APPLICATION OF ARBITRARILY PRIMED POLYMERASE CHAIN REACTION (AP-PCR) FOR THE DETECTION OF *Vibrio parahaemolyticus* IN SHELLFISH

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INTRODUCTION

Vibrio parahaemolyticus is recognized as a causative microorganism for gastroenteritis since 1950 when a large outbreak of food poisoning in Japan led to the discovery of the organism. Seafood-borne outbreaks and sporadic cases of gastroenteritis occur worldwide and are usually associated with the consumption of contaminated seafood, specially bivalve mollusks (Mcnulty et al. 1989). The bacterium is a natural inhabitant of temperate marine environments around the world and it is a well-recognized cause of seafood-related gastroenteritis worldwide (DePaola et al. 1990; Balows 1991). The pathogenic effect to humans following infection has been associated with those strains that produce thermostable direct hemolysin (TDH) (Nishibuchi and Kaper 1985). Production of TDH by a strain of this pathogen is referred as Kanagawa phenomenon (K+) (Nishibuchi and Kaper 1985) and can be identified by ß-type hemolysis on Wagatsume blood agar (Tada et al. 1992). However, the microbiological assay for the Kanagawa phenomenon is time consuming and not determinative since many of the clinical isolates are Kanagawa negative (K-) and do not produce TDH (Taniguchi et al. 1986). Similarly, the immunological methods, although shown to be highly sensitive for detecting TDH, fails to detect clinical strains that lack the TDH toxin. Almost all strains of *V. parahaemolyticus* are reported to produce thermolabile direct hemolysin toxin (TL). The TL does not show ß-hemolysis on Wagatsuma blood agar (Taniguchi et al. 1986). Incidence of *V. parahaemolyticus*-related gastroenteritis could occur by a specific strain of K+ phenotype. The identification of a disease-causing strain in a contaminated oyster bed could help to track the source of the pathogen, thereby specific measures can be taken to prevent further outbreak of the disease. Applications of conventional polymerase chain reaction (PCR) methodologies can be useful for the species- or genus-specific detection of this pathogen (Bej et al. 1998; Tada et al. 1992). However, applications of arbitrarily-primed polymerase chain reaction (AP-PCR) can be used to generate genomic fingerprints of a specific strain of a microbial pathogen. AP-PCR is a variation of the standard PCR (Saiki 1989) in which a short single stranded DNA oligomer is allowed to anneal to denatured template genomic DNA under low stringency (Welsh and McClelland 1991). At some frequency, some primers will anneal to the template relatively close to one nucleotide and DNA polymerase the oligomers are extended to form a new copy of the target DNA. By then increasing the stringency of the reaction, only those products formed in the first few low stringency reactions are amplified. By repeating this process many times, specific fragments of DNA can be amplified, thereby becoming relatively abundant in the resulting mixture of DNA. Different randomly-amplified fragments are generated in different bacteria. When the amplified fragment are separated based on size by using electrophoresis, they result in the production of a readily distinguishable pattern of DNA bands. These bands represent a DNA fingerprint that can be used to identify even various strains of a bacterial species. The AP-PCR fingerprinting can be useful to identify various strains of *V. parahaemolyticus* in contaminated seafood including bivalve mollusks. This will help to trace the source of contamination by this pathogen. Once the contaminating strain are identified, appropriate precautions can be taken to selectively discard the seafood samples, thus helping the seafood industry from financial losses and help further spreading of the disease. The objectives of the present study were to optimize the AP-PCR approach to generate genomic DNA fingerprints of pathogenic K+ and non-pathogenic K- strains of *V. parahaemolyticus* and test the applicability of this approach in artificially contaminated oyster tissue homogenates.

MATERIALS AND METHODS

Bacterial Strains and Growth Media

The *V. parahaemolyticus* strains used in this study, growth media and optimum growth temperatures are described in Table 1. For determination of Kanagawa phenomenon, all *V. parahaemolyticus* strains were grown on Wagatsuma blood agar (Atlas 1996; Miyamoto 1969). The Kanagawa phenomenon was determined for those *V. parahaemolyticus* strains which showed characteristic "halo" surrounding the growth due to ß-hemolysis.

DNA Purification

Total genomic DNA from all pure culture bacterial strains were purified by following the procedure described by Ausubel et al. (1987). Briefly, 2 ml of an overnight grown culture from each of the strains of *V. vulnificus* was collected by centrifugation and cell pellets resuspended in

567 μl of Tris-EDTA buffer [10 mM Tris·Cl (pH 8.0), 1 mM EDTA]. Next, 30 μl of 10% (w/v) sodium dodecyl sulfate and 3 μl from a 20 mg per ml solution of proteinase K (Sigma) were added and the mixture incubated at 37°C for 1 h. The samples were then treated with 100 μl of 5 M NaCl and 80 μl of hexadecyltrimethyl ammonium bromide (CTAB)/NaCl, and incubated at 65°C for 10 min, and purified first with an equal volume of chloroform:isoamyl alcohol (24:1 v/v) and then with phenol:chloroform:isoamyl alcohol (25:24:2 v/v). The DNA was precipitated with 0.6 volume of 100% cold isopropanol and washed with 1 ml of 70% cold ethyl alcohol. The DNA pellet was dried in a DNA 120 speedvac (Savant) for 10 min and resuspended in Tris·EDTA buffer (pH 8.0). An aliquot (typically 1-2 μl) of the sample was subjected to spectrophotometric analysis at OD_{260} and OD_{280} nm wavelengths to determine the purity and the efficiency of recovery.

Oligonucleotide Primer

An arbitrarily chosen single oligonucleotide primer, R-PSE-420, 18 nucleotide long, consisting of 12 AT and 6 GC with the melting temperature (Tm) of 48°C was used for AP-PCR amplification.

AP-PCR Amplification Reaction

Each AP-PCR reaction consisted of a 5 μl PCR reaction buffer (10 x buffer contained 500 mM Tris.Cl, pH 8.9; 500 mM KCl; and 25 mM $MgCl_2$); 8.0 μl dNTP mix (200 μM of each of the dNTPs); 5.0 μl (1 μM) R-PSE 420 primer; 2 μg of purified genomic DNA; 5 U of AmpliTaq DNA polymerase (Perkin Elmer), and deionized sterile distilled water to a total volume of 50 μl. To prevent evaporation, 60 μl of sterile mineral oil.

The following AP-PCR parameters were followed for generating fingerprints of the *V. parahaemolyticus* strains: initial denaturation for 3 min at 94°C followed by 2 cycles of amplification of the target DNA in which each cycle consisting of denaturation at 94°C for 3 min; primer annealing at 35°C for 1 min; primer extension at 72°C for 3 min. Immediately following initial 2 cycles, 10 cycles of amplification was carried on in which each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 45°C for 1 min and primer extension at 72°C for 2 min. A final 30 cycles of amplification was performed with denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and primer extension at 72°C for 2 min. All PCR amplifications were performed in a Perkin Elmer Model 420 thermal cycler.

Following amplification, the amplified DNAs were analyzed by using 1% (w/v) agarose gel [a mixture of 0.4 g of agarose + 0.1 g of Synergel® (Diversified Biotechnology, MA)] stained with ethidium bromide. The gels were visualized on a UV transilluminator and the results were documented using Polaroid™ type 67 film.

Seeding Oyster Tissue Homogenate With *V. parahaemolyticus*

All oyster samples were purchased from local seafood restaurant. The oyster samples were washed with tap water and 70% alcohol to remove any contaminating microorganisms on the outer surface of the shell. The oysters were shucked and the meat samples were homogenized using a Tissue Tearer (Fisher). Oyster tissue homogenate (1 g) was used for seeding target microbial cells. *V. parahaemolyticus* ATCC 35118 (K^+), were grown exponentially (OD_{450} = 0.15) in nutrient broth supplemented with 3% (w/v) NaCl, and spot plated to determine the viable plate count. The cell culture was serially diluted in nutrient broth supplemented with 3% (w/v) NaCl and an aliquot (0.1 ml) from 10^{-1} ($5x10^3$ cells), 10^{-3} ($5x10^1$ cells), and 10^{-5} (no cell) dilutions were added 3 separate 1 g of oyster tissue homogenates. The samples were incubated at 35°C for 30 min and an aliquot of 0.1 ml from each of the artificially seeded oyster tissue homogenate was plated on Wagatsuma blood agar. The plates were incubated at 37°C overnight. Several colonies were selected randomly. In one of the oyster tissue homogenate no *V. parahaemolyticus* cell was added and used as a negative control. Genomic DNA from each colony was purified as described earlier. Purified DNAs were subjected to AP-PCR amplification using the PCR reaction and thermal cycling parameters described earlier. The AP-PCR amplified DNA fingerprints were analyzed by agarose gel electrophoresis methods described earlier.

RESULTS AND DISCUSSION

All strains of *V. parahaemolyticus* (both K+ and K-) showed a common amplified DNA band of molecular weight of 0.615 kbp (Figure 1). This suggests that species specific AP-PCR amplification can be used for the detection of all *V. parahaemolyticus strains*. The K- strains can be identified by analyzing an additional amplified DNA band of molecular weight of 0.862 kbp (Figure 1). However, only one strain of *V. parahaemolyticus* ATCC 35117 showed very faint or no amplified DNA band of the same molecular weight which could be due to the impurities associated with the DNA. The K+ strains of *V. parahaemolyticus* can be distinguished from the K- strains by the absence of the 0.861 kbp DNA band. Additional DNA bands were noticed in both K+ and K- strains which did not show similarities when compared with one strain with another.

Among the colonies isolated from the artificially contaminated oyster tissue homogenates, three similar DNA banding patterns matched with the *V. parahaemolyticus* K+

(Figure 2). However, the other three DNA fingerprint profiles from three other isolated colonies did not match with either K- or K+ strains of *V. parahaemolyticus* suggesting that these isolates were not *V. parahaemolyticus*. A total of 50 *V. parahaemolyticus* cells were inoculated from the 10^{-3} dilution and following plating on Wagatsuma agar, 30 colonies were recovered. Therefore, the recovery was only 60%. Since, the unseeded oyster meat showed 10 colonies per gram of oyster meat, the recovery can be estimated as 40%. Therefore, the sensitivity of detection by this approach can be estimated 50-30 cells per g of oyster tissue homogenate.

CONCLUSIONS

In this study we have optimized AP-PCR method of amplification of genomic DNA to generate DNA fingerprints of both pathogenic (K+) and non-pathogenic (K-) strains of *V. parahaemolyticus* using a single primer, R-PSE. The amplified DNA fingerprints were analyzed in an agarose gel electrophoresis stained with ethidium bromide. All strains of *V. parahaemolyticus* showed a single amplified DNA band of molecular weight of 0.615 kbp. All non-pathogenic K- strains of *V. parahaemolyticus* showed an additional amplified DNA band of molecular weight of 0.861 kbp. The AP-PCR approach was applied in the detection of K+ and K- strains of *V. parahaemolyticus* in artificially contaminated oyster meat. This approach has the potential to identify the source of contamination by this pathogen in oysters and other bivalve mollusks, therefore, helping to reduce the risk of *V. parahaemolyticus*-related disease outbreaks.

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Table 1. List of *Vibrio parahaemolyticus* strains used in this study. Kanagawa phenomenon manifested by them, and their source are described.

Strain	Kanagawa Phenomenon	Source	Growth Temperatures and Growth Media
1. *Vibrio parahaemolyticus*	K+	ATCC 33844	Marine agar/broth (30°C)
2. *V. parahaemolyticus*	K+	ATCC 33846	Marine agar/broth (30°C)
3. *V. parahaemolyticus*	K+	ATCC 33847	Marine agar/broth (30°C)
4. *V. parahaemolyticus*	K+	ATCC 35118*	Nutrient agar/broth+3% NaCl (37°C)
5. *V. parahaemolyticus*	K+	ATCC 43996	Marine agar/broth+3% NaCl (30°C)
6. *V. parahaemolyticus*	K+	ATCC 17802	Nutrient agar/broth+3% NaCl (37°C)
7. *V. parahaemolyticus*	K+	ATCC 17803	Nutrient agar/broth+3% NaCl (37°C)
8. *V. parahaemolyticus*	K+	ATCC 27519	Brain Heart Infusion agar/broth+3% NaCl (37°C)
9. *V. parahaemolyticus*	K+	ATCC 27969	Modified Sea Water Extract (MSWE) (30°C)
10. *V. parahaemolyticus*	K+	ATCC 35845	Nutrient agar/broth+3% NaCl (37°C)

* Strains used for contamination to oyster tissue homogenates

Fig. 1

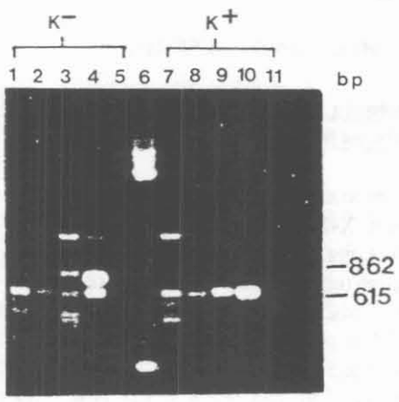

Figure 1. Agarose gel electrophoresis of AP-PCR amplified genomic DNA fingerprints of *Vibrio parahaemolyticus*. Lanes 1 through 5, *V. pahaemolyticus* K+ strains; lanes 7 through 11, *V. parahaemolyticus* K- strains; lane 6, 123 bp DNA ladder as size marker (GIBCO BRL). bp, base pair; K+, Kanagawa positive; K- Kanagawa negative.

Fig. 2

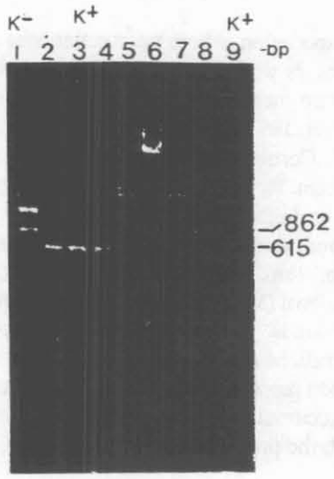

Figure 2. Agarose gel electrophoresis of AP-PCR amplified genomic DNA fingerprints from the bacterial colonies isolated from the artificially contaminated oyster tissue homogenates. Lane 1, *V. parahaemolyticus* K- strain ATCC 17802 as a control; lane 9, *V. parahaemolyticus* K+ strain ATCC 35118 as a control; lane 6, 123 bp DNA ladder as size marker (GIBCO BRL); lanes 2-4 and 7, 8 bacterial isolates from the artificially contaminated oyster tissue homogenates. Lanes 2-4 showed DNA fingerprint profiles similar to *V. parahaemolyticus* K+ strain ATCC 35118 which was used to seed the oyster tissue homogenates. Lanes 4, 7 and 8 showed DNA fingerprint profiles for non-*V. parahemolyticus* bacterial species present indigenous to the oyster tissue homogenates. bp, base pair; K+, Kanagawa positive; K- Kanagawa negative.