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Evaluation of PCR Amplification-based Detection of Heat-killed *Escherichia coli* and Cell-free DNA in Shellfish

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Abstract

Presence of *E. coli* is a useful indicator of fecal contamination in estuarine shellfish. Polymerase chain reaction using primers for *uidA* were used to detect DNA released from dead *E. coli* cells and from heat-killed cells in seeded oyster tissue homogenate. Two different methodologies were adopted for DNA extraction from the seeded oyster homogenate: Chelex™ 100 method and a modified cell-lysis method. Results showed PCR amplification of DNA purified by the modified cell-lysis method was able to eliminate detection of cell-free DNA or heat-killed non-viable cells at various concentrations tested as opposed to the Chelex™ 100 method. DNA-PCR detection was positive for *uidA* using the Chelex™ 100 DNA extraction method on oyster samples with high concentrations (6-100μg) of cell-free DNA or heat-killed cells (1 X 10^6 cells/ml). This suggests that a difference in DNA extraction techniques can eliminate false-positive results by PCR, thus making it a reliable and rapid diagnostic tool to differentiate viable and non-viable cells in shellfish samples. It would greatly enhance the microbiological safety of shellfish and the quality of risk-assessment involved in the detection of viable microbial pathogens in seafood samples.

Introduction

Outbreak of diseases from the consumption of contaminated shellfish, especially raw oysters, is one of the major concerns of the seafood industry and public health agencies. Identification and characterization of the etiologic agents of seafood-related illnesses are often impeded by the extended time required to conduct culture-based conventional microbiological assays. Over the last several years, numerous seafood industries throughout the United States have suffered financially due to the frequent incidents of disease outbreaks, which resulted in poor consumer confidence. Shellfish concentrate microorganisms in their tissues from surrounding water during the filter-feeding process. It is also known that estuarine filter-feeders including shellfish are prone to contamination by fecal pathogens from sewage polluting the waters in which they grow (Roberts, 1990; Rippey, 1994; Wilson and Moore, 1996). American Public Health Association mandates monitoring shellfish for fecal contamination by the identification of *Escherichia coli* as an indicator microorganism (APHA, 1986). Although the microbiological culture-based methods are conventionally used for the detection of *E. coli* in shellfish, rapid detection by PCR amplification has also been adopted by a number of agencies. PCR amplification method of detection of microorganisms provides an alternative approach to the conventional microbiological culture-based assays, and has the ability to detect the targeted microorganism with high specificity and sensitivity within hours instead of days in various biological, food and environmental samples (Bej *et al*., 1991a,b; Atlas and Bej, 1994; Bej and Mahbubani, 1994; Jones and Bej, 1994; Mahbubani and Bej, 1994; Brasher *et al*., 1998; Rijpens and Herman, 2002). It is known that microbial cells can remain in the environment in the dead or non-viable state (Kaprelyants *et al*., 1993). Since conventional PCR method uses the DNA as a template for amplification, it is possible that positive results are obtained from non-viable target microorganisms or cell-free DNA released from dead cells. Therefore, the conventional DNA-based PCR approach cannot distinguish the detection of viable from non-viable microorganisms. In fact, several reports have indicated that heat-killed *E. coli, Listeria* and *Salmonella* cells or the cell-free DNA from these microorganisms can be detected in beef, seawater, or in pure cultures by conventional DNA-PCR (Josephson *et al*., 1993; Masters *et al*., 1994; Dupray *et al*., 1997; Uyttendaele *et al*., 1999). So far, application of the PCR approach for detection of non-viable microorganisms or their cell-free DNA in shellfish has not been reported. Detection of viable and culturable microorganisms is becoming an important issue to the seafood industry, as this would help establish guidelines for a relatively accurate risk assessment for the microbiological safety of seafood (Masters *et al*., 1994; Desenclos, 1996; Lipp and Rose, 1997; Sumner and Ross, 2002). In this study, we have examined whether the application of the conventional DNA-PCR amplification approach has the potential to detect heat-killed non-viable *E. coli* or its cell-free DNA in shellfish. This would help the seafood industry and the government agencies to make appropriate decisions whether to implement the DNA-PCR-based detection of *E. coli* as an indicator microorganism for fecal contamination of consumable shellfish.

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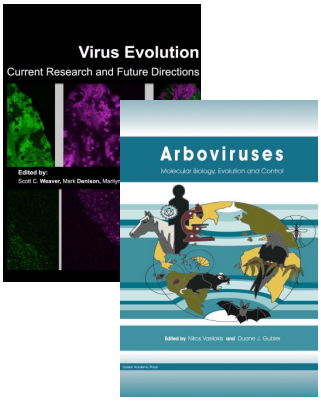

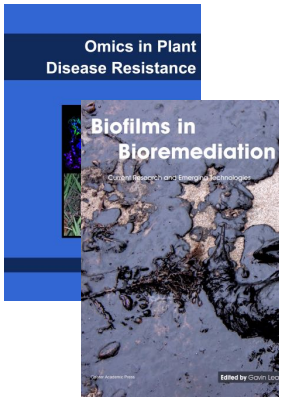

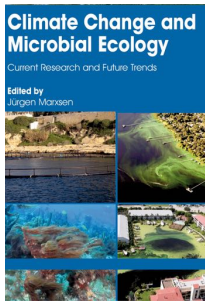

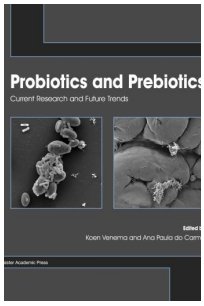

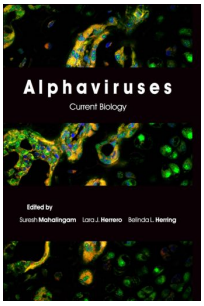

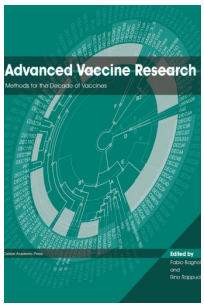

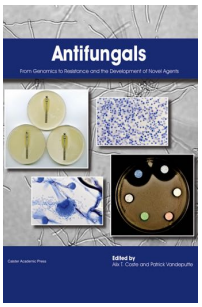

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Table 1. Results of PCR amplification to detect "cell-free" *E. coli* DNA seeded in oyster tissue homogenate.

Type of DNA Purification	Amount of DNA added											Positive control
	100 μg	6 μg	3 μg	1.2 μg	0.6 μg	0.3 μg	30 ng	3 ng	300 pg	30 pg	3 pg	Purified *E. coli* DNA
Cell-lysis method	-	-	-	-	-	-	-	-	-	-	-	+
Chelex™ 100 method	+	+	-	-	-	-	-	-	-	-	-	+

+ = Positive PCR amplification of the *uid*A
– = No amplification of the *uid*A

Results

Detection of cell-free E. coli genomic DNA from seeded oyster tissue homogenate

PCR amplification of the cell-free DNA purified from the seeded oyster tissue homogenate by the modified cell-lysis method exhibited no amplification of the template DNA (Figure 1a; Table 1). In contrast, extraction by Chelex™ 100 method exhibited positive PCR amplification of an expected 0.147 kbp DNA band from the oyster tissue homogenates that were seeded with 100 μg and 6 μg of template DNA (Figure 1b; Table 1). These amounts of purified genomic DNA are equivalent to 1.6 x 10^{10} and 1 x 10^9 *E. coli* cells, respectively (Atlas and Bej, 1990). The positive control experiment showed detection of 10^9 *E. coli* cells (Figure 1a,b - lane 14) but not 10^3 cells (Figure 1a,b - lane 15) for both modified cell-lysis and Chelex™ 100 DNA purification methods. This suggests that both DNA purification protocols used here allow detection of viable cells in oyster tissue homogenate. No amplification was noticed in the sample where no DNA was added (the negative controls; Figure 1a,b – lane 13).

Detection of heat-killed E. coli cells in seeded oyster tissue homogenate

The non-viability of the heat-killed *E. coli* culture was confirmed by no growth on both LB and an *E. coli*-specific VRB agar with MUG media. In addition, epifluorescent microscope study of the *Bac*Light™ stained cultures exhibited red fluorescent-labeled cells indicating that in fact the cells were non-viable following heat-treatment (Figure 2a). The untreated control *E. coli* culture exhibited green fluorescent-labeled cells suggesting that the cells were viable (Figure 2b).

PCR amplification of the DNA purified from oyster tissue homogenate seeded with heat-killed *E. coli* by modified cell-lysis method did not exhibit amplification of the targeted gene (Figure 3a; Table 2). However, PCR

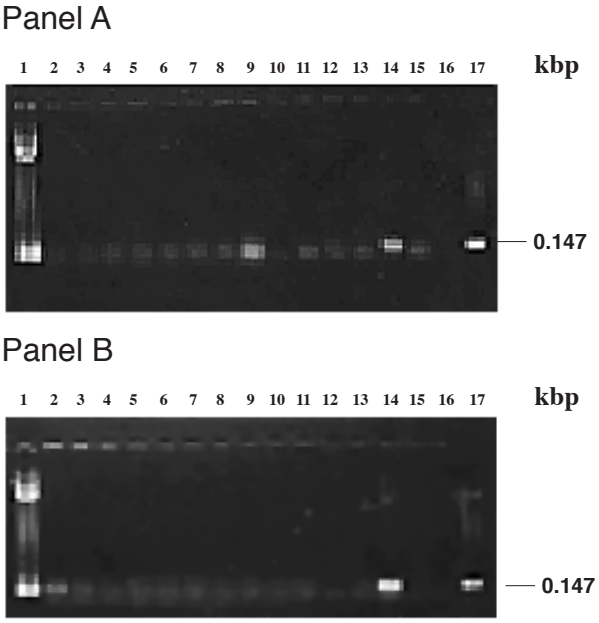

Figure 1. Analysis of PCR amplification to detect "cell-free" *E. coli* DNA seeded in oyster tissue homogenate. **Panel A,** PCR amplification of DNA purified by using the "modified cell lysis" method; and **Panel B,** PCR amplification of DNA purified by using the Chelex™ 100 method. Lane 1, 123 bp DNA ladder (GIBCO BRL, Gaithersburg, MD) as size standard; lane 2, 100 μg DNA was added in oyster tissue homogenate; lane 3, 6 μg DNA was added; lane 4, 3 μg DNA was added; lane 5, 1.2 μg DNA was added; lane 6, 0.6 μg DNA was added; lane 7, 0.3 μg DNA was added; lane 8, 30 ng DNA was added; lane 9, 3 ng DNA was added; lane 10, 300 pg DNA was added; lane 11, 30 pg DNA was added; lane 12, 3 pg DNA was added; lane 13, negative control (unseeded oyster tissue); lane 14, 6 x 10^9 *E. coli* cells; lane 15, 6 x 10^3 *E. coli* cells; lane 16, negative control (no template DNA); lane 17, PCR amplification purified *E. coli* genomic DNA as a positive control. Following Chelex™ 100 purification, "cell-free" *E. coli* DNA was detected at both 100 μg and 6 μg while the modified cell lysis method did not detect any "cell-free" DNA. Both methods detected 6 x 10^9 viable *E. coli* cells, but not 6 x 10^3 cells.

Table 2. Results of PCR amplification to detect heat-killed *E. coli* DNA seeded in oyster tissue homogenate.

Type of DNA Purification	Number of Heat-killed *E.coli* cells added (cells/ml)						Positive control Viable *E. coli* Cells	
	$1x10^6$	$1x10^5$	$1x10^4$	$1x10^3$	$1x10^2$	$1x10^1$	$6x10^9$	$6x10^3$
Cell-lysis method	-	-	-	-	-	-	+	-
Chelex™ 100 Method	+	-	-	-	-	-	+	-

+ = Positive PCR amplification of the *uid*A
– = No amplification of the *uid*A

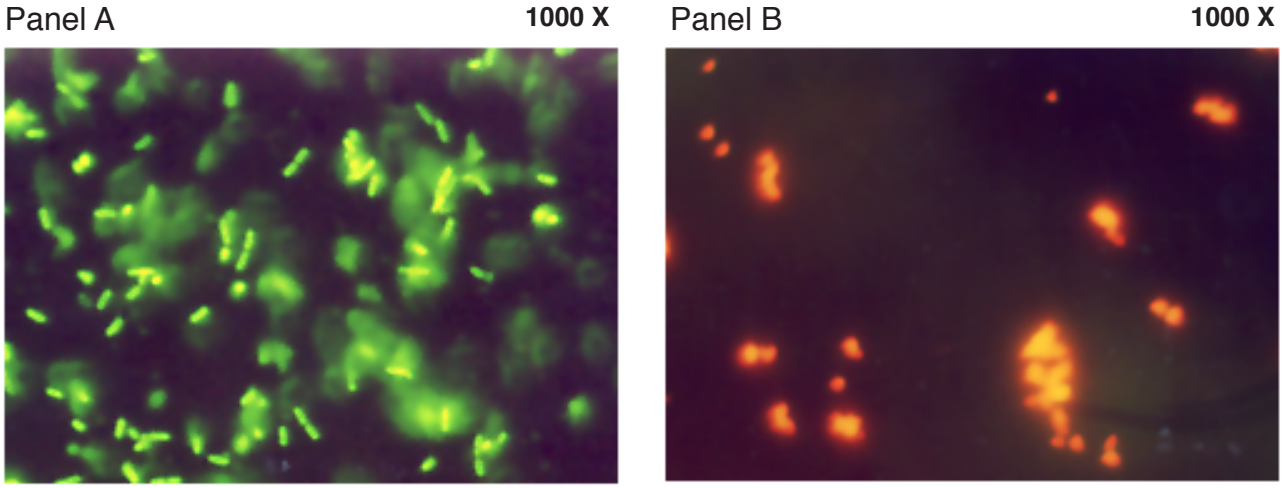

Figure 2. Heat-killed and untreated *Escherichia coli* cells examined under an epifluorescent microscope shown at 1000x magnification. The cells were stained with the using Live/Dead *Bac*Light™ (Molecular Probes) staining kit and viewed under an epifluorescent microscope. The kit works on the principle that propidium iodide, a red dye, only penetrates bacterial cells with damaged membranes, causing a reduction in the Syto9 green stain fluorescence (taken up equally by living and dead cells) when both dyes are present. **Panel A,** The control *E. coli* culture emit a green fluorescence due to the Syto9 stain suggesting that the cells are viable. **Panel B**, The heat-killed *E. coli* culture fluoresces red due to the propidium iodide stain suggesting that these cells are non-viable.

amplification of DNA purified from the sample seeded with 10^7 cells by the Chelex™ 100 method exhibited amplification of an expected 0.147 kbp DNA fragment (Figure 3b; Table 2).

Discussion

Detection of viable *E. coli* with high specificity and sensitivity in shellfish is important to determine possible fecal contamination and accurately estimate the risk of becoming infected if the contaminated batch of shellfish is consumed. Since conventional PCR approach uses DNA as template, presence of cell-free DNA or DNA from non-viable cells could generate amplified DNA resulting in the false-positive detection of the microorganism of interest. In this study, we have investigated the detection of heat-killed non-viable *E. coli* cells in seeded oyster tissue homogenates by conventional DNA-PCR method. Previously, it has been shown that exposure of *E. coli*, *Listeria monocytogenes* and *Salmonella typhimurium* to high temperatures has relatively no effect on DNA amplification by PCR (Master *et al*., 1994; Dupray *et al*., 1997). In these reports, various

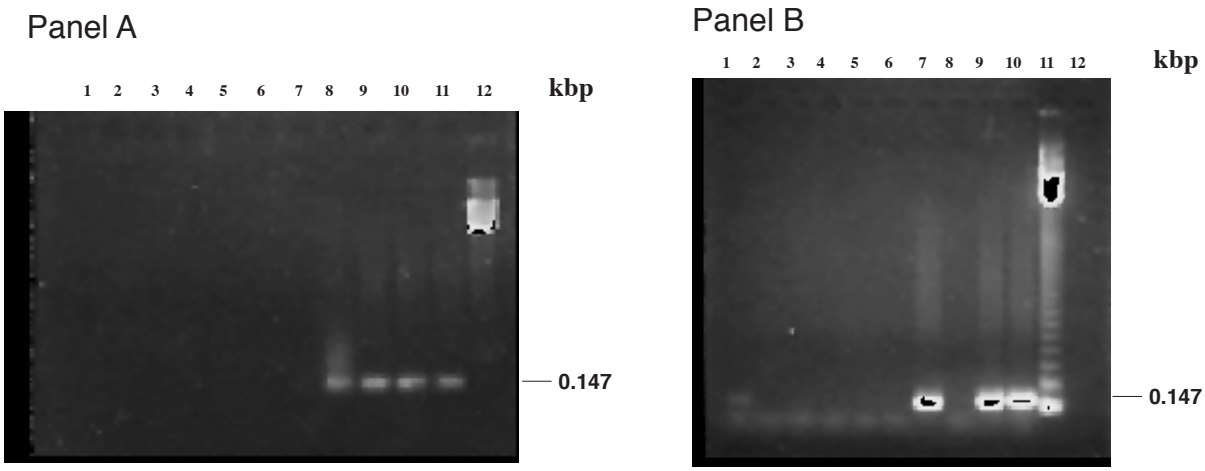

Figure 3. Analysis of PCR amplification to detect "heat-killed" *E. coli* cells added to oyster tissue homogenate in oyster tissue homogenate. **Panel A,** PCR amplification of DNA purified using the "modified cell lysis" method. Lane 1, 1 x 10^6 cells; lane 2, 1 x 10^5 cells; lane 3, 1 x 10^4 cells; lane 4, 1 x 10^3 cells; lane 5, 1 x 10^2 cells; lane 6, 1 x 10^1 cells; lane 7, negative control (no cells added to oyster tissue); lane 8, positive control (1 x 10^6 viable *E. coli* cells added without heat treatment); lane 9, positive control (1 x 10^6 live *E. coli* cells + 6 μg purified cell-free *E. coli* DNA); lane 10, PCR positive control of 0.5 μg purified *E. coli* genomic DNA; lane 11, PCR positive control of 1 μg purified *E. coli* genomic DNA; lane 12, 123 bp DNA ladder as size marker. **Panel B,** PCR amplification of DNA purified by using the Chelex™ 100 method. Lane 1, 1 x 10^6; lane 2, 1 x 10^5 cells; lane 3, 1 x 10^4 cells; lane 4, 1 x 10^3 cells; lane 5, 1 x 10^2 cells; lane 6, 1 x 10^1 cells; lane 7, positive control (1 x 10^6 cells *E. coli* added without heat-treatment); lane 8, negative control (no cell added to oyster tissue); lane 9, PCR positive control (0.5 μg purified *E. coli* genomic DNA); lane 10, PCR positive control on 1 μg purified *E. coli* genomic DNA); lane 11, 123 bp DNA ladder (GIBCO BRL, Gaithersburg, MD) as size marker.

sample preparation techniques have been tried in order to reduce the false-positive results. Bouyant density centrifugation (BDC) proved to be unfeasible in distinguishing viable and non-viable food pathogens by PCR (Lindqvist *et al*., 1997). PCR assay of heat-killed *E. coli* 0157:H7 in ground beef, on the other hand, did not detect any dead cells subjected to various DNA extraction methods like centrifugation, BDC, immunomagnetic separation and Chelex™ 100, as long as the inocula did not exceed 10^7 cfu/g and a wash-step is included (Uyttendaele *et al*., 1999). Longer time intervals for pre-enrichment in *L. monocytogenes* seemed to play a role in avoiding detection of high numbers of non-viable bacteria (Agersborg *et al*., 1997). These studies indicate that depending on the food matrix and the organism in question, the protocols for eliminating false-positives may be varied. The results from our study suggest that the modified cell lysis method of DNA extraction eliminates false-positive detection by PCR. In contrast, the Chelex™ 100 method of DNA purification could detect heat-killed *E. coli* cells or cell-free DNA when present in high numbers. Therefore, the modified cells lysis method of DNA purification from oyster tissue homogenate may have an advantage over the Chelex™ 100 DNA purification method for the detection of viable *E. coli*. Detection of viable *E. coli* cells used in the positive PCR control reactions suggests that both purification methods produce sufficiently pure DNA for successful PCR amplification. However, the detection level of viable *E. coli* was found to be relatively high for both DNA extraction methods. Therefore, it may be necessary to further modify the methods to improve the sensitivity of detection. The experimental results from the current study suggest that in order to avoid false-positive detection of *E. coli* by DNA-PCR method and to estimate risk associated with the consumption of fecal contaminated shellfish, an appropriate DNA purification method should be adopted.

Experimental Procedures

Bacterial strains and growth media

Escherichia coli MG1655 was used for all experiments in this study. The cells were maintained on LB agar or broth (Miller, 1972) at 37°C. Alkaline peptone water (APW) (pH 8.5) used for the enrichment study was prepared by adding 10 g NaCl and 10 g Peptone to deionized water to a volume of 1 L. The solution was mixed, pH adjusted to 8.5, and autoclaved at 121°C for 10 min at 15 psi (Atlas, 1993). Violet red bile (VRB) agar with 4-Methylumbelliferyl-ß-D-glucuronide (MUG) was also used as an *E. coli*- specific agar. It was prepared by rehydrating 41.6 g of pre-mixed VRB with MUG (Difco Laboratories) in 1 L sterile water followed by boiling for less than 2 min. (Atlas, 1993).

Genomic DNA extraction

Genomic DNA was extracted from exponential cultures of the organism by first resuspending in 567 μl of TE buffer, pH 8.0 (10 mM Tris.Cl, 1.0 mM EDTA). Alkaline lysis was performed with 30 μl of 10% (w/v) sodium dodecyl sulfate and 3 μl of 20 mg/ml proteinase K (Sigma) using a procedure adapted from Ausubel *et al*. (1987). After 1 hr incubation, 100 μl of 5 M NaCl was added along with 80 μl CTAB/NaCl solution to complex with polysaccharides. DNA was purified from proteins and other cellular constituents using an equal volume (780 μl) chloroform-isoamyl alcohol (24:1) followed by centrifugation at 10,000 x g for 5 min. Further purification of the supernatant, which was transferred to a new tube, was achieved by adding an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) followed by centrifugation. Then, 0.6 volume (300 μl) of isopropanol was used to precipitate the DNA. The DNA was pelleted by centrifugation at 10,000 x g for 5 min in a microcentrifuge and washed once with 1 ml cold 70% (v/v) ethanol before being dried under vacuum. When ready for use, the purified DNA was resuspended in 50 μl TE buffer, pH 8.0.

Selection of target and oligonucleotide primers

The target gene for the PCR detection of *E. coli* was *uid*A that codes for β-glucuronidase (Blanco *et.al*., 1985). This gene was previously described to be unique in *E. coli* and used in conjunction with the MUG test for monitoring microbiological safety of drinking water (Bej *et al*., 1991 a,b). The oligonucleotide primer sequences [L-uid739: 5'-TGGTAATTACCGACGAAAACGGC-3'(Tm=68°C); R-uid900: 5'-ACGCGTGGTTACAGTCTTGCG-3' (Tm=70°C)] are located within the open reading frame of the *uidA* gene as previously described by Bej *et al.* (1991a). These primers were shown to be specific for the detection of *E. coli* in the environmental and food samples (Cleuziat and Robert-Baudouy, 1990; Bej *et al*., 1991a,b; Iqbal *et al*., 1997; Brasher *et al*., 1998). The oligonucleotide primers were custom-synthesized by Integrated DNA Technology, Inc., ID.

PCR reaction and cycling parameters

All PCR reactions were performed by using 5 μl of 10x Buffer C [10x Buffer C consisted of 25 mM $MgCl_2$, 500 mM KCl, 500 mM Tris.Cl (pH 8.9)], 8 μl of dNTP mix (200 μM of each of the dNTP), 2.5 μl of each of the oligonucleotide primers (1 μM of each of the primers), 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer) and an appropriate amount of template DNA. Total reaction volume was adjusted to 50 μl with sterile distilled water.

The PCR cycling parameters were as follows: initial denaturation of the template DNA was at 94°C for 3 min; then 25 cycles of amplification of which each cycle consisted of denaturation at 94°C for 1 min, primer annealing at 60°C for 1.5 min, primer extension at 72°C for 2 min; and final extension of the incompletely synthesized DNA was at 72°C for 5 min. The amplified DNA was detected by agarose gel electrophoresis (1% w/v) using Tris.Acetate/EDTA buffer (pH 8.3) (Ausubel *et al*., 1987) at 5 V/cm constant voltage. After separation, the DNA was stained with ethidium bromide ($2x10^{-4}$ μg/ml) and visualized under a Photoprep I UV transilluminator (Fotodyne, Inc.). The results were photographed using Polaroid™ Type 55 Film.

Detection of purified cell-free DNA or heat-killed E. coli cells in seeded oyster tissue homogenate

Oysters were collected from Gulf of Mexico near Dauphin

Island Bay, Alabama. The oysters were shucked and tissue homogenate prepared by the recommended standard method (APHA, 1986; Kaysner and DePaola, 2001). The oysters and the tissue homogenates were kept on ice until used. Purified genomic DNA (3 μg) from *E. coli* MG1655 was serially diluted to 3 pg in Tris.EDTA (pH 8.0) buffer. The individual dilutions (0.1 ml) were then added to 1 g oyster tissue homogenate in 20 ml APW (pH 8.5). Then the samples were enriched in APW (pH 8.5) at 35°C for 6 hrs. Oyster tissue homogenate with the added genomic DNA was then subjected to DNA purification either by modified cell lysis or Chelex™ 100 methods as described in later sections.

In another experiment, a pure culture of *E. coli* MG1655 was grown to mid-log phase (OD_{450}= 0.3). The culture was then 10-fold serially diluted in APW (pH 8.5) and 0.1 ml of each dilution was plated on LB agar medium to determine viable plate count. An aliquot (2 ml) of the undiluted culture was then exposed to 70°C for 2h. Following heat treatment, 3 aliquots of 0.1 ml of the sample were plated on LB agar to ensure no colony forming units (CFUs) remained. Also, an aliquot (0.05 ml) of the heat-killed culture was stained with *Bac*Light™ live vs. dead cell fluorescent dye (Molecular Probes, Inc.) and examined under a *Leitz™* Diaplan epifluorescent microscope. The heat-killed *E. coli* culture was then vortexed for 0.5 min and 10-fold serially diluted in APW (pH 8.5). Then, 0.1 ml of each serial dilution was added to 1 g oyster tissue homogenate. The samples were enriched in APW (pH 8.5) for 6 hrs in a 35°C incubator on a rotary shaker at 150 rpm. Following enrichment, aliquots (0.1 ml each) were plated onto *E. coli*-specific VRB-MUG agar to ensure reliable results. Rest of the enriched cultures were subjected to DNA purification by Chelex™ 100 or direct cell lysis methods and PCR amplification and detection was performed as described earlier.

Chelex™ 100 Method for DNA Purification

Following enrichment, the seeded oyster tissue was centrifuged and the tissue pellet resuspended in 0.5 ml of sterile distilled water, washed and centrifuged. The pellet was then resuspended in 0.2 ml of sterile distilled water and total DNA was purified by the "Chelex™ 100" (BioRad) method. In this method, the resuspended oyster tissue homogenate was mixed with 18% (w/v) Chelex™ 100 (BioRad) by vortexing for 10-15 secs. The samples were then incubated at 58°C for 10 min and boiled for 20 min. The samples were then cooled to room temperature and mixed with solid ammonium acetate (3 M final concentration). The DNA from each sample was subsequently purified by treatment with an equal volume of chloroform:isoamyl alcohol (24:1 v/v). Following centrifugation, the aqueous phase was transferred to a new tube and DNA precipitated with 0.6 volumes of isopropanol. The precipitated DNA was washed once with 70% (v/v) ice-cold ethanol and dried under vacuum. The DNA was resuspended in 50 μl TE (pH 8.0) buffer. An aliquot (typically 3 μl for 50 μl of PCR reaction) of the purified DNA was used for PCR amplification.

Cell-Lysis Method for DNA Purification

This procedure was originally described by Gannon *et al.* (1992) to detect *E. coli* in ground beef samples. This approach was tested and found to be unreliable for purification of PCR-amplifiable DNA from target microbial pathogens in shellfish (unpublished). Therefore, a modification of the original procedure was developed which was ascertained to be effective for both shellfish, ground beef and possibly other food matrices (Lett *et al.*, 1995; Wilson and Moore, 1996). Briefly, 0.5 ml of each of the pre-enriched cultures was treated with 0.2 ml of a lysis buffer consisting of 10 mM EDTA, 100 mM Tris.Cl (pH 8.0), 5 μl of 20 mg/ml proteinase K (Sigma), and 50 mg/ml of Sarkosyl (Fisher) at 65°C for 10 min. Following incubation, 100 μl of ice cold 3 M NaOAc (pH 4.6) was added and the tube inverted several times to mix**.** Then an equal volume (800 μl) of chloroform:isoamyl (24:1 v/v) was added to the sample and the tube shaken rapidly until the sample became cloudy. The mixture was centrifuged at 10,000 x g for 5 min and the clear supernatant transferred to a new microcentrifuge tube without disturbing the interphase. Total DNA was precipitated with 1 ml of ice-cold ethanol and centrifuged at 12,000 x g for 5 min. The supernatant was discarded and the pellet washed with 0.1 ml of ice-cold 70% (v/v) alcohol, centrifuged, and dried in a DNA 20 speed-vac drier (Savant). The DNA was resuspended in 10 μl of TE buffer (pH 8.0) and incubated at 65°C for 30 min before being subjected to PCR amplification. Typically, 3-5 μl of the resuspended DNA was used for PCR amplification using the PCR reaction parameters described before.

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