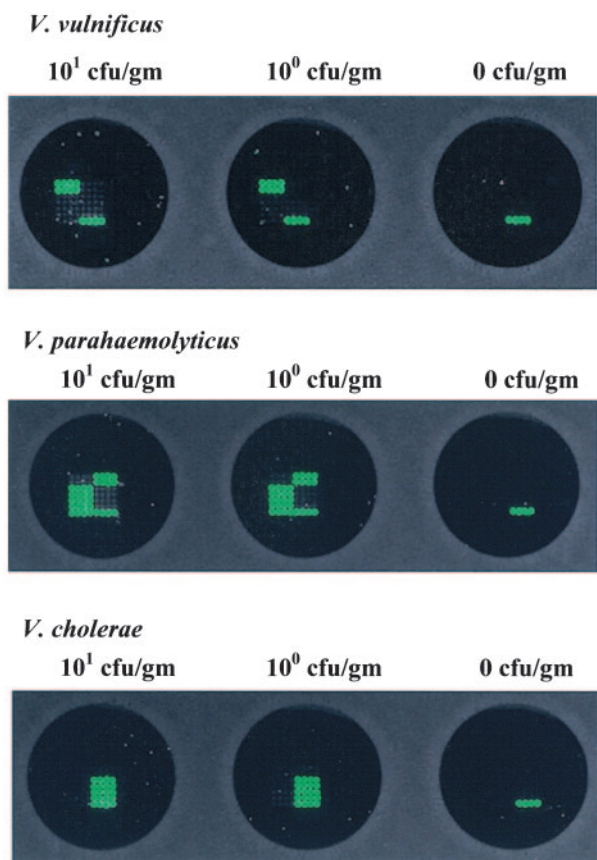
FIG. 3. Sensitivity of detection of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in seeded oyster tissue homogenates following 5 h of enrichment. The arrays shown here represent detection in samples at dilutions ranging from an initial cell count of 10^1 CFU/g to extinction. The results shown here are representative of experiments carried out in duplicate.
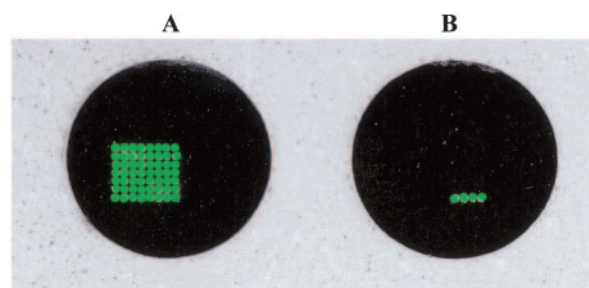

FIG. 4. DNA array showing simultaneous hybridization of all 10 PCR-amplified targets of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in seeded oyster tissue homogenates following 5 h of enrichment. The results shown here are representative of experiments carried out in duplicate. (A) 10^0 CFU of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* seeded in 1 g of oyster tissue homogenates. (B) Unseeded oyster tissue homogenates.

V. parahaemolyticus and *V. cholerae*, consistent amplification of all targets was observed at 100 pg for oyster tissue spiked with DNA. This result could have been due to inefficient amplification of four targeted genes with higher molecular weights in the presence of the oyster matrix resulting in a lower sensitivity of detection.

Sensitivity of detection in oysters following enrichment. The Instagene matrix used for DNA extraction from seeded oyster tissue homogenates resulted in sufficient DNA for PCR detection followed by DNA array hybridization of all targeted genes at an initial cell count of 1 CFU/g for each *Vibrio* sp. (Fig. 3). In addition, simultaneous hybridization of 10 PCR-amplified targeted genes from 5-h-enriched *Vibrio* spp. at an initial cell count of 1 CFU/g resulted in the detection of all targeted genes on a single DNA array (Fig. 4). These results indicated that a single DNA array hybridization reaction can be used for a comprehensive, reliable, and sensitive analysis of PCR-amplified genes from all three *Vibrio* spp. and potentially pathogenic genes in shellfish with a level of sensitivity that is in compliance with the Interstate Shellfish Sanitation Conference (ISSC) guideline (24).

Detection of *Vibrio* spp. in natural oysters. The specificity of PCR-amplified targeted genes from natural oyster samples en-

riched for 5 h was confirmed by DNA array hybridization (Table 2 and Fig. 5). The water temperature ranged from 24 to 26°C, and the salinity was recorded at 16 to 22 ppt. All 30 samples were found to be positive for *vvh*, whereas only 20% were found to be positive for *viuB* (Table 2). *V. parahaemolyticus* was also present in most samples (83.3%); 13 to 20% of the strains were

TABLE 2. Results of multiplex PCR and DNA array hybridization of natural oyster samples

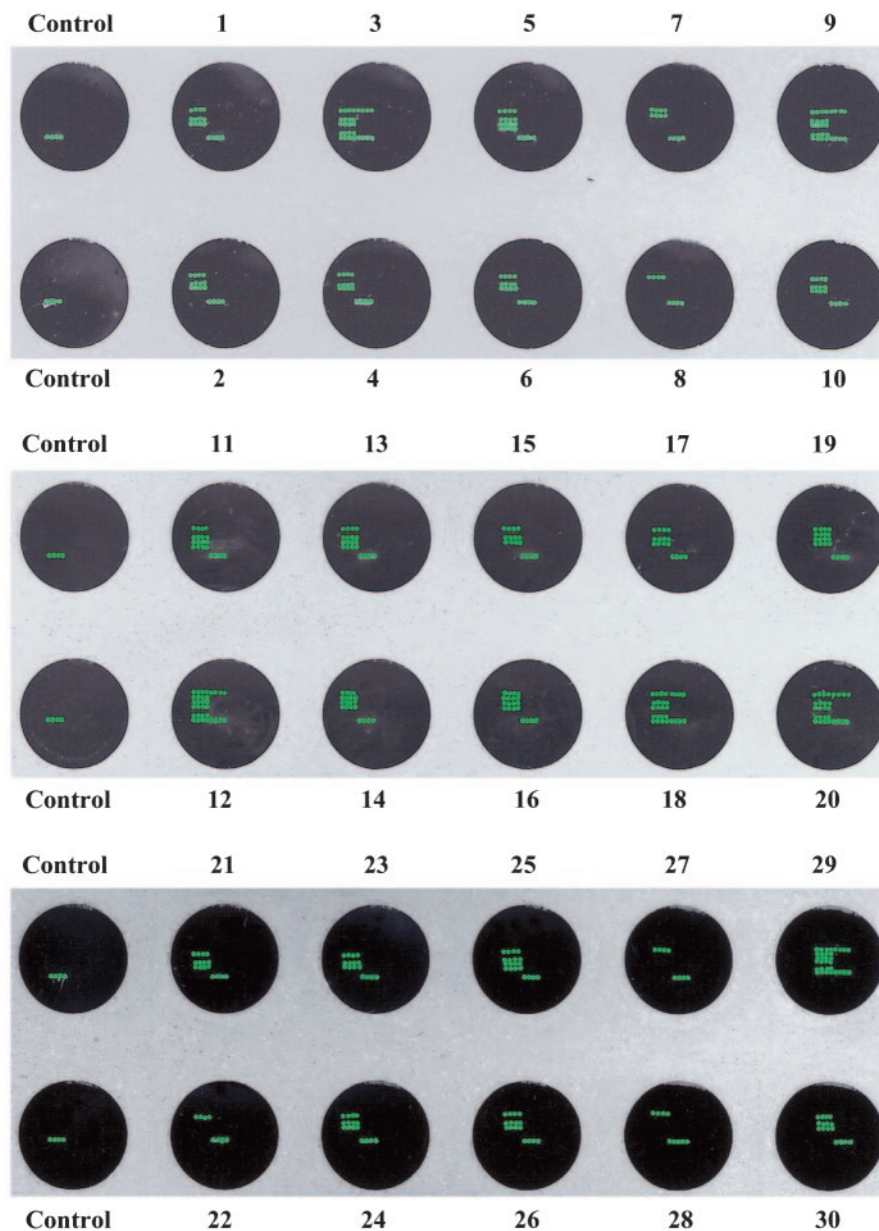| Sample | Result[a] for: | | | | | | | | | |
| | *V. vulnificus* | | *V. parahaemolyticus* | | | | *V. cholerae* | | | |
	vvh	viuB	tlh	tdh	trh	ORF8	toxR	ompU	tcpI	hlyA
1	+	−	+	−	−	−	−	−	−	−
2	+	−	+	−	−	−	−	−	−	−
3	+	−	+	−	+	−	−	−	−	−
4	+	−	+	−	−	−	−	−	−	−
5	+	−	+	+	−	−	−	−	−	−
6	+	−	+	−	−	−	−	−	−	−
7	+	+	−	−	−	−	−	−	−	−
8	+	−	−	−	−	−	−	−	−	−
9	+	−	+	−	+	−	−	−	−	−
10	+	−	+	−	−	−	−	−	−	−
11	+	−	+	+	−	−	−	−	−	−
12	+	+	+	−	+	−	−	−	−	−
13	+	−	+	+	−	−	−	−	−	−
14	+	+	+	−	−	−	−	−	−	−
15	+	−	+	−	−	−	−	−	−	−
16	+	+	+	−	−	−	−	−	−	−
17	+	−	+	−	−	−	−	−	−	−
18	+	−	+	−	+	−	−	−	−	−
19	+	+	+	−	−	−	−	−	−	−
20	+	−	+	−	+	−	−	−	−	−
21	+	−	+	−	−	−	−	−	−	−
22	+	−	−	−	−	−	−	−	−	−
23	+	−	+	−	−	−	−	−	−	−
24	+	−	+	−	−	−	−	−	−	−
25	+	−	+	+	−	−	−	−	−	−
26	+	−	+	−	−	−	−	−	−	−
27	+	−	−	−	−	−	−	−	−	−
28	+	−	−	−	−	−	−	−	−	−
29	+	+	+	−	+	−	−	−	−	−
30	+	−	+	−	−	−	−	−	−	−
% Positive samples	100	20	83.3	13.3	20	0	0	0	0	0

[a] +, positive; −, negative.

FIG. 5. Application of the DNA array-based detection of PCR-amplified gene segments from *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in natural oyster samples collected from gulf water. Arrays 1 to 30 represent DNA array hybridization of PCR-amplified targets from 30 oyster samples collected from June through August 2003. The control was a PCR-negative control that was also hybridized to an individual array. The results shown here are representative of experiments carried out in duplicate.

pathogenic strains possessing either *tdh*, *trh*, or both (Table 2). ORF8 was not detected in any of the oyster samples that were found to be positive for *V. parahaemolyticus*. The distribution of pathogenic strains observed in this experiment for *V. vulnificus* and *V. parahaemolyticus* concurs with the results of previously published reports (21, 40). None of the samples was found to be positive for the *ompU*, *hylA*, *toxR*, and *tcpI* targeted genes, suggesting that *V. cholerae* is not prevalent in gulf water.

DISCUSSION

The seafood industry, especially the Gulf Coast oyster industry, continues to suffer from elevated *V. vulnificus* levels in uncooked oysters and associated fatal illnesses (22). Based on the severity of this problem, the ISSC has proposed that illnesses caused by *V. vulnificus* due to the consumption of raw or poorly cooked shellfish must be reduced at least 60% by the year 2007 (24). Besides *V. vulnificus*, the presence of high levels of pathogenic *V. parahaemolyticus* in shellfish during the summer months causes sporadic as well as extensive outbreaks of gastroenteritis in humans (18). Although *V. cholerae*-related incidents are rare in the United States, routine monitoring of *V. cholerae* in shellfish is essential, as this species has been identified as a category B organism in the list of pathogens released by the U.S. government (www.niaid.nih.gov/biodefense

/bandcpriority.htm). Moreover, frequent outbreaks of cholera in Central America, South America, Africa, and Southeast Asia could cause accidental transfer of this pathogen into coastal waters of the United States through planktonic attachment, ship ballast water, or unauthorized import of seafood products not kept at appropriate temperatures (10).

Previous studies showed that in order to identify a potentially pathogenic strain, it is necessary to target multiple genes for PCR amplification. In this study, species-specific *tlh*, pathogenic strain-specific *tdh* and *trh*, and serotype O3:K6-specific ORF8 were selected for the detection of *V. parahaemolyticus* in shellfish by multiplex PCR (4, 32). Similarly, species-specific *vvh* (35) and clinical strain-specific *viuB* (34) were selected for the detection of *V. vulnificus* in this study. For a comprehensive detection of various serogroups of *V. cholerae*, targeted genes were selected based on a previous study by Rivera et al. (37). In their study, *ompU*, *tcpI*, *hlyA*, and *toxR* were found to be present in *V. cholerae* strains including O1, non-O1, nontoxigenic O1, O139, and non-O139 serovars. Although there are differences in the nucleotide sequences of *hlyA* in *V. cholerae* classical and El Tor biotypes, the oligonucleotide probe used in this study was selected from the segment of the gene containing the consensus sequence, enabling the detection of both biotypes. In addition, PCR amplification of multiple genes instead of a single gene from each of the *Vibrio* spp. would help to reduce false-negative identification during testing of a complex matrix such as shellfish tissue. However, multigene PCR amplification is limited to individual *Vibrio* spp.

In this study, we established a DNA array hybridization assay that enabled us to confirm the post-PCR detection of these naturally occurring pathogenic vibrios and selected serogroups in shellfish that have a history of causing disease outbreaks, often with fatalities. This single DNA array hybridization assay would save valuable time in the detection of these pathogens, thus helping to protect consumer health by reducing shellfish-related disease outbreaks.

During this study, we were able to detect PCR products that were biotinylated via a 5′ conjugated primer. In some instances, however, terminally labeled products were detected only when they were also nick translated. The difference in detection likely was due to secondary structures on the target DNA (28). Secondary structures can interfere with microarray hybridization, whereas the nick translation process fragments the DNA and thereby disrupts interfering secondary structures. An alternative labeling approach would be to incorporate labeled nucleotides during PCR followed by sonication of amplicons to disrupt any secondary structures. While this latter approach includes a more expensive PCR, we expect some time savings from the elimination of the need for nick translation before hybridization (1 to 2 h).

These pathogens were detected in a mixed culture without enrichment at 10^2 to 10^3 CFU/ml, a level which was not sufficient to meet the recommended minimum detection level needed to declare a food product safe for human consumption (24). Therefore, in order to achieve a higher level of detection, it was necessary to enrich bacterial cells in shellfish tissue homogenates. In previous studies, enrichment of oyster tissue homogenates was used to achieve the detection of single cells of *V. vulnificus* and *V. parahaemolyticus* (5, 35). A 5-h enrichment step followed by a relatively simple DNA purification

step of boiling in Instagene matrix enabled us to achieve reliable detection of a single pathogen in shellfish tissue homogenate samples. Although PCR inhibition by the oyster matrix was evident in this study, the inclusion of a 5-h enrichment step resulted in a sufficient number of cells to achieve detection of the targeted pathogens.

Agarose gel electrophoresis analysis of amplified products was found to be consistent with the positive hybridization signals of gene-specific probes on the DNA array. In addition, the use of tyramide signal amplification with DNA array hybridization enabled us to attain high colorimetric signals from samples containing 1 CFU of each of the targeted pathogens. The level of detection achieved in this study was sufficient to meet the required ISSC guideline for the detection of these pathogens in shellfish samples. The robustness of the assay was further confirmed by the detection of these vibrios with high degrees of specificity and sensitivity in natural oyster samples.

To our knowledge, this is the first report describing a comprehensive DNA array-based analysis of multiplex PCR-amplified gene segments of *Vibrio* spp. that are present in shellfish samples and that are considered to be the primary etiologic agents of shellfish-related bacterial illnesses in humans. The estimated cost for the assay described here is almost half that of Taqman real-time PCR or other commercially available conventional microbiological culture and biochemical methods followed by confirmatory tests, such as DNA-DNA colony hybridization. The combination, developed in this study, of DNA array hybridization with multiplex PCR of targeted genes from pathogenic vibrios in shellfish can be further expanded in the future as needed for the detection of enteric viruses, protozoans, and coliform bacteria as indicators of fecal contamination of shellfish and shellfish harvest sites. Rapid, reliable, and comprehensive detection of multigene amplicons from pathogenic vibrios and other etiologic agents in a single DNA array hybridization assay with required specificity and sensitivity is important for a steady supply of microbiologically safe seafood products in order to guard consumer health and protect the shellfish industry from financial loses.

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