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Evaluation of DNA Probes for Detection of Shiga-Like-Toxin-Producing *Escherichia coli* in Food and Calf Fecal Samples

MANSOUR SAMADPOUR,[1]* JOHN LISTON,[1] JERRY E. ONGERTH,[2] AND PHILLIP I. TARR[3]

Institute for Food Science and Technology, School of Fisheries,[1] Department of Environmental Health, School of Public Health and Community Medicine,[2] and Department of Pediatrics, School of Medicine,[3] University of Washington, Seattle, Washington 98195

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The use of DNA probes for Shiga-like toxin I (SLT-I) and SLT-II for detection of SLT-producing *Escherichia coli* (SLTEC) in foods and calf fecal samples was evaluated. Enrichment cultures were prepared from food or fecal samples. Colonies formed by plating of enrichment cultures were probed for SLTEC by colony hybridization. Alternatively, enrichment cultures were analyzed for SLTEC presence by dot blot. The lowest detected concentration of SLTEC in sample homogenates inoculated with *E. coli* O157:H7 corresponded to 1.3 CFU/g of sample. Of the 44 food samples and 28 fecal samples from dairy calves tested by the colony hybridization method, 4 food samples, including ground beef, raw goat milk, blueberries, and surimi-based delicatessen salad, and 9 calf fecal samples were positive with the SLT probes. The dot blot technique yielded results within 48 h and can be used as a fast and sensitive method of detection for SLTEC in foods and calf fecal samples. The colony hybridization technique took 3 to 4 days but permits recovery of the positive colonies when desired.

Strains of *Escherichia coli* which produce potent cytotoxins known as Shiga-like toxin I (SLT-I) and SLT-II, also known as verocytotoxins I and II, are emerging as important causes of diarrhea (1, 10–12). These organisms are associated with a painful bloody diarrhea, designated hemorrhagic colitis. This condition can progress to the serious systemic microangiopathic hemolytic anemias, hemolytic uremic syndrome, or thrombotic thrombocytopenic purpura (5). The source of these organisms has not been established with certainty. The SLT-producing *E. coli* (SLTEC) isolated most frequently in clinical cases, *E. coli* O157:H7, has also been recovered from cattle (R. Clarke, S. McEwens, N. Harnet, H. Lior, and E. Gyles, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, P48, p. 282), retail meats (2), asymptomatic humans, and water (K. L. McGowan, E. Wickersham, and N. A. Strockbine, Letter, Lancet **i**:967–968, 1989). Serotypes of SLTEC other than O157:H7 have been recovered from a wide variety of farm animals (5).

The identification of SLTEC involves diverse microbiological techniques. Among SLTEC, only serotype O157:H7 does not ferment sorbitol within 24 h (13), a characteristic which facilitates its identification in clinical specimens. Using a hydrophobic-grid-filter immunoblot technique and polyclonal antibody raised against a crude SLT preparation, Doyle and Schoeni (2) identified this pathogen in retail meats from supermarkets in Calgary, Alberta, Canada, and Madison, Wisconsin. Todd et al. (14) used a similar method in conjunction with an anti-O157 monoclonal antibody for specific detection of O157 *E. coli* in food.

Identification of SLTEC strains of serotypes other than O157:H7 depends on demonstrating cytotoxic activity of culture or specimen supernatants (10). Recently, DNA probes which can sensitively and specifically identify colonies of organisms capable of producing the similar but distinct toxins SLT-I and SLT-II have become available (9).

In this study, we evaluated the use of the DNA probes for SLT-I and -II for detection of SLTEC by either colony hybridization or dot blot in overnight enrichment cultures of food and calf fecal samples, with the purpose of identifying SLTEC of all serotypes.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. An *E. coli* O157:H7 strain containing genes for SLT-I and -II was isolated from a patient with hemorrhagic colitis at Children's Hospital and Medical Center, Seattle, Washington. Recombinant plasmids containing SLT-I and -II probe fragments were obtained from John Newland and Roger Neill (9). Modified Trypticase soy broth (mTSB) and agar (mTSA) (BBL Microbiology Systems, Cockeysville, Md.) (2) were used for enrichment of food samples and plating of bacterial enrichment cultures. Vancomycin (20 μg/ml) was substituted for novobiocin. Bacteria containing recombinant plasmids were grown in Luria broth (LB) media (7) with the appropriate antibiotic added. All cultures were incubated at 37°C.

Food samples, calf fecal samples, and enrichment cultures. All food samples were obtained from local grocery stores and returned to the laboratory for immediate processing. Calf fecal samples were obtained from a dairy farm near Seattle, Washington. By using sterile specimen containers, samples of fresh feces were collected from all calves between 7 and 21 days old. Samples were refrigerated and returned to the laboratory for immediate processing. A 10-g portion of each food or fecal sample was aseptically removed and added to a 250-ml flask containing 90 ml of mTSB and incubated at 37°C with agitation (150 rpm) for 16 to 24 h.

Preparation of filters for colony hybridization. Serial log dilutions of enrichment cultures were made, and 100 μl of the 10^{-4}, 10^{-5}, and 10^{-6} dilutions of each enrichment culture was spread on individual mTSA plates. Of the three dilution plates from each sample, the two containing the highest numbers of isolated colonies were selected after overnight incubation at 37°C. Typically, these plates contained between 50 and 1,000 colonies. Filters for colony hybridization were prepared as previously described (6). Briefly, a piece of Whatman 541 filter paper (Whatman, Inc., Clifton, N.J.) was

labeled and marked asymmetrically with three dots and placed on the colonies. The asymmetrical dot locations were copied on the back of each plate. To transfer colonies to the Whatman filters, the filters were pressed gently onto the agar surface. They were next lifted and placed colony side up on a tray lined with Whatman no. 1 paper saturated with 0.5 N NaOH and 1.5 N NaCl. The tray was then covered and put in a steamer for 10 min, after which the filters were neutralized by transferring to a solution of 0.5 N Tris (pH 8) and 1.5 N NaCl for 10 min. They were then air dried on paper towels, colony side up, and stored dry until hybridization. Plates were stored at 4°C in a sealed plastic bag for later recovery of positive colonies.

Inoculation and recovery test. Two ground beef samples and two jars of oysters (fresh shucked) were used for inoculation studies. A 10-g portion of each sample was blended aseptically with 90 ml of peptone water. Aerobic plate counts (APC) were made on serial log dilutions of each sample. Portions (9 ml) of each homogenate were added to a series of tubes. Tubes were seeded with 1 ml of serial log dilutions (in peptone water) of an overnight culture of the *E. coli* O157:H7 strain, the concentration of which was determined by APC. Each of the seeded homogenates was added to a separate flask containing 90 ml of mTSB and incubated at 37°C with agitation for 16 to 24 h, after which they were prepared for hybridization in the same way as the enrichment cultures.

Dot blot. A 100-μl portion of each oyster enrichment culture (seeded and nonseeded) was spotted on a Nytran filter by placing the filter on sintered glass and applying vacuum. The cells were lysed by placing the Nytran filter on Whatman no. 1 filter paper saturated with 0.5 N NaOH and 1.5 N NaCl for 10 min at room temperature, and then neutralized by placing the filter on Whatman no. 1 paper saturated with a solution of 0.5 N Tris (pH 8) and 1.5 N NaCl for 10 min. The filter was then air dried and baked in a vacuum oven at 80°C for 1 h.

Toxin gene probes and hybridization conditions. Toxin gene probes consisting of the 1,142-base-pair *Taq*I-*Hinc*II fragment of parts of the SLT-IA and SLT-IB subunits and the 842-base-pair *Sma*I-*Pst*I fragment of the SLT-IIA subunit were excised and purified from their respective vectors and labeled with [α-^{32}P]dATP and [α-^{32}P]dCTP (Dupont, NEN Research Products, Boston, Mass.) by extension of random hexanucleotide primers as previously described (3). Filters were placed in prehybridization solution consisting of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (7), 5× Denhardt solution (7), 0.1% sodium dodecyl sulfate, 1 mM EDTA, 50% formamide, and 100 μg of boiled, sheared salmon sperm DNA per ml for 5 to 30 min at room temperature before transfer to hybridization solution of the same composition but containing 300,000 to 400,000 total cpm/ml per filter of an equal amount of the SLT-I and -II probes. The probes were boiled immediately before use. The hybridization reaction was incubated overnight at 37°C. Filters were subjected to three 20-min washes in 2× SSC and 0.1% sodium dodecyl sulfate at 65°C, air dried, and exposed to an X-ray film (X-Omat; Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen.

Filters found to be positive with the combined probes were, in selected cases, reprocessed to determine their reactivity with the individual probes. Filters were prepared for reprobing by washing twice, 15 min each time, at 95°C in 0.1× SSC with 0.1% sodium dodecyl sulfate and then air drying. The filter was then hybridized with a single probe by the procedures described above. The reprobing sequence was repeated with additional probes as desired.

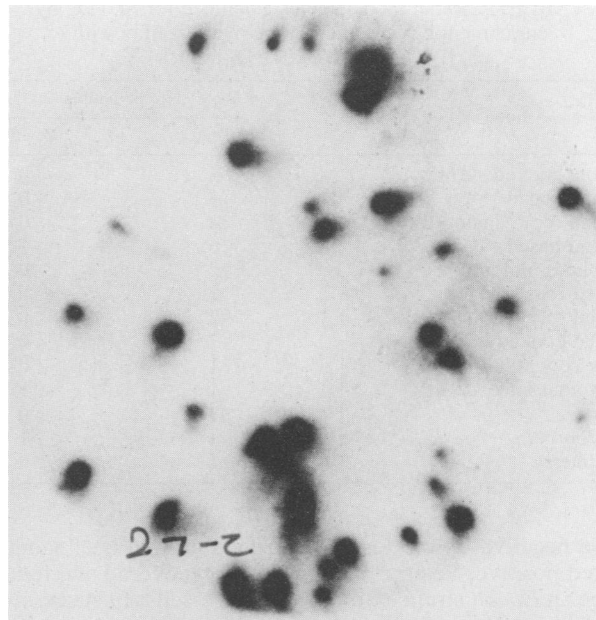

FIG. 1. Autoradiogram resulting from colony hybridization of an enrichment culture of a food sample naturally contaminated with SLTEC.

Recovery of SLTEC from positive samples. Autoradiograms with signals indicating probe-positive colonies (Fig. 1) were aligned with the filters and the plates from which they were derived by using the three asymmetric dots on the exposed Whatman 541 filters and on the petri dishes as guides. A sterile inoculating loop was used to remove the colonies from the areas of the positive signal and then to streak the colonies on a plate of mTSA. After overnight incubation at 37°C, 48 isolated colonies were picked and then subcultured on a premarked grid pattern on each of duplicate mTSA plates. Positive (*E. coli* O157:H7) and negative (a noncytotoxic *E. coli* strain) controls were added to each plate. After overnight incubation at 37°C, the colonies from one of these plates were transferred to Whatman 541 paper, steam denatured, neutralized, hybridized, washed, and exposed as described above. A pure culture of positive colonies was established by isolation from the corresponding colony on the duplicate plate.

Cytotoxicity assay. Supernatants of aerobically incubated cultures of probe-positive isolates were tested for toxicity to Vero cells by the method of Gentry and Dalrymple (4), in which Vero cells were substituted for HeLa cells.

RESULTS

Two oyster and two ground beef samples were used as examples of complex food matrices on which to test the SLTEC identification method. Background APC levels in the oyster samples were ca. 10^3 CFU/g, and levels in the ground beef samples were ca. 10^6 CFU/g. In each of the inoculated samples of both the oysters and the ground beef, SLTEC were clearly detected at all inoculum dilutions above extinction, which corresponded to an SLTEC concentration in sample homogenates of 1.3 CFU/g of sample. The two uninoculated oyster samples and one ground beef sample

TABLE 1. Retail food samples examined for SLTEC by enrichment culture and colony hybridization with SLT-I and SLT-II DNA probes

Food	No. of samples	
	Tested	Positive
Whole cooked shrimp	3	0
Shrimp salad	3	0
Surimi (crab analog)	14	0
Surimi-based salad	6	1
Smoked salmon dip	2	0
Sliced roast beef	1	0
Sliced roast turkey breast	2	0
Sliced cooked ham	2	0
Raw cow milk	1	0
Raw goat milk	1	1
Tofu	3	0
Blueberry	1	1
Raspberry	1	0

were negative by the test. The second ground beef sample tested positive. Positive colonies were recovered and found to be an *E. coli* strain cytotoxic for Vero cells. In successive reprobings, the original filter from the positive ground beef sample was positive with the SLT-II probe and negative with the SLT-I probe. Similar results were obtained when the oyster enrichment cultures were tested by the dot blot method. The Nytran filter containing the denatured lysates of the uninoculated enrichment cultures did not yield any signals. However, lysates from the inoculated homogenates seeded at SLTEC levels corresponding to as few as 1.3 CFU/g of sample yielded clear positive signals.

Overnight enrichment of the oyster homogenates seeded with *E. coli* O157:H7 at levels ranging from 1.3 to 1.3×10^4 CFU/g resulted in SLTEC titers of from 8.5×10^7 to 5.3×10^8 CFU/ml. Overnight enrichment of the SLTEC-negative ground beef homogenate seeded at the same levels resulted in SLTEC titers ranging from 3.8×10^7 to 2.0×10^8 CFU/ml. Overnight enrichment of uninoculated, naturally contaminated samples resulted in SLTEC titers ranging from 3×10^8 to 1.5×10^8 CFU/ml.

To determine the applicability of the method to other types of samples in addition to the two oyster and two ground beef samples tested above, we used the colony hybridization procedure to test another 40 food samples (Table 1) and fecal samples from 28 dairy calves. Of the total of 44 food samples tested in this study, 4 were positive with the SLT gene probes. These included one sample each of ground beef, raw goat milk, blueberries, and a surimi-based delicatessen salad.

Of the 28 calf fecal samples tested, 9 (30%) were positive with the probes. Several positive colonies were isolated from two of the samples. All the calf fecal isolates were found to be *E. coli* cytotoxic for Vero cells. All SLTEC colonies from one of the two samples reacted only with the SLT-I DNA probe, while the SLTEC isolates from the second sample reacted only with the SLT-II probe.

DISCUSSION

Assays of food and calf fecal samples for SLTEC by enrichment culture of sample homogenates followed by colony hybridization or dot blot were very sensitive. Uninoculated enrichment cultures of oyster and ground beef samples that were negative for SLTEC tested positive by this method when seeded with as little as 1.3 CFU of *E. coli* O157:H7 per g. In oyster and ground beef samples with APC levels of ca. 10^3 and ca. 10^6 CFU/g, respectively, seeded with *E. coli* O157:H7 at 1.3 CFU/g, overnight enrichments resulted in SLTEC levels of 8.5×10^7 and 3.2×10^7 CFU/ml, respectively. Levels of SLTEC reached in overnight enrichments of naturally contaminated samples ranged from 3×10^5 to 1.5×10^8 CFU/ml. The method is thus clearly suitable for detection and recovery of SLTEC strains, even when they constitute a very minor portion of the APC.

Although SLTEC strains have been isolated from foods of bovine origin (2), this is the first time that these organisms have been detected in fruit, raw goat milk, and a surimi-based product. None of the 14 surimi analog samples tested were positive, which may indicate that the organism was introduced into the surimi-based delicatessen salad through other ingredients or through the mixing process by a contaminated utensil or infected handler. Blueberries are picked by hand, and an infected picker may have been the source of contamination. Fecal samples from 9 (30%) of the 28 dairy calves tested positive with SLT DNA probes. This is similar to observations reported previously (8).

These data indicate the ease and sensitivity with which toxin gene probes for SLT-I and -II can be applied to screening of food and environmental samples for the detection of organisms bearing the SLT genes. This technique has two definite advantages. First, all SLT-producing organisms, regardless of serotype, can be detected and recovered. Though the extent of the problem of these potential pathogens in humans has yet to be defined, the probing technique will enable the source of these organisms to be identified. Also, our technique avoids of problem of cross-reactive organisms that could be detected by antibodies to the O157 antigen. Second, after hybridization and exposure to X-ray film, the colony hybridization and dot blot filters can be washed and reprobed (we have reprobed the filters up to five times). This makes the method suitable for detection of a wide range of pathogens for which specific DNA probes are available and which would grow well in the enrichment media.

The time between initiation of the enrichment culture and the detection of the SLTEC in food samples was 3 to 4 days. By the dot blot method, we were able to detect the presence of *E. coli* O157:H7 in 2 days. Unfortunately, the dot blot technique does not allow the recovery of the organism containing the SLT gene, though it can be used for the rapid screening of food samples. The dot blot method could be used with a nonradiolabeled nucleic acid probe, which would make it more accessible for users in food microbiology laboratories. Finally, with the characterization of additional gene sequences to identify a wider range of food-borne pathogens, the combination of enrichment culture with either colony hybridization or dot blot or both may augment or replace traditional microbiological methods for the detection of a variety of food-borne pathogens.

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