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Foodborne Enterotoxigenic *Escherichia coli*: Detection and Enumeration by DNA Colony Hybridization

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Four methods were compared for detecting heat-labile toxin production by *Escherichia coli*: DNA colony hybridization, two enzyme-linked immunosorbent assays, and the mouse Y-1 adrenal cell reaction. Although results of the methods were in general agreement, there were some differences in specificity and sensitivity. DNA colony hybridization was used to detect and enumerate enterotoxigenic *E. coli* isolates in artificially contaminated food without enrichment. Sensitivity level was 100 cells per g.

Although most *Escherichia coli* strains exist in harmony with their human hosts, some can cause diarrheal illness. *E. coli* isolates which elaborate toxins and express species-specific colonization factors (15, 42) may be acquired from contaminated foods (32, 46). About 5 to 10% of *E. coli* strains obtained from cheeses, ground meats, and seafoods were found to be enterotoxigenic (39, 43).

The enrichment for *E. coli*, its isolation from foods, and the demonstration of enterotoxigenicity and colonization factors are lengthy processes not well suited for large numbers of samples. Furthermore, current enrichment strategies (33) may result in the loss of plasmid-associated virulence determinants (22). In *E. coli*, the genes for toxin production and colonization are located on plasmids (13), and it has been difficult to recover pathogenic strains efficiently from foods (34).

With the advent of recombinant DNA methodologies, genes for the heat-stable and heat-labile toxins (LT) produced by *E. coli* have been cloned (37, 52, 53). DNA colony hybridization, although useful for identifying pathogenic strains of bacteria without enrichment, can detect only organisms themselves and is unable to determine the presence of gene products such as preformed toxins. Therefore, this method is not useful for detecting contaminated foods responsible for illness caused by ingestion of preformed toxins such as those of *Clostridium botulinum*, *Staphylococcus aureus*, and *Bacillus cereus*.

One method for detecting LT uses an enzyme-linked immunosorbent assay (ELISA) and is based on the cross-reaction of *E. coli* LT with cholera toxin (CT); another method uses the binding of toxin to gangliosides (5, 23, 24).

Colony hybridization has been used to detect *E. coli* strains with the genetic potential to produce LT in a background of nonproducing cells (21). This report shows that colony hybridization can reliably detect toxin producers both in pure culture and in experimental mixtures of producers and nonproducers. The method can be used to detect strains which elaborate only minute quantities of toxin since efficient gene expression is not required. For this reason, we compared colony hybridization with immunological and biological assays that require release of toxin from cells.

MATERIALS AND METHODS

Bacterial strains. Cultures of *E. coli* used in this study are listed in Table 1. Strain ATCC 25922 was purchased from Difco Laboratories, Detroit, Mich.

Plasmid DNA isolation. A starter culture of C600(pEWD299) was grown overnight at 37°C with shaking in brain heart infusion broth containing 10 μg of ampicillin per ml. Ten liters of F-buffer (44) containing 0.4% glucose, 0.2% Casamino Acids, and 2 μg of thiamine per ml were inoculated to an absorbance at 550 nm (A_{550}) of 0.03 and incubated with agitation until A_{550} reached 0.4. Powdered chloramphenicol (Sigma Chemical Co., St. Louis, Mo.) was added to a concentration of 100 μg per ml of growth medium, agitation was reduced to 75 rpm, and incubation was continued for 18 h (6). Cells were harvested by centrifugation, cleared lysates were prepared, and plasmid DNA was purified by ultracentrifugation. The techniques have been described (21) with the following exceptions. After the plasmid DNA was collected from cesium chloride-ethidium bromide gradients, the DNA was rebanded in an SW50.1 rotor at 35,000 rpm

TABLE 1. Comparison of four methods for detecting *E. coli* strains which elaborate heat-labile toxin

Strain	Reference	Colony hybridization	ELISA result		Mouse Y-1 adrenal cell assay
			CT toxin	Ganglioside binding	
739056	12	+	+	+	+
ATCC 25922	4	−	−	−	−
C600	1	−	−	−	−
C600(pEWD299)	9	+	+	+	+
C600(pBR322)	3	−	−	−	−
CG146	48	+	−	−	+
E2534	30	+	+	+	+
H10407	49	+	+	+	+
H10407P	14	+	−	−	+
HB101(pBR313)	2	−	−	−	−
JC1569(pSC101)	7	−	−	−	−
K334C2	40	+	+	+	+
LE392(pEWD299)	9	+	+	+	+
P233	51	+	−	−	+
TD225C4	36	+/−[b]	+/−	+/−	+/−
TD462C1	36	−	−	−	−
TD514C1	36	−	−	−	−
V517	31	−	−	−	−

[a] ELISA based on (i) the cross-reaction of LT with CT, and (ii) the binding of LT to gangliosides.
[b] Original isolate was positive (see the text).

for 2 days. The DNA was harvested and extracted with isopropanol; 2 volumes of water and 6 volumes of −20°C ethanol were added, and the sample was stored at −20°C overnight. It was then centrifuged in an SS34 rotor at 10,000 rpm for 15 min at −15°C, drained well, suspended in TE buffer (10 mM Tris [pH 8.0]–1 mM disodium EDTA) and stored at 4°C until use.

Restriction endonuclease digestion and DNA fragment purification. The restriction endonuclease *Hin*dIII (Bethesda Research Laboratories, Rockville, Md.) was used according to the specified reaction conditions. The digest was fractionated on a vertical 10% polyacrylamide gel (39 acrylamide:1 bis-acrylamide) for 4 h at 150 V in the buffer system of Meyers et al. (35). After it was stained with 2 μg of ethidium bromide per ml, the band containing the 850 base-pair *Hin*dIII fragment (9) was sliced from the gel, inserted into a dialysis bag, and electroeluted at 50 V for 16 h. After a second electrophoresis and electroelution, the purified DNA fragment was passed through a DE52 column (Whatman, Inc., Clifton, N.J.) for removal of contaminating substances that might inhibit subsequent enzymatic reactions (55). DE52 was equilibrated in loading buffer (150 mM NaCl–10 mM Tris [pH8.0]–1 mM EDTA), and the DNA was bound to a 0.3-ml column. After being rinsed with 10 column volumes of loading buffer, the DNA fragment was removed from the column with eluting buffer (1.0 M NaCl–10 mM Tris [pH 8.0]–1 mM EDTA). Fractions containing the DNA fragment were pooled, precipitated with ethanol, and resuspended in TE buffer. This DNA fragment contained all of the B subunit and about one-third of the A subunit of the *E. coli* LT genes (9).

Labeling of the toxin gene DNA fragment. The 850 base-pair toxin gene DNA fragment was radiolabeled in vitro by the nick translation method (26) with reagents from Bethesda Research Laboratories. Toxin gene DNA (50 ng in 7.5 μl of TE buffer) was added to 16 μl of aqueous stabilized deoxycytidine triphosphate [α-^{32}P] (3,000 Ci/mmol, 10 mCi/ml; Amersham Corp., Arlington Heights, Ill.), 0.5 μl each of the other three unlabeled deoxyribonucleoside triphosphates (1 mM), 3 μl of 10-fold concentrated reaction buffer, and 2 μl of DNase I (100 ng/ml). After 10 min at 15°C, 2 μl of DNA polymerase I (1 U/μl) was added. The reaction was terminated after 2 h of incubation, and unincorporated nucleotides were separated from the DNA by chromatography through a 2-ml Sephadex G-50 column (Sigma). The specific activity of this labeled DNA was usually 2×10^8 to 4×10^8 cpm/μg.

Colony hybridization filters. About 75 μl of each pure *E. coli* culture, grown overnight, was added to a sterile microtiter plate well. A stainless steel replicator with 1.6-mm diameter prongs was used to transfer the cultures to sterile nitrocellulose filters (BA85; Schleicher & Schuell Co., Keene, N.H.) on MacConkey agar. The replicator simplifies the preparation of multiple filters and helps to achieve a relatively uniform inoculum. After incubation at 37°C for 4 to 6 h, when the inoculated areas were faintly but clearly visible, the cells were lysed or stored overnight at 4°C. The method of Grünstein and Hogness (18) as modified by Moseley et al. (38) was used to lyse cells with the following changes. Colonies on nitrocellulose filters were placed on filter papers soaked with 0.5 M NaOH for 10 min before three 1-min transfers on filters wetted with a solution of 1.0 M ammonium acetate and 0.02 N NaOH (50). After 10 min on a fourth change of the latter solution, filters were air dried for at least 30 min, baked in vacuo for 2 h at 80°C, and stored under vacuum at room temperature for up to 1 year.

DNA colony hybridization. DNA hybridization reactions were carried out by the method of Moseley et al. (38) as modified by Hill (21), with the following exceptions. Radiolabeled toxin-gene DNA fragments were of a specific activity of 2×10^8 to 4×10^8

cpm/μg, and filters were washed at 70°C after hybridization. Autoradiograms were exposed for 1 to 3 days at −70°C by using Kodak XAR film and regular intensifying screens.

Microbiological analysis of food. Food samples were analyzed by the method of Mehlman and Andrews (33). To inoculate colony hybridization filters, 0.1 ml of a particular dilution was spread-plated onto a sterile filter which had been placed on aerobic plate count agar. The plates were incubated for 24 h, and the colonies were lysed as described above.

Detection of heat-labile toxin. Tests for toxin were carried out by several methods. Strains of *E. coli* were grown at 37°C with shaking for 18 to 24 h in Casamino Acids-yeast extract medium containing 0.2% sucrose (28). To induce higher levels of LT synthesis (29), these strains were also grown in this medium supplemented with 45 or 90 μg of lincomycin per ml. Filtered culture supernatants were tested for LT on mouse Y-1 adrenal cells by a modification of the methods of Donta et al. (11) and Sack and Sack (41). After the Y-1 cells were exposed overnight to culture supernatants, the tissue culture medium was replaced with fresh medium. Incubation was continued for 2 h before Y-1 cells were examined microscopically. Our experience has been that the replacement of the medium reduces the nonspecific changes in Y-1 cell morphology which are observed with some strains.

LT was also detected by an ELISA based on the cross reaction of LT with antibodies to CT (5, 24). A multivalent antiserum directed against CT was detected by protein A conjugated to horseradish peroxidase (Zymed Laboratories, Burlingame, Calif.). Some assays were carried out by using a "double antibody sandwich assay," with antibodies to purified *E. coli* LT (47; Shah et al., manuscript in preparation). In addition, the ability of toxin to bind to gangliosides was determined by an indirect ELISA (8, 23, 27). Antibody to CT was added to ganglioside-coated microtiter plate wells, and binding was detected by the protein A-peroxidase system described above.

RESULTS

Pure cultures. The strains of *E. coli* listed in Table 1 were grown in pure culture, inoculated onto a nitrocellulose filter, and examined by colony hybridization for the presence of genes encoding LT (Fig. 1). The large spots indicated that the radioactively labeled toxin gene DNA was bound to a complementary nucleotide sequence found in a lysed colony. This radioactivity caused an autoradiogram to be exposed, resulting in the appearance of a spot. In this report, only about 30 cultures had been spotted on an 82-mm-diameter filter; several hundred cultures can be grown on a single 150-mm filter (17).

These results were compared with those of three other methods for detection of *E. coli* LT: one biological and two immunological methods (Table 1). Although the results were in general agreement, there were exceptions. Two porcine strains, CG146 and P233, had the toxin gene but did not react in the two immunological tests based on sera produced against CT. The ST-negative derivative of H10407 (designated H10407P) still produced LT, as shown by the reaction of Y-1 cells; however, this toxin could not be demonstrated by the ganglioside binding assay.

Mixed cultures. To determine whether a few enterotoxigenic *E. coli* cells could be detected against a large background of cells not producing toxin, strains H10407 and C600 were grown overnight, mixed in varying ratios, and spread-plated on nitrocellulose filters (Fig. 2). About 10 distinct colonies of strain H10407 were observed by DNA colony hybridization.

Experimentally contaminated food. The aerobic plate count of a 50-g sample of scallops was 1.9×10^5 cells per g, and the three-tube most-probable-number estimates of total coliforms and *E. coli* cells in the sample were 23 and ≤3 per g, respectively. Aliquots A to D of the homogenized food sample were inoculated with strain H10407 to about 14,500, 1,450, 145, or 15 cells per ml, respectively. On autoradiograms of filters spread-plated with 0.1 ml of each aliquot, darkened spots represented colonies of cells with the genetic potential to produce LT (Fig. 3). Table 2 compares the expected and the observed numbers of enterotoxigenic *E. coli* for aliquots A to D of the inoculated food sample. As a control, strain H10407 was also plated on aerobic plate count agar with or without nitrocellulose filters. The titer of CFU per milliliter on these filters was 83% of that without filters (data not shown). Therefore, it appears that most cells of this strain can form colonies on nitrocellulose filters.

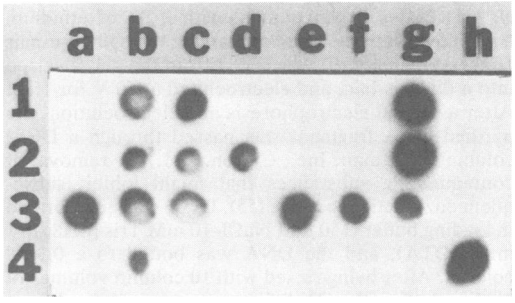

FIG. 1. Autoradiogram of colony hybridization filters inoculated with pure cultures of *E. coli* strains. 1-b, P233; 1-c, H10407; 1-d, V517; 1-e, JC1569(pSC101); 1-f, C600(pBR322); 1-g, C600(pEWD299); 2-b, CG146; 2-c, K334C2; 2-d, TD225C4; 2-e, TD514C1; 2-f, TD462C1; 2-g, C600(pEWD299); 2-h, HB101(pBR313); 3-a, H10407; 3-b, 739056; 3-c, E2534; 3-d, TD225C4; 3-e, LE392(pEWD299); 3-f, H10407; 3-g, H10407P; 4-b, C600(pEWD299); 4-c, ATCC 25922; 4-h, C600(pEWD299). Positions not listed were not inoculated.

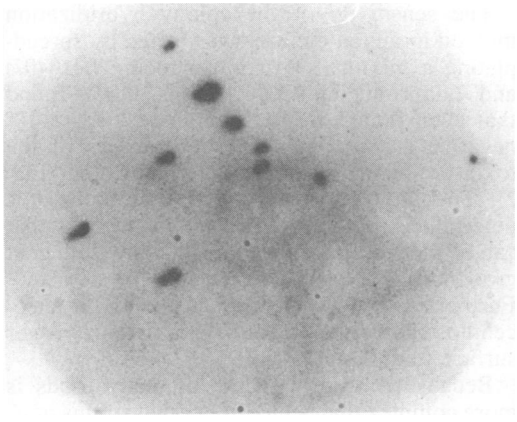

FIG. 2. Autoradiogram of colony hybridization filter inoculated with about 20 cells of *E. coli* H10407 and 1.2×10^6 cells of strain C600.

DISCUSSION

Colony hybridization is a method for determining whether bacterial cells contain specific nucleotide sequences. Enterotoxigenic *E. coli* isolates from clinical sources have been detected by this method (38). However, although strains of pathogenic *E. coli* are present in relatively large numbers in the stools of persons with diarrheal illness, *E. coli* in food samples may be present in low numbers and in much more heterogeneous environments. Therefore, a physiologically demanding enrichment procedure has been devised (33). It is far from certain whether all strains of *E. coli* pathogenic to humans can be recovered (34; Hill, unpublished data) and whether they will still retain their determinants of pathogenicity. The possibility of using DNA hybridization to detect a few cells (21) with the genetic potential for producing LT (20) prompted us to apply this method to food samples in an effort to detect and enumerate pathogenic strains without the need for selective enrichment.

Pure cultures of *E. coli* from a number of sources were tested by colony hybridization for the genetic potential to produce toxin by the mouse Y-1 adrenal cell assay and by two ELISAs, one based on the affinity of LT for CT antibody, the other based on the binding of toxin to gangliosides. A clone of TD225C4 (Fig. 1, 3-d), which in the laboratory had spontaneously lost the ability to produce toxin, was also negative for toxin gene nucleotide sequences (this report and Myron Levine, Center for Vaccine Development, University of Maryland, Baltimore, Md., personal communication).

Although there was general agreement among results of these three tests (Table 1), several differences were observed. The positive response of H10407P in the Y-1 cell assay, with and without lincomycin, was a typical cell rounding and not a cytopathic effect. When tested with a double antibody technique, after colonies had been treated with EDTA, lysozome, and chloroform (47), this strain produced a weakly positive response to anti-LT antibody (data not shown). However, H10407P did not

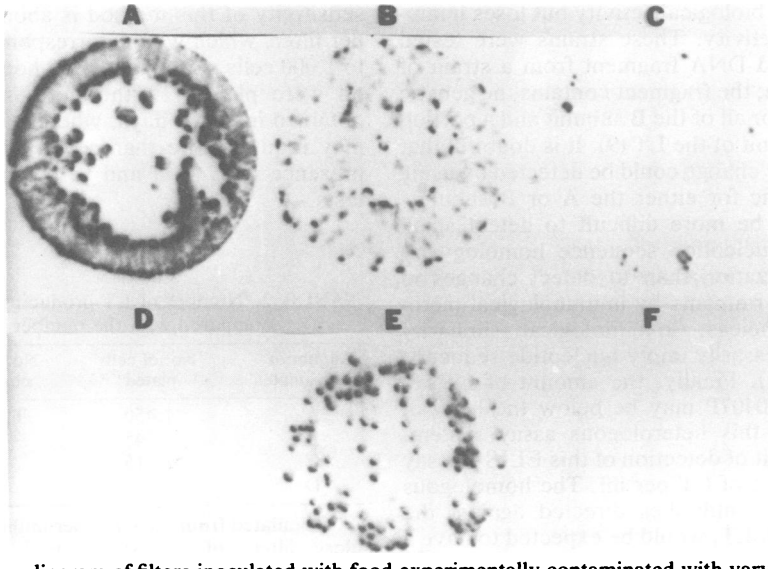

FIG. 3. Autoradiogram of filters inoculated with food experimentally contaminated with varying numbers of enterotoxigenic *E. coli* cells. A, 1,450 cells; B, 145 cells; C, 15 cells; D, 1 cell; E, 10^{-6} dilution of pure culture of strain H10407; F, 0.1 ml of food sample before inoculation with strain H10407.

produce levels of toxin that could be detected by the heterologous ELISA (Table 1).

Two strains isolated from pigs and containing the LT gene, as revealed by DNA colony hybridization and the mouse Y-1 cell response, were negative in the ELISA that is based on the immunological cross-reaction of *E. coli* LT with CT antiserum. Honda et al. (25) reported that LTs elaborated by *E. coli* strains of porcine and human origin are antigenically related but not identical. Similarly, antibodies produced against the LT of an *E. coli* strain of human origin had high neutralization titers when tested with the homologous toxin and CT (P. H. Gilligan et al., Int. Congr. Microbiol. Symp. 1982, Abstract, p. 112). However, the anti-LT antibodies only weakly neutralized LT elaborated by an *E. coli* strain isolated from pigs. Similarly, Gustafsson and Möllby (19) reported that when a GM1 ganglioside ELISA was used to detect LT, 19 of 20 *E. coli* strains of human origin were negative if heterologous antisera (raised against LT from porcine isolates) were used. Most of these antigenic differences seem to reside in the B subunit (16).

De Mol and co-workers (10), using *E. coli* strains isolated from humans, found that of 60 cultures that were positive in the mouse Y-1 adrenal cell assay, 2 were negative in an ELISA based on the binding of LT to GM1 ganglioside. Bound LT was detected by a guinea pig antibody to LT labeled with peroxidase. Therefore, if isolates from various sources are to be examined, both antihuman and antiporcine LT sera should be used.

It seems that mutations can alter the toxin so that it retains biological activity but loses immunological reactivity. These strains were tested with a labeled DNA fragment from a strain of porcine origin; the fragment contains the genetic information for all of the B subunit and a portion of the A subunit of the LT (9). It is doubtful that such a genetic change could be detected by using probes specific for either the A or B subunits since it may be more difficult to detect small changes in nucleotide sequence homology by DNA hybridization than to detect changes in antigenic determinants by immunological methods. Nevertheless, immunological similarity does not necessarily imply nucleotide sequence homology (45). Finally, the amount of LT released by H10407P may be below the level of detection for this heterologous assay system. The lower limit of detection of this ELISA assay is 0.5 to 1.0 ng of CT per ml. The homologous system, using antibodies directed against the purified *E. coli* LT, would be expected to have a greater sensitivity even though the toxin subunits are similar enough to form biologically active hybrids in vitro (54).

The sensitivity of the colony hybridization method in mixed cultures was tested by spread-plating a mixture of toxin-producing (H10407) and -nonproducing (C600) strains. It was found that even though a filter contained $\geq 1.0 \times 10^6$ negative cells, about 50% of the cells of the toxin-producing strain could be detected (Fig. 2). The limit of sensitivity of this method is determined in part by the number of cells that can be placed on a filter and still allow sufficient growth so that positive cells may be detected. Furthermore, the efficiency of plating on nitrocellulose filters was about 80% of that on an agar surface (data not shown).

Because the normal flora of many foods is more complex than a mixture of two strains of *E. coli*, this method was tested on an experimentally contaminated food. Homogenates of scallops were inoculated with different numbers of enterotoxigenic *E. coli* strain H10407. This strain was chosen for several reasons: it was isolated from a human, it might be found as a foodborne contaminant, and it may not have been recovered by standard enrichment procedures (34). The strain also contains only a few (one to five) copies per cell of the genes for LT and in this characteristic probably represents most LT-producing strains. The strain C600(pEWD299) may contain 20 to 50 copies of the LT gene, and though it serves well as a strongly positive control, it is not typical of the strains usually found in foods.

Colony hybridization was effective in detecting and estimating the number of enterotoxigenic *E. coli* cells present in a laboratory-contaminated food (Table 2; Fig. 3). The sensitivity of this method is about 1 to 10 cells per filter, which would correspond to about 100 to 1,000 cells per g in the food homogenate if 0.1 ml were plated. Furthermore, results can be obtained in 2 to 3 days, whereas other methods may require more than a week to confirm the presence of *E. coli* and to complete the toxin tests.

TABLE 2. Number of LT-producing *E. coli* strains compared with the number plated

Aliquot of food sample:	No. of cells plated[a]	No. of cells observed[b]	%
A	1,450	TNTC[c]	
B	145	112	77
C	15	13	87
D	1	1	

[a] Calculated from the CFU per milliliter on nitrocellulose filters of the strain used for inoculation (H10407).

[b] Spots observed on filters (see Fig. 3).

[c] TNTC, Too numerous to count.

Isolation of pure cultures of enterotoxigenic *E. coli* from foods without selective enrichment would be useful in studying and testing these isolates by immunological or biological assay. When the original spread-plated filter is grown, a replica filter is made by pressing a second sterile nitrocellulose filter on the one containing colonies. The second filter is then used for the colony hybridization. When positive colonies are located on the autoradiogram, corresponding areas on the original filters are streaked onto a nitrocellulose filter and incubated. A replica of the second filter is made and used for colony hybridization, and isolated positive colonies may be picked to establish culture lines for the second master filter. If no isolated positive colonies are observed, the process can be repeated by picking and streaking from positive areas on the second master filter.

In summary, colony hybridization can detect strains of *E. coli* which have the genetic potential to produce LT. This method yields results similar to those based on immunological and biological tests and may be more sensitive. If only small amounts of toxin are elaborated, colony hybridization will still give a positive result because it measures gene dosage and is not dependent on the level of gene expression. Furthermore, enterotoxigenic *E. coli* can be detected and enumerated in food at levels of 100 cells per g without the need for enrichment. The parameters of this method are being explored, especially with respect to the numbers and types of indigenous flora and to the effect of the food itself on the results.

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