Direct Extraction of Bacterial Plasmids from Food for Polymerase Chain Reaction Amplification

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In this report we describe a simple and rapid technique using DNA affinity columns that permits direct extraction of bacterial plasmids from a variety of foods for polymerase chain reaction amplification. The procedure was used to detect virulent enteroinvasive Escherichia coli in several artificially seeded matrices, including seafoods, greens, dairy products, enrichment media, and water. Polymerase inhibitors present in both foods and enrichment media were removed efficiently.

Enteroinvasive Escherichia coli (EIEC), like Shigella spp., produce an invasive, dysenteric diarrheal illness in humans (2). EIEC have been identified as the causative agents of two major food-associated outbreaks in the United States in 1971 and 1981. The first outbreak was traced to imported French Brie and Camembert cheeses and involved at least 387 persons (9). The second major outbreak was caused by consumption of contaminated potato or macaroni salad (11). To prevent and limit future food-associated outbreaks, food microbiology laboratories would benefit from rapid, specific, and sensitive methods to detect foodborne pathogenic bacteria such as EIEC. The U.S. Food and Drug Administration Bacteriological Analytical Manual describes a two-step enrichment process to recover E. coli from foods (10). However, this type of culture-based method is often not specific enough to selectively enrich a particular strain of bacteria from high numbers of indigenous bacteria. Furthermore, Hill et al. (5) showed that some strains of pathogenic E. coli do not survive enrichment and that, among those that do survive, loss of plasmid-borne virulence genes ranges from 20 to 95%. Since EIEC rely on a 220-kbp plasmid for virulence (4), EIEC may not be reliably detected using currently established procedures.

Recently, the polymerase chain reaction (PCR) has received increased consideration as a highly sensitive procedure for detecting pathogenic bacteria in foods. However, efforts to detect food-borne pathogens with the PCR have been hindered by the lack of methods enabling consistent and rapid purification of amplifiable DNA directly from foods (13). We evaluated several plasmid purification schemes to eliminate the PCR-inhibiting components present in foods and enrichment media. The Magic Minipreps DNA purification system (Promega, Madison, Wis.) proved to be quite effective and facilitated the development of an extremely rapid PCR-based assay.

Fourteen foods were artificially contaminated with an overnight culture of EIEC strain M4163 (4). Samples of 25 g of the following foods were added to 225 ml of phosphate-buffered dilution water (62.5 mM KH2PO4, [pH 7.2]): water, bean sprout, lettuce, raw oyster meat, terrific broth (8), cooked shrimp, Havarti cheese, cooked crab meat, coconut milk, soft tofu, dried milk and egg powder, raw mushroom, and macadamia nut meat. Each mixture was homogenized by blending for 2 min. Homogenates were split and seeded with either EIEC or plasmid-deficient E. coli ATCC 25922 at a level of 105 CFU/ml.

Samples (300 μl) of each seeded homogenate were removed and added to 1.5-ml microfuge tubes containing 300 μl of cell lysis solution (0.2 M NaOH, 1% sodium dodecyl sulfate). Cells were lysed by heating the solutions to 50°C and vortexing slowly for 10 min. The lysed cell mixtures were neutralized by adding 300 μl of neutralization solution (2.55 M potassium acetate [pH 4.8]) to each tube. Food particles and cell debris were removed by centrifugation at 10,000 x g for 30 s in an Eppendorf microcentrifuge. Samples (600 μl) of each cleared supernatant were added to 1.0 ml of Magic Minipreps DNA purification resin in a 3-ml Luer-Lok syringe. A Magic Minipreps column was attached to the syringe barrel, and the slurry was pushed into the column with the syringe plunger. The column was washed with 2.0 ml of column wash solution (100 mM NaCl, 10 mM Tris-HCl [pH 7.5], 2.5 mM EDTA, 50% ethanol). Residual column wash solution was removed by centrifugation at 10,000 x g for 10 s, and plasmids were eluted in 100 μl of 50 mM NaOH. The eluates were neutralized with 100 mM HCl and 10 mM Tris-HCl (pH 7.0).

PCR master mixes were prepared from a GeneAmp PCR reagent kit with AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.) as recommended by the manufacturer. For detection of the invasion plasmid, a 748-bp region that overlaps most of the invasion-associated locus (ial) (3) was amplified with sense primer KL1 and antisense primer KL8 (7). Figure 1 shows a partial restriction map of the 2.5-kbp HindIII fragment from the 220-kbp EIEC plasmid and the location of these primers with respect to the ial gene. In optimization experiments, it was observed that a concentration of 2.5 mM MgCl2 in the PCR reaction buffer resulted in the greatest yield and highest specificity of PCR product (data not shown). Reagent controls with no template were run with each set of master mix. All reactions were performed in 50-μl volumes and were cycled in a Perkin Elmer Cetus DNA Thermal Cycler 480. The cycle parameters included an initial incubation at 94°C for 1 min followed by 50 cycles of 30-s incubations at 94, 60, and 72°C. A 5-min final incubation was performed at 72°C.

Foods seeded with virulent EIEC and subjected to column purification demonstrated positive amplification of the pre-
Directed 748-bp region of the ial gene (Fig. 2). As a negative control, foods were seeded with plasmid-deficient E. coli; extracts from these foods did not yield any detectable PCR product (data not shown). EIEC templates were successfully extracted and amplified from foods (such as shrimp) that have a high DNA/weight ratio. In addition, foods (such as raw oysters) that had $\geq 10^5$ CFU of indigenous bacteria per g before seeding consistently yielded positive amplification of the 748-bp region of the EIEC plasmid. To validate the specificity of amplification reactions, PCR products were subjected to restriction endonuclease digestion with PstI and AccI (U.S. Biochemical, Cleveland, Ohio), which produced fragments of the predicted sizes (data not shown).

Although strict quantitative issues have not been addressed directly, PCR-based approaches are widely recognized for their great sensitivity. In the current study, we found this assay to be effective in detecting as few as 10^3 CFU/ml in some types of artificially seeded food samples (data not shown). However, the procedures described herein could easily be modified to enhance sensitivities even further. For example, sensitivities could be increased manyfold by initially sampling a larger volume of the homogenate and/or employing a second round of PCR.

Two unrelated foods, bean sprouts and oyster meat, were chosen to demonstrate the presence of polymerase inhibitors. Samples (5 µl) of $10^{-3}$, $10^{-2}$, and $10^{-1}$ dilutions of bean sprout or oyster meat homogenate were added to PCR reactions that contained constant amounts of EIEC virulence plasmid (approximately 20 pg or 80,000 copies) isolated from pure culture. Both oyster and bean sprout tissue inhibited PCR amplification; oyster meat was slightly more inhibitory than bean sprout tissue. The addition of 5 µl of the $10^{-1}$ dilution of sprout tissue per PCR reaction was completely inhibitory, whereas just 5 µl of the $10^{-2}$ dilution of oyster meat homogenate greatly inhibited PCR reactions (Fig. 3). In another study (1), several enrichment media were determined to be inhibitory to PCR amplification.

In summary, the technique presented here overcomes many of the problems associated with culture-based analyses of foods and provides an alternative for detecting pathogens with plasmid-borne virulence genes. Other investigators have attempted to extract nucleic acids from food. However, their efforts were culture dependent and focused on extracting DNA from only one particular food matrix such as cheese (12), oysters (6), or lettuce (7). For example, Wernars et al. (12) found that the detection limit of Listeria monocytogenes in soft cheeses largely depended on the brand of cheese used. He also reported that phenol extraction usually removed inhibitors coisolated with DNA but resulted in a great reduction in the amount of DNA recovered.

The method presented here is superior to previous methods mainly because it is not limited to any one food matrix. Moreover, this procedure is much faster, much more economical, and avoids extractions with organic solvents and
ethanol precipitations. Unlike other protocols, this technique can be used to isolate plasmid templates directly from food homogenates without enrichment. Furthermore, this method can be used together with current Bacteriological Analytical Manual techniques because small portions of food homogenates, before or after enrichment, can be removed and quickly screened to determine which culture-based methods will be productive. This procedure has the potential of greatly increasing the productivity of food microbiology laboratories with a concomitant saving of time and money.

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REFERENCES