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Detection of Microbial Pathogens in Shellfish with Multiplex PCR

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Abstract. Multiplex PCR amplification of *uidA*, *cth*, *invA*, *ctx*, and *tl* genes was developed enabling simultaneous detection in shellfish of *Escherichia coli*, an indicator of fecal contamination and microbial pathogens, *Salmonella typhimurium*, *Vibrio vulnificus*, *V. cholerae*, and *V. parahaemolyticus*, respectively. Each of the five pairs of oligonucleotide primers was found to support PCR amplifications of only its targeted gene. The optimized multiplex PCR reaction utilized a PCR reaction buffer containing 2.5 mM $MgCl_2$ and primer annealing temperature of 55°C. Oyster tissue homogenate seeded with these microbial pathogens was subjected to DNA purification by the Chelex™ 100 (BioRad) method. The sensitivity of detection for each of the microbial pathogens was $\leq 10^1$–10^2 cells following a ''double'' multiplex PCR amplification approach. Amplified target genes in a multiplex PCR reaction were subjected to a colorimetric GeneComb™ (BioRad) DNA-DNA hybridization assay. This assay was rapid and showed sensitivity of detection comparable to the agarose gel electrophoresis method. The colorimetric GeneComb™ assay avoids use of hazardous materials inherent in conventional gel electrophoresis and radioactive-based hybridization methods. Multiplex PCR amplification, followed by colorimetric GeneComb™ DNA-DNA hybridization, has been shown to be an effective, sensitive, and rapid method to detect microbial pathogens in shellfish.

Shellfish, particularly raw oysters, contaminated with microbial pathogens such as *Salmonella* spp. and *Vibrio* spp., have been implicated in gastroenteritis, cholera, and life-threatening septicemia [21]. In susceptible individuals, the first signs of illness often occur in less than 24 h after ingestion. *Vibrio vulnificus* infections may cause death within 1–2 days. Shellfish concentrate microbial pathogens in their tissues from the surrounding contaminated water during the filter-feeding process. Because estuarine waters are now frequently contaminated by anthropogenic activities, filter-feeders inhabiting polluted waters are prone to contamination by fecal pathogens [32,33,38]. Consequently, the presence of *Escherichia coli* in shellfish can be used as an indication of fecal contamination [1]. Incidence of illness from the consumption of contaminated shellfish, especially raw oysters, is a primary concern of the seafood industry and public health agencies. Identification and characterization of the etiologic agents of seafood-related disease outbreaks are often impeded by extended time required to conduct conventional, microbiological culture assays. Such assays require multiple subculturing and biotype- or serotype-identification steps that may take 5–7 days, making these methods inefficient [14,16,32]. Although gene-probe DNA–DNA hybridization methodology can be used to detect these pathogens, its sensitivity of detection of microbial pathogens in food samples is inadequate. Protecting public health requires early and rapid diagnosis of pathogens in contaminated seafood. The advent of genetic-based technologies makes a reliable, sensitive, and economical approach for the detection of microbial pathogens feasible. As a result, economy of the seafood industry and safety of seafood workers as well as consumers would benefit from these improved monitoring procedures.

Polymerase chain reaction (PCR) methodology has been used successfully for rapid detection with high specificity and sensitivity of various microbial pathogens [2,3] from environmental waters [5,8], clinical samples [7,24], and various food products [12,19,23]. PCR is a rapid, non-cultural, oligonucleotide primer-directed in vitro method for replicating defined DNA sequences from

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Table 1. Descriptions of oligonucleotide primers and probes used for multiplex PCR amplification detection of microbial pathogens in shellfish

Pathogen	Target gene	Primer[a]	Tm (°C)	Amplicon size (kbp)	References
Escherichia coli	*uidA*	L-UIDA: 5′-tggtaattaccgacgaaaacggc-3′	68	0.147	[9–11, 18]
		R-UIDA: 5′-acgcgtggttacagtcttgcg-3′	70		
		P-UIDA: 5′-tgccgggatccatcgcagcgtaatgctc-3′			
Vibrio vulnificus	*cth*	L-CTH: 5′-ttccaacttcaaaccgaactatgac-3′	70	0.205	[39, 40]
		R-CTH: 5′-gctactttctagcattttctctgc-3′	68		
		P-CTH: 5′-gaagcgcccgtgtctgaaactggcgtaacg-3′			
Salmonella typhimurium	*invA*	L-INVA: 5′-ctctacttaacagtgctcgtttac-3′	68	0.273	[17, 25]
		R-INVA: 5′-ttgataaacttcatcgcaccgtca-3′	68		
		P-INVA: 5′-ctgaattactgattctggtactaatggtga-3′			
Vibrio cholerae	*ctx*	L-CTX: 5′-ctcagacgggatttgttaggcacg-3′	74	0.302	[8, 26]
		R-CTX: 5′-tctatctctgtagccggtattacg-3′	70		
		P-CTX2: 5′-attccatactccccaaatata			
Vibrio parahaemolyticus	*tl*	L-TL: 5′-aaagcggattatgcagaagcactg-3′	68	0.450	[29, 37]
		R-TL: 5′-gctactttctagcattttctctgc-3′	68		
		P-TL: 5′-acggacgcaggtgcgaagaacttcatgttg-3′			

[a] L, oligonucleotide primer sequence located on the upstream of the gene; R, oligonucleotide primer sequence located on the downstream of the gene; P, oligonucleotide probe.

target organisms [28,34]. Although microbial pathogens such as *S. typhimurium* [12,20,25], *V. cholerae* [13], *V. vulnificus* [15] and *V. parahaemolyticus* [22,29,36] have been detected in shellfish with PCR amplification, identification of individual microbial pathogens can be relatively costly and time-consuming. However, simultaneous detection of these microbial pathogens in a single reaction with the multiplex PCR approach would be relatively rapid and cost-effective. The objective of this research was to develop a multiplex PCR amplification followed by a colorimetric DNA-DNA hybridization-based detection of microbial pathogens such as *E. coli*, *S. typhimurium*, *V. cholerae*, *V. vulnificus*, and *V. parahaemolyticus* in seeded oyster tissue homogenate.

Materials and Methods

Bacterial strains. The bacterial strains used in this work were as follows: *V. cholerae* 569B ATCC 25870, *S. typhimurium* ATCC 19585, *V. vulnificus* MO6-24 (a human patient isolate), *V. parahaemolyticus* 8338335 (a human patient isolate), and *E. coli* MG 1655 (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Microbiological media. *V. parahaemolyticus* was grown on nutrient agar (NA) (Difco) supplemented with 3% NaCl; *V. vulnificus* was cultured on marine agar (MA) (Difco, Detroit, MI), and *V. cholerae*, *S. typhimurium*, and *E. coli* on Luria broth (LB) or LB agar [27].

Genomic DNA extraction. Genomic DNA was extracted from log-phase cultures of the pathogens following the procedure described by Ausubel et al. [4]. Briefly, the cells were centrifuged, the pellet was resuspended in 567 µl TE buffer, pH 8.0 (10 mM Tris · Cl, 1.0 mM EDTA, pH 8.0), and then lysed with 30 µl of 10% (wt/vol) sodium dodecyl sulfate and 3 µl of 20 mg/ml proteinase K (Sigma). After 1 h incubation, 100 µl of 5 M NaCl was added along with 80 µl of CTAB/NaCl solution to complex with polysaccharide. DNA was purified with an equal volume (780 µl) chloroform-isoamyl alcohol (24:1) followed by centrifugation at 10,000 *g* for 5 min. Further purification of the DNA in the supernatant, which was transferred to a new tube, was achieved by extracting with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1). Then, a 0.6 × volume (300 µl) of isopropanol was added to precipitate the DNA. The purified DNA was pelleted by centrifugation at 10,000 *g* for 5 min in a microcentrifuge and washed once with 1 ml of cold 70% (vol/vol) ethanol before being dried under vacuum. The dried DNA was resuspended in 50 µl of TE buffer [10 mM Tris · Cl (pH 8.0), 1 mM EDTA] and the DNA concentration determined with a Lambda II spectrophotometer (Perkin-Elmer) set at 260 nm wavelength.

Selection of targets, oligonucleotide primers, and probes. The target genes for each pathogen, nucleotide sequences of the primers, melting temperatures (Tm) and the amplicon sizes are described in Table 1. The Tm value for each of the primers was estimated by using the equation, Tm (°C) = 2(A + T) + 4 (G + C) [35]. All oligonucleotide primers were custom-synthesized by Integrated DNA Technology, Inc. (Coralville, ID). Each primer set was tested for specificity of amplification of the intended target to ensure that false-positive results would not be generated in multiplex PCR reactions. Similarly, each of the probes listed in Table 1 was based on the nucleotide sequence internal to the amplified segment of the respective target genes.

Optimization of multiplex PCR and specificity of oligonucleotide primers. For optimization of the multiplex PCR amplifications, 200 µM of each dNTP, 1 µM of each primer, 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT), 1 µg of template DNA, and 1 × reaction buffer were used. The 1 × PCR reaction buffer consisted of 50 mM Tris · Cl (pH 8.9), 50 mM KCl, along with either 2.5 mM $MgCl_2$ (buffer C), 4.0 mM $MgCl_2$ (buffer D), 6.0 mM $MgCl_2$ (buffer E), or 8.0 mM $MgCl_2$ (buffer F). The final volume of all reaction mixtures was adjusted to 100 µl with sterile MilliQ™ (Millipore, Bedford, MA) water. All multiplex PCR amplification reactions were performed in a DNA thermal cycler (Perkin Elmer) with the following PCR temperature-cycling parameters: initial denaturation at 94°C for 3 min followed by 30 cycles of amplifications of the target genes. Each amplification cycle consisted of denaturation of the target genes at 94°C for 1 min, primer annealing at 55°C or 60°C for 2 min, and primer extension at 72°C for 3

min. Following amplification, the final extension of the incompletely synthesized DNA was carried on at 72°C for 10 min.

Also, PCR reactions were performed to test for the specificity of each set of oligonucleotide primers to its targeted gene segment. For example, *uidA* primers were used in separate PCR reactions with the genomic DNA from *E. coli*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *S. typhimurium*. Similarly, the primer sets for *invA*, *cth*, *ctx*, and *tlh* primers were each used individually to test their specificity for all five pathogens in separate PCR reactions.

Sensitivity of detection of multiplex PCR. One hundred picograms (10^{-10} g) of purified genomic DNA from each of the microbial pathogens was serially diluted (1:10 vol/vol) in sterile distilled water up to 0.1 fg (10^{-16} g). The lowest amount of DNA of each of the targeted genes was tested in the multiplex PCR reaction. Initially, 30 cycles of PCR amplification were performed with the aforementioned temperature cycling parameters, with the primer annealing temperature at 55°C. Then another 30 cycles of multiplex PCR amplification were carried on in a separate reaction with 5 µl of the previously amplified DNA as targets. All PCR reactions assessing limits of detection were performed in triplicates.

Detection of microbial pathogens in seeded oysters. Oysters, obtained from local seafood restaurants, were shucked, and, following standard methods [1], the shellstocks were homogenized in a sterilized Waring blender, the homogenates exposed to UV light for 1 h, and then subjected to three cycles of freezing at −80°C followed by thawing at room temperature to reduce the indigenous target microbial population. This was done to reduce background PCR amplification signals (unpublished). Cells from each of the five microbial strains were inoculated into 5 ml of *A*lkaline *P*eptone *W*ater [APW (pH 7.8)] (Difco, Detroit, MI) and grown to an optical density (OD_{450nm}) of 0.15 as determined by a spectrophotometer (Lambda 2, Perkin Elmer). The pH (7.8) for the APW was found to be optimum for growth of all microorganisms selected in this study (unpublished). Each culture was then serially diluted in APW (pH 7.8), and viable plate counts from each dilution were determined. Microbial pathogens from each dilution were used to seed approximately 1 g of homogenized shell stock and then grown in 30 ml APW (pH 7.8) at 35°C for 6 h to enrich the target microbial population. Following enrichment, the samples were subjected to DNA purification with the ''Chelex™ 100'' (BioRad) [6] method. Accordingly, the seeded oyster tissue homogenates were centrifuged at 10,000 *g* and the pellets resuspended in 0.5 ml of sterile distilled water, washed, and centrifuged. The washed pellets were resuspended in 0.2 ml of sterile distilled water and mixed with 18% (wt/vol) Chelex™ 100 (BioRad) by vortexing for 10–15 s. The treated samples were then incubated at 58°C for 10 min, boiled for 20 min, cooled to room temperature, and mixed with ammonium acetate (3 M final concentration). The DNA from each sample was subsequently purified by first adding an equal volume of chloroform:isoamyl alcohol (24:1 vol/vol) followed by centrifugation. The partitioned aqueous phase was transferred to a new microcentrifuge tube and DNA precipitated with 0.6 volume of ice-cold isopropanol. The precipitated DNA was then washed once with 70% (vol/vol) ice-cold ethanol and dried under vacuum. The dry, purified DNA was resuspended in 50 µl TE (pH 8.0) buffer. An aliquot (typically 5 µl) of the purified DNA was used for multiplex PCR amplification. To achieve maximum sensitivity, a 5-µl aliquot of the initial multiplex PCR-amplified DNAs was subjected to re-amplification by a second (or ''double'') PCR reaction [30] using the same PCR reagents and cycling parameters.

Detection of amplified DNA. All PCR-amplified DNAs were separated at a constant voltage of 5 V/cm in 2% (wt/vol) NuSieve 3:1 or 2% (wt/vol) SeaKem™ agarose (FMC Bioproducts) with 1 × TAE [40 mM Tris · Cl (pH 8.0), 1.18 ml acetic acid, 2 mM Na_2EDTA per liter] [4]. The separated DNAs in the gel were stained with 2×10^{-4} µg/ml ethidium bromide and visualized on a FotoPrep I (Fotodyne, Inc.) UV transilluminator. For documentation and further analysis, the amplified DNA bands in the gel were photographed with Polaroid™ Type 55 film.

Alternatively, amplified DNAs were detected by a GeneComb™ (BioRad, CA) [31] colorimetric hybridization assay kit. Amplified DNAs were labeled by using oligonucleotide primers designated as R-UID, R-CTH, R-CTX, R-INVA, and R-TL which were biotinylated at their 5′-ends in combination with the non-biotinylated L-UID, L-CTH, L-CTX, L-INV, and L-TL primers. Following PCR amplification, the DNA strands extended at the 3′-ends of R-UID, R-CTH, R-CTX, R-INV, and R-TL primers were biotinylated at the 5′-ends. An aliquot (typically 10 µl from a 100-µl PCR reaction) of the amplified DNAs was subjected to electrophoresis in agarose gel and analyzed as described above.

The identity of each of the multiplex-amplified gene fragments specific for a given microbial pathogen was determined colorimetrically based on the DNA-DNA hybridization assay by use of Genecomb™ kit (BioRad). In this method, oligonucleotide probes were diluted in Binding Buffers™ A and B to a final concentration of 50 ng/µl. Each of the oligonucleotide probes (0.5 µl) (see Table 1) was spotted onto a given nitrocellulose membrane tooth-strip of the GeneComb™. The probes were covalently cross-linked to the membrane by exposure to a UV light for 3 min (200–500 mJ/cm^2) and air dried for 5 min.

Biotinylated amplified individual genes or multiplex PCR-amplified DNA of all five target genes (10 µl from 100-µl of a PCR reaction) was mixed with 50 µl of HybriRun™ solution and then denatured by boiling for 5 min, followed by quick chill for 2–3 min on ice. Denatured, amplified DNA of each gene target was transferred in the following order to the first row of wells of a microtiter plate: first well with *uidA*, second with *cth*, third with *invA*, fourth with *ctx*, fifth with *tl*, sixth well with multiplex PCR-amplified DNA from oyster tissue homogenate seeded with all five microbial pathogens, and the seventh well with multiplex PCR amplified DNA from oyster tissue homogenate in which no cells had been added. Then, each well of the 2nd row of the microtiter plate was filled with 50 µl of streptavidin alkaline phosphatase solution, those in the 3rd row with 50 µl of chromogenic substrate, and those in the 4th row with 50 µl of stop solution. To determine the specificity of the probes to their amplified DNA target, the first five strips [A to E in Fig. 4(b)] were incubated with DNA that was amplified by PCR in individual reactions directed toward *uidA*, *cth*, *invA*, *ctx*, and *tl* target genes, respectively. The 6th Genecomb™ strip was exposed to multiplex PCR-amplified DNA purified from seeded oyster tissue homogenate, and the 7th strip was incubated with the reaction mixture from the multiplex PCR amplification reaction on DNA extracted from oyster tissue homogenate in which no cells were added. The oligonucleotide probes bound to the strips on the GeneComb™ were hybridized for 15 min at 37°C. All subsequent reactions were performed at room temperature. The next reaction was accomplished by transferring the GeneComb™ to the 2nd row. After the DNA hybrids were allowed to conjugate with streptavidin alkaline phosphatase for 5 min, the GeneComb™ was transferred to row 3 containing the chromogenic substrate and incubated for 7 min for color development. The reaction was completed by incubating the GeneComb™ in the 4th row containing the stop solution for 3 min. The color spots representing the positive hybridization reactions on the GeneComb™ were documented with a Polaroid™ film.

Results and Discussion

Specificity of oligonucleotide primers. PCR amplification with each set of oligonucleotide primers yielded a

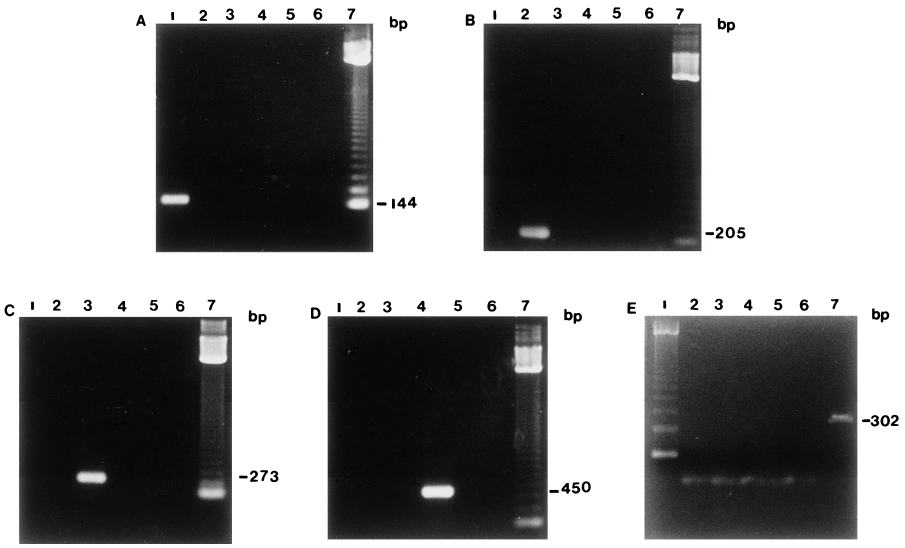

Fig. 1. Agarose gel electrophoresis analysis of the PCR amplification of the purified genomic DNA from microbial pathogens to determine specificity of the oligonucleotide primers to their respective target genes. Panel A–D: lane 1, *Escherichia coli*; lane 2, *Vibrio vulnificus*; lane 3, *Salmonella typhimurium*; lane 4, *Vibrio parahaemolyticus*; lane 5, *Vibrio cholerae*; lane 6, PCR negative control; and lane 7, 123 bp DNA ladder (GIBCO BRL) as DNA size standard; Panel E, lane 1, 123 bp DNA ladder (GIBCO BRL) as DNA size standard; lane 2, *Escherichia coli*; lane 3, *Vibrio vulnificus*; lane 4, *Salmonella typhimurium*; lane 5, *Vibrio parahaemolyticus*; lane 5, PCR negative control; lane 6, *Vibrio cholerae*. Genomic DNAs in Panel A were amplified with the L-UIDA and R-UIDA primers showing amplification of a 144-bp DNA band from *E. coli uidA* gene; Panel B with LCTH and RCTH primers showing amplification of a 205-bp DNA band from *V. vulnificus cth* gene; Panel C with LINV and RINV primers showing amplification of a 273-bp DNA band from *Salmonella typhimurium invA* gene; Panel D with LTL and RTL primers showing amplification of a 450-bp DNA band from *V. parahaemolyticus tl* gene; and Panel E with LCTX and RCTX primers showing amplification of a 302-bp DNA band from *V. cholerae ctx* gene.

single, detectable DNA fragment of the expected molecular weight only in the presence of each of their respective template DNAs (Fig. 1). These results indicated that each of the selected oligonucleotide primer sets for each of the targeted gene segments was specific for its respective target microbial pathogen. This specificity provided an appropriate foundation on which PCR-based detection of the pathogens could be developed, and in which ''false-positive'' signals would be unexpected.

Optimization of multiplex PCR reaction and cycling parameters. Simultaneous amplification of all five target genes was achieved with comparable band intensities for each target DNA using PCR cycling parameters with a primer annealing temperature of 55°C (Fig. 2). An increase in primer annealing temperature to 60°C resulted in relatively weaker amplified DNA fragments of some of the target genes, whereas primer annealing temperature below 50°C resulted in the appearance of spurious DNA bands (data not shown). Use of PCR reaction buffers with various concentrations of $MgCl_2$ revealed that amplification of all five target gene segments yielded comparable band intensities (Fig. 2) only when buffer C (2.5 mM $MgCl_2$) was used. Hence, of all four buffers tested, only buffer C was used in subsequent multiplex PCR reactions.

Sensitivity of PCR detection on purified target DNA. The sensitivity of detection after ''double'' PCR amplification for all five target genes was between 0.1 and 1 pg of total genomic DNA (Fig. 3). This level of detection is equivalent to $\leq 10^1$–10^2 cells for each of the target pathogens and is determined on the basis of the previously reported studies [2,3,9–11]. Although the copy number of each of the genes per cell is not known, the variation of the sensitivity of multiplex PCR detection could be the result of the presence of higher copy number of some of the target genes compared with others.

Detection of microbial pathogens in seeded oysters. Use of Chelex™ 100 to purify DNA from seeded oyster tissue homogenate showed positive PCR amplifications of all target genes with relatively equal intensities. Following 6 h of enrichment in APW (pH 7.8), the minimum level of detection, as determined by gel electrophoresis, of each of the target microbial pathogens in a single multiplex PCR reaction was ≤100 cells/g of oyster tissue homogenate. However, the level of detection was improved to ≤10 cells for each of the pathogens per gram of oyster tissue homogenate following ''double'' PCR amplification (Fig 4A). These levels of detection were based on the initial inoculum size of each of the microbial

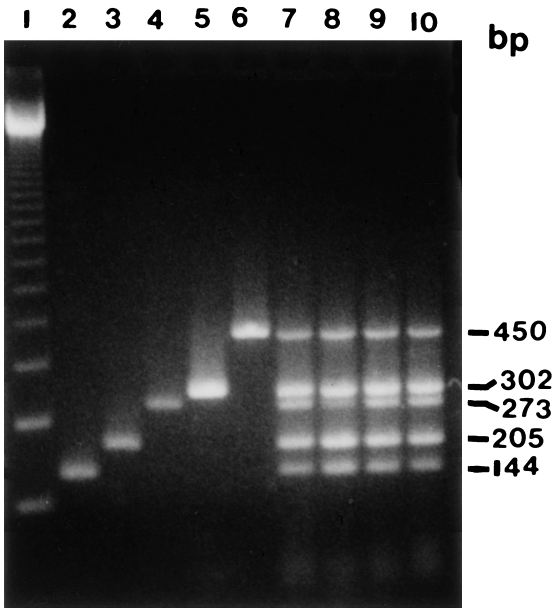

Fig. 2. Agarose gel electrophoretic analysis of the optimization of multiplex PCR amplification from purified DNA from 5 of the 7 proposed microbial pathogens. Lane 1, 123-bp DNA ladder as size marker; lane 2, 144-bp *uidA* amplicon from *E. coli*; lane 3, 205-bp *cth* amplicon from *V. vulnificus*; lane 4, 273 bp *invA* amplicon from *S. typhimurium*; lane 5, 302-bp *ctx* amplicon from *V. cholerae*; lane 6, 450-bp *tl* amplicon from *V. parahaemolyticus*; lane 7, multiplex PCR amplification of all five target genes using PCR reaction buffer C consisting of 2.5 mM $MgCl_2$; lane 8, multiplex PCR amplification of all five target genes using PCR reaction buffer D consisting of 4.0 mM $MgCl_2$; lane 9, multiplex PCR amplification of all five target genes using PCR reaction buffer E consisting of 6.0 mM $MgCl_2$; lane 10, multiplex PCR amplification of all five target genes using PCR reaction buffer F consisting of 8.0 mM $MgCl_2$.

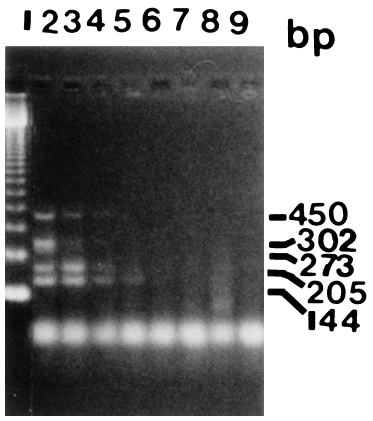

Fig. 3. Agarose gel electrophoresis analysis showing the lowest level of detection of target genes from *E. coli*, *V. vulnificus*, *S. typhimurium*, *V. cholerae*, and *V. parahaemolyticus* using multiplex PCR amplification. Lane 1, 123 bp DNA ladder (GIBCO BRL) as DNA size standard; lanes 2, multiplex PCR amplification of 100 pg genomic DNA from each of the microbial pathogens; lane 3, multiplex PCR amplification of 10 pg genomic DNA from each of the microbial pathogens; lane 4, multiplex PCR amplification of 1 pg genomic DNA from each of the microbial pathogens; lane 5, multiplex PCR amplification of 100 fg genomic DNA from each of the microbial pathogens; lane 6, multiplex PCR amplification of 10 fg genomic DNA from each of the microbial pathogens; lane 7, multiplex PCR amplification of 1 fg genomic DNA from each of the microbial pathogens; lane 8, multiplex PCR amplification of 100 ag genomic DNA from each of the microbial pathogens; Lane 9, PCR negative control in which no genomic DNA was added to the multiplex PCR reaction. Note that the *tl* (450 bp), *ctx* (302 bp), and *invA* (273 bp) target genes were amplified up to 1 pg level of genomic DNA, whereas *uidA* (144 bp) and *cth* (205 bp) target genes amplified up to 100 fg level of genomic DNA.

pathogens to oyster tissue homogenates prior to pre-enrichment.

The GeneComb™ colorimetric assay enabled detection of multiplex PCR-amplified target genes from individual microbial pathogens as revealed by positive hybridizations with only corresponding oligonucleotide probes. This suggests that the oligonucleotide probes and the colorimetric hybridization reactions were specific and can be used without any cross-hybridization reactions (Fig. 4B).

Conclusion

Selection of appropriate target genes, oligonucleotide primers, PCR-reaction and cycling-parameters resulted in the amplification of five target genes simultaneously in a single PCR reaction with a sensitivity of detection of $\leq 10^1$–10^2 cells/g of oyster tissue homogenate. The oligonucleotide primers and probes were specific for their target microbial pathogens as shown by positive amplifications, followed by specific DNA-DNA hybridizations using the GeneComb™ assay. The multiplex-PCR-amplified DNA from seeded oyster tissue homogenate showed hybridizations with all five target genes at the same level of sensitivity as obtained by the gel electrophoresis method, suggesting that the GeneComb™ assay is a possible alternative for detecting multiplex-PCR-amplified DNA. It is a useful and rapid approach since the use of hazardous material, such as ethidium bromide, is avoided. Total time from seeding the oyster tissue homogenate to detection by agarose gel electrophoresis or by colorimetric GeneComb™ hybridization of the amplified DNA was determined to be between 36 and 48 h (≤2 days). Use of conventional, single-target amplification methods would be relatively costly; however, targeting multiple microbial pathogens simultaneously in a single PCR reaction is more time-efficient and cost-effective.

In this study we have demonstrated that detection of multiple microbial pathogens simultaneously from seeded oyster tissue homogenate by multiplex PCR, followed by GeneComb™ colorimetric hybridization, could also be

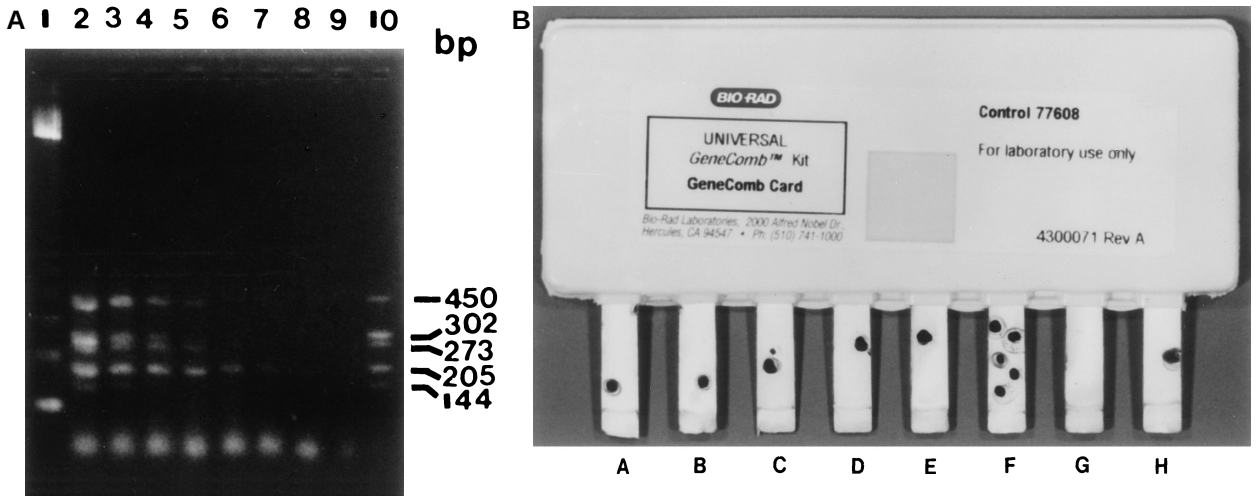

Fig. 4. (A) Agarose gel electrophoresis analysis showing the lowest level of detection of *E. coli*, *V. vulnificus*, *S. typhimurium*, *V. cholerae*, and *V. parahaemolyticus* from seeded oyster tissue homogenate by multiplex PCR amplification. Lane 1, 123 bp DNA ladder (GIBCO BRL) as DNA size standard; lanes 2, multiplex PCR amplification of 10^4 cells of each of the five microbial pathogens; lanes 3, multiplex PCR amplification of 10^3 cells of each of the five microbial pathogens; lanes 4, multiplex PCR amplification of 10^2 cells of each of the five microbial pathogens; lanes 5, multiplex PCR amplification of 10^1 cells of each of the five microbial pathogens; lanes 6, 7, 8, multiplex PCR amplification of 10^0 cells of each of the five microbial pathogens; Lane 9, PCR negative control in which no target microorganism was added to the oyster tissue homogenate; lane 10, multiplex PCR positive control in which purified DNA (0.2 µg) from each of the 5 microbial pathogens was used as targets. (B) GeneComb® colorimetric DNA-DNA hybridizations on target genes which were amplified separately in single PCR reactions or in a multiplex format. Strip A, hybridization with the amplified DNA from the *uidA* target gene; Strip B, hybridization with the amplified DNA from the *cth* target gene; Strip C, hybridization with the amplified DNA from the *invA* target gene; Strip D, hybridization with the amplified DNA from the *ctx* target gene; Strip E, hybridization with the amplified DNA from the *tl* target gene; Strip F, hybridizations of a mixture of all five of the target genes which were amplified simultaneously in a single tube using the multiplex PCR amplification method; Strip G, hybridization with the multiplex amplified reaction in which no target DNA was added (negative control); Strip H, hybridization with a positive control DNA supplied in the GeneComb® kit.

achieved with high specificity and sensitivity in a relatively short period of time. Compared with conventional microbiological culture methods, multiplex PCR provides a rapid means for accurately detecting microbial pathogens in shellfish and can be used to monitor seafood-borne microbial pathogens.

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